Kupffer cells potentiate liver sinusoidal endothelial cell injury in sepsis by ligating programmed cell death ligand-1

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ABSTRACT

PD-1 and PD-L1 have been reported to provide peripheral tolerance by inhibiting TCR-mediated activation. We have reported that PD-L1−/− animals are protected from sepsis-induced mortality and immune suppression. Whereas studies indicate that LSECs normally express PD-L1, which is also thought to maintain local immune liver tolerance by ligating the receptor PD-1 on T lymphocytes, the role of PD-L1 in the septic liver remains unknown. Thus, we hypothesized initially that PD-L1 expression on LSECs protects them from sepsis-induced injury. We noted that the increased vascular permeability and pSTAT3 protein expression in whole liver from septic animals were attenuated in the absence of PD-L1. Isolated LSECs taken from septic animals, which exhibited increased cell death, declining cell numbers, reduced cellular proliferation, and VEGFR2 expression (an angiogenesis marker), also showed improved cell numbers, proliferation, and percent VEGFR2 levels in the absence of PD-L1. We also observed that sepsis induced an increase of liver F4/80 PD-1−expressing KCs and increased PD-L1 expression on LSECs. Interestingly, PD-L1 expression levels in LSECs decreased when PD-1−expressing KCs were depleted with clodronate liposomes. Contrary to our original hypothesis, we document here that increased interactions between PD-1−/− KCs and PD-L1−/− LSECs appear to lead to the decline of normal endothelial function—essential to sustain vascular integrity and prevent ALF. Importantly, we uncover an underappreciated pathological aspect of PD-1/PD-L1 ligation during inflammation that is independent of its normal, immune-suppressive activity. J. Leukoc. Biol. 94: 963-970; 2013.

Introduction

Sepsis is the 10th leading cause of death in the United States and the leading cause of death in critically ill patients [1]. Sepsis often progresses to MODS, which is characterized by the physiological breakdown of two or more organs. Whereas liver dysfunction is not the most common form of organ injury encountered in the septic patient, when it culminates into ALF, this becomes a grave complication [2]. Therefore, it is important to understand the pathophysiological changes that contribute to liver dysfunction associated with the development of MODS, which has been defined as the combination of cellular injury in addition to heightened inflammation.

ECs contribute to the complex pathology associated with MODS [3]. EC activation and dysfunction play major roles in MODS. We have shown recently that LSECs undergo Fas-mediated apoptosis and are susceptible to injury during sepsis [4]. We have also shown that KCs protect LSECs from further sepsis-induced injury [4]. LSECs, along with KCs, remain as the predominant NPC types of the hepatic sinusoid [5]. In addition to LSECs and KCs, the hepatic sinusoid contains an array of other immunologically active cells, including DCs. All (LSECs, DCs, and KCs) have been shown to recognize endotoxin, express TLR4, and play a role in liver immune tolerance [5].

Under normal conditions, the liver does not respond to food and microbial antigens that come from the intestine; all of the blood that passes through the intestine and spleen is eventually delivered to the liver. Instead, the liver’s metabolic functioning allows it to produce neoantigens and maintain a liver-tolerance effect [6]. This “liver tolerance effect”, classified in 1969, is known as the liver’s ability to induce an antigen-specific immune tolerance [7]. Contrary to vascular ECs, LSECs have the capacity to function as APCs and through their presentation of “neoantigens”, are considered to be major contributors of the liver immune-tolerance state [8].

It has been proposed that LSECs also contribute to the state of immune homeostasis through their expression of PD-L1.

Abbreviations: −/−=deficient, ALF=acute liver failure, CLP=cecal ligation and puncture, EBD=Evan’s blue dye, EC=endothelial cell, FasL=Fas ligand, HBV/HCV=hepatitis B/C virus, HCC=hepatocellular carcinoma, KC=Kupffer cell, LSEC=liver sinusoidal endothelial cell, MODS=multiorgan dysfunction syndrome, NPC=nonparenchymal cell, PBST=PBST-Tween-20, PD-1=programmed death receptor-1, PD-L1=programmed death ligand-1, pSTAT3=phosphorylated STAT3

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

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Twenty-four hours post-Sham and/or CLP surgery, animals were killed by Western blotting—protein expression of total STAT3 but the cecum was neither ligated nor punctured [17]. The site of incision was numbed with lidocaine. Animals were resuscitated ceral and skin layers were sewn back into place with a 6.0-nylon suture, and silk thread, and then punctured twice with a 22-gauge needle. One puncture was made to administer anesthesia with isoflurane, and a midline incision was made below the diaphragm. The cecum was exposed, ligated with a sterile silk thread, and then punctured twice with a 22-gauge needle. One puncture occurred close to the site of ligation, whereas the other was distal to the site of ligation. Fecal material was allowed to exude from the punctured cecum, and then it was put back into the abdominal cavity. The visceral and skin layers were sewn back into place with a 6.0-nylon suture, and the site of incision was numbed with lidocaine. Animals were resuscitated with 0.5 mL lactate Ringer’s solution. Sham control surgery was performed, but the cecum was neither ligated nor punctured [17].

Western blotting—protein expression of total STAT3 and pSTAT3
Twenty-four hours post-Sham and/or CLP surgery, animals were killed by CO2 overdose. Liver tissues from each animal were then harvested and placed into lysis buffer (containing 50 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 10 mM Na2HPO4, 10 mM NaF, 1 mM Na-orthovanadate, 0.5% Triton X-100, and 10 mM PMSF, in addition to a protease inhibitor cocktail). Samples were homogenized, left on ice for 30 min, and spun at 9300 g for 10 min at 4°C. Cell lysates (45 μg protein, as determined by a Bradford protein assay) were loaded equally onto 10% polyacrylamide gels (Life Technologies). Membranes were blocked for 1 h at room temperature in 10% nonfat dry milk in PBST and then probed with anti-mouse pSTAT3 (Tyr705) and total STAT3 antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C in 5% BSA + PBST. After primary antibody incubation, membranes were washed three times in PBST, and rabbit anti-mouse secondary antibody (Cell Signaling Technology) was added at a concentration of 1:2000 in 3% BSA + PBST. Membranes were developed by chemiluminescence using an Amersham prime ECL Plus detection system (GE Healthcare Life Sciences, Pittsburgh, PA, USA). GAPDH was used as a loading control, and densitometric analyses were performed as reported previously by our laboratory [18].

Vascular leakage assay
Twenty-four hours post-Sham and/or CLP surgery, animals were i.v. injected with 200 μL 0.5%/w/v EBD (Sigma-Aldrich, St. Louis, MO, USA); we performed this liver vascular leakage procedure according to the methods of Zellweger et al. [19]. The dye was allowed to percolate to the subendothelial spaces for 15 min, and then, the mice were killed by CO2 overdose. Whole livers were perfused with 1× PBS, weighed, and then dissociated with formamidine (Sigma-Aldrich) for 48 h at 37°C. After 2 days, supernatants were spun down and read on a spectrophotometer at 610 nm. The amount of vascular leakage or EBD extracted from each liver sample was calculated by the OD value, and concentration (mg/mL) was normalized to the amount of EBD (mg/mL) collected in the serum.

NPC isolation and LSEC (CD146+)* magnetic bead enrichment
Animals were killed by CO2 asphyxiation, 24 h post-CLP and/or -Sham surgery. We have reported the NPC isolation protocol previously [1], modified from Katz et al. [20]. In brief, liver tissue was perfused with collagenase buffer, extracted, and digested for 30–35 min at 37°C. Single-cell suspension samples were then collected in 40-μm strainers, and hepatocyte pellets were removed by slow centrifugation at 50 g for 10 min at 4°C. Cells in supernatant were enriched by 30% histodenz (Sigma-Aldrich) and spun down at 1650 g for 30 min at 4°C. NPCs, at the interface layer, were collected, washed, counted, and set aside for magnetic bead enrichment—CD146+ cells were separated and purified from NPCs by positive-selection CD146+ beads (Miltenyi Biotec, Auburn, CA, USA). Phenotype and purity were confirmed by flow cytometry on a BD FACSArray—50,000 events.

Flow cytometry
NPCs and/or CD146+–enriched LSECs were blocked with Fc Receptor block antibody (CD16/CD32: eBioscience, San Diego, CA, USA) for 15 min at room temperature. For PD-L1 staining, NPCs were stained for extracellular markers, anti-mouse CD45-PE/CY7 (eBioscience), and anti-mouse CD166-PE (BioLegend, San Diego, CA, USA), in combination with anti-mouse PD-L1-allophycocyanin (R&D Systems, Minneapolis, MN, USA) for 45 min at 4°C. For KC, PD-L1, and pSTAT3 staining, NPCs were stained with extracellular markers, anti-mouse F4/80-allophycocyanin (eBioscience), PD-L1-PE (eBioscience), and/or PD-L1-PE (eBioscience) for 45 min at 4°C. For Annexin V staining, enriched LSECs were stained initially for extracellular markers, anti-mouse CD45-PE/CY7, and anti-mouse CD166-PE for 30 min at room temperature. Next, cells were spun down, resuspended in 1× binding buffer (BD Biosciences, San Jose, CA, USA), and stained with Annexin V (BD Biosciences) for 15 min at room temperature. For Fas staining, enriched LSECs were stained for anti-mouse CD146-PE, CD45-PE/CY7, and Fas biotin (eBioscience) for 30 min at room temperature. Next, streptavidin-allophycocyanin was added for 15 min at room temperature. For VEGFR2

MATERIALS AND METHODS

Animals and animal use
Experimental protocols performed within this study have been approved by the Animal Care Usage Committee at Rhode Island Hospital (Providence, RI, USA) and meet the standards set forth by the U.S. National Institutes of Health’s Guide for Laboratory Animal Use and Care. PD-L1−/− (or B7-H1−/− on C57BL/6 background) animals were bred in-house; the C57BL/6 male mice (7–9 weeks of age) were ordered from The Jackson Laboratory (Bar Harbor, ME, USA).

CLP animal surgery
Male mice were anesthetized by isoflurane, and a midline incision was made below the diaphragm. The cecum was exposed, ligated with a sterile silk thread, and then punctured twice with a 22-gauge needle. One puncture occurred close to the site of ligation, whereas the other was distal to the site of ligation. Fecal material was allowed to exude from the punctured cecum, and then it was put back into the abdominal cavity. The visceral and skin layers were sewn back into place with a 6.0-nylon suture, and the site of incision was numbed with lidocaine. Animals were resuscitated with 1.0 mL lactate Ringer’s solution. Sham control surgery was performed, but the cecum was neither ligated nor punctured [17].
staining, enriched LSECs were stained with anti-mouse CD146-PE, CD45-PECy7, and VEGFR2-allophycocyanin (eBioscience) for 45 min at 4°C. For Ki67 staining, enriched LSECs were stained initially for extracellular markers, anti-mouse CD146-PE, and CD45-PECy7 for 30 min at room temperature. After extracellular staining, cells were then fixed and permeabilized in 1× eBioscience fixation/permeabilization solution for 15 min at 4°C. Cells were then washed twice in 1× permeabilization buffer and stained for anti-mouse Ki67-allophycyanin, an intracellular marker (eBioscience). Isotype controls for each staining condition were also stained at the same time as indicated by each manufacturer. After each staining condition, cells were washed 2× and then read on a BD FACSArray—50,000 events. Flow cytometry data were analyzed by FlowJo software (TreeStar, Ashland, OR, USA).

**KC depletion**

Clodronate and PBS control liposomes (provided by Dr. Nico van Roojen, Amsterdam, Netherlands) were mixed, diluted 1:5 in 1× PBS, and then i.v.-injected (total volume of 200 µl) into C57BL/6 animals [21]. Two days later, Sham and/or CLP surgery were performed on these mice.

**Statistical analyses**

All data collected in this study were analyzed and graphed by Prism v5 software (GraphPad, La Jolla, CA, USA). Graphs are displayed as the mean ± SEM. Significant differences were confirmed between groups when \( P < 0.05 \). \( P \) values were determined by a nonparametric Mann-Whitney U-test for two groups and a nonparametric one-way ANOVA test for more than two groups.

**Online Supplemental material**

Typical gating strategies (representative flow cytometry dot-plots and histogram overlays) for enriched LSECs (gated on the CD146+CD45− population), stained for Ki67, are displayed in Supplemental Fig. 1, along with a detailed legend description.

**RESULTS**

**PD-L1 modulates pSTAT3 expression in the liver**

Interestingly, it has been proposed that STAT3 regulates PD-L1 expression of tolerogenic APCs (primarily DCs) by binding to the PD-L1 promoter [22]. STAT3 has also been shown to protect the whole liver from Fas-induced injury [23]. As we have shown that sepsis-induced injury leads to an up-regulation of Fas and FasL in the liver [24], we decided to determine how STAT3 activation (increased pSTAT3) was affected in the presence or absence of PD-L1 in the livers, taken from septic animals. Here, we found that pSTAT3 increased in WT (C57BL/6) CLP mouse liver tissues when compared with Sham controls. However, whereas no change was observed between Sham and CLP PD-L1−/− mice, there was a trend toward a decrease in CLP PD-L1−/− compared with CLP WT mouse livers (Fig. 1A-C). This data suggest that the whole liver might be protected partially from sepsis-induced injury in the absence of PD-L1. As we looked at the levels of pSTAT3 in whole liver tissue homogenates, which include hepatocytes, we decided to determine whether PD-L1 directly affects the sinusoidal endothelium in our next set of experiments.

**CLP-induced PD-L1 expression on LSECs affects liver tissue vascular permeability**

LSECs have been reported to constitutively express PD-L1 [25]. To assess if sepsis affects PD-L1 expression on LSECs, we isolated NPGs and phenotyped the expression of PD-L1 on LSECs by flow cytometry (defined as CD146+CD45−PD-L1+ expressing cells). Initially, we found that LSECs up-regulated PD-L1, 24 h after CLP in WT mice (Fig. 2A). We then determined how liver tissue permeability was affected in the presence and/or absence of PD-L1. With the use of a vascular leakage assay, we found that there was more EBD extravasation into the tissues of CLP WT mouse livers compared with Sham WT controls, and this decreased in CLP PD-L1−/− animals (Fig. 2B). We also noted that sepsis induced an overall increase in liver tissue vascular permeability, as CLP PD-L1−/− animals also exhibited an increase of EBD leakage compared with their respective Sham controls (Fig. 2B). Collectively, these results suggest that not only is PD-L1 expression on LSECs increased in response to sepsis, but also, the expression of this gene directly contributes to elevated vascular permeability and localized tissue edema in sepsis. These processes are thought to play a significant role in organ dysfunction.

**PD-L1−/− attenuates CLP-induced LSEC apoptosis and cell loss, independent of Fas expression**

PD-1 was isolated originally from a T cell hybridoma that was undergoing programmed cell death [26]. Although it was isolated in the context of hunting for genes that regulate apoptosis, it was subsequently revealed that the function of PD-1 is not directly related to immune cell apoptosis but instead, promotes T cell anergy [27]. We have reported previously that...
LSECs undergo Fas-mediated apoptosis in sepsis [4]. We have also shown that LSECs have increased Fas expression after CLP in WT mice [4]. To confirm further that PD-L1-PD-L1 signaling and Fas:FasL signaling are independent events, we chose to determine Fas expression on LSECs from WT and CLP PD-L1−/− animals. Isolated LSECs ex vivo from CLP WT and CLP PD-L1−/− animals had an up-regulation of the Fas death receptor compared with their corresponding Sham controls (Fig. 3A). Whereas Fas:FasL and PD-L1-PD-L1 may remain as independent signaling events, whole liver tissue permeability was still affected. Therefore, we decided to stain isolated LSECs ex vivo for an apoptosis marker, Annexin V, in WT and PD-L1−/− mice. Here, we found that LSECs from septic WT animals had an increase of Annexin V staining, and this was mitigated in LSECs from CLP PD-L1−/− animals (Fig. 3B). When we looked at total cell numbers, we also found that there was a twofold decrease in LSEC numbers from CLP WT animals, and these numbers were restored in CLP PD-L1−/− animals (Fig. 3C). These data collectively indicate that whereas Fas-mediated apoptosis of LSECs is independent of PD-L1 in sepsis, PD-L1 expression on LSECs still does impact sepsis-induced endothelium injury and cell loss.

**Lack of PD-L1 expression restores LSEC angiogenesis and proliferation**

PD-L1 has been shown to regulate EC angiogenesis and proliferation in vitro but not in vivo [16]. Therefore, we decided for the next set of experiments to determine if PD-L1 expression on LSECs would directly affect their ability to undergo angiogenesis and cellular proliferation—important EC functions in maintaining vascular integrity. We noted initially that isolated LSECs from CLP WT had a significant decrease in VEGFR2, classified as CD146+CD45−VEGFR2−expressing cells, as determined by flow cytometry (Fig. 4A). Yet, the percentage of VEGFR2 expression on LSECs was restored in the CLP PD-L1−/− animals (Fig. 4A). VEGFR2 is not only a marker of angiogenesis, but also, it can be a good indicator of EC permeability, barrier function, and vitality. VEGFR2 is the receptor to the growth factor VEGF, which has been shown to be a crucial regulator of vascular development during embryogenesis, and new blood vessel formation in adults [28]. The down-regulation of VEGFR2 on LSECs in sepsis suggests that these cells are less viable.

We decided further to confirm our earlier observations that LSECs from CLP PD-L1−/− animals are protected from sep-
Figure 4. PD-L1 expression on mouse LSECs modulates angiogenesis and cellular proliferation in response to CLP. (A) There is a decrease of VEGFR2 expression on LSECs from CLP versus Sham WT animals, and this returns to Sham WT levels in LSECs from CLP PD-L1−/− animals, n = 3–8; *P < 0.05, CLP WT versus Sham WT; #P < 0.05, CLP PD-L1−/− versus CLP WT. P values were determined by a non-parametric one-way ANOVA test. (B) LSECs from CLP versus Sham WT animals proliferate less, n = 3–4; *P < 0.05, CLP WT versus Sham WT; there is greater proliferation in LSECs from CLP PD-L1−/− animals; #P < 0.05, CLP PD-L1−/− versus CLP WT (see Supplemental Fig. 1 for Ki67+ gating strategy). P values determined by a non-parametric one-way ANOVA. All data in this panel are expressed as mean ± sem. The numbers on the bars within this panel reflect the animals in each experimental group.

Figure 5. PD-1+ expressing KCs potentiate LSEC injury in sepsis

Our initial hypothesis was that PD-L1 expression on LSECs would protect them from sepsis-induced injury, as this molecule should be involved in driving immune suppression. However, up until this point, our data have suggested the opposite of this hypothesis and that PD-L1 expression on LSECs was actually mediating detrimental affects. Next, we decided to determine if the PD-1+ expressing cell population was potentially responsible for the observed functional changes in LSECs during sepsis, and if so, what were the changes? Initially, we noted an increase in the percent of and F4/80+PD-L1+ and F4/80+PD-1+ expressing cells in the liver, 24 h post-CLP (Fig. 5A and B). These cells are typically classified as KCs [32], and we chose to determine what effect ablating them by clodronate liposomes would have on the expression of PD-L1 on LSECs. We observed that the marked rise in the ex vivo frequency of PD-L1+ expression on LSECs derived from CLP WT animals was attenuated in CLP clodronate-treated (KC-depleted) animals (Fig. 5C). Overall, these data imply that PD-1+ KCs are likely interacting with PD-L1 on LSECs during sepsis, and this ligation alters normal EC functions, culminating in increased vascular permeability and liver injury.

DISCUSSION

The PD-1-PD-L1 pathway has not only been shown to promote septic morbidity but also to contribute to sepsis-induced immunosuppression—a key aspect of critical illness found in septic shock patients [21]. We have reported that PD-L1−/− animals are pro-

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ected from sepsis-induced lethality and have decreased immune suppression [13]. Zhang et al. [15] have reported that when an anti-PD-L1-blocking antibody was administered to septic mice, their overall survival improved. They also found that this correlates with a decrease in T lymphocyte apoptosis and the anti-inflammatory cytokine, IL-10. Yet, in lieu of these findings, the absence of PD-1 and more specifically, PD-L1 has not been linked directly to the pathology of MODS and how its expression on ECs contributes to this process. In this study, we demonstrate that PD-L1−/− also has beneficial effects on EC vitality and survival, which directly impact overall liver tissue permeability. Yet, these results are also potentially contrary to the proposed normal or homeostatic functions that have been attributed to PD-L1 found in the liver.

Under normal or homeostatic conditions, LSECs are considered to be unique APCs that not only cross-present antigen but also through their PD-L1 expression, can interact with PD-1 on T cells to promote immune tolerance and dampen local inflammation [33]. For example, PD-L1 on LSECs has also been shown to inhibit cytokine release of T117 and T11 cells by ligating its receptor, PD-1 [34]. Outside of the context of the liver, vascular ECs in vitro have been also shown to inactivate CD8+ T cell cytokine secretion through the expression of PD-L1s [35]. Therefore, our original hypothesis was that PD-L1 on LSECs would protect them from sepsis-induced injury and help to attenuate local inflammation. However, we believe, based on data presented here, that the normal functions of LSECs to provide immune-tolerant signals eventually become lost in response to sepsis. As a result of the up-regulated expression of PD-L1 on LSECs, increased interactions with PD-1+expressing leukocytes, such as KCs, eventually lead to their demise.

LSECs have been reported to constitutively express PD-L1 [25]. We have noted that LSECs up-regulate PD-L1 expression during the response to sepsis (Fig. 2B). These findings correlate with studies in other liver viral infection models, which have suggested that PD-L1 may be a marker of chronic inflammation, where there are indices of active liver disease [36]. PD-L1 has been shown to be up-regulated on chronically inflamed livers, as evidence by liver biopsies from HBV and HCC human patients [37]. It has also been reported that there are increased PD-L1 expression levels during the active phases of HBV infection, in contrast to decreased expression levels of PD-L1 on LSECs and KCs during inactive/quiescent phases [38]. Up-regulation of PD-L1 and PD-L1 also corresponds with viral persistence during active HCV infection [39].

We also found that there is a partial decrease in pSTAT3 protein expression in whole liver tissue homogenates taken from septic PD-L1−/− animals compared with WT septic animals alone (Fig. 1). STAT3 has been shown to not only protect hepatocytes from Fas-induced injury but also to be required for liver regeneration [23]. In the absence of PD-L1, the whole liver tissue trends toward a decrease in pSTAT3 expression compared with septic WT livers but still increased in comparison with its own Sham control (CLP PD-L1−/− vs. Sham PD-L1−/−; Fig. 1B). This might be explained by the marked rise of Fas expression on LSECs in the presence and absence of PD-L1 (Fig. 4A). Although the expression profiles of Fas and PD-L1 are distinct, both molecules are considered to maintain peripheral immune tolerance in select situations. The primary difference, however, resides in how FasL and PD-L1 are proposed to function. FasFasL signaling has been shown to cause T cell deletion and work independently of the costimulatory signal CD28, whereas PD-L1:PD-L1 signaling requires CD28 [40–42]. In addition, their downstream signaling cascades are unique: FasFasL signaling uses the recruitment of death receptor-associated caspases and kinases, whereas PD-L1:PD-L1 signaling uses phosphatases [43, 44].

Interestingly, the most relevant topic for EC viability and survival is maintenance of their angiogenic capacity. Angiogenesis is required for liver regeneration after partial hepatectomy [45]. Here, we show that LSECs from WT septic animals have less ex vivo evidence of cellular proliferation compared with Sham controls or CLP PD-L1−/− animals (Fig. 4). In fact, LSEC progenitor cells have been shown to be required for liver regeneration in rats [46], making continual EC proliferation and replenishment of LSECs essential for restoring liver tissue function in cases of injury, such as sepsis. PD-L1 on vascular ECs has been shown to modulate angiogenesis in vitro, but it has not been implicated in a sepsis model or in vivo [16]. Here, we found that VEGFR2 expression decreases dramatically on LSECs during sepsis, and this is restored in CLP PD-L1−/− animals (Fig. 4). We also noted that LSECs from CLP PD-L1−/− mice exhibited decreased cell loss and apoptosis (Fig. 3B and C). These results are consistent that PD-L1 ligation modulates angiogenesis in vascular ECs [16] but also indicate that sepsis causes a loss of LSEC numbers and down-regulation of VEGFR2 and overall, affects the sinusoidal endothelium’s ability to respond to VEGF and form new blood vessels. In addition, our findings of decreased VEGFR2 levels on septic LSECs correlate with a study that indicated that there are increased circulating levels of VEGF in blood-serum samples from septic patients and animals [47]. Perhaps there is an increase of VEGF, as the receptor has been down-regulated on ECs, as we have observed. Even though the functional significance of the rise in blood VEGF levels is debated, it appears to be a good indicator of developing septic morbidity and/or organ injury [47].

Although we found that PD-L1−/− promotes LSEC survival and angiogenesis, we also believe that PD-1+expressing KCs drive the pathological process of LSEC dysfunction. The interaction between PD-L1 on the LSEC and PD-1 on the KC promotes the associated pathology of immune-tolerance breakdown and overall cell injury. Whereas most studies have indicated that LSECs and KCs are the primary cell populations that express PD-L1, we show here that KCs express PD-L1 and found that there is an increased frequency of F4/80+PD-L1+ cells in the liver during sepsis (Fig. 5B) [48]. There are also supporting data from other studies that have indicated that monocytes and/or KCs up-regulate their PD-L1 expression during septic shock, autoimmune liver disease, and HCC (Fig. 5A) [49–51]. For example, PD-L1 expression on KCs has been shown to interact with PD-1+expressing CD8+ T cells and down-regulate their autoreactivity in autoimmune liver disease [50]. PD-L1 expression on KCs has also been proposed to suppress T lymphocyte effector functions during heightened inflammation, such as HCC. When a PD-L1-blocking antibody was incubated with CD8+ T cells from HCC patients ex vivo, T cells exhibited better effector functions, such as cytokine secretion and proliferative ability. Perhaps in these forms of liver injury, PD-L1 on KCs does serve to balance local inflammation by signaling to
PD-1⁺-expressing CD8⁺ T cells [51]. We have shown that CD8⁺ T cells are not involved directly in LSEC injury [4]. This could also explain why we see that sepsis results in an increased percentage of F4/80⁺PD-L1⁺ cells in the liver, but these cells are not responsible for the observed effects on hepatic endothelial function, as they are signaling to CD8⁺ T lymphocytes [52]. Yet, it has also not been shown that KCs express PD-1, making our results novel. We have reported, however, that peritoneal macrophages express PD-1, and this cell population is essential for the protection from septic-induced mortality seen in PD-L1⁻/⁻ animals [13]. We further add to the story, however, by also confirming that PD-1⁺-expressing KCs (F4/80⁺PD-1⁺) do contribute to EC dysfunction by ligating PD-L1 on LSECs. In the absence of KCs, there is an attenuation of PD-L1 expression on isolated LSECs from septic animals, which under septic conditions, becomes up-regulated (Figs. 2B and 5C).

In summary, we show here the first evidence that PD-L1 on LSECs potentially impacts and contributes to the pathology of ALF that occurs during septic shock. We believe this process to be dependent on the interactions between PD-1⁺-expressing KCs and PD-L1 on the sinusoidal endothelium. Most studies have suggested that targeting and/or blocking the PD-1/PD-L1 pathway would be a good strategy to combat immune suppression seen in critically ill patients [53], but we now also think that this stands true for the protection of EC function, hepatic microcirculation, and indirectly, the suppression of MODS that develops during septic challenge. Whereas LSECs are prone to Fas-mediated apoptosis during septic inflammation, this may not be the sole mechanism of injury. We propose that the increased expression of Fas and PD-L1 on the endothelium may synergize to elicit marked increased interactions with FasL⁺ and PD-1⁺ leukocytes. The Fas/FasL and PD-1/PD-L1 signaling pathways are discreet from one another, but their combined effects could exacerbate local LSEC injury and/or cell death.

The potential clinical implications of this work not only confirm that PD-L1 is a marker of liver inflammation, as proposed previously [36] but also may predict disease outcome based on the expression levels of PD-1 and PD-L1 on liver macrophage populations. KCs may have differential effects on the liver during inflammation, and inflammation, in general, reorganizes the expression levels of PD-L1, shifting the liver from away from its normal immune-quietescent state. Inflammatory monocytes have been reported to express high levels of PD-L1, whereas resident macrophages, such as KCs, express low levels [54]. Yet, perhaps during inflammation or situations similar to sepsis, infiltrating monocytes can modulate the expression levels of PD-1 and PD-L1 on resident macrophage populations. This may be why we see two distinct F4/80⁺ populations (Fig. 5A and B). In fact, it has been proposed in other liver-infection models, such as an experimental animal model of fibrosis, that distinct subpopulations of macrophages play both roles in fibrosis progression and regression [55, 56]. One population acts in an anti-inflammatory fashion, and the other secretes proinflammatory cytokines to activate other liver cell populations. Thus, it would be worthwhile in future studies to investigate how modulating PD-L1-expression levels in the liver impacts KCs and ultimately, disease progression.

AUTHORSHIP
N.A.H. designed and executed all experiments presented herein, as well as conducted statistical data analyses, prepared figures, and wrote the manuscript. C.S.C. assisted with experiments and edited the written manuscript. F.W. performed flow cytometry experiments to determine the percentage of PD-1⁺ on F4/80⁺ NPCs (Fig. 5B). Y.W. performed flow cytometry experiments to determine the percentage of PD-L1 on F4/80⁺ NPCs (Fig. 5A). A.A. oversaw the study, as well as reviewed and edited the manuscript.

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DISCLOSURES
The authors declare no conflicts of interests.

REFERENCES


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