Contribution of programmed cell death receptor (PD-1) to Kupffer cell dysfunction in murine polymicrobial sepsis

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Wang F, Huang X, Chung CS, Chen Y, Hutchins NA, Ayala A. Contribution of programmed cell death receptor (PD)-1 to Kupffer cell dysfunction in murine polymicrobial sepsis. Am J Physiol Gastrointest Liver Physiol 311: G237–G245, 2016. First published June 10, 2016; doi:10.1152/ajpgi.00371.2015.—Recent studies suggest that coinhibitory receptors appear to be important in contributing sepsis-induced immunosuppression. Our laboratory reported that mice deficient in programmed cell death receptor (PD)-1 have increased bacterial clearance and improved survival in experimental sepsis induced by cecal ligation and puncture (CLP). 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.

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 upregulationexpression 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 (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.
METHODOLOGY 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 puncturing, 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 µg/mL gentamycin), layered on top of 30% Histodenz (Sigma Aldrich), and spun at 1,650 × g for 25 min at 4°C, and cells at the interface layer were collected, washed, and counted (the NPC suspension). While for a few flow cytometric studies the NPCs were used, for most studies adherent macrophage monolayers on plastic tissue culture plates were established and stimulated without or with 1 µg LPS per milliliter of DMEM medium, supplemented with 10% FBS for various analyses.

Flow cytometry. Mouse liver leukocytes/NPC suspensions were isolated as described above. The leukocytes were stained with fluorochrome-conjugated anti-F4/80 (clone BMS), anti-CD163 (clone J43), anti-major histocompatibility complex (MHC) II (clone M5/114.15.2) antibodies (purchased from eBioscience, San Diego, CA), or anti-CD80 (clone 16-10A1), anti-CD86 (clone GL1) antibodies (purchased from BD Bioscience), along with the appropriate hamster/rat isotype controls, and then assessed for frequency and extent of cellular fluorescence on FACSAarray flow cytometer (BD Biosciences). FlowJo software (Tree Star, Ashland, OR) was used to analyze the data acquired on the FACSAarray, as previously described (30).

Cytokine determination. Adherent liver macrophage (Kupffer) cell monolayers were incubated with LPS (1 µg/mL) for 24 h; cell supernatants were then collected and stored at −80°C until the concentrations of murine cytokines were measured by ELISA (BD Biosciences), as previously described (30).

Western immunoblotting. After LPS stimulation for 24 h, Kupffer cell monolayers were washed, the cells lysed in lysis buffer, and the protein content established for Western immunoblotting analysis (13). In brief, samples were separated on 16% SDS polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Life Technologies, Grand Island, NY). The membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.05% Tween 20 and incubated antibody specific to the dually phosphorylated forms of MAPK p38 (p-p38), Akt (p-Akt), or cleaved caspase-3 (Cell Signaling Technology, Danvers, MA) overnight at 4°C. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody. After washing, proteins were visualized by ECL and densitometrically assessed by Alpha-Innotech image analyzer (San Leandro, CA). Antibody against total MAPK p38 or Akt were used to determine basal expression of these proteins, and anti-GAPDH or anti-β-actin were used as a loading control. For transcription factor PU.1 expression, F4/80+ cells were isolated from the livers of sham or CLP mice at 2, 4, and 24 h after surgery using magnetic beads (Myltenyi Biotec). Cell lysate was collected and probed with anti-PU.1 (Biologend, San Diego, CA).

Terminal deoxynucleotidyl transferase dUTP nick end-labeling assay. Stained Kupffer cell monolayers were washed and fixed in 10% buffered formaldehyde. Hematoxylin and eosin staining was performed by Core Research Laboratories at Rhode Island Hospital. The terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) staining was performed according to the manufacturer’s instructions (Roche Applied Science, Indianapolis, IN) (17). The images were collected with a fluorescent microscope (Nikon Eclipse 80i) using a ×20 objective and a Spot RT3 camera. Image processing and analysis was performed using ImageJ software.

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 expressors of F4/80 typically than Kupffer cells (39, 57).

PD-1 contributes to the changes of antigen-presenting molecule expression of Kupffer cells in septic mice. Inasmuch as we found that the percentage and number of liver macrophage expressing PD-1 was significantly increased in response to polymicrobial septic challenge, we asked whether or not this change in expression associated with functional changes in the Kupffer cells derived from these mice. With respect to the capacity of these liver macrophages to express cell-surface molecules important for the process of antigen presentation in response to a proinflammatory stimulus, we found that PD-1...
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).

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**Fig. 1.** The %PD-1+ Kupffer cells was augmented in liver nonparenchymal cells (NPCs) by sepsis. A: representative flow cytometry dot plots of PD-1 expression on F4/80+/PD-1+ Kupffer cells (circled population) among mouse liver NPCs. B: there was an upregulation of PD-1 expression on Kupffer cells in liver NPCs 24 h after CLP in WT mice. C: of note, there was no marked change in the total number of NPCs obtained from sham as opposed to CLP WT mouse livers at this time point. Values are expressed as means ± SE; n = 6/group. *P < 0.05, WT CLP vs. WT sham by Mann-Whitney nonparametric t-test.

**Fig. 2.** PD-1 gene deficiency increased the expression of MHC II and CD86, while it decreased CD80 expression of septic Kupffer cells. CLP induced significantly decreased expression of MHC II (A) and CD86 (B), while it increased CD80 (C) expression on WT F4/80+ Kupffer cells. PD-1 deficiency partially reversed the expression of MHC II, CD86, and CD80 on F4/80+ 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.
CLP mice were not reduced and were even higher than in sham mice. Values from PD-1 gene-deficient CLP mice compared with WT CLP mice, LPS-stimulated apoptosis decreased in Kupffer cells from CLP-induced changes in susceptibility. What we report is that Kupffer cells, but if PD-1 gene deficiency could affect these apoptosis in response to LPS challenge compared with sham not only more susceptible to undergoing the process of apoptosis or not the Kupffer cells derived from these CLP-WT mice were (14, 15, 60). In light of this, we attempted 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, 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, much of what we know has been gleaned from isolated human blood monocyte or the mouse peritoneal tissue resident macrophage. 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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 expression in mind, we attempted to determine what the effect of the expression of PD-1 was on their functional capacity (the development of immune suppression/dysfunction) by examining the impact of PD-1 gene 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 conditions 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.
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 effected by PD-1 gene deficiency. In this respect, a marked reduction of PU.1 has been correlated with alveolar macrophage dysfunction, including phagocytosis (50). Here we report that PD-1 gene deficiency restored PU.1 expression in CLP mice (to WT mouse sham levels), in accordance with recovery of phagocytic activity in liver macrophages after sepsis.

Regarding changes of Kupffer cell susceptibility to undergoing cell death, while not showing marked evidence of altered apoptosis in vivo, here we documented, much as we have done before (14, 15, 60), that liver macrophage derived from septic mice are significantly more susceptible to undergoing apoptosis when subsequently stimulated, as determined by increased cleavage of caspase-3 and TUNEL-positive staining when exposed to LPS in ex vivo culture. Importantly, we showed that this susceptibility was strongly dependent on the expression of PD-1 gene/protein as the capacity of LPS to induce these changes in apoptotic markers was markedly attenuated in cells derived from CLP PD-1−/− mice cells. Since the ligation of PD-1:PD-L1 (B7-H1) is also reported to affect the expression of key proteins, such as bcl-2, bcl-xL, and Fas (10), it is tempting to hypothesize that the altered expression of these genes may be contributing to the preservation of these cells. This again would need further experimentation to establish. Finally, it is worth pointing out that one result of PD-1 ligation, at least in T-lymphocytes (49), contributes to the suppression of cell survival signals, such as activation of Akt (p-Akt). Here we document not only that in LPS-stimulated liver macrophage derived from WT sham vs. CLP mice a decline in Akt activation is evident, but that this is mitigated by the loss of PD-1 gene expression/signaling. The protection this might afford the liver macrophage from undergoing cell death in response to septic challenge is supported by previous reports by Bomhard et al. (11), linking the restoration of Akt activation to improved survival and/or reduced leukocyte cell death following CLP.

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., X.H., C.-S.C., and A.A. interpreted results of experiments; F.W., C.-S.C., and A.A. prepared figures; F.W., C.-S.C., N.A.H., and A.A. edited and revised manuscript; F.W., X.H., C.-S.C., N.A.H., and A.A. approved final version of manuscript.

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