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Liver Sinusoidal Endothelial Cell Lectin Inhibits CTL-Dependent Virus Clearance in Mouse Models of Viral Hepatitis

Biao Liu,*1 Min Wang,*1 Xiaowen Wang*,†1 Danhuan Zhao,* Di Liu,* Jing Liu,* Pei-Jer Chen,* Dongliang Yang,* Fuchu He,*† and Li Tang*

Liver sinusoidal endothelial cell lectin (LSECtin) was recently reported to suppress intrahepatic T cell immunity and to limit immune-mediated liver injury. However, its role in the outcome and pathogenesis of viral infection has not yet been elucidated. Using a mouse model infected with a hepatotropic adenovirus, we found that the absence of LSECtin led to a higher frequency of intrahepatic effector CTLs. These cells produced higher levels of antiviral cytokines and cytotoxic factors and exhibited an increased expression of the transcription factors T-bet and Runx3. This phenotype observed in the LSECtin-knockout cells mediated a more efficient virus-specific cytotoxicity compared with that of wild-type cells. As a consequence, LSECtin deficiency significantly accelerated liver adenovirus clearance. In contrast, LSECtin upregulation in the liver delayed viral clearance; this delayed clearance was accompanied by the downregulation of the antiviral activity of CTLs. We further constructed an immunocompetent mouse model of acute hepatitis B viral infection to demonstrate that LSECtin significantly delayed the clearance of hepatitis B virus from blood and infected hepatocytes by limiting the frequency of hepatitis B virus–specific IFN-γ–producing cells. Consistent with this function, LSECtin was upregulated in the liver of mouse models of viral hepatitis. Taken together, our results suggest that LSECtin may facilitate the reduction of liver inflammation at the cost of delaying virus clearance and that this effect might be hijacked by the virus as an escape mechanism. The Journal of Immunology, 2013, 190: 000–000.

The liver is an attractive target site of infections by non-cytopathic pathogens, such as hepatitis B virus (HBV) and hepatitis C virus. It has become increasingly accepted that virus-specific T cell responses play a major role in the outcome and pathogenesis of viral infections in the liver (1, 2). The activities of these T cells must be tightly regulated to maintain the balance between immunopathology and viral control (3). However, the continuous activation of these regulation mechanisms may perpetuate the deactivation of the already diminished antiviral immune response, which would permit the immune escape of the virus (4).

Thus, the investigation of the mechanisms that dampen T cell responses during liver infections would provide important insights into liver immunopathology and viral control (5–7). Although most previous studies have focused on the inhibitory molecules found on peripheral T cells, the contribution of local suppressors derived from the liver itself is only just beginning to be appreciated. Liver sinusoidal endothelial cell lectin (LSECtin), which is a cell-surface molecule that belongs to the C-type lectin receptor family, is constitutively and specifically expressed in LSECs and Kupffer cells (8–10). We recently reported that LSECtin suppresses intrahepatic T cell immunity and limits immune-mediated liver injury during Con A–induced experimental acute hepatitis (10). The liver-specific expression profile of LSECtin and its ability to limit hepatic T cell immunity inspired us to investigate whether this molecule regulates antiviral immunity and virus invasiveness in the liver and to determine how its expression is modulated in the context of viral infection.

Because hepatitis B virus does not propagate in rodents, we used mouse models of adenovirus infection and HBV replication to mimic a viral infection of the liver. An i.v. injection of a replication-defective adenovirus that encodes the LacZ gene causes efficient infection and viral gene expression in the liver. The CD8+ T cell–mediated immune system responds to the virus-coded gene products, ultimately causing the elimination of the infected hepatocytes (11). Therefore, this model is suitable for the examination of intrahepatic CTL effector mechanisms (12, 13). The hydrodynamic injection (HDI) of a plasmid containing HBV super-genomic DNA into BALB/c mice initiates higher-titer HBV replication in the liver, which results in acute self-limiting viral hepatitis, and the HBV clearance in these mice depends on their potent virus-specific CTL immunity (14). This HDI-HBV–treated model gives us the unique opportunity to characterize the immu-
In this study, we found that LSECtin-knockout (KO) animals exhibited accelerated liver viral clearance due to a higher frequency of intrahepatic CTLs with higher antiviral activities. In contrast, LSECtin upregulation in the liver had the opposite effect. In addition, the LSECtin expression in the liver was upregulated in the context of viral infection and in response to stimulation with either IFN-γ or IL-10.

Materials and Methods

Animals

For Ad-LacZ infection, mice were injected i.v. with 1.5–2 × 10⁶ PFU Ad-LacZ. For HBV infection, mice were injected hydrodynamically with 10 μg pAAV-1.2HBV plasmids via the tail vein.

Cells preparation

Intrahepatic lymphocytes (IHL) and LSECs/Kupffer cells were prepared as described previously (17, 18). CD8+ T cells were sorted by the BD FACSAria III system (BD Biosciences). LSECs and Kupffer cells were treated with various stimuli: IL-10 (50 ng/ml), IFN-γ (50 ng/ml), IL-4 (200 ng/ml), TNF-α (20 ng/ml), and IL-6 (20 ng/ml) from PeproTech.

Abs and flow cytometry

Abs used were as follows: allophycocyanin-labeled H2-Kb βgal tetramer (ICPMYAVR) was provided by ProfImmune. The following reagents were used for surface and intracellular staining: anti-mouse CD8α allophycocyanin (clone 53-6.7), anti-mouse CD25-FITC (clone PC6.5), anti-mouse CD62L-PE (clone MEL-14), anti-mouse CD127-FITC (clone A7R34), anti-mouse Granzyme B–FITC (clone 16G6), anti-mouse perforin-FITC (eBioOMAK-PE (clone MEL-14), anti-mouse CD127-PE (clone A7R34), anti-mouse F4/80-FITC (clone 50.2.4), and anti-mouse CD146-FITC (clone ME-9F1) were purchased from BioLegend. Western blot Abs were as follows: rabbit polyclonal to T-bet and rabbit polyclonal to Runx-3 were from Abcam. Intrahepatic lymphocytes were used for surface and intracellular staining: anti-mouse CD8a allophycocyanin (clone 53-6.7), anti-mouse CD3-PE (clone 145-2C11), and anti-mouse F4/80-PE (clone 50.2.4), anti-mouse CD146-FITC (clone ME-9F1) were purchased from BioLegend. Western blot Abs were as follows: rabbit polyclonal to T-bet and rabbit polyclonal to Runx-3 were from Abcam. Intrahepatic lymphocytes were prepared for cell-surface or intracellular staining. For FACS-based cytotoxicity assay, EL4 target cells were loaded with β-Gal peptide before coincubation with intrahepatic CD8+ T cells. The percentage of Annexin V+ target cells was determined. For in vivo BrdU labeling, at days 0 or 7 of adenovirus infection, mice were i.p. injected with 10 mg/ml BrdU (BD Pharmingen). One hour after BrdU injection, the liver were harvested for BrdU detection by flow cytometry. For detection apoptosis of IHLs in vivo, CD8+ T cells were isolated 4 h after BrdU injection, the liver were harvested for BrdU detection by flow cytometry. For detection apoptosis of IHLs in vivo, CD8+ T cells were isolated 1 h after BrdU injection, the liver were harvested for BrdU detection by flow cytometry.

RT-PCR assays

Liver tissue was harvested and total RNA extracted by the phenol/chloroform method by using TRIzol Reagent (Invitrogen). RNA (5 μg) was reverse-transcribed to cDNA in a 50 μl reaction mixture with Superscript II RNase H− reverse transcriptase and random primers (Life Technologies, Rockville, MD). For semiquantitative PCR, the specific primers for LacZ GAPDH and murine LSECtin (mLSECtin) were from Invitrogen: LacZ sense, 5′-CAAGTCCTG-3′; and probe, 5′-ACTOCAAGAGCGTCCTGACCC-3′. For SYBR Green Q-PCR, LacZ sense, 5′-GCAAGCCTGGTTCTGCATTGCAAGG-3′ and antisense, 5′-TGGACCACTTCTCGGCACGACC-3′; GAPDH sense, 5′-TAACAGAGAAGCTCCTACTGG-3′ and antisense, 5′-GAGGTCACACAA-GGTGTCATTGAG-3′; mLSECtin sense, 5′-GATCGTCCTGCAGTGAGAGGCAGA-3′; and probe, 5′-ACTGTTGTCATTGAG-3′ and antisense, 5′-AACCTGTGTCAGAGGTTGC-3′; mLSECtin sense, 5′-GGAAAGCAGCAAAATCAT-3′ and antisense, 5′-GGACCTGGACCACTGGA-3′; and mLSECtin sense, 5′-GGTGCGTATGCCTGCA-3′ and antisense, 5′-GCCTGTAAGGGAGACGCCGCA-3′.

ELISPOT assay

Liver lymphocytes were cocultured with or without 10 μg/ml peptides (HBsAg peptide 28–39; IPQSLDSSWMTSL for H-2Kd-restricted CTLs and HBCAg peptide 87–95; SVYNTMNGL for H-2Kd-restricted CTLs). Membranes of 96-well ELISPOT plates (MultiScreenTM ELISPOT; Millipore, Bedford, MA) were prewet with 70% ethanol and then washed and coated overnight at 4°C with anti-mouse IFN-γ mAb (Mabtech, Mariemont, OH). The following day, plates were washed and blocked. Stimulated cells were transferred to the ELISPOT plate at 3 × 10⁹ viable cells per well, and cells were allowed to incubate for 24 h. The following day, plates were washed and labeled with secondary monoclonal biotinylated anti-mouse IFN-γ (Mabtech) and then streptavidin-alkaline phosphatase conjugate (Mabtech). Plates were then washed, and BCIP/NGE plus developer (Mabtech) was added. Plate reactions were stopped, and data were collected using an ELISPOT plate reader (Autoimmun Diagnostika, Strassberg, Germany) and analyzed by ELISPOT Software Version 3.1 (Autoimmun Diagnostika).

Plasmids for hydrodynamic injection

SolubleFc or Fc-LSECtin fragments were each cloned into the pLIVE vector. Screening of effective small interfering RNA for silencing mouse LSECtin expression in the liver was described previously (10). mLSECtin short hairpin RNA (shRNA) were cloned into the pSIREN-DNR-DsRed vector (Clontech). The HBV replication-competent recombinant plasmid pAAV/HBV1.2 containing 1.2 copies of HBV supergenomic DNA (adw subtype) was provided by Prof. P.J. Chen (National Taiwan University, Taipei, Taiwan).

Detection of HBV Ag

Sera from individual mice were obtained at indicated time points. The levels of HBsAg and HBeAg were determined using an electrochemiluminescence immunoassay (Modular Cobas 6000; Roche Diagnostics).

Statistical analyses

Comparison between groups was made using the two-tailed unpaired t test. Cochran t test and Wilcoxon test were used to analyze the clearance of HBsAg: *p < 0.05, **p < 0.01, ***p < 0.001.

Results

Increased effector and cytolytic functions of the liver CD8+ T cells of LSECtin−/− mice upon viral infection

Mice were injected i.v. with 1.5–2 × 10⁶ PFU of Ad-LacZ, and the liver tissue was collected on days 0, 7, 10, and 14 postinfection. The infectious particles were monitored using X-Gal staining. A large proportion of hepatocytes were infected between days 7 and 10, and the viral particles were almost completely cleared by day 14 (Supplemental Fig. 1A). The viral gene expression was assessed through PCR amplification of the LacZ mRNA on day 10 (Supplemental Fig. 1B). We analyzed the cellular infiltration of the liver using BrdU labeling of the T cells. There was obvious T cell proliferation in the liver 7 d postinfection (dpi) (Supplemental Fig. 1C). Moreover, the serum ALT and AST levels were elevated on days 7, 10, and 15, which suggests that Ad-LacZ causes a certain degree of liver damage (Supplemental Fig. 1D). Consistent with previous reports, our findings show that we have successfully established a mouse model of acute liver adenovirus infection with a measurable T cell immune response.

To characterize the LSECtin function, wild-type (WT) and LSECtin−/− mice were injected with Ad-LacZ; the IHLs were then separated, and the phenotypes of the CD8+ T cells of these two mice were compared 7 dpi. We found that the CD8+ T cells of CAAGTCCTG-3′; and probe, 5′-ACTOCAAGAGCGTCCTGACCC-3′. For SYBR Green Q-PCR, LacZ sense, 5′-GCAAGCCTGGTTCTGCATTGCAAGG-3′ and antisense, 5′-TGGACCACTTCTCGGCACGACC-3′; GAPDH sense, 5′-TAACAGAGAAGCTCCTACTGG-3′ and antisense, 5′-GAGGTCACACAA-GGTGTCATTGAG-3′; mLSECtin sense, 5′-GATCGTCCTGCAGTGAGAGGCAGA-3′; and probe, 5′-ACTGTTGTCATTGAG-3′ and antisense, 5′-AACCTGTGTCAGAGGTTGC-3′; mLSECtin sense, 5′-GGAAAGCAGCAAAATCAT-3′ and antisense, 5′-GGACCTGGACCACTGGA-3′; and mLSECtin sense, 5′-GGTGCGTATGCCTGCA-3′ and antisense, 5′-GCCTGTAAGGGAGACGCCGCA-3′.
the LSECtin−/− mice exhibited increased CD25 (p < 0.05) but decreased CD62L and CD127 (p < 0.01) expression (Fig. 1A), which is indicative of an activated or effector phenotype. To determine whether the absence of LSECtin signaling induces increased effector functions in the virus-induced CD8+ T cells, we analyzed the production of cytokines and cytotoxic molecules by these cells. The percentages of intrahepatic CD8+ T cells expressing TNF-α (p < 0.01), IFN-γ (p < 0.001), granzyme B (p < 0.001), and perforin (p < 0.01) were significantly increased in the LSECtin−/− mice compared with the WT mice (Fig. 1B, 1C).

**FIGURE 1.** Increased effector and cytolytic functional properties of the liver CD8+ T cells of LSECtin−/− mice upon adenovirus infection. WT and LSECtin−/− mice were injected with 1.5–2 × 10⁹ PFU of Ad-LacZ, and the IHLs were harvested 7 dpi. (A) Dual staining and flow cytometry were used to analyze the expression of CD25, CD62L, and CD127 in the CD8+ T cells. A two-tailed unpaired t test was used (n = 6). Significant differences in the levels of CD25 (p < 0.05), CD62L (p < 0.01), and CD127 (p < 0.01) were found between the WT and LSECtin−/− cell populations.

The IHLs were dual-labeled for CD8 and intracellular TNF-α, IFN-γ, granzyme B (GrB), or perforin (B, C), and the numbers of CD8+ T cells that produced TNF-α, IFN-γ, granzyme B (GrB), or perforin (D, E) are shown. A two-tailed unpaired t test was used (n = 4). Significant differences in the levels of TNF-α (p < 0.01), IFN-γ (p < 0.001), perforin (p < 0.01) and granzyme B (p < 0.001) were found between the WT and LSECtin−/− cell populations. (F and G) The IHLs were harvested 7 dpi, and the CD8+ T cells were sorted using flow cytometry. The expression of T-bet, Runx3, and Eomes was evaluated by Q-PCR (F) and Western blot (G). A two-tailed unpaired t test was used (n = 3; p < 0.01). *p < 0.05, **p < 0.01, ***p < 0.001.
This result was further confirmed by the analysis of the absolute numbers of these effector T cells (Fig. 1D, 1E).

The T-box transcription factors T-bet and Eomes have been linked to the regulation of genes encoding effector cytokines and genes that are important for cytolytic function (19). In acute infection, the Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs (20). We tested whether these transcription factors were involved in the LSECtin-controlled regulation of virus-induced CD8+ T cells. WT and LSECtin-KO mice were either injected or not injected with the Ad-LacZ virus.

**FIGURE 2.** Increased Ag-specific cytolytic function of the liver CD8+ T cells of LSECtin−/− mice upon adenovirus infection. WT and LSECtin−/− mice were injected with 1.5–2 × 10⁹ PFU of Ad-LacZ, and the IHLs were harvested 7 dpi. (A) The IHLs were dual-labeled with anti-CD8 and the H2-Kb β″gal tetramer. A two-tailed unpaired t test was used (n = 3; p < 0.05). (B and C) The EL4 target cells were loaded with 0 or 1 μM of the β-Gal peptide for 2 h. The target cells were then coincubated with the sorted intrahepatic CD8+ T cells at the indicated E:T ratios for 3.5 h. The percentage of Annexin V+ target cells in the CD8-negative EL4 target population was determined (B). A representative flow diagram (E:T ratio = 30:1) is shown (C). ***p < 0.001.

**FIGURE 3.** Increased proliferation of the liver CD8+ T cells of LSECtin−/− mice upon adenovirus infection. LSECtin−/− or WT mice were i.v. injected with Ad-LacZ. (A) On day 7 postinfection, the mice received an i.p. injection of BrdU and were sacrificed 1 h later. The IHLs were surface-labeled with anti-CD8 and stained for intracellular BrdU. A two-tailed unpaired t test was used (n = 4). A significant difference in the level of BrdU (p < 0.05) was found between the WT and LSECtin−/− cell populations. (B) The IHLs were harvested 7 dpi and dual-stained with Annexin V and anti-CD8. A two-tailed unpaired t test was used (n = 6). There was no significant difference in the CD8+ T cell apoptosis between the WT and LSECtin−/− mice. **p < 0.01.
The hepatic CD8⁺ T cells were separated 7 dpi; the total RNA was then extracted, and the cell lysates were prepared. T-bet, Eomes, and Runx3 mRNAs were not detectable in either the WT or the LSECtin-KO CD8⁺ T cells that were not infected, but all of these mRNAs were strongly induced upon virus challenge. Compared with the WT CD8⁺ T cells, the LSECtin-KO CD8⁺ T cells expressed higher levels of T-bet and Runx3 (Fig. 1F; p < 0.01). This result was further confirmed by Western blot (Fig. 1G). However, there was no significant difference in the Eomes expression between the two groups (Fig. 1F).

Because the CD8⁺ T cells of LSECtin⁻²/⁻ mice produced higher levels of cytokines and cytolytic factors, we examined their Ag-specific cytolytic functions. The frequency of β-gal-specific CD8⁺ T cells that were positive for the class I MHC H2-Kb-restricted β-gal tetramer (ICPMYARV) was higher in the LSECtin⁻²/⁻ mice than in the WT mice (Fig. 2A; p < 0.001). In a short-term assay in which targeted cell death was measured within 3.5 h, the CD8⁺ T cells of LSECtin⁻²/⁻ mice displayed a significantly higher Ag-specific cytolytic function, as determined by their ability to induce apoptosis in a larger number of Ag-pulsed target cells at different E:T cell ratios (Fig. 2B, 2C).

Increased proliferation of liver CD8⁺ T cells of LSECtin⁻⁻/⁻ mice upon viral infection

We previously reported that LSECtin inhibited T cell immune responses, including decreased T cell activation and proliferation and the downregulation of T cell-derived cytokines (10, 21). Therefore, to understand the mechanism by which LSECtin suppresses the intrahepatic effector functions of CD8⁺ T cells in a mouse model of adenovirus infection, we compared the T cell proliferation in the liver through in vivo BrdU labeling. On day 0, there were almost no proliferating cells found in the liver of either LSECtin⁻⁻/⁻ or WT mice (Supplemental Fig. 1C). On day 7 postinfection, the frequency of BrdU⁺ CD8⁺ T cells was increased in the liver of LSECtin⁻⁻/⁻ mice compared with WT mice (Fig. 3A; p < 0.05). Furthermore, we investigated whether the CD8⁺ T cells in the liver of LSECtin⁻⁻/⁻ mice were resistant to apoptosis. However, the percentage of Annexin V⁺ CD8⁺ T cells in...
the liver of LSECtin−/− mice upon adenovirus infection was similar to that observed in WT mice (Fig. 3B). These results suggest that LSECtin may inhibit the proliferation of, but does not induce the apoptosis of, intrahepatic effector CD8+ T cells upon viral infection.

**LSECtin deficiency does not affect the expression of programmed cell death ligand-1 and Galectin-9 in the liver**

It is noteworthy that the suppressive molecules programmed cell death ligand-1 (PD-L1) and Galectin-9 are expressed in the liver and that these molecules play crucial roles in the regulation of T cell immunity during viral infection in the liver (22–24). Therefore, we evaluated the expression of PD-L1 and Galectin-9 on the LSECs and Kupffer cells of LSECtin−/− mice. LSECtin deficiency did not affect the incidence of LSECs or Kupffer cells expressing PD-L1 and Galectin-9 nor the level of expression of these molecules, as determined by the mean fluorescence intensity (Fig. 4A–D). Upon adenovirus infection, there were also no major differences in the expression of these molecules, as determined by the mean fluorescence intensity (Fig. 4A–D). Upon adenovirus infection, there were also no major differences in the expression of these molecules, as determined by the mean fluorescence intensity (Fig. 4A–D).

**LSECtin deficiency or knockdown accelerates the adenoviral clearance of liver viral infection**

To investigate the role of LSECtin during the elimination of hepatocytes infected with adenovirus, Ad-LacZ was administered to LSECtin−/− and WT mice. The duration of the LacZ gene expression was evaluated in liver samples by X-Gal staining. Remarkably, a rapid clearance of the adenovirus was observed in the livers of LSECtin−/− mice, whereas a strong LacZ expression was sustained in the liver of WT mice until day 10 post-infection (Fig. 5A). Comparable results were obtained when the viral clearance was evaluated by RT-PCR (Supplemental Fig. 2A). The mice were administered the Ad-LacZ virus and then received the mLSECtin-shRNA plasmid (i.v.) on days 0, 4, and 8 post–viral infection. A rapid clearance of the adenovirus in the liver was observed in the mice treated with the shRNA plasmid, whereas a strong LacZ expression was sustained in the livers of the control mice (Fig. 5D). Comparable results were obtained when the LacZ gene expression was measured using semiquantitative PCR or Q-PCR analysis (Fig. 5E, 5F; p < 0.001). These results support our hypothesis that the induction of the downregulation of LSECtin expression, or the potential blockage of the function of LSECtin, promotes viral clearance in the liver.

**Upregulation of LSECtin delays adenoviral clearance by inhibiting intrahepatic CD8+ T cell effector function**

As an alternate approach to understanding the role of LSECtin in viral clearance, we examined animals that overexpressed LSECtin in the liver. The pLIVE vector is designed for the induction of a high-level specific and prolonged expression of transgenes in the mouse liver (25). Control pLIVE-Fc and LSECtin overexpression (pLIVE-Fc-mLSECtin) plasmids were developed. The expression of the LSECtin plasmid in the liver and serum was estimated (Supplemental Fig. 2B, 2C). We found that the upregulation of LSECtin in the liver delayed adenoviral clearance (Fig. 6A–C). This delayed clearance was accompanied by a decrease in the percentage of intrahepatic CD8+ T cells expressing the cytokines TNF-α (p < 0.05) and IFN-γ (Fig. 6D; p < 0.01) and the cytotoxic factors granzyme B and perforin (Fig. 6E; p < 0.001).

**LSECtin deficiency accelerates liver HBV clearance**

To more closely mimic viral infection in the human liver, we constructed an immunocompetent mouse model of acute hepatitis B viral infection. The pAAV-HBV1.2 plasmid was injected into BALB/c mice using HDI. The HBV Ags (HBsAg and HBeAg) could be detected in the sera, and the activation of hepatic T cells could be detected 10 dpi (Supplemental Fig. 3). To identify the role of LSECtin in viral clearance, we injected HBV-HBV1.2 plasmid was injected into WT and LSECtin−/− mice. The sera of these mice were collected, and the presence of HBsAg and HBeAg in the sera was analyzed. The results show that HBsAg (Fig. 7A; p < 0.01) and HBeAg (Fig. 7B, p < 0.05) were cleared significantly faster from

![FIGURE 5. LSECtin deficiency or knockdown accelerates liver adenoviral clearance.](http://www.jimmunol.org/)

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The image contains a figure with the following contents:

**A** WT and LSECtin−/− mice were injected with Ad-LacZ virus. The mice were sacrificed at the indicated time points, and the liver sections were analyzed by X-Gal staining (original magnification ×200). (A). Liver homogenates (10 dpi) were prepared to evaluate the LacZ gene expression by semiquantitative PCR (B) or Q-PCR (C). (D–F) Mice were injected with the Ad-LacZ virus after the administration of 10 μg/1.5 ml saline containing mLSECtin-shRNA/control plasmids. The mice were sacrificed at the indicated time points, and the liver sections were analyzed by X-Gal staining (original magnification ×200). (D). Liver homogenates (10 dpi) were prepared to evaluate the expression of the LacZ gene by semiquantitative PCR (E) or Q-PCR assay (F). A two-tailed unpaired t test was used (n = 3; p < 0.001), **p < 0.001.
the serum of LSECtin−/− mice and that the positive rate of HBsAg was lower (16.6% in LSECtin−/− mice compared with 100% in WT mice on day 28 postinfection) (Fig. 7C). Moreover, a marked reduction in the number of HBV core-positive hepatocytes was observed in LSECtin−/− mice on day 14 postinfection (Fig. 7D). We also analyzed the phenotypes of the CD8+ T cells of HDI-HBV–treated WT and LSECtin−/− mice. The CD8+ T cells of HDI-HBV–treated LSECtin−/− mice exhibited higher levels of CD25 (p, 0.05) and lower levels of CD62L (p, 0.001) and CD127 (Fig. 7E, p, 0.01). We further examined the frequency of HBcAg/HBsAg-specific IFN-γ–producing cells in the IHLs of BALB/c mice 10 dpi using the ELISPOT assay. The number of IFN-γ–producing cells in the LSECtin-KO mice was significantly higher than that observed in the WT mice (Fig. 7F, 7G; p < 0.001). These results suggest that the suppressed effector T cells may partly contribute to the delayed clearance of HBV from the blood and infected hepatocytes in an HDI-HBV–treated mouse model.

**Regulation of LSECtin expression in the context of viral infection**

The ability of LSECtin to affect antiviral immunity and viral clearance prompted us to investigate the regulation of endogenous LSECtin during viral infection. We evaluated the expression of LSECtin in the liver following adenovirus or HBV plasmid injection at various time points. The LSECtin transcript (Fig. 8A, 8C; p, 0.001) and protein expression (Fig. 8B, 8D) levels progressively increased during the course of infection, regardless of the
FIGURE 7. LSECtin delays liver HBV clearance. WT and LSECtin−/− mice on a BALB/c background were injected hydrodynamically with 10 μg of the pAAV/HBV1.2 plasmid. At the indicated time points, blood was collected, and the serum HBs (A, B) and HBe (C) Ags were quantified. Positivity for HBsAg was defined as cutoff index = 1. Cochran t test and Wilcoxon test were used. Representative immunohistochemical (Figure legend continues)
infection model. We further examined the relevant stimuli that induced the expression of LSECtin in endogenous liver cells. LSECs and Kupffer cells were separated and cultured in the presence of a panel of stimuli, including IFN-γ, IL-10, IL-4, TNF-α, and IL-6, which are important cytokines during viral infection and immune regulation. The exposure of LSECs and Kupffer cells to TNF-α or IL-6 resulted in decreased LSECtin expression. However, the exposure of these cells to IFN-γ, IL-10, or IL-4 upregulated LSECtin expression (Fig. 8E; p < 0.01).

**Discussion**

Our recent research has revealed that LSECtin is specifically expressed in the liver and that it is an important negative regulator of hepatic T cells (10). In this study, we further investigated the role of LSECtin in antiviral immunity and viral control in the liver and examined the regulation of LSECtin expression in the context of viral infection.

Because hepatitis B virus does not propagate in rodents, we used a mouse adenovirus to model viral infection in the liver. Numerous reports have confirmed that this model is suitable for the examination of intrahepatic CTL effector mechanisms (12, 13). During acute adenovirus infection, the hepatic CD8+ T cells of LSECtin−/− mice exhibited a phenotype with higher CD25 expression and lower expression of CD62L and CD127. After Ag stimulation, most CD8+ T cells began to downregulate CD127, and most Ag-specific effector cells expressed significantly lower levels of CD127 at the peak of the immune response to the infection. Thus, CD127low cells predominate during the acute effector phase. These cells appear to represent end-stage effector CD8+ T cells, which express a number of activation markers (e.g., CD25), exhibit downregulated levels of lymph node homing receptors (e.g., CD62L), and display strong immediate effector function (e.g., IFN-γ production and CTL activity) (26, 27). Therefore, LSECtin−/− hepatic CD8+ T cells may possess higher effector and cytolytic functional properties. Our continued investigation confirmed this hypothesis. The absence of the LSECtin signal induces an increased effector function in virus-induced CD8+ T cells, which express a number of activation markers (e.g., CD25), exhibit downregulated levels of lymph node homing receptors (e.g., CD62L), and display strong immediate effector function (e.g., IFN-γ production and CTL activity) (26, 27). Therefore, LSECtin−/− hepatic CD8+ T cells may possess higher effector and cytolytic functional properties.

A number of transcription factors have been implicated in the regulation of effector CD8+ T cell differentiation. The expression of T-bet is rapidly induced in CD8+ T cells by signaling through the TCR. T-bet then induces the expression of the effector mole-

![FIGURE 8. LSECtin expression in the liver is upregulated upon viral infection or exposure to IFN-γ, IL-10, or IL-4. LSECtin expression in the liver postinfection with adenovirus or HBV. Mice were injected with Ad-LacZ (A, B) or the HBV expression plasmid (C, D). At the indicated time points, liver homogenates and lysates were prepared, and the LSECtin expression was evaluated by Q-PCR and Western blot. (E) RT-PCR analysis of the LSECtin mRNA expression in LSECs and Kupffer cells exposed to various stimuli. The data are presented relative to the level of GAPDH mRNA. *p < 0.05, **p < 0.01, ***p < 0.001 compared with control LSECs and Kupffer cells (two-tailed unpaired t test).](http://www.jimmunol.org/)
cules IFN-γ, perforin, and granzyme B. In the absence of T-bet, CD8+ T cells fail to acquire a normal profile of effector cell-surface markers, which leads to the generation of a greater proportion of effector and memory cells with higher CD127 and CD62L expression (19, 28). Runx3, which is thought to be important for the establishment of the CD8+ T cell lineage, cooperates with the T-box factors to mediate effector differentiation (20). In our study, the LSECtin-KO CD8+ T cells expressed higher levels of T-bet and Runx3 mRNAs and proteins upon viral infection compared with WT cells. This result is consistent with the fact that the CD8+ T cells of LSECtin-KO mice exhibited the CD25highCD62LlowCD127low phenotype, higher levels of IFN-γ, TNF-α, granzyme B, and perforin, and higher Ag-specific cytolytic functions.

Because LSECtin appeared to dampen the antiviral CD8+ T cell immune and cytotoxic functions, we examined the function of LSECtin in viral control. Consistent with the function of LSECtin in limiting the number of cytolytic CD8+ T cells, LSECtin deficiency accelerated the clearance of adenovirus from the liver. We further analyzed the effect of LSECtin on viral clearance using HDI-HBV–treated models. A more rapid clearance of the HBV infection compared with WT mice. All of these results suggest that LSECtin may delay viral clearance by controlling the effector functions of intrahepatic CD8+ T cells.

Intriguingly, some coinhibitory molecules and soluble immunosuppressive factors can be induced in acute hepatic infections. During infection, these molecules facilitate the reduction of the immune response, limit organ damage, and maintain viral control. In contrast, the continuous activation of these mechanisms during persistent infection may perpetuate the deactivation of the already diminished antiviral immune responses (4, 29–32). LSECtin inhibits the intrahepatic CD8+ T cell effector function and controls viral clearance, which prompted us to investigate the regulation of endogenous LSECtin during viral infection.

The expression of LSECtin progressively increased during the course of infection. This increase was parallel to the onset of liver inflammation in both acute adenovirus and HBV infection models. Moreover, LSECtin expression was induced by IL-10, IFN-γ, or IL-4. IL-10 is a potent immunosuppressive cytokine with tissue-protective and immunoregulatory effects in acute HBV infection and other infections (29, 30). IFN-γ, which is generally perceived as an immune-stimulatory cytokine, can also induce the expression of inhibitory molecules, including B7-H1,IDO, and arginase, which may participate in a feedback mechanism to efficiently downregulate the immune response and avoid tissue and organ damage (31). Thus, we hypothesize that the liver might upregulate LSECtin expression during acute liver infection to restrain excessively harmful immune responses and limit immunopathology (Supplemental Fig. 4) at the cost of delaying viral control.

If LSECtin is continually induced and activated in the setting of a persistent infection by immunoregulatory cytokines, such as IL-10 or IL-4, it could perpetuate the deactivation of the already diminished antiviral immune responses. This effect, in combination with other tolerance mechanisms, would contribute to the maintenance of viral persistence. However, although this study clearly confirmed that LSECtin delays viral clearance in acute adenovirus and HBV infection models, further work is needed to determine the role of LSECtin in the regulation of the immune response during chronic and persistent viral infections in the liver.

The accumulated observations suggest that a complex interplay of immunological factors contributes to hepatic T cell immune dysfunction during chronic infection. Nakamoto et al. (33) demonstrated that the blockade of programmed cell death-1 alone was unable to restore the function of liver-derived hepatitidis virus–specific CD8+ T cells. However, the targeting of additional inhibitory signaling pathways reinvigorated the antiviral function of hepatic T cells (33). It is noteworthy that recent studies have proposed that the Tim-3/Galectin-9 pathway plays a crucial role in the regulation of T cell immunity during both hepatitis B and hepatitis C infections (23, 24). Moreover, the blockade of both Tim-3 and PD-1 has been found to have an additive or synergistic effect on the recovery of HBV-specific CD8+ T cells in some patients (23). In this study, we showed that LSECtin is modulated by the viral environment and that its upregulation delayed the clearance of the virus from the liver. Considering the fact that LSECtin, PD-L1, and Galectin-9 are all expressed in the liver, it would be interesting to examine the interplay between these molecules that contributes to the T cell–suppressive effects in a virus-infected liver. It is possible that the immune suppression could be reversed by the combined modulation of these intrahepatic inhibitory pathways.

Disclosures

The authors have no financial conflicts of interest.

References


