With respect to this, the development of this immune dysfunctional state does not appear to be simply a reflection of generalized loss of all macrophage functions, as concomitant evidence of macrophage retaining the ability to actively produce various anti-inflammatory mediators has been observed by a number of laboratories, including our own (1, 40, 42, 56). Furthermore, these findings of altered mediator release are underpinned by observations that septic mouse macrophages appear to be differentially utilizing/activating various signaling pathways (21, 23, 24, 41, 52, 53) and the upregulation/expression of inhibitory receptors like programmed cell death receptor-1 (PD-1), CD40, etc. (22, 25, 30, 54). Also, while the loss of immune cells via apoptosis is evident in these animals (28, 46, 59), this is likely, at least in part, a response to the actions of immune-suppressive cell populations or the anti-inflammatory mediators they release (3, 40, 58). Thus, while enhanced apoptosis is clearly one of the results of this process and the clearance of these apoptotic cells is suggested to have immune-suppressive potential (27, 55), it is not the sole cause for the immune dysfunction seen in sepsis. Our laboratory (9, 43) and others (31, 44, 47, 61) have shown that the presence of marked tissue injury/damage (in the form of cecal ligation-no puncture, or burn wound) alone is not sufficient to cause marked mortality, yet does appear to play a role in stimulating the differentiation of anti-inflammatory/immune-suppressive macrophage or dendritic cell phenotypes (9, 36, 37). We have also found that much of the proinflammatory response in cecal ligation and puncture (CLP) mice is mediated through differential activation of various tissue macrophages (7, 8), and it appears that the subsequent induction of these divergent immune-suppressive macrophage phenotypes is differentially controlled (16, 51).

Recent studies suggest that coinhibitory receptors, including PD-1, cytotoxic T-lymphocyte-associated protein-4, and B- and T-lymphocyte attenuator, appear to be important in contributing to sepsis-induced immunosuppression (33, 38). Our laboratory reported that mice deficient in PD-1 have increased bacterial clearance and improved survival in experimental sepsis (4, 18, 30). In response to infection, the liver clears the blood of bacteria and produces cytokines. Kupffer cells, the resident macrophages in the liver, are strategically situated to perform the above functions. However, it is not known if PD-1 expression on Kupffer cells is altered by septic stimuli, let alone if PD-1 ligation contributes to the altered microbial handling seen. Here we report that PD-1 is significantly upregulated on Kupffer cells during sepsis. PD-1-deficient septic mouse Kupffer cells displayed markedly enhanced phagocytosis and restoration of the expression of major histocompatibility complex II and CD86, but reduced CD80 expression compared with septic wild-type (WT) mouse Kupffer cells. In response to ex vivo LPS stimulation, the cytokine productive capacity of Kupffer cells derived from PD-1−/− CLP mice exhibited a marked, albeit partial, restoration of the release of IL-6, IL-12, IL-1β, monocyte chemoattractant protein-1, and IL-10 compared with septic WT mouse Kupffer cells. In addition, PD-1 gene deficiency decreased LPS-induced apoptosis of septic Kupffer cells, as indicated by decreased levels of cleaved caspase-3 and reduced terminal deoxynucleotidyl transferase dUTP nick end-labeling-positive cells. Exploring the signal pathways involved, we found that, after ex vivo LPS stimulation, septic PD-1−/− mouse Kupffer cells exhibited an increased Akt phosphorylation and a reduced p38 phosphorylation compared with septic WT mouse Kupffer cells. Together, these results indicate that PD-1 appears to play an important role in regulating the development of Kupffer cell dysfunction seen in sepsis.

Kupffer cells, the resident macrophages in the liver, are strategically situated to perform the above functions (18, 26). However, it is not known if PD-1 expression on Kupffer cells is altered by septic stimuli, let alone if PD-1 ligation contributes to the altered microbial handling seen after sepsis. Inasmuch, the goal of this study was to investigate the effect of PD-1 gene expression has on Kupffer cells in mice with sepsis.
METHODS AND MATERIALS

Mice. Male 8- to 10-wk-old C57BL/6j [wild-type (WT)] mice (from Jackson Laboratories, Bar Harbor, ME) or PD-1 gene-deficient mice (PD-1–/–; kindly provided by Tasuku Honjo, Kyoto University, Kyoto, Japan, via Megan Sykes at the Massachusetts General Hospital, Charlestown, MA, and bred at the Central Res. Facility at Lifespan-RI Hospital) (35) were utilized for all the studies described here. All protocols carried out with these animals (between 8 AM to 11 AM; no prior fasting) were done according to National Institutes of Health Guide for Animal Use and Care and were approved by the Lifespan-Rhode Island Hospital Institutional animal care and use committee (AWC no. 0228-13).

CLP. For studies here, we performed CLP or sham-CLP on mice, as previously described, to produce a state of acute polymicrobial septic challenge (4). In brief, male mice, 8–10 wk old, were anesthetized using isoflurane. A midline incision (1–2 cm) was made below the diaphragm. The cecum was exposed, ligated with a sterile silk thread, and punctured twice with a 22-gauge needle. After puncture, the fecal material was extruded into the abdominal cavity. The wound site was nubbed with lidocaine, and the viscera and skin layers were closed with a 6.0 nylon silk suture. The animals were resuscitated with 1.0 ml of lactate Ringer solution. Sham controls were exposed to the same surgery; however, their cecum was neither ligated nor punctured.

Cell preparations. Animals were killed by CO2 asphyxiation, 24 h following CLP/sham. Then as we have previously described (32), the liver was perfused with a collagenase IV (Sigma Aldrich, St. Louis, MO) buffer (1% collagenase IV and 0.02% DNase I in 10 mM HEPES-NaOH pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, and 1.8 mM CaCl2 in PBS). After perfusion, the liver was excised, minced, and digested for 30 min at 37°C. The collagenase was neutralized with ice-cold PBS, and the hepatocytes were separated by slow-speed centrifugation at 30 g for 10 min. The supernatants were collected and spun at 300 g for 10 min to pellet the nonparenchymal cell (NPC) fraction. The pellet was resuspended in DMEM complete media (10% FBS, 500 cell (NPC) fraction. The pellet was resuspended in DMEM complete media (10% FBS, 500

G238 PD-1 AND KUPFFER CELL FUNCTION IN SEPSIS

Phagocytosis assay. Adherent Kupffer cells were co-cultured with pHrodo-conjugated E. coli (Life Technologies) in PBS at 37°C for 1 h, as described previously (4). Cells were harvested by gently scraping the cells off the monolayer, washed, and detected by flow cytometry.

Statistical analysis. Data are shown as the means ± SE and analyzed with the Mann-Whitney rank-sum test for two groups and a one-way ANOVA to test presence of a significant difference for more than two groups, along with a Holms-Sidak multiple comparisons. Groups were considered statistically significantly different at a P value of <0.05.

RESULTS

PD-1 expression on Kupffer cells is increased in septic mice. To the extent that CLP induced an increase in the percentage of cells expressing PD-1, NPCs were isolated 24 h post-CLP [a time point at which our laboratory has previously documented marked changes in ex vivo CLP mouse Kupffer cell function(s)/phenotype(s) (14) and at which most of the mice are still alive], stained with anti-PD-1 plus anti-F4/80 and analyzed by flow cytometry. Results of a typical dot-plot gated on F4/80+PD-1+ cells are shown in Fig. 1A. We found that there was a marked increase in the frequency of Kupffer cells expressing PD-1 in septic WT mice (Fig. 1B). Importantly, this increase took place in the absence of a significant change in the total viable NPC yield from the livers of CLP, as opposed to the sham-surgery mice [a result in keeping with prior reports for this model (6, 29); see Fig. 1C], thus arguing against the result of infiltrating blood monocytes, which are also lower by 10.220.33.6 on September 21, 2017 http://ajpgi.physiology.org/ Downloaded from
gene deficiency in mice partly prevented CLP-induced decline in these cells’ capacity to express MHC II and CD86, as well as CLP-induced rise in the expression of CD80 typically encountered in the CLP WT mouse cells (Fig. 2).

**PD-1 contributes to Kupffer cell dysfunction in septic mice.** As our laboratory has previously reported following the onset of experimental septic challenge in WT mice, we see reduced bacterial clearance capacity both in vivo (in whole blood levels and peritoneal fluid levels of bacteria) and ex vivo/in vitro compared with sham animals (4, 30). However, it has not been established if a similar phenomenon is evident in the macrophages derived from the CLP mouse liver, let alone the role of PD-1 in Kupffer cell’s ability to phagocytize. Here we observed that PD-1 gene deficiency not only restored the septic mouse Kupffer cell’s capacity to phagocytize labeled gram-negative bacteria, but also markedly potentiated them above the levels seen in cells taken from WT or PD-1−/− sham mice (Fig. 3A).

PU.1, a transcription factor that is involved in myeloid and B cell development/differentiation, has been reported to also regulate alveolar macrophage functions, including phagocytosis [review in Shibata et al. (50)]. To delineate the possible mechanism by which the expression of PD-1 contributes to the changes in the phagocytic capacity of liver macrophages here, the expression of PU.1 by F4/80+ cells derived from the livers of WT and PD-1−/− mice sham or CLP surgery was examined. We found that PU.1 expression was down regulated at 2 h after CLP in WT mice. PD-1 gene deficiency restored PU.1 expression at 2 h after CLP (Fig. 3B). However, at 4 or 24 h after surgery, there were no changes observed in PU.1 expression in these cells.

**PD-1 gene deficiency partially restores LPS-stimulated Kupffer cell cytokine release capacity.** To assess the impact of PD-1 gene expression on septic mouse Kupffer cell functions, we subjected mice deficient in PD-1 gene expression (PD-1−/−) (35), as opposed to WT animals, to CLP, isolated the liver macrophages, and examined their ex vivo/in culture ability to release pro-/anti-inflammatory cytokines, phagocytosis bacteria, express cell-surface molecules important for the process of antigen presentation, as well as initiate the process of cell death/apoptosis, in response to the inflammatory stimulus LPS. What we found was that the PD-1 gene deficiency typically was able to partially reverse the decline in LPS-stimulated cytokine production of that commonly seen in Kupffer cell cultures derived from WT CLP mice (Fig. 4).
PD-1 gene deficiency reduces the CLP-induced increase in Kupffer cell apoptotic frequency when exposed to LPS. From a functional perspective, while we have not seen overt evidence of in vivo (and/or ex vivo in the absence of stimulation) liver macrophage cell programmed cell death, we have established previously that, along with the decline in the experimental septic mouse’s capacity of their peritoneal macrophages to produce cytokines and express cell-surface receptors important in potentiating the process of antigen presentation, we observed that these same cells appeared to be predisposed to undergoing the process of programmed cell death/apoptosis (14, 15, 60). In light of this, we attempted to determine whether or not the Kupffer cells derived from these CLP-WT mice were not only more susceptible to undergoing the process of apoptosis in response to LPS challenge compared with sham Kupffer cells, but if PD-1 gene deficiency could affect these CLP-induced changes in susceptibility. What we report is that LPS-stimulated apoptosis decreased in Kupffer cells from PD-1 gene-deficient CLP mice compared with WT CLP mice, as evidence by both suppression in the increase of the expression of the cleaved form of caspase-3 (Fig. 5A), as well as in the extent of TUNEL-positive cells seen in the CLP mouse-derived liver macrophage monolayers (Fig. 5B).

PD-1 gene deficiency restores Kupffer cell survival signaling through Akt, while it suppresses the rise in p38 MAPK in septic mice. Finally, since previous studies have noted that the decline in the capacity of macrophages to produce proinflammatory cytokines in response to LPS challenge, as well as their ability to undergo apoptosis, is associated with activation of signaling through the MAPK p38 pathway (increased p-p38) (52, 53) with concomitant decline in survival signaling through the Akt pathway (decreased p-Akt), we set out to determine whether these changes in LPS-induced functional responsiveness seen in our septic mouse Kupffer cells were associated with changes in the respective activation of MAPK p38 and Akt. What we found was that, compared with sham Kupffer cells, LPS stimulation decreased p-Akt (Fig. 6A) and increased p-p38 (Fig. 6B) in Kupffer cells derived from septic WT mice. Alternatively, PD-1 gene deficiency reversed LPS-stimulated p-Akt and p-p38 in Kupffer cells isolated from CLP mice compared with cells taken from CLP WT animals (Fig. 6).

DISCUSSION

Macrophages are purported to play a central role, not only in regulating significant aspects of innate and adaptive immune responsiveness to foreign pathogens, but also in shaping the response to tissue/organ injury, remodeling, and wound healing. Inasmuch, understanding their role in the response to septic challenge like polymicrobial sepsis here and the processes/mechanisms that underpin such reactions should elucidate not only novel insight into the macrophage functions under such conditions, but could point to novel therapeutic targets for the treatment of this condition. With respect to the macrophage’s response to septic challenge, much of what we know has been gleaned from isolated human blood monocyte or the mouse peritoneal tissue resident macrophage. One of the interesting observations that has been consistently made concerning either the human blood monocyte and/or the peritoneal tissue resident macrophage is that, rapidly following their initial exposure to septic challenge, they shift from a cell that is characterized by the release of substantial amounts of proinflammatory mediators to a cell that responds poorly to (insensitive to/tolerant of) subsequent stimuli, like LPS, lipotichoic acid, muramyl dipeptide, etc. (1, 30, 40, 42, 56). Since these changes are also associated with a decline in their ability to serve as antigen-presenting cells, this has often been ascribed to the development of immune suppression/dysfunction in the septic patient/experimental septic animal (20, 42, 58). While several mechanisms have been identified that may contribute to the evolution of this condition, our laboratory has recently reported that the upregulation of the immune co-inhibitory cell surface receptor, PD-1, appears to play an unanticipated role in regulating some of the changes induced in blood and peritoneal macrophage derived from mice subjected to polymicrobial sepsis in the form of CLP. In those studies, we reported that mouse peritoneal macrophage and blood monocytes, as well as human blood monocytes, could upregulate the expression of PD-1 in response to sepsis, which was otherwise not normally evident in cells from naive (other-
erwise untreated/healthy) animals (25, 30). Furthermore, we demonstrated that the presence of PD-1 on these specific cell types from septic mice had an effect on a variety of functions, such as suppressing their cytokine release capacity, depressing their bacterial phagocytic ability, and potentiating their random/nondirected migratory ability (4, 30). And while such findings are intriguing and point to a mechanism that might and has been therapeutically targeted (12, 33), much remains to be understood about the generalizability of these observations beyond blood monocytes and peritoneal macrophages. In this regard, we thought it was important to understand whether or not PD-1 expression was similarly upregulated on the resident macrophages of the liver, the Kupffer cells, as the liver is not only the potentially largest source of tissue macrophage, but is an important source of macrophage-derived mediators produced during experimental sepsis (5, 34, 45) and has been reported to respond in a distinct fashion from other resident tissue macrophage populations, e.g., peritoneal, splenic, and blood macrophages (7, 8).

Here we report that, by 24 h following the onset of sepsis, there is a marked upregulation in the proportion of Kupffer cells that could be detected as staining positively for PD-1 by flow cytometry. The change in the percent of macrophages in the liver that are positive for PD-1 is in keeping with what our laboratory has previously reported for both monocytes and macrophages derived from mouse blood or the peritoneum, respectively, at 24 h post-CLP (30). Importantly, unlike the peritoneum and blood, this increase took place in the absence of a significant change in the total viable NPC yield from the livers of CLP as opposed to the sham-surgery mice (6, 29). This, in turn, argues against the change seen in %F4/80/PD-1 liver macrophages being the result of infiltrating blood monocytes (39, 57). Interestingly, with respect to bio/pathological significance of this upregulation of PD-1 expression, our laboratory has previously shown that depletion of Kupffer cells expressing PD-1 with liposomal chlodronate before the induction of CLP sepsis not only markedly improved the survival and function of liver sinusoidal endothelial cells via ligation of PD-L1 (which the liver sinusoidal endothelial cell expresses), but also reduced proinflammatory IL-6 release (34). With these changes in septic mouse Kupffer cell PD-1 deficiency on macrophage function of Kupffer cells derived from sham or

Fig. 4. PD-1 gene deficiency restored cytokine release capacity in Kupffer cells after sepsis and subsequent ex vivo stimulation without or with LPS. Kupffer cell monolayers were established by adherence of NPCs on plates and stimulated without or with LPS (1 μg/ml) for 24 h, and supernatants were collected for cytokine level analysis by ELISA. Kupffer cells produced lower levels of TNF-α, IL-6, IL-12, IL-1β, MCP-1, and IL-10 after CLP in both WT and PD-1−/− mice without or with LPS stimulation compared with their respective shams. However, septic PD-1−/− mouse Kupffer cells produced significantly higher levels of the above cytokines than septic WT mouse Kupffer cells with and without LPS stimulation. Values are means ± SE; n = 4−6/group. *P < 0.05, CLP vs. sham, and #P < 0.05, PD-1−/− CLP vs. WT CLP, by ANOVA followed by Holm-Sidak’s test.
CLP mice. Here we report that PD-1 gene/protein expression appears to play an important role in regulating the development of changes in numerous aspects, i.e., cytokine release capacity, phagocytosis, and cell death/survival. Interestingly, while similar to our experience with ex vivo peritoneal macrophage assessment of cytokine release capacity in response to LPS challenge (30), we were able to document a partial restoration of the septic mouse macrophage’s capacity to release proinflammatory cytokines, IL-6, IL-12 p40, IL-1β, monocyte chemoattractant protein-1, and anti-inflammatory IL-10. This implies that, while PD-1 expression is contributing to dysfunction in cytokine production, this does not appear to be PD-1’s primary effect/action. Nonetheless, the strongest effects were evident with the restoration of IL-10 release capacity. In this respect, it is tempting to speculate that this is regulated, at least in part, by the concomitant change in signaling through p38 MAPK, as our laboratory has documented before in macrophage (52, 53). Here, while we saw a potentiation of WT CLP

Fig. 5. PD-1 gene deficiency decreased active caspase-3 levels and TUNEL staining of septic Kupffer cells after ex vivo stimulation with LPS. Kupffer cell monolayers were established by adherence of NPCs on plates and stimulated without or with LPS (1 μg/ml) for 24 h, and cells were washed and collected for cleaved caspase-3 determination by Western blot analysis. A. top: representative blot of cleaved caspase-3 expression in Kupffer cells from WT and PD-1−/− sham and CLP mice. Bottom: semiquantitated of intensity by densitometry, expressed as integrated density (IDT), values of caspase-3 relative to IDT values of GAPDH are shown. Caspase-3 was increased significantly in LPS-stimulated Kupffer cells in septic WT and PD-1−/− mice compared with their sham controls. PD-1 deficiency markedly reduced caspase-3 levels in Kupffer cells after CLP. Values are means ± SE; n = 4–6/group. *P < 0.05, CLP vs. sham, and #P < 0.05, PD-1−/− CLP vs. WT CLP, by ANOVA followed by Holm-Sidak’s test. B: TUNEL staining of Kupffer cell monolayers showed that PD-1 deficiency had fewer apoptotic cells compared with WT mice after sepsis. Original magnifications, ×400.
mouse liver macrophage phosphorylated p38 MAPK, no such change was evident in sham vs. CLP PD-1−/− mouse cells, suggesting that it may be involved. This does not preclude that PD-1 may be acting directly/indirectly through other signaling pathways, like NF-κB, Toll/IL-1 receptor domain-containing adaptor-inducing interferon-β, etc.; however, this remains to be established.

Alternatively, as with our prior ex vivo examination of septic mouse peritoneal macrophage’s ability to phagocytize opsonized labeled heat-killed *E. coli* (4, 30), we were able to document that the decline in the septic mouse’s liver macrophage’s ability to take up labeled bacteria was also markedly attenuated in Kupffer cells derived from mice that lacked PD-1 gene expression. In fact, the capacity of Kupffer cells to phagocytize *E. coli* was markedly potentiated compared with either WT sham or PD-1−/− sham mice. To further investigate the mechanism by which PD-1 may be involved in the phagocytic function of liver macrophages, we attempted to determine whether the expression of the transcription factor PU.1, which has been reported to be important in myeloid cell development/differentiation (50), not only was altered by CLP, but was not only immune, but general, liver dysfunction associated with sepsis.

Fig. 6. PD-1 gene deficiency increased Akt, while it decreased p38 MAPK, activation of septic Kupffer cells. Kupffer cell monolayers were established by adherence of NPCs on plates and stimulated without or with LPS (1 μg/ml) for 24 h, and cells were washed and collected for determination of Akt and p38 MAPK activation by Western blot analysis. A: upon LPS stimulation, Akt activation in Kupffer cells from WT and PD-1−/− CLP mice was decreased compared with their sham controls. Semiquantitation of intensity by densitometry, expressed as integrated density (IDT), values of phosphorylated Akt relative to IDT values of total Akt are shown. However, PD-1 deficiency restored Akt activation in septic mouse Kupffer cells. B: p38 MAPK activation in Kupffer cells, stimulated with LPS, from WT and PD-1−/− mice was increased significantly compared with their sham controls. Again, PD-1 deficiency markedly reduced p38 MAPK activation in Kupffer cells after CLP. Semiquantitation of intensity by densitometry, expressed as IDT, values of phosphorylated p38 MAPK relative to IDT values of total p38 MAPK are shown. Values are means ± SE; n = 4–6/group. *P < 0.05, CLP vs. sham, and #P < 0.05, PD-1−/− CLP vs. WT CLP, by ANOVA followed by Holm–Sidak’s test.

In conclusion, PD-1 gene expression appears to play an important role in regulating the development of changes in numerous aspects, i.e., cell death, receptor expression, cytokine release, phagocytosis, etc., of Kupffer cell innate and/or adaptive immune responsiveness, which likely contribute to not only immune, but general, liver dysfunction associated with sepsis.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
F.W., X.H., Y.C., N.A.H., and A.A. conception and design of research; F.W., C.-S.C., and Y.C. performed experiments; F.W. and Y.C. analyzed data; F.W., C.-S.C., and A.A. prepared figures; F.W., C.-S.C., N.A.H., and A.A. drafted manuscript; F.W., X.H., C.-S.C., N.A.H., and A.A. edited and revised manuscript; F.W., X.H., C.-S.C., Y.C., N.A.H., and A.A. approved final version of manuscript.

REFERENCES