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In Hepatic Fibrosis, Liver Sinusoidal Endothelial Cells Acquire Enhanced Immunogenicity

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The normal liver is characterized by immunologic tolerance. Primary mediators of hepatic immune tolerance are liver sinusoidal endothelial cells (LSECs). LSECs block adaptive immunogenic responses to Ag and induce the generation of T regulatory cells. Hepatic fibrosis is characterized by both intense intrahepatic inflammation and altered hepatic immunity. We postulated that, in liver fibrosis, a reversal of LSEC function from tolerogenic to proinflammatory and immunogenic may contribute to both the heightened inflammatory milieu and altered intrahepatic immunity. We found that, after fibrotic liver injury from hepatotoxins, LSECs become highly proinflammatory and secrete an array of cytokines and chemokines. In addition, LSECs gain enhanced capacity to capture Ag and induce T cell proliferation. Similarly, unlike LSECs in normal livers, in fibrosis, LSECs do not veto dendritic cell priming of T cells. Furthermore, whereas in normal livers, LSECs are active in the generation of T regulatory cells, in hepatic fibrosis LSECs induce an immunogenic T cell phenotype capable of enhancing endogenous CTLs and generating potent de novo CTL responses. Moreover, depletion of LSECs from fibrotic liver cultures mitigates the proinflammatory milieu characteristic of hepatic fibrosis. Our findings offer a critical understanding of the role of LSECs in modulating intrahepatic immunity and inflammation in fibro-inflammatory liver disease. The Journal of Immunology, 2010, 185: 000–000.

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iver sinusoidal endothelial cells (LSECs) are an integral part of the hepatic reticuloendothelial system (1). LSECs are morphologically identified by their fenestrations, which are transcytoplasmic canals arranged in sieve plates (2). In addition, LSECs express the CD45−CD146+ surface phenotype that makes their ex vivo purification from bulk hepatic nonparenchymal cells (NPCs) feasible (3, 4). Primary roles of LSECs include scavenger function and blood clearance. LSECs are equipped with specialized receptors, including those for endocytosis of acetylated low density lipoprotein and mannosylated proteins, and are uniquely equipped to eliminate viruses, colloids, and macromolecular waste from the circulation (1).

In addition to their role in clearance of toxins, a unique immunological role has been attributed to LSECs. They express modest levels of MHC class I and class II and costimulatory molecules (CD40, CD80, and CD86), and can induce Ag-restricted T cell proliferation (5). However, rather than mediate immunogenic T cell responses to Ag, LSECs induce hepatic immunologic tolerance (6). In fact, the phenomenon of oral tolerance to Ag introduced via the oral route or by portal venous injection has been attributed in part to LSEC-mediated tolerogenicity (7). In multiple experimental models, including presentation of oral Ag, cross-presentation of soluble protein, and uptake of apoptotic tumor cells, T cells that have been engaged by LSECs deviate either toward a CD4+ T regulatory cell (Treg) phenotype or toward CD8+ T cell tolerance, respectively (6–8). In addition, beyond actively inducing T cell anergy via Ag presentation, LSECs have been shown to veto T cell activation by other professional APCs (9). The cellular and molecular mechanisms of LSEC-mediated T cell anergy are not entirely understood. However, Diehl et al. (10) recently reported that B7-H1 signaling on LSECs is required for induction of CD8+ T cell tolerance via PD-1 ligation.

Whereas the hallmark of LSEC immune function in the unperturbed liver is the induction of tolerance, LSEC function has not been studied in disease states. Hepatic fibrosis is characterized by intense intrahepatic inflammation following liver insult, ultimately resulting in activation of hepatic stellate cells (HSCs) that produce extracellular matrix and fibrillar collagen (11). We have previously shown that the immunogenicity of liver APCs is a primary determinant of the intrahepatic inflammatory milieu in fibrotic liver disease (12). We reported that liver dendritic cells (DCs) transform from mediators of hepatic tolerance in the normal liver to purveyors of potent immunogenicity in hepatic fibrosis. The newly immunogenic DCs govern the hepatic inflammatory environment in fibrosis by inducing proinflammatory innate and adaptive immune responses in the liver (12). However, the changes in LSEC immune function in hepatic fibrosis, and their contributions toward the hepatic inflammatory milieu, have not been previously studied. In the current study, we report that, in fibrosis, LSECs become highly proinflammatory and are a critical component of intrahepatic inflammation. Moreover, in the fibrotic liver, LSECs transform from mediators of tolerance into potent stimulators of adaptive immunity.
Our findings have important relevance to the understanding of intrahepatic immunity and inflammation in hepatic fibrosis.

Materials and Methods

Animals and procedures

Male C57BL/6, OT-I (B6.Cg-RAG2tm1Fwa-TgN), OT-II (B6.Cg-RAG2tm1Alt-TgN), and RAG\(^{-2}\) mice were purchased from Taconic Farms (Germantown, NY). To induce hepatic fibrosis, mice were treated with thrice-weekly injections of thioacetamide (250 mg/kg; Sigma-Aldrich, St. Louis, MO) and leptin (1.5 mg/kg; Biomyx, San Diego, CA) for 6 wk, as described (12, 13). Alternatively, in selected experiments, mice received biweekly injections of CCl\(_4\) (0.5 ml/kg; Sigma-Aldrich) for 12 wk, as described (12). Animal procedures were approved by the New York University Institutional Animal Care and Use Committee.

Cellular isolation and culture

Liver NPCs were isolated, as we have previously described (14). Briefly, the portal vein of mice was infused with 3 ml 1% collagenase IV (Sigma-Aldrich), followed by hepatectomy and mechanical digestion. Low speed (30 \(\times\) \(g\)) centrifugation of liver suspensions was performed to exclude hepatocytes, followed by high speed (400 \(\times\) \(g\)) centrifugation to isolate the NPCs. NPCs were further enriched over an Optiprep (Sigma-Aldrich) gradient. Leukocytes, including liver NK cells, T cells, NKT cells, B cells, macrophages, Kupffer cells, and neutrophils, were excluded using anti-CD45 immunomagnetic beads (Miltenyi Biotec, Bergisch-Gladbach, Germany) and passage through depletion columns. LSECs were then purified using anti-CD146 immunomagnetic beads and passage through positive selection columns or by FACS sorting CD45\(^{-}\)CD146\(^{+}\) cells. This technique resulted in a >95% pure LSEC population (Supplemental Fig. 1). Other liver NPC subsets were similarly isolated either by FACS sorting or using immunomagnetic beads and passage through positive selection columns. HSCs were isolated by density centrifugation over a three-layer Optiprep gradient, as we have described (12). Splenocytes were harvested by mechanical disruption of spleens. Splenic T cells were purified using anti-CD90, anti-CD8, or anti-CD4 immunomagnetic beads and passage through positive selection columns.

Flow cytometry and cytokine analysis

Flow cytometry was performed using the FACSCalibur (BD Biosciences, Franklin Lakes, NJ) after incubating 5 \(\times\) 10\(^5\) cells/tube with 1 \(\mu\)g anti-Fc\(\gamma\)RIII/II Ab (2.4G2, Fc block; mAb Core, Sloan-Kettering Institute, New York, NY) and then labeling with 1 \(\mu\)g fluorescently conjugated Ab to CD4 (RM4-5), CD8\(\alpha\) (53-6.7), CD11b (M1/70), CD11c (N418), CD25 (PC61.5), CD31 (390), CD40 (HM40-3), CD44 (IM7), CD45 (Ly5.2), CD62L (MEL-14), CD86 (GL1), CD105 (M170), CD146 (ME-9F1), CD178 (MFL3), CD146 (ME-9F1), CD178 (MFL3)
MHC class II (I-Ab), and Foxp3 (FJK-16s) (all from eBiosciences, San Diego, CA, except CD146 [Miltenyi Biotec]). For cytokine analysis, cell suspensions were cultured in complete RPMI (RPMI 1640 with 10% heat-inactivated FBS, 2 mM L-glutamine, and 0.05 mM 2-ME) at a concentration of $1 \times 10^6$ cells/ml for 24 h before supernatant harvest and analysis using either a cytometric bead array (BD Biosciences) or the Milliplex immunoassay (Millipore, Billerica, MA). Intracellular cytokine staining was performed using an intracellular cytokine detection kit, according to the manufacturer’s protocol (BD Biosciences). TGF-β was assayed by ELISA (Bender MedSystems, Vienna, Austria).

**In vitro T cell assays**

For CD4+ T cell proliferation assays, LSECs were pulsed with OVA323–339 peptide (Abcam, Cambridge, MA; LSEC.OVA323) for 90 min before washing and plating in various concentration with OT-II T cells for 72 h in 96-well plates. For the last 24 h, 1 μCi [3H]thymidine was added to each well and radioactivity uptake was measured using a MicroBeta counter (PerkinElmer, Waltham, MA). To assess LSEC induction of Tregs, LSECs were cultured in equal numbers with syngeneic spleen CD4+ T cells before assay of T cell coexpression of CD25 and Foxp3 at 96 h. To examine LSEC-mediated activation of CD8+ T cells, LSECs were loaded with OVA257–264 (Abcam; LSEC.OVA257) before coculture with OT-I T cells and analysis of T cell surface phenotype at 24 h.

**In vivo T cell assays**

To test LSEC induction of T cell proliferation in vivo, C57BL/6 mice were administered $2 \times 10^6$ OT-I T cells i.v., followed by footpad immunization with LSEC.OVA257 ($2 \times 10^5$) at 24 h. T cell proliferation in the draining ipsilateral popliteal lymph node was measured at 96 h by counting the number of CD8+OVAtetramer+ T cells by flow cytometry. To determine whether LSECs can enhance endogenous CTL responses, Rag2−/− mice were reconstituted with $1 \times 10^7$ CD8+ OT-I T cells. On day 10, reconstituted mice were immunized with $1 \times 10^6$ LSEC.OVA257. On day 17, splenocytes were harvested from recipient mice, restimulated in vitro with OVA257-264, and tested for Th1 and Th2 cytokine production. To determine the ability of LSECs to produce de novo CTL, C57BL/6 mice were i.p. twice at weekly intervals with $1 \times 10^6$ LSEC.OVA257 or mock immunized. One week after the second immunization, mice were challenged i.v. with a 1:1 ratio of CFSEhigh-labeled splenocytes (Invitrogen, Carlsbad, CA) loaded with OVA257-264 and CFSElow-labeled splenocytes that were mock loaded. CTL-specific lysis in the spleen or liver was determined at 4 h, according to the following formula: $100 - \frac{\text{ratio(CFSEhigh cells/CFSElow cells)}_\text{OVA immunized}}{\text{ratio(CFSEhigh cells/CFSElow cells)}_\text{mock immunized}}$.

**Ag capture assays**

For in vitro Ag uptake assays, DCs or LSECs ($5 \times 10^5$) were incubated with FITC-dextran, FITC-albumin, or FITC-mannose albumin (1 mg/ml; all Sigma-Aldrich) at 37°C for various time intervals. Ag uptake was determined by flow cytometry. Assays were performed in triplicate.

**Histology and immunohistochemistry**

For histological analysis, livers were fixed with 10% buffered formalin, dehydrated in ethanol, embedded in paraffin, and stained with H&E or picric acid-Sirius red. For immunohistochemical studies, paraffin-embedded liver sections were stained with anti-CD45 (BD Biosciences) or anti-α-smooth muscle actin (SMA; Sigma-Aldrich). Images were captured using an Axiovert 40 microscope (Zeiss, Thornwood, NY) and a Jenoptik digital camera (Jena, Germany).

**Statistics**

Data are presented as mean ± SEM. Statistical significance ($p < 0.05$) was determined by the Student t test and the log rank test.

**Results**

**Model of fibrosis**

Livers of mice treated with hepatotoxin demonstrated an irregular surface contour on morphologic examination (Fig. 1A). Histologic

[FIGURE 3. Ag capture by LSECs is altered in hepatic fibrosis. LSECs ($5 \times 10^5$) were tested for their ability to capture dextran (A), albumin (B), and mannose-albumin (C) in vitro at various time points of incubation. Similarly, DCs ($5 \times 10^5$) from normal and fibrotic livers were tested for their ability to capture albumin (D) and mannose-albumin (E). Experiments were repeated more than three times and performed in triplicate. *p < 0.05.]

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analysis revealed that in treated animals the organized hepatic architecture was replaced by regenerative nodules bounded by fibrous septa (Fig. 1B). On picric acid-Sirius red staining, collagen deposition was seen surrounding and bridging the central portal triads. α-SMA staining confirmed HSC activation in vivo in livers of treated mice. Fibrosis was accompanied by a modest, but distinct CD45+ leukocytic infiltrate (Fig. 1B).

**LSEC surface phenotype in fibrosis**

Because liver fibrosis was accompanied by intrahepatic leukocytosis, the fraction of CD45+CD146+ LSECs among all NPCs was lower in the fibrotic liver compared with the normal liver (Fig. 2A). However, the actual number of LSECs was similar in both normal and fibrotic mice. Overall, ~2–4 × 10^6 LSECs were isolated per mouse. We compared the surface phenotype of normal LSECs (N-LSECs) and fibrotic LSECs (F-LSECs) by flow cytometry. Surface phenotype was similar for both groups (Fig. 2B). In particular, CD31 and CD105 were expressed highly on LSEC populations regardless of the presence of fibrosis. Conversely, there was low expression of MHC class II, CD40, CD86, and CD11b.

**F-LSECs are capable of enhanced Ag capture in vitro**

A primary function of LSECs is Ag capture, both for blood clearance and for the initiation of immune responses (2). We tested the capacity of LSECs from normal or fibrotic livers to capture Ag by both generalized macropinocytosis and via specialized mannose receptors. F-LSECs had greater capacity to capture dextran (Fig. 3A), albumin (Fig. 3B), and mannose-albumin (Fig. 3C) compared with N-LSECs. In contrast to LSECs, DCs from fibrotic livers were deficient at capturing albumin (Fig. 3D) and mannosylated albumin (Fig. 3E), consistent with the mature phenotype that DCs acquire in liver fibrosis (12).

**LSECs produce elevated levels of inflammatory mediators in fibrosis**

Because liver NPCs produce intense intrahepatic inflammation in liver fibrosis (11) and LSECs constitute a high fraction of NPCs, we postulated that F-LSECs were proinflammatory. To test this, freshly isolated N-LSECs and F-LSECs were cultured for 24 h, and supernatants were assayed for chemokines and cytokines with established import in fibrogenesis. F-LSECs produced markedly elevated levels of TNF-α as well as a number of chemokines, including MIP-1α, MIP-1β, MIP-2, MCP-1, RANTES, and KC compared with N-LSECs. In particular, CD31 and CD105 were expressed highly on LSEC populations regardless of the presence of fibrosis. Conversely, there was low expression of MHC class II, CD40, CD86, and CD11b.

**F-LSECs produce enhanced T cell activation**

Because LSECsd become proinflammatory in fibrosis, we postulated that they would have greater immunogenic potential. To test this, LSEC.OVA257 from normal or fibrotic mice were cocultured with equal numbers of CD8+ OT-I T cells for 24 h before analysis of T cell activation by flow cytometry. F-LSECs induced greater downregulation of CD62L and upregulation of CD25 and CD178 expression on OT-I T cells and modest upregulation of CD44 compared with the effects of N-LSECs (Fig. 5A). Similar enhancement of LSEC ability to activate CD8+ T cells was seen using the CCL4 model of liver fibrosis (Supplemental Fig. 4).

In normal livers, LSECs are associated with immunologic tolerance via the generation of Tregs (5). To test these effects in fibrosis, LSECs were coincubated with spleen CD4+ T cells before assessment of T cell coexpression of CD25 and Foxp3. Notably, after coculture with N-LSECs, 15% of T cells were CD25+Foxp3+, indicative of Tregs. Conversely, only 1% of CD4+ T cells incubated with F-LSECs exhibited the Treg phenotype (Fig. 5B).
T cells. *F*-LSECs induced higher T cell proliferation at the two highest concentrations tested (Supplemental Fig. 5). To test LSEC induction of T cell proliferation in vivo, LSECs from normal or fibrotic livers were loaded with OVA257–264 and administered into the footpad of mice that had been given an i.v. injection of OT-I T cells 24 h earlier. Treatment with *F*-LSEC.OVA257–264 induced more robust Ag-restricted T cell proliferation than *N*-LSECs (Fig. 6A, 6B). In addition, CD44 was more highly expressed on CD8+OV A tetramer+ T cells after transfer of *F*-LSEC.OVA257 compared with *N*-LSEC.OVA257 or mock adoptive transfer (Fig. 6C). Immunization using LSECs that had not been Ag loaded did not affect either tetramer cell number or activation status (data not shown).

In liver fibrosis, LSECs enhance Ag-specific CTL

In the normal liver, LSECs inhibit Ag-restricted CTL (7). To test whether LSECs from fibrotic livers either prevent or augment CTL, we reconstituted Rag mice with OT-I T cells and subsequently used either *N*-LSEC.OVA257 or *F*-LSEC.OVA257 for immunization. One week after i.p. immunization, splenocytes were harvested from recipient mice, restimulated in vitro with OVA257–264 for 4 d, before assay of CTL supernatant for immunogenic cytokines. CTL supernatant derived from reconstituted Rag mice that had been immunized with *F*-LSEC.OVA257 produced significantly elevated levels of IFN-γ, TNF-α, MCP-1, and IL-6 (Fig. 7A–D). Conversely, *N*-LSEC.OVA257–264 immunization failed to augment production of immunogenic cytokines in CTL cultures. Moreover, immunization with peptide-pulsed *F*-LSECs, but not *N*-LSECs, resulted in decreased levels of IL-10, an inhibitory cytokine, in CTL cultures (Fig. 7E).

**F**-LSECs do not inhibit DC immunogenicity

The tolerogenic effects of LSECs have recently been extended to active disruption of T cell stimulation by professional APCs. In particular, LSECs from normal livers have been shown to prevent Ag-restricted DC stimulation of T cells in vitro (9). To determine whether *F*-LSECs have similar inhibitory effects on DCs, freshly isolated spleen DCs were loaded with OVA257–264 and used to stimulate OT-I T cells. In selected wells, nonpeptide-pulsed LSECs were added in equal numbers to DCs. Notably, whereas *N*-LSECs inhibited T cell proliferation, as previously reported (9), *F*-LSECs were noninhibitory of DC induction of T cell proliferation (Fig. 9).

**Discussion**

Intrahepatic immunity has been studied extensively in the normal liver. The liver is the paradigm for immunologic tolerance and, in that capacity, is the center for important physiologic processes, such as oral tolerance to ingested Ag (15). Similarly, the acceptance of hepatic allografts with minimal immune suppression, hepatic autoimmune disease, and the disproportional occurrence of metastatic disease to the liver have all been linked, in part, to tolerogenic immune processes within the liver (16). Both professional APCs, such as...
as plasmacytoid DCs, and nonprofessional APCs, such as LSECs, are primary immunologic mediators underlying hepatic tolerance (7, 17). However, whereas intrahepatic immunity within the normal liver is the subject of intense study, there is little information regarding adaptive immunity in chronic fibroinflammatory liver disease. In particular, the immune-stimulatory capabilities of both professional and nonprofessional hepatic APCs in liver fibrosis are poorly understood. This is a very relevant area of study as our group and others have recently shown that primary events in hepatic fibrosis, such as HSC activation, and even the degree of fibrosis, may be contingent on intrahepatic immunity (11, 12).

In the current study, we found that the immunogenic function of LSECs is reversed in fibrosis compared with the properties of N-LSECs. F-LSECs produce an array of proinflammatory mediators (Fig. 4), capture Ag at a higher rate (Fig. 3A–C), and induce higher Ag-specific T cell proliferation (Fig. 6). F-LSECs also induce an activated T cell surface phenotype (Fig. 5A) while preventing the generation of Tregs (Fig. 5B). Moreover, in contrast to their counterparts in the normal liver, LSECs from the fibrotic liver do not veto DC-mediated T cell activation (Fig. 9) and are capable of enhancing CTL responses (Fig. 7) and generating de novo CTL in vivo (Fig. 8). We have previously reported that, in hepatotoxin-induced models of liver fibrosis, DCs also acquire enhanced immunogenic capacity and produce potent T cell responses to Ag (12). The mechanism for enhanced APC-related immunogenicity in fibrosis relate to the elevated intrahepatic inflammatory milieu in fibrosis. For example, we found that depletion of proinflammatory F-LSECs significantly lowers the levels of multiple cytokines (IL-1α, TNF-α, IL-6) and chemokines (RANTES, MCP-1, MIP-1α) and lay down extracellular matrix proteins, including fibrillar collagen. The physiologic consequences of higher APC-related immunogenicity in fibrosis is that undetermined factors produced by F-LSECs are responsible for their altered functional profile.

Liver fibrosis is a complex wound healing-type response to hepatic injury. After hepatic insult, an array of inflammatory mediators and reactive oxygen species is produced in the liver by both hepatic leukocytes, including T cells, as well as hepatocytes and biliary epithelial cells (11). The end result of the intense intrahepatic inflammation is activation of HSCs, which further produce high levels of cytokines (IL-1α, TNF-α, IL-6) and chemokines (RANTES, MCP-1, MIP-1α, MIP-1β) and lay down extracellular matrix proteins, including fibrillar collagen. The physiologic consequences of higher APC-related immunogenicity in fibrosis is that undetermined factors produced by F-LSECs are responsible for their altered functional profile.

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Similarly, we have previously shown that in hepatic fibrosis, DCs control the inflammatory milieu within the fibrotic liver by activation of immune effector cells, including NK and T cells (12). Therefore, heightened APC immunogenicity in both LSECs and DCs appears to profoundly affect the very inflammatory environment in the liver, which is characteristic of hepatic fibrosis.

Our data do not prove, but raise the specter that, given their prominent role in intrahepatic inflammation, F-LSECs may affect the actual fibrotic process within the liver. Consistent with this notion, Deleve et al. (18) reported that whereas in the unperturbed liver LSECs actively promote HSC quiescence, in fibrosis, capillarized LSECs allow for HSC activation. However, definitive determination of the importance of LSECs in fibrogenesis is hampered by the inability to deplete LSECs in vivo in experimental models of liver fibrosis.

These findings offer a critical understanding of the role of LSECs in intrahepatic immunity and inflammation in hepatic fibrosis and may also have implications to liver allograft tolerance (19, 20). However, a limitation to this study is that modeling LSEC function in vitro may not reflect their function in the hepatic milieu. LSEC immune phenotype may also be unavoidably modified upon isolation. The study of LSECs is thus hampered by the inability to deplete LSECs in vivo and thereby distinguish their contributions to

\[ \text{FIGURE 7. } F\text{-LSECs enhance endogenous CTL. Rag mice were reconstituted with OT-I T cells (1 x 10^7) before either mock immunization or immunization using LSEC.OVA}_{257}\text{ (1 x 10^6). One week postimmunization, splenocytes from treated animals were restimulated in vitro with OVA}_{257-264} \text{ before measurement of IFN-\gamma (A), TNF-\alpha (B), IL-6 (C), MCP-1 (D), and IL-10 (E) in day 5 CTL cultures. Experiments were repeated twice using two to four mice per experiment. }^* p < 0.05. \]
hepatic function from other NPCs in vivo. Furthermore, the physiologic correlate to LSEC-mediated tolerance in the human liver and its reversal in human liver fibrosis are uncertain. Karrar et al. (21) reported that sinusoidal endothelial cells from the normal human liver induce apoptosis in activated T cells after direct cellular contact, consistent with their role in tolerance induction in rodents.

Neutralizing Abs to LSECs have also been surmised to facilitate acute liver allograft rejection by downregulating the immune regulatory cytokine TGF-β and thus upregulating alloreactive T cell proliferation (19). However, very little is known about the immunogenicity of human LSECs in disease states, such as fibrosis. Yeli gar et al. (22) recently reported that chronic alcohol consumption increases RANTES production in human endothelial cells via activation of NF-κB, hypoxia-inducible factor-1α, and AP-1. An important area for investigation would be to isolate LSECs from the fibrotic human liver and determine their role in hepatic immunity and inflammation. However, any potential investigation into LSEC immune function in cirrhosis is confounded by the fact that the majority of cases of hepatic cirrhosis in humans are related to hepatitis C virus infection, which alone modulates intrahepatic immunity, even in the absence of parenchymal fibrosis (23, 24).

Disclosures
The authors have no financial conflicts of interest.

References


