Severe Bacteremia Results in a Loss of Hepatic Bacterial Clearance

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Rationale: Although it has been postulated that liver injury results in impaired clearance of bacteria from the blood, no prior study has evaluated hepatic bacterial clearance during sepsis.

Objectives: We hypothesized that liver injury during the evolution of sepsis would result in impaired hepatic bacterial clearance.

Methods: Mild and severe bacteremia were generated in C57BL/6 mice by low- and high-dose intratracheal inoculation with *Pseudomonas aeruginosa.*

Measurements and Main Results: The mortality rates with mild and severe bacteremia were 20% and 60%, respectively. Hepatic bacterial clearance was preserved throughout the evolution of mild bacteremia but was lost late with severe bacteremia. The loss of hepatic bacterial clearance resulted in increased systemic bacteremia and mortality. Pretreatment with a caspase inhibitor resulted in preservation of hepatic bacterial clearance with severe bacteremia and eventual control of the bacteremia. When Kupffer cells were ablated before the onset of bacteremia, there was a loss of hepatic bacterial clearance. This converted an initially mild bacteremia into severe bacteremia with increased organ injury and mortality.

Conclusions: These observations suggest that hepatic bacterial clearance may be lost during the evolution of sepsis, resulting in a failure to control bacteremia. Thus, the capacity of the liver to clear bacteria is an important determinant of the outcome in sepsis.

Keywords: apoptosis; bacteremia; infection

In the United States, there are approximately 250,000 cases of sepsis with bacteremia annually (1). Approximately 50% of cases of bacteremic sepsis develop evidence of organ injury, including liver dysfunction, and this is an important determinant of survival (1). During the course of infection, the liver clears the blood of bacteria and produces cytokines in response to the infection (2). It clears bacteria and other particulates, like endotoxin, from the blood via the hepatic reticuloendothelial system. Kupffer cells, the resident macrophages of the hepatic reticuloendothelial system, are strategically situated to perform this function because they are located in the periportal region where blood enters the liver.

Acute and chronic liver diseases are associated with an increased risk of bacteremia (3–5). In addition, it has been shown that impaired clearance of injected particulates from the blood by the liver is associated with subsequent increased severity of infection (6). A recent study found that liver disease is an independent risk factor for the development of bacteremia in patients with community-acquired pneumonia (7). Although it is clearly documented that preexisting liver disease is associated with increased severity of infection, no study has directly investigated the evolution of hepatic bacterial clearance during the evolution of sepsis. Therefore, we evaluated hepatic bacterial clearance during the evolution of bacteremia in the setting of normal preexisting liver function. We used a murine model of pneumonia and bacteremia with *Pseudomonas aeruginosa* strain PA103. This strain of *Pseudomonas* causes acute epithelial injury and bacterial dissemination via the production of type III secreted toxins (8). The type III secretion system is a major determinant of virulence and allows the bacteria to inject toxins into the host cells (9).

We evaluated the evolution of hepatic bacterial clearance using models of mild and severe bacteremia. Mild and severe bacteremias were triggered by intratracheal inoculation of 5×10^3 and 10^4 colony-forming units (cfu) of PA103, respectively. We found that hepatic injury increased over time only with the severe bacteremia model. This was associated with a prolonged hepatic proinflammatory response and loss of bacterial clearance by the liver. Furthermore, we found that inhibition of caspase activity with the caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk) resulted in preservation of hepatic bacterial clearance with severe bacteremia. When Kupffer cells were ablated with gadolinium chloride (GdCl₃) before the onset of bacteremia, the mild form of bacteremia evolved into a more severe form of bacteremia. In all settings, when bacterial clearance by the liver was lost, it was associated with increased injury to other organs and mortality. Some of the results of these studies have been previously reported in the form of abstracts (10, 11).

METHODS

Pneumonia and Bacteremia Models

After anesthesia with ketamine/xylazine, C57BL/6 mice (female, age 6–8 wk) (Harlan Laboratories, Indianapolis, IN) underwent intratracheal intubation with a 20-gauge Angiocath as previously described (12, 13). A 50-μl volume of *P. aeruginosa*, strain PA103, was instilled into the trachea. Mild and severe bacteremias were induced with 5×10^3 CFU and 5×10^4 CFU, respectively. To inhibit apoptosis, separate mice were pretreated with z-VAD-fmk (Sigma-Aldrich, St. Louis, MO) at a concentration of 10 mg/kg dissolved in dimethyl sulfoxide 1 h before generation of bacteremia. To ablate Kupffer cells, separate mice were treated with 7.5 mg/kg GdCl₃ (Sigma-Aldrich) daily for 3 d before generation of bacteremia as previously described (14). Survival studies were performed on separate animals and used temperature as a surrogate endpoint for death. The criteria used were based upon prior studies showing severe hypothermia as a marker of death (15, 16). Briefly, temperature was monitored every 4 h for 24 h and then every 6 h. Animals were considered deceased if they had an absolute temperature below 27°C or a temperature below 30°C that failed to improve to above 30°C over the next 12 h. Animal studies were approved by the University of Iowa Institutional Animal Care and Use Committee.

Tissue Harvest and Homogenization

At predetermined time points, animals were killed according to Animal Care Guidelines. Blood was obtained from the portal vein and right ventricle. Organs were harvested and perfused free of intravascular
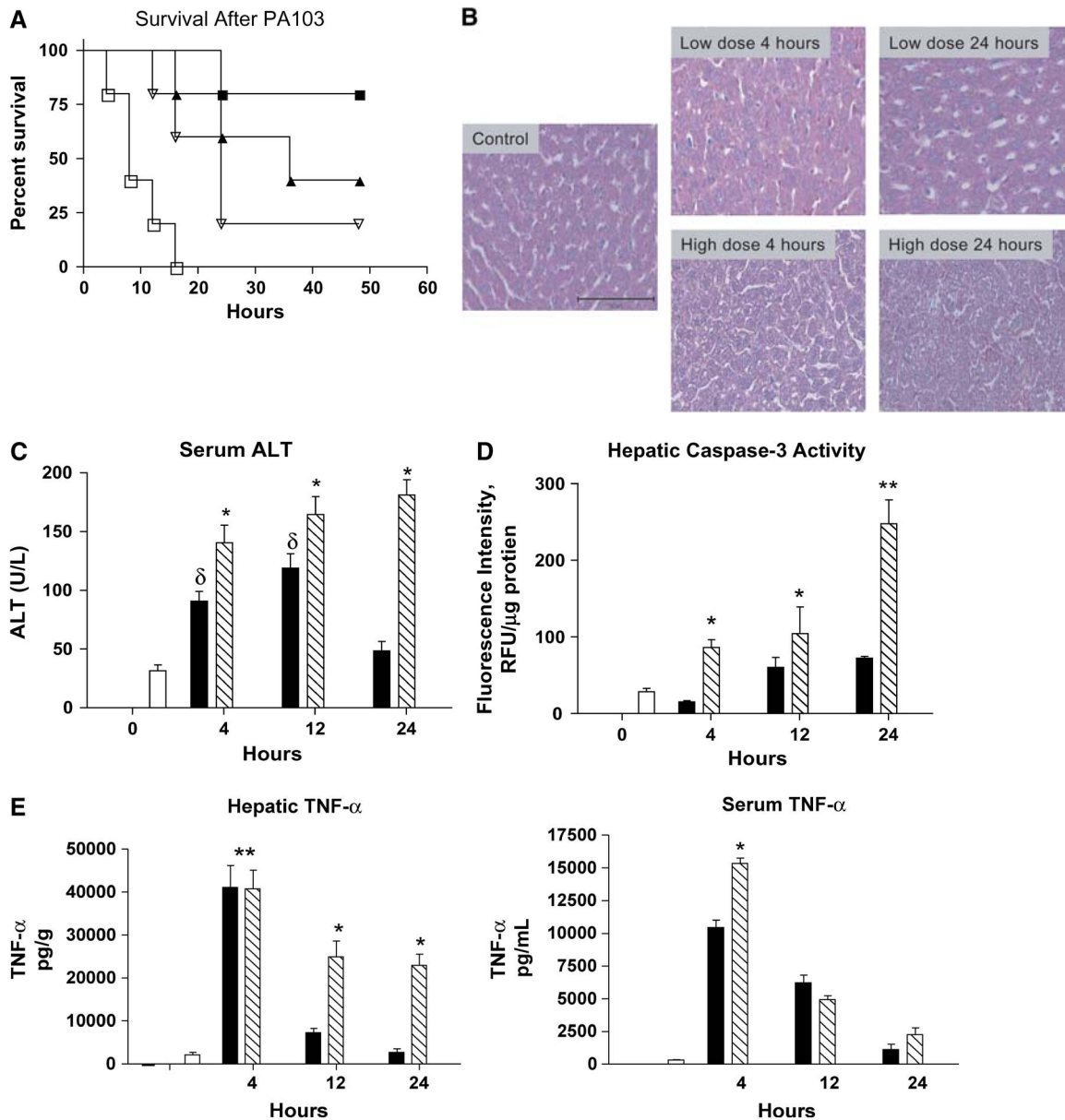
Figure 1. Severity of liver injury differs in mild and severe bacteremia. (*A*) C57BL/6 mice (n = 40) underwent endotracheal intubation and instillation of increasing doses of *P. aeruginosa* (strain PA103). Animals were monitored as described in METHODS. *Solid squares*, 5×10^3; *solid triangles*, 5×10^4; *inverted open triangles*, 5×10^5; *open squares* 5×10^6 cfu. (*B*) At 4 and 24 h after generation of mild and severe bacteremia, livers were fixed in 4% paraformaldehyde. All images are hematoxylin and eosin stained. (Original magnification: $\times$20.) (*C*) Serum alanine aminotransferase (ALT) was measured after generation of mild and severe bacteremia. Each group represents seven mice. Severe bacteremia results in increased ALT compared with control and mild infection at all time points (*$p < 0.001$). In mild bacteremia, there was an increase in ALT compared with control at 4 and 12 h ($\delta p < 0.001$), but this returned to baseline by 24 h. (*D*) The caspase-3 assay shows an increase in caspase-3 activity in liver lysates at 12 and 24 h in the severe bacteremia model (*$p < 0.01$ and **$p < 0.001$, respectively). (*E*) Tumor necrosis factor α (TNF-α) was measured in liver lysates by ELISA. There was a significant increase in TNF-α levels at 4 h in mild and severe bacteremia compared with control (**$p < 0.001$). There was also an increase in hepatic TNF-α in severe bacteremia compared with mild bacteremia at 12 and 24 h (*$p < 0.001$). Serum TNF-α was increased in severe bacteremia at 4 h (*$p < 0.05$), but there was no difference at the later time points. *C–E*: *solid bars*, 5×10^3 organisms; *hatched bars*, 5×10^4 organisms; *open bars*, control.

cells. Tissues were homogenized, and supernatants were collected. In separate animals, livers were harvested and fixed in 4% paraformaldehyde for histologic and immunohistochemical analysis.

Serum Measurements

Interleukin 1β (IL-1β) and tumor necrosis factor α (TNF-α) were measured by ELISA (R&D Systems, Minneapolis, MN). Serum alanine aminotransferase (ALT) was determined by a kinetic assay (Ther-

moTrace, Melbourne, Australia). Serum creatine kinase mb fraction (CK-mb) was determined by an immunoinhibition assay (ThermoTrace).

Quantitative Real-Time Polymerase Chain Reaction

For real-time polymerase chain reaction (PCR) analysis, 2 ng of experimental sample DNA was added to 48 μl of reaction mixture containing 1$\times$ iQ SYBR Green Supermix (BioRad, Hercules, CA) and 0.2 μM each of sense and antisense primers (IDT, Coralville, IA). Primers used
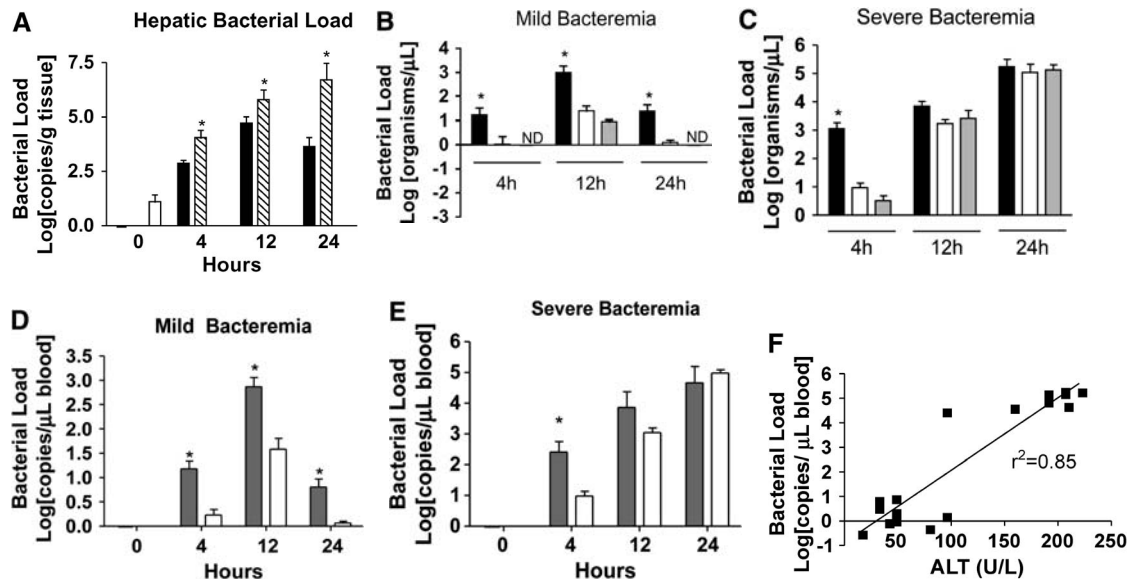
Figure 2. Loss of bacterial clearance occurs in severe bacteremia. (*A*) Bacterial load was measured in liver lysates after generation of mild or severe bacteremia by quantitative real-time PCR with primers specific for *P. aeruginosa*. Each group represents seven mice. A log transformation was performed to correct for unequal variances. There was increased bacterial load in severe bacteremia compared with mild bacteremia at all time points (*$p < 0.001$). *Solid bars*, 5×10^3 organisms; *hatched bars*, 5×10^4 organisms; *open bars*, control. (*B*) Bacterial load was measured in the portal vein (PV), right ventricle (RV), and hepatic vein (HV). In mild bacteremia, the use of the RV as a measure of hepatic bacterial clearance slightly underestimates the degree of bacterial clearance by the liver (*$p < 0.05$ comparing PV with RV and HV at all time points). ND = none detected. (*C*) In severe bacteremia, use of the RV slightly underestimates bacterial clearance at 4 h; however, bacterial clearance by the liver is lost at 12 h using HV and RV bacterial load. *B, C: solid bars*, PV; *open bars*, RV; *shaded bars*, HV. (*D*) In mild bacteremia, PV bacterial load is greater than RV bacterial load at all time points (*$p < 0.05$) *Shaded bars*, PV; *open bars*, RV. (*E*) In severe bacteremia, PV bacterial load is greater than that in the RV at 4 h (*$p < 0.05$). However, there is no difference at 12 or 24 h, suggesting ineffective bacterial clearance. (*F*) Serum ALT was compared with RV bacterial load at 24 h after infection. Linear regression analysis shows a significant correlation between degree of liver injury and the amount of bacteria in the RV ($r^2 = 0.85$).

to amplify the outer membrane lipoprotein gene, *oprL*, are as follows (5′ to 3′): forward, ATGGAAATGCTGAAATTCGGC; reverse, CTTCTTCAGCTCGACGCGACG. These primers are specific for *Pseudomonas* spp. (17). Amplification, specificity, and quantification were determined as previously described (18). Sensitivity of the assay was determined to be 50 copies per 2-ng sample.

Caspase-3 Activity Assay

A master mix of 45-μl caspase buffer and 5-μl caspase-3 substrate (Calbiochem, San Diego, CA) per sample was added to a black 96-well plate. Baseline fluorescence was read (excitation 355 nm, emission 460 nm). After incubation for 1 h at 37°C in the dark, fluorescence was read. Baseline values were subtracted to determine relative fluorescence intensity.

Statistical Analyses

Statistical analyses were performed using Prism GraphPad software (San Diego, CA). Data are presented as mean ± SEM. Comparisons were made using analysis of variance followed by Tukey's test for multiple comparisons unless otherwise stated.

RESULTS

Mortality after Infection with PA103

To establish appropriate models of mild and severe bacteremia, we performed survival studies using increasing intratracheal doses of PA103. Using body temperature as a surrogate endpoint for death, we found 20% and 60% mortality at 36 h with doses of 5×10^3 and 5×10^4 cfu, respectively (Figure 1A). On the basis of the survival studies, the remainder of this article uses 5×10^3 cfu as a model of mild bacteremia and 5×10^4 cfu as a model of severe bacteremia.

Hepatic Inflammation Is Prolonged and Hepatic Cell Death Is Increased with Severe Bacteremia

Using these models of mild and severe sepsis, we evaluated liver injury by histology and serum ALT levels during the evolution of bacteremia. Histologic analysis shows mild hepatic injury at 4 h in the mild bacteremia model, with return of normal hepatic architecture by 24 h (Figure 1B). There is increased liver injury after 4 h of severe bacteremia and extensive hepatic cell death after 24 h as compared with control and mild bacteremia livers (Figure 1B). After 24 h of severe bacteremia, the liver shows evidence of microthrombi formation. The sections shown in Figure 1B are representative of the entire liver and were reviewed by a pathologist and a hepatologist.

Paralleling the histology, we found that serum ALT increased progressively in the severe bacteremia model (Figure 1C). In contrast, mild bacteremia was associated with an early increase in serum ALT that resolved by 24 h. The transient increase in serum ALT in mild bacteremia corresponds to the histologic evidence of mild hepatic injury that subsequently resolved. To evaluate for evidence of hepatic apoptosis during bacteremia, we measured caspase-3 activity in liver lysates. We found that hepatic caspase-3 activity increased during the course of severe bacteremia, concomitant with the increase in serum ALT (Figure 1D). This suggests that hepatic apoptosis accounts for at least some of the hepatic cell death seen during severe bacteremia.

We next evaluated hepatic levels of TNF-α during the course of sepsis. We found that mild and severe bacteremia are associated with an early and equal hepatic TNF-α response (Figure 1E). In mild bacteremia, TNF-α rapidly decreases and returns toward baseline by 12 h. In contrast, severe bacteremia is associated

with a sustained elevation of TNF-α. We also found that there was increased serum TNF-α in the severe bacteremia group compared with the mild bacteremia group at 4 h, but the levels were no different at the later time points (Figure 1E). Amounts of IL-1β in liver and serum mirrored the levels of TNF-α (data not shown). These observations suggest that the degree and duration of the hepatic inflammatory response is associated with the severity of hepatic parenchymal injury.

Severe Bacteremia Is Associated with Loss of Hepatic Bacterial Clearance

Hepatic bacterial load was evaluated using quantitative real-time PCR with primers specific for *P. aeruginosa* (17). We found that hepatic bacterial load increases in mild bacteremia at 12 h and trends down by 24 h (Figure 2A). In contrast, severe bacteremia is associated with increasing hepatic bacterial load over time.

To evaluate bacterial clearance by the liver, we measured the bacterial concentrations in portal vein and right ventricular blood. Because the right ventricle also receives blood from the superior vena cava (SVC), we compared SVC and portal vein bacterial loads at all time points and found no significant difference (data not shown). This suggests that bacterial load in the right ventricle primarily represents bacterial clearance by the liver rather than dilution by SVC blood. To further investigate whether right ventricular bacterial load would underestimate bacterial clearance, we measured simultaneous bacterial loads in the portal vein, hepatic vein, and right ventricle in separate

mice with mild and severe bacteremia. We found that in the setting of effective bacterial clearance during mild bacteremia, the use of the right ventricle slightly underestimates bacterial clearance by the liver (Figure 2B). However, loss of bacterial clearance by the liver during severe bacteremia occurs at the same time point in hepatic vein and right ventricular measurements (Figure 2C). Therefore, right ventricular bacterial load was used as a measurement of hepatic bacterial clearance in subsequent studies.

We found that in mild bacteremia there is a gradient of bacteria between the portal vein and right ventricle, suggesting effective bacterial clearance by the liver (Figure 2D). In contrast, severe bacteremia is associated with equalization of bacterial loads in the portal vein and right ventricle by 24 h, suggesting a loss of hepatic bacterial clearance late during severe bacteremia (Figure 2E). At 24 h, serum ALT correlates with right ventricular bacterial load (Figure 2F).

Caspase Inhibition Preserves Hepatic Bacterial Clearance in Severe Bacteremia

We postulated that a preservation of Kupffer cells would prevent the loss of hepatic bacterial clearance during severe bacteremia. We pretreated animals with z-VAD-fmk 1 h before generation of severe bacteremia. Pretreatment with z-VAD-fmk resulted in preservation of hepatic bacterial clearance (Figure 3A) and a decrease in bacterial load at 24 h after infection. This suggests that the loss of bacterial clearance seen during hepatic injury may be related to apoptosis of liver cells, including Kupffer cells.
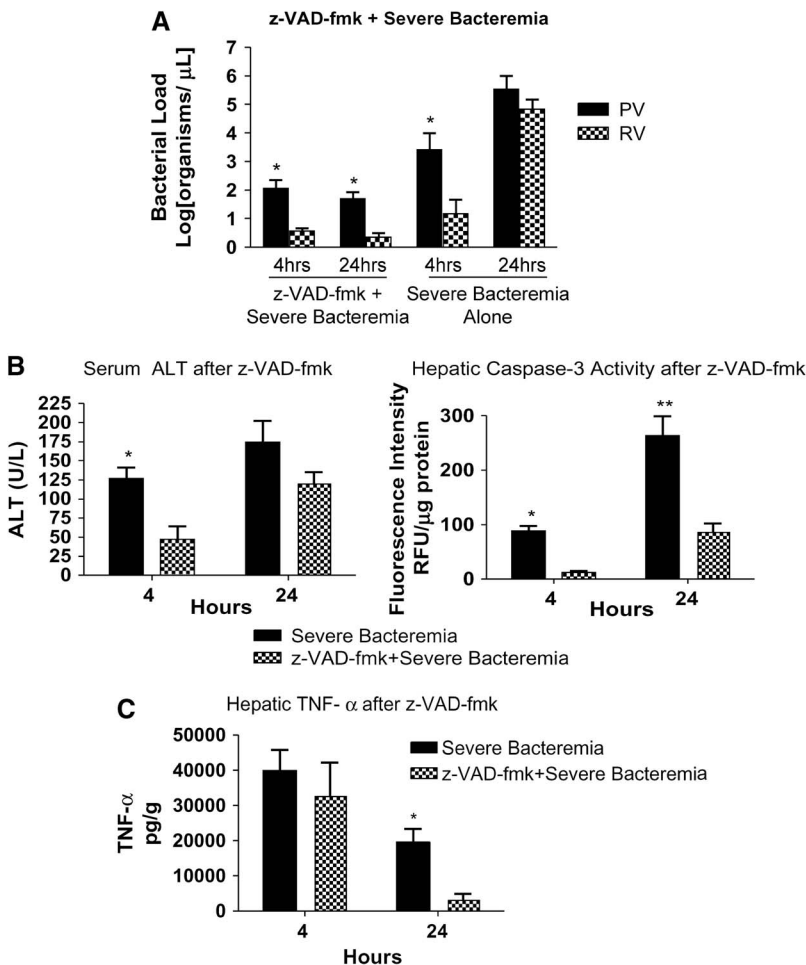

Figure 3. Caspase inhibition results in preserved hepatic bacterial clearance. (*A*) After pretreatment with z-VAD-fmk, mice were infected with PA103 10^4 cfu (severe bacteremia) and killed at 4 and 24 h. Control animals received dimethyl sulfoxide followed by PA103 10^4 cfu. Pretreatment with z-VAD-fmk resulted in preserved bacterial clearance at 4 and 24 h compared with severe bacteremia alone (*p < 0.01). (*B*) At 4 h, the severe bacteremia–alone animals had increased serum ALT compared with the animals pretreated with z-VAD-fmk (*p < 0.01). Caspase-3 activity in the liver was increased in the severe bacteremia alone mice at 4 (*p < 0.01) and 24 (**p < 0.001) h. (*C*) TNF-α was measured in liver lysates. At 4 h, there was no difference in hepatic TNF-α in the mice treated with z-VAD-fmk and the mice treated with severe bacteremia alone. At 24 h, there was increased TNF-α in the mice treated with severe bacteremia alone (*p < 0.01).

We found that serum ALT was decreased at 4 h in mice that received z-VAD-fmk compared with mice that were not exposed to the caspase inhibitor (Figure 3B). This provides further evidence that liver injury in sepsis is at least in part due to apoptosis. To confirm that the effect of z-VAD-fmk on the liver was associated with decreased caspase activity, we measured caspase-3 activity in liver lysates. We found that there was significantly less caspase-3 activity in the liver in animals pretreated with z-VAD-fmk (Figure 3B).

There was no difference in levels of hepatic TNF-α at 4 h between animals that were untreated and those that were pretreated with z-VAD-fmk (Figure 3C). However, over time the z-VAD-fmk mice released less hepatic TNF-α, whereas the untreated mice had a more prolonged hepatic TNF-α response. Because z-VAD-fmk preserves Kupffer cell numbers and function, this observation suggests that the increased bacterial load drives the prolonged hepatic TNF-α response in severe bacteremia.

Kupffer Cell Ablation with Gadolinium Results in a Loss of Hepatic Bacterial Clearance and Converts Mild Bacteremia to Severe Bacteremia

To directly evaluate the importance of Kupffer cells in bacterial clearance during bacteremia, we ablated Kupffer cells by treating animals with GdCl₃. Treating animals with GdCl₃ resulted in a 70% reduction in the number of Kupffer cells seen with immunohistochemistry (Figure 4A). Ablation of Kupffer cells before the onset of infection resulted in a loss of hepatic bacterial clearance even in the setting of mild bacteremia (Figure 4B). In the absence of Kupffer cells, the mild bacteremia model was converted to one of severe bacteremia. These observations show that Kupffer cells are important for the clearance of bacteria in this model.

Serum ALT levels were not elevated in the animals pretreated with GdCl₃ at 4 h (Figure 4C). However, there was evidence of liver injury 16 h after generation of bacteremia in the animals pretreated with GdCl₃. We also found that caspase-3 activity increased at 16 h in the GdCl₃-treated animals compared with the mild bacteremia alone model (Figure 4C). This suggests that a portion of the hepatic cell death seen in this model of bacteremia is due to apoptosis. The decrease in Kupffer cell number resulted in decreased hepatic TNF-α at 4 h after infection, and it did not increase at 16 h (Figure 4D). With GdCl₃, liver injury occurred in the setting of low hepatic TNF-α, suggesting that liver injury in this setting is not mediated solely by the production of TNF-α. Serum TNF-α levels were also decreased in the GdCl3-treated mice (data not shown), suggesting that the Kupffer cells are an important source of systemic TNF-α with bacteremia.

Loss of Bacterial Clearance Is Associated with Increased End-Organ Damage

To assess whether loss of hepatic bacterial clearance predisposed to the development of end-organ damage, we measured serum CK-mb and myocardial caspase-3 activity of the left ventricle to evaluate myocardial injury. Serum CK-mb did not increase in the mild sepsis model. In contrast, serum CK-mb increased after 24 h in the severe bacteremia model (Figure 5A). This was similar to the caspase-3 activity in the severe bacteremia model.

Inhibition of caspase activity with z-VAD-fmk before infection with the severe bacteremia model resulted in no detectable cardiac injury or apoptosis at 24 h compared with severe bacteremia alone (Figure 5B). This could be secondary to improved hepatic bacterial clearance driving the bacterial burden down and to local inhibition of apoptosis by z-VAD-fmk (19, 20). We found that there was a trend toward increased CK-mb and an earlier increase in cardiac caspase-3 activity at 16 h in the GdCl₃-treated animals, whereas the animals in the mild bacteremia
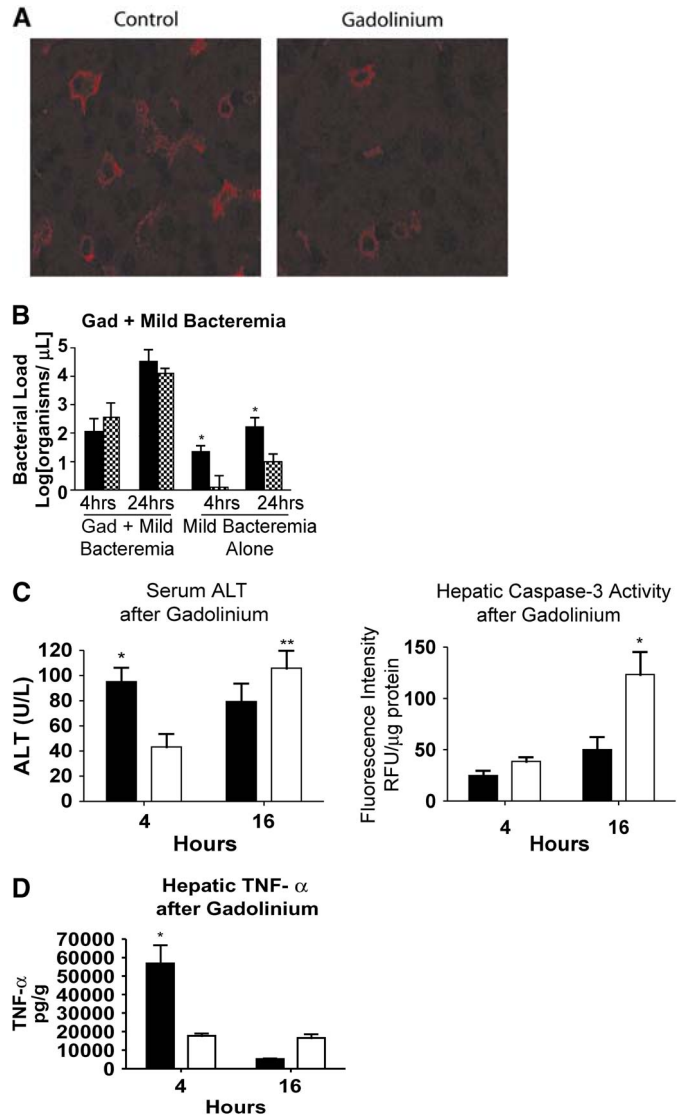

Figure 4. Kupffer cell ablation results in loss of hepatic bacterial clearance. (A) After pretreatment with GdCl₃, livers were harvested and stained with F4/80 macrophage antibody. Compared with control, there were decreased macrophages in the GdCl₃-treated liver. (B) After pretreatment with GdCl₃, mice were infected with PA103 10³ cfu (mild bacteremia) and killed at 4 and 16 h. Control animals received PBS followed by PA103 10³ cfu. Pretreatment with GdCl₃ resulted in impaired bacterial clearance at 4 and 16 h compared with mild bacteremia (*p < 0.01). *Solid bars*, PV; *cross-hatched bars*, RV. (C) At 4 h, serum ALT was increased in mice treated with mild bacteremia alone compared with mice treated with GdCl₃ mice (*p < 0.001). In mice treated with GdCl₃, there was increased ALT at 16 h compared with 4 h (**p < 0.001). Hepatic caspase-3 activity increased in mice treated with GdCl₃ at 16 h compared with mice treated with mild bacteremia alone (*p < 0.001). (D) TNF-α was measured in liver lysates. There was significantly less TNF-α in the liver in mice treated with GdCl₃ at 4 h compared with mice treated with mild bacteremia alone (*p < 0.001). C, D: solid bars, mild bacteremia; open bars, gadolinium + mild bacteremia.

alone group had no increase in CK-mb or cardiac caspase activity (Figure 5C).

Because serum creatinine could be representative of volume status rather than kidney injury, we chose to evaluate kidney lysates for evidence of bacteria and evidence of apoptosis. We were unable to detect *P. aeruginosa* in the kidney until 24 h
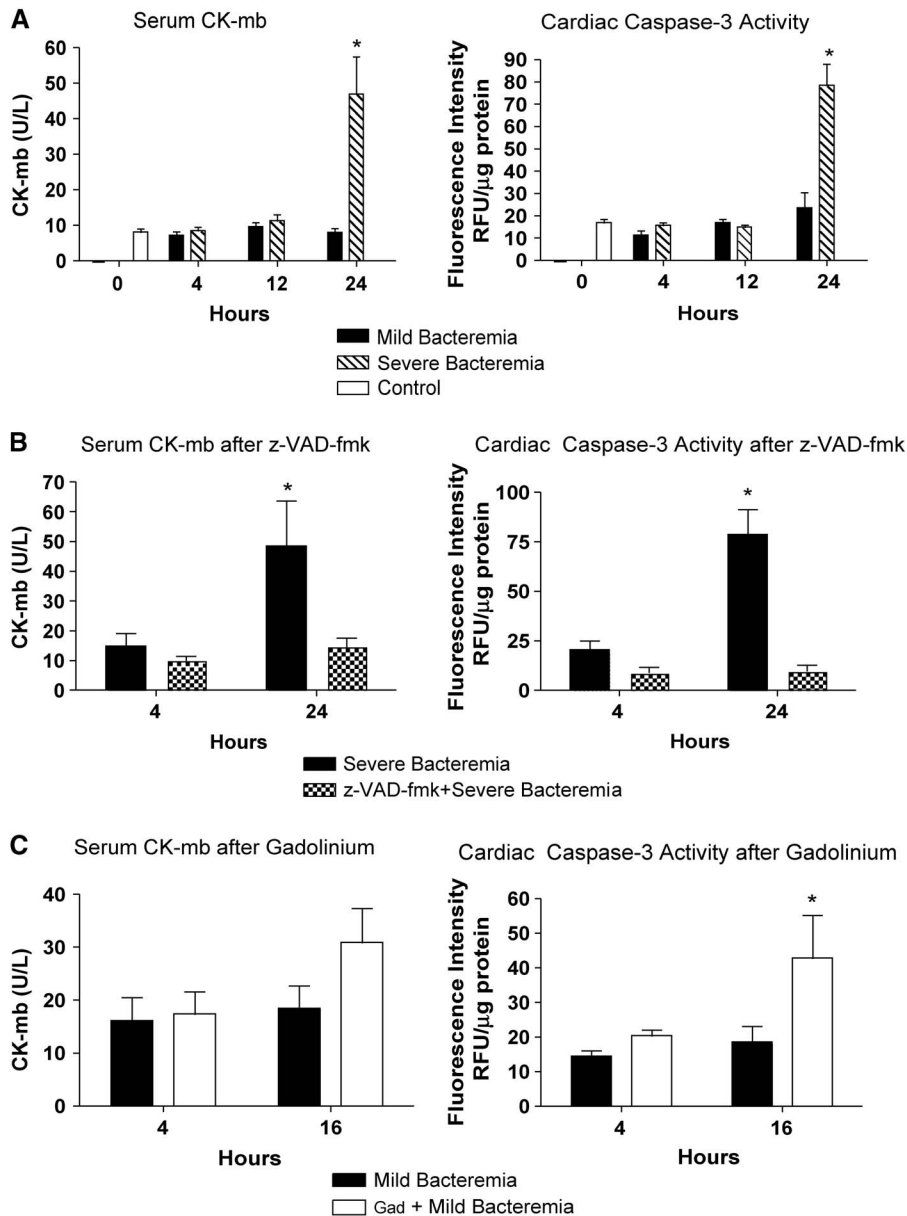
Figure 5. Cardiac injury in bacteremia is abated by caspase inhibition and exacerbated by Kupffer cell ablation. (*A*) Serum CK-mb levels were measured during mild and severe bacteremia. Serum CK-mb increased only at 24 h in the severe bacteremia model (*$p < 0.001$). Similarly, there was an increase in caspase-3 activity in myocardial lysates at 24 h in the severe bacteremia model (*$p < 0.001$). (*B*) Pretreatment with z-VAD-fmk followed by infection with the severe bacteremia model resulted in no evidence of increased CK-mb or cardiac caspase-3 activity. (*C*) Pretreatment with GdCl$_3$ followed by infection with the mild bacteremia model resulted in a trend toward increased CK-mb levels at 16 h and evidence of increased cardiac caspase-3 activity at 16 h (*$p < 0.01$) compared with mice infected with mild bacteremia alone.

in the severe bacteremia model (Figure 6A). This time point correlates with the loss of hepatic bacterial clearance. There was no evidence of *P. aeruginosa* in kidney lysates in the mild bacteremia model. The amounts of bacteria in the kidney paralleled the amounts of caspase-3 activity.

We examined renal injury in animals pretreated with z-VAD-fmk or GdCl$_3$ followed by infection with severe or mild bacteremia, respectively. Animals with severe bacteremia that were pretreated with z-VAD-fmk had no increase in kidney bacterial load (Figure 6B). This observation is consistent with the preservation of hepatic bacterial clearance in animals pretreated with z-VAD-fmk. Animals pretreated with z-VAD-fmk had no increase in kidney caspase-3 activity. In contrast, animals with mild bacteremia pretreated with GdCl$_3$ had an increase in kidney bacterial load at 16 h compared with the mild bacteremia alone animals, in which no bacteria was detected in the kidney (Figure 6C). The increase in kidney bacterial load in the mice treated with GdCl$_3$ occurred earlier than the increase seen in the severe bacteremia model. These animals had an increase in kidney

caspase-3 activity at the same time point. These data suggest that as hepatic bacterial clearance worsens, more bacteria are allowed systemic access, resulting in increased organ injury.

Caspase Inhibition Improves Survival and Kupffer Cell Ablation Decreases Survival in Bacteremia

To evaluate the effect of z-VAD-fmk on mortality from sepsis, we pretreated animals with z-VAD-fmk 1 h before generation of severe bacteremia. We found that inhibition of caspase activity significantly improved survival up to 48 h compared with severe bacteremia alone (Figure 7A). Although our data suggest that inhibiting apoptosis improves hepatic bacterial clearance by decreasing apoptosis of Kupffer cells, this only in part contributes to the increased survival. The decrease in organ injury seen with z-VAD-fmk is likely due to inhibition of apoptosis at the level of the individual tissues, and this likely contributes to the increase in survival.

To further evaluate the role of hepatic bacterial clearance by Kupffer cells in survival from bacteremia, we pretreated animals
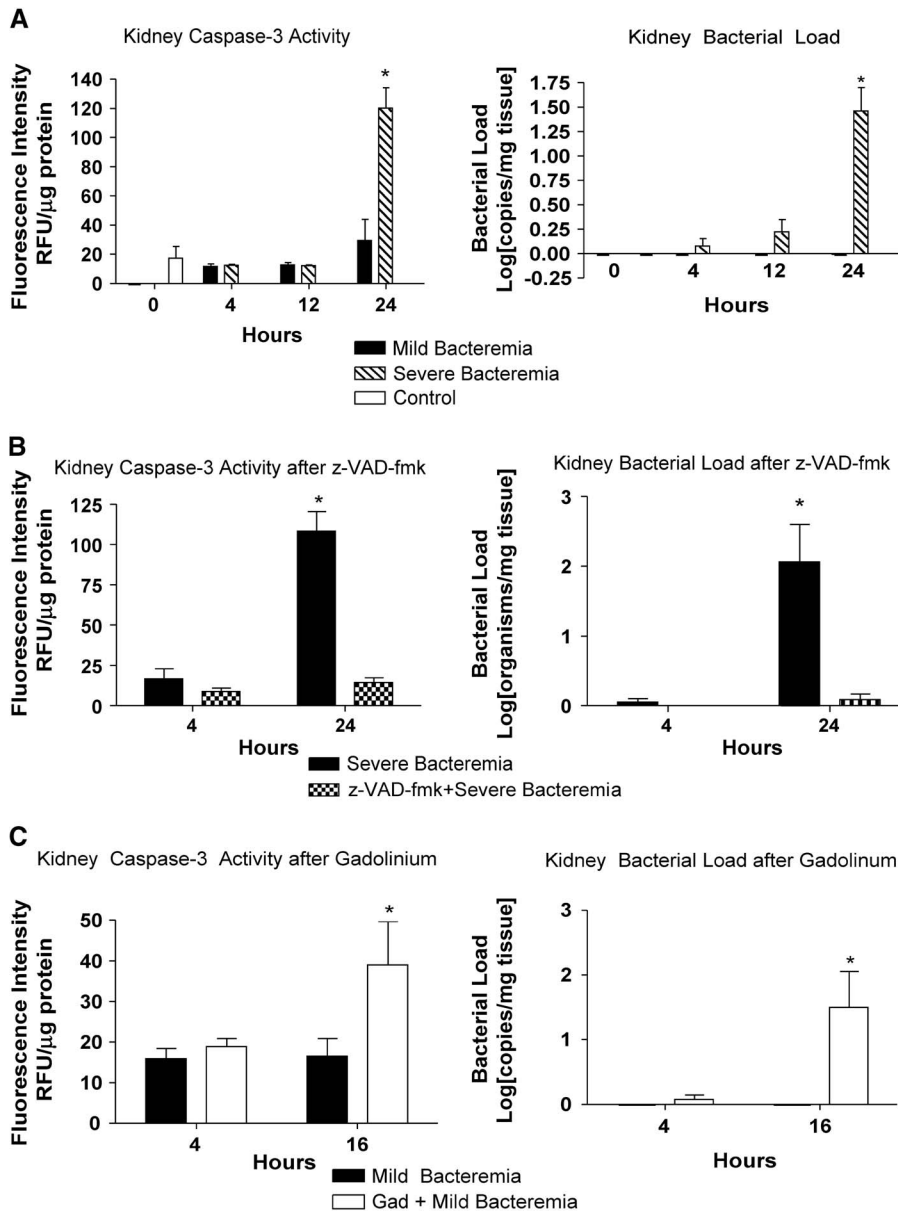
Figure 6. Renal injury in bacteremia is prevented by caspase inhibition and worsened by Kupffer cell ablation. (*A*) Kidney injury was evaluated by bacterial load and caspase-3 activity in kidney lysates. There was an increase in kidney caspase-3 activity in the severe bacteremia model at 24 h (*$p < 0.001$). Bacterial load was determined by quantitative real-time PCR with primers specific for *P. aeruginosa* and is reported as a log transformation. There was no *P. aeruginosa* detected in kidney lysates in the control animals or in the mild bacteremia model. There was a significant increase in bacterial load in the kidney in the severe bacteremia model at 24 h (*$p < 0.001$). (*B*) Pretreatment with z-VAD-fmk followed by infection with the severe bacteremia model resulted in no increase in kidney caspase-3 activity or kidney bacterial load at 24 h. (*C*) Pretreatment with GdCl₃ followed by infection with the mild bacteremia resulted in increased kidney caspase-3 activity (*$p < 0.01$) and kidney bacterial load (*$p < 0.01$) at 16 h.

with GdCl₃ before the generation of mild bacteremia. We found that ablation of Kupffer cells followed by infection with the mild bacteremia model resulted in decreased survival compared with mild bacteremia alone (Figure 7B). This model resulted in decreased survival compared with the severe bacteremia model as well. These data provide evidence that hepatic bacterial clearance by Kupffer cells is a determinant of the outcome of bacteremia.

DISCUSSION

In this study, we directly evaluated *in vivo* hepatic bacterial clearance during bacteremia in mice with normal baseline liver function. We also determined whether severe bacteremia results in a loss of hepatic bacterial clearance. We found that hepatic bacterial clearance, manifested as a gradient of bacteria between the portal vein and right ventricle, is effective in mild bacteremia. These animals experience less organ injury and mortality. In contrast, late in severe bacteremia, there is a loss of hepatic bacterial clearance, manifested as equalization of bacterial loads

between the portal vein and right ventricle. These animals have a prolonged hepatic inflammatory response, experience more liver and other organ injury, and have increased mortality. We validated the use of right ventricular blood to measure bacterial clearance by comparing bacterial loads in right ventricular blood with hepatic vein blood.

A potential mechanism of decreased hepatic bacterial clearance is apoptosis of Kupffer cells. To further test this, we pretreated animals with a nonspecific caspase inhibitor and found that inhibition of apoptosis resulted in a preservation of hepatic bacterial clearance and better control of the bacteremia in severe bacteremia. In contrast, ablation of Kupffer cells resulted in a loss of hepatic bacterial clearance, increased severity of infection, and increased mortality in the setting of an initially mild bacteremia. These findings strongly suggest that hepatic bacterial clearance is an important determinant of the outcome of bacteremia. To our knowledge, these are the first studies to evaluate bacterial clearance during the evolution of bacteremia and to relate these findings to the outcome of this infection.
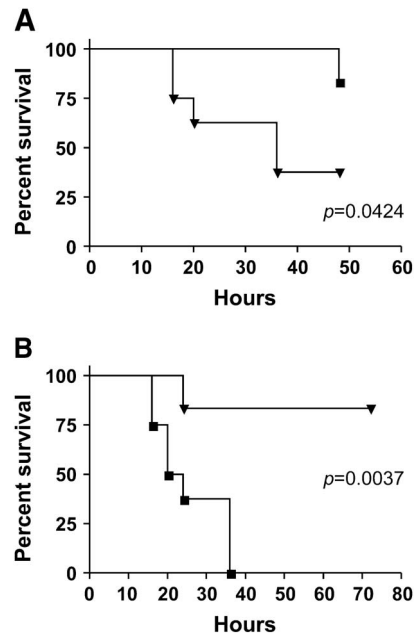
Figure 7. Caspase inhibition improves survival and Kupffer cell ablation decreases survival after bacteremia. (*A*) Pretreatment with z-VAD-fmk followed by infection with severe bacteremia resulted in decreased mortality compared with severe bacteremia alone out to 48 h by log-rank test (p < 0.0424). *Squares,* z-VAD-fmk + severe bacteremia; *inverted triangles,* severe bacteremia. (*B*) Pretreatment with GdCl₃ followed by infection with mild bacteremia resulted in 100% mortality at 36 h. This is significantly increased compared with mild bacteremia alone by log-rank test (p < 0.0037). *Squares,* gadolinium + mild bacteremia; *inverted triangles,* mild bacteremia.

We used a murine model of *P. aeruginosa* pneumonia with bacteremia. Animals treated with PA103 developed evidence of systemic inflammation manifested by cytokine production and evidence of hypothermia. Although this does not meet the strict criteria for sepsis (21, 22), it is similar to sepsis seen in other murine models (15). This model has limitations in that it is not an exact reflection of human sepsis. The liver injury seen in this model was more severe that than normally seen in humans. The inflammatory response in this model was greater than that seen in studies of human sepsis (23), although it was similar to other animal studies (24). A potential explanation for this difference is the early timing of the TNF-α peak, which may be missed in human sepsis. Nonetheless, this model is an effective way to study hepatic clearance of circulating bacteria and its effect on end-organ injury.

We used quantitative real-time PCR with primers specific for *P. aeruginosa* to determine bacterial load. PCR is more sensitive than standard plating techniques in that it can quantify exact numbers of bacteria and is able to detect low-level bacteremia (18, 25). In addition, sepsis is associated with an active cellular and humoral response, resulting in bacterial killing. Standard culture techniques, which rely on bacterial viability, may not represent the true bacterial load in the setting of a brisk bactericidal response (26). PCR also allowed us to detect and quantify differences in bacterial load between the portal vein and hepatic vein or right ventricle. Using primers specific for the bacteria responsible for the primary infection allowed us to evaluate hepatic bacterial clearance of the primary infection.

Our data indicate that there is loss of hepatic bacterial clearance during severe bacteremia. We also found that this could be prevented by the inhibition of caspase activity. This suggests that apoptosis of Kupffer cells may be an important mediator of decreased bacterial clearance in sepsis. This is consistent with a prior study showing that inhibition of caspase activity improved Kupffer cell function, as determined by phagocytosis of fluorescent latex particles (27). We recognize that z-VAD-fmk has effects that are not specific to the liver. Treatment with z-VAD-fmk has been shown to prevent cardiac injury (19, 20) and improve lymphocyte survival (28) during sepsis. Caspase inhibition has also been shown to improve overall survival in murine sepsis (29). It is likely that pretreatment with z-VAD-fmk in our study resulted in decreased apoptosis of other organs and that this likely played a role in improving survival.

To further investigate the importance of Kupffer cells in sepsis, we ablated Kupffer cells with GdCl₃ before mild infection. Several studies have established the efficacy of GdCl₃ in ablating Kupffer cells in mice (14, 30, 31). We found a loss of hepatic bacterial clearance at all time points. Furthermore, we found that, in the absence of Kupffer cells, infection with the mild bacteremia model resulted in the generation of severe bacteremia with earlier organ injury and increased mortality. Ablation of Kupffer cells with GdCl₃ also resulted in decreased hepatic and serum TNF-α. This is similar to other studies showing decreased TNF-α with Kupffer cell ablation (32–34) and suggests that Kupffer cells are an important source of TNF-α during sepsis. We found significantly increased mortality when Kupffer cells were ablated. This is consistent with a prior study showing increased mortality after cecal ligation and puncture in the setting of Kupffer cell ablation (30). Ablation of Kupffer cells before infection resulted in increased organ injury and mortality despite lower TNF-α levels. This is consistent with a prior study showing that ablation of alveolar macrophages before *P. aeruginosa* pneumonia resulted in increased lung injury 48 h after infection (35).

Our data suggest that apoptosis of Kupffer cells may lead to loss of bacterial clearance during the evolution of sepsis. Several studies have evaluated the importance of apoptosis of immune cells in sepsis (36–38). Hotchkiss and colleagues have shown an increase in splenic lymphocyte apoptosis in a murine model of sepsis (39). They found that this was associated with increased mortality. Furthermore, they found that this could be prevented by caspase inhibition (28). Studies have also shown evidence of macrophage apoptosis during sepsis (37). Inhibition of apoptosis has been associated with improved outcomes in animal models of sepsis (37, 40–43).

There are several potential mechanisms that could lead to Kupffer cell apoptosis in sepsis. First, TNF-α has been implicated in apoptosis during sepsis (36, 38). It is well known that sustained elevations of TNF-α can induce apoptosis via caspase activation (38). Although we have not shown that sustained TNF-α caused impaired hepatic bacterial clearance, a notable difference between our two models is the prolonged hepatic TNF-α response seen in severe bacteremia. Second, it is possible that the circulating bacteria have a direct toxic effect on Kupffer cells. Pathogen-induced macrophage apoptosis has been extensively studied (44). The YopP exotoxin of *Yersinia* spp. has been shown to induce macrophage apoptosis via inhibition of nuclear factor-κB (45). *P. aeruginosa* has been shown to induce apoptosis in a macrophage cell line via the elaboration of type III secretions (46). It is possible that the loss of hepatic bacterial clearance in our model was due to a combination of bacterial toxins and prolonged inflammation. Whatever the mechanism, hepatic bacterial clearance is an important determinant of outcome in bacteremia.

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