

## NONINVASIVE IMAGING OF STORED RED BLOOD CELL-TRANSFUSION AGGRAVATING SEPSIS-INDUCED LIVER INJURY ASSOCIATED WITH INCREASED ACTIVATION OF M1-POLARIZED KUPFFER CELLS

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**ABSTRACT**—Liver injury has a critical effect on the severity and outcome of sepsis. The impact of stored red blood cells (RBCs) on the pathogenesis of sepsis-associated hepatic injury is not well understood. Therefore, to investigate the effects of stored-RBC transfusion on sepsis-induced liver damage as well as the associated mechanism, we constructed a sepsis mouse model enabling noninvasive imaging of bacterial infection caused by *Pseudomonas aeruginosa*, a common gram-negative respiratory pathogen. We showed that transfusions with stored RBCs enhanced sepsis-induced liver injury *in vivo*, and liver injury exacerbated the severity of sepsis and decreased survival in *P aeruginosa*-infected mice. Stored-RBC transfusions enhanced the production of proinflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$ , interleukin 6 (IL-6), and IL-1 $\beta$ , which play important roles in sepsis-associated liver injury in *P aeruginosa*-infected mice. Further study showed that the enhanced inflammation observed was associated with increased activation of M1-polarized Kupffer cells, which produce many inflammatory cytokines, including TNF- $\alpha$  and IL-6. Moreover, the M1-polarized Kupffer cells and secreted proinflammatory cytokines exerted their effects on hepatocytes through enhanced Jun N-terminal kinase activation and inhibited nuclear factor-kappaB activation, demonstrating that transfusion with stored RBCs disrupted the balance between cell survival and cell death in the liver. Understanding the mechanisms whereby stored RBCs might contribute to these complications will likely be helpful in providing guidance toward making transfusions safer.

**KEYWORDS**—Bacterial, liver damage, luciferase, M1-polarized, NF- $\kappa$ B, stored RBCs, survival

### INTRODUCTION

Sepsis, severe sepsis, and septic shock are syndromes resulting from infection that have progressively increasing severity and mortality (1). The primary circulatory treatment of patients may be supplemented with a transfusion of red blood cells (RBCs) in the case of persistent hypoperfusion (2). Although potentially life-saving in individual patients, the practice of transfusion is associated with increased morbidity and/or mortality in different sepsis/septic shock patients (2–4).

The altered properties of stored blood products might partly explain this paradoxical effect. During storage, significant biochemical, structural, and morphologic changes take place in RBCs. In many observational studies, transfusion of RBCs stored for longer durations had adverse outcomes, such as

increased risk for infectious disease complications (5–11). Previous studies have shown that transfusion of older RBCs exacerbates infection with gram-negative bacteria (12). However, the mechanisms whereby transfusion with older RBCs exacerbates infection are poorly understood.

The liver plays essential roles in both sepsis and the elimination of old RBCs. Liver injury before or after the onset of sepsis has a critical effect on the severity and outcome of sepsis in patients (13–15). Acute liver injury is common in severe sepsis and substantially increases the risk of death (16–19). In addition to its important role in sepsis, recently, the liver selectively accumulated monocyte-derived transient macrophages (tM $\phi$ s) that were capable of propagating on-demand, high-capacity erythrophagocytosis, and iron recycling (20). The modulatory activity on both sepsis and eliminating old RBCs supports the potential contribution of older RBCs in exacerbating infection.

However, the impact of stored RBCs on the pathogenesis of sepsis-associated hepatic injury is not well understood. Therefore, in the present study, we studied the effects and mechanism of stored-RBC transfusion on sepsis-induced liver damage. A sepsis mouse model enabling noninvasive imaging of bacterial infection was constructed. We showed that transfusions with stored RBCs enhanced *P aeruginosa*-induced liver injury *in vivo*, and liver injury exacerbated the severity of sepsis and decreased survival in *P aeruginosa*-infected mice.

Understanding the mechanisms by which stored RBCs contribute to these complications will likely provide guidance in making transfusions safer.

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## MATERIALS AND METHODS

Male C57BL/6 mice, 6 to 8 weeks of age, were purchased from the Academy of Military Medicine Science Animal Center. Animals used in the study followed the National Institutes of Health's Guidelines for the Humane Treatment of Laboratory Animals. The ethics committee of the National Beijing Center for Drug Safety Evaluation and Research (NBCDSER) approved this protocol.

### RBCs

C57BL/6 mice were bled aseptically by cardiac puncture into citrate-phosphate-dextrose-adenine (CPDA)-1. RBCs (approximately 15 mL) were placed in 50-mL Falcon tubes and stored at 4°C for up to 14 days.

### Bacteria

The bioluminescent *P aeruginosa* Xen 13 strain (PerkinElmer, Waltham, Mass) was used in this study. Bacteria were washed twice in phosphate-buffered saline (PBS) and resuspended to an appropriate number of colony-forming units (CFUs) in PBS.

### RBC transfusions and bacterial infections

RBC suspensions (400  $\mu$ L at 17.0–17.5 g/dL hemoglobin; 2 equivalent human units) were transfused through the retro-orbital plexus of isoflurane-anesthetized mice. Concurrently, cohorts of mice were infected by tail-vein injection of bacteria.

### Plasmids and hydrodynamics-based gene delivery

The pattB-NF- $\kappa$ B-Fluc and attB-ANLuc(DEVD)BCLuc plasmids were generated previously (21–23). Briefly, different amounts of plasmid DNA were injected rapidly into the tail vein in <5–8 s, using a 27-gauge needle.

### Imaging luciferase activity in vivo

Bioluminescence imaging was performed using an IVIS Spectrum system (PerkinElmer Inc., Waltham, Mass). Quantitation of luciferase activity and data analysis were performed using Living Image software 4.4 (Xenogen, Alameda, Calif).

### Histology and immunohistochemistry

Livers were fixed overnight with 10% neutrally buffered formalin and then embedded in paraffin. Images were captured using a CI-S microscope and a DS-FI2 imaging system (Nikon, Tokyo, Japan).

### Preparation of bone marrow-derived macrophages (BMDMs) and coculture experiments

BMDMs were prepared as previously described (24). For coculture experiments,  $1 \times 10^6$  cells in complete medium were incubated with fresh or stored RBCs (RBC:macrophage ratio of 50:1) at 37°C for 4 h in 12-well plates. Subsequently, the cells were stimulated with 10 ng/mL LPS (Sigma, St Louis, Mo) and incubated for an additional 4 h at 37°C.

### Cytokine assays on serum and BMDM supernatants

ELISA kits (R&D Systems, Minneapolis, Minn) were used to measure the production levels of interleukin (IL)-1 $\beta$ , IL-6, and tumor necrosis factor (TNF)- $\alpha$  in the serum and in BMDM culture supernatants.

### Electrophoretic mobility-shift assays (EMSAs)

Hepatic nuclear extracts were analyzed in DNA-binding assays and EMSAs were performed following the manufacturer's protocol.

### Real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from mouse livers, RNA (1  $\mu$ g) was reverse-transcribed into cDNA, according to the manufacturer's instructions. The sequences of the primers used in this study are shown in Table S1, <http://links.lww.com/SHK/A574>.

### Western blot analysis

Proteins in hepatic cell extract were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Detection was achieved using secondary antibodies, followed by imaging on a Bio-Rad machine.

### Statistical analysis

Data are shown as the mean  $\pm$  SD. Statistical comparisons between different groups were assessed by *t* tests, one-way ANOVA, and the Mann–Whitney *U* test. A *P* value of less than 0.05 was considered significant. All significance levels are two-sided.

## RESULTS

### Stored-RBC transfusions exacerbated sepsis and decreased survival in *P aeruginosa*-infected mice

*In vitro* imaging was performed to confirm that the luciferase activity correlated positively with the number of bacterial CFUs (Figure S1, <http://links.lww.com/SHK/A574>). As shown in Figure 1A, bioluminescence readings correlated with bacteria CFUs numbers. The mortality was dose- and time-dependent in *P aeruginosa*-treated mice infected with  $1 \times 10^7$ ,  $2 \times 10^7$ ,  $3 \times 10^7$ , or  $4 \times 10^7$  CFUs/body weight (Fig. 1B).

We found that the survival of mice transfused with old RBCs was significantly lower than that of mice infused with fresh RBCs ( $P < 0.05$ ; Fig. 1C). Bioluminescence imaging also showed that the area under the curve of bioluminescent flux during the first 24 h postinfection was significantly increased in mice treated with stored RBCs (Fig. 1D). Taken together, these data indicated that transfusing stored RBCs could exacerbate sepsis and decrease survival in *P aeruginosa*-infected mice.

### Transfusions of stored RBCs enhanced *P aeruginosa*-induced liver injury in vivo

The data demonstrated that *P aeruginosa* injection induced the highest luciferase signal in the hepatic region (Fig. 2A). These observations were in agreement with previous reports (3, 16) (Figure S2, <http://links.lww.com/SHK/A574>).

The serum levels of alanine aminotransferase (ALT) and aminotransferase (AST) in mice treated with fresh RBCs increased, but were significantly reduced compared with those of mice treated with stored RBCs (Fig. 2B), and the levels of ALT and AST with RBCs stored for 3 days were not different with those for 7 days (Fig. S3, <http://links.lww.com/SHK/A574>). The difference of the ALT and AST of the early and late after transfusion in the CPDA-1 group was not significant (Fig. S4, <http://links.lww.com/SHK/A574>). The difference of the ALT and AST of the transfusion at the beginning and later of sepsis was not significant (Fig. S5, <http://links.lww.com/SHK/A574>). Only spotted necrosis was observed in the livers of mice treated with fresh RBCs, whereas livers from mice treated with stored RBCs showed massive necrosis (Fig. 2C). These results indicated that transfusions with stored RBCs enhanced *P aeruginosa*-induced liver injury *in vivo*, and liver injury exacerbated the severity of sepsis.

### Stored RBC transfusions enhanced the production of proinflammatory cytokines in *P aeruginosa*-infected mice

When *P aeruginosa*-infected mice were transfused with stored RBCs, the release of TNF- $\alpha$ , IL-6, and IL-1 $\beta$  was

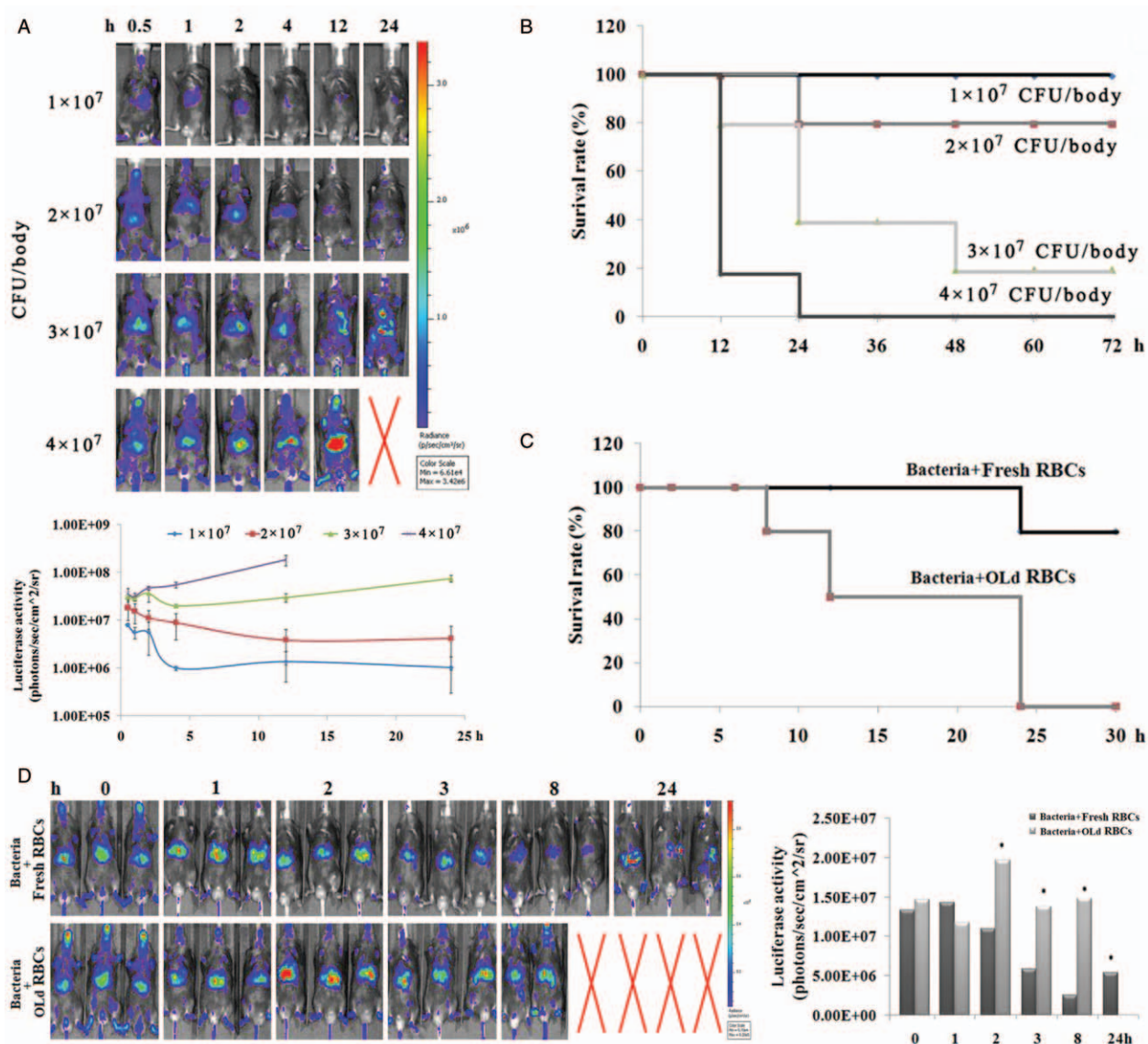


FIG. 1. **Stored-RBC transfusions exacerbated sepsis and decreased survival in *Paeruginosa*-infected mice.** A, Bioluminescence imaging at 24 h after injection with different numbers of bacteria CFUs ( $n = 5$ /group). B, Survival curves at 72 h after injection with different numbers of bacteria CFUs ( $n = 10$ /group). C, Survival curves after bacteria injection and RBC transfusion after 24 h are shown ( $n = 10$ /group). D, Luciferase activities were measured by noninvasive bioluminescence imaging at multiple time points for up to 24 h post-transfusion. \* $P < 0.05$  ( $n = 5$ /group). CFUs indicates colony-forming units; RBC, red blood cell.

significantly higher than the levels in mice transfused with fresh RBCs at 2 h postinfection (Fig. 3). These results indicated that stored-RBC transfusions could amplify systemic inflammatory responses to bacterial infection during sepsis and that excessive inflammatory responses resulted in liver injury.

#### **Increased levels of M1-polarized Kupffer cells were responsible for the enhanced proinflammatory responses mediated by transfused, stored RBCs**

Previous findings showed that Kupffer cells are believed responsible for producing inflammatory cytokines during early sepsis and for mediating sepsis-induced liver injury (25). As shown in Figure 4A, the IL-6 and TNF- $\alpha$  levels in the supernatant of the stored-RBC-treated group were significantly higher than those observed in the fresh-RBC-treated group,

suggesting that incubation with stored RBCs leads to markedly enhanced macrophage activation.

It has been reported that diversity and plasticity are hallmarks of macrophages (26). As determined by flow cytometry, among F4/80+ macrophages, CD86 and CD197 (M1-specific markers) were more highly expressed by cells treated with stored RBCs, compared with those treated with fresh RBCs (88% vs. 79.24% for CD86; 38.59% vs. 25.95% for CD197) (Fig. 4B), whereas CD209 (an M2-specific marker) showed no difference (23.88% vs. 21.91%). These results indicated that M1-polarized macrophages played an important role in the enhanced proinflammatory responses mediated by transfused stored RBCs.

As shown in Figure 5A, the transcript levels of F4/80 were higher in the stored-RBC-treated groups than in fresh-RBC-

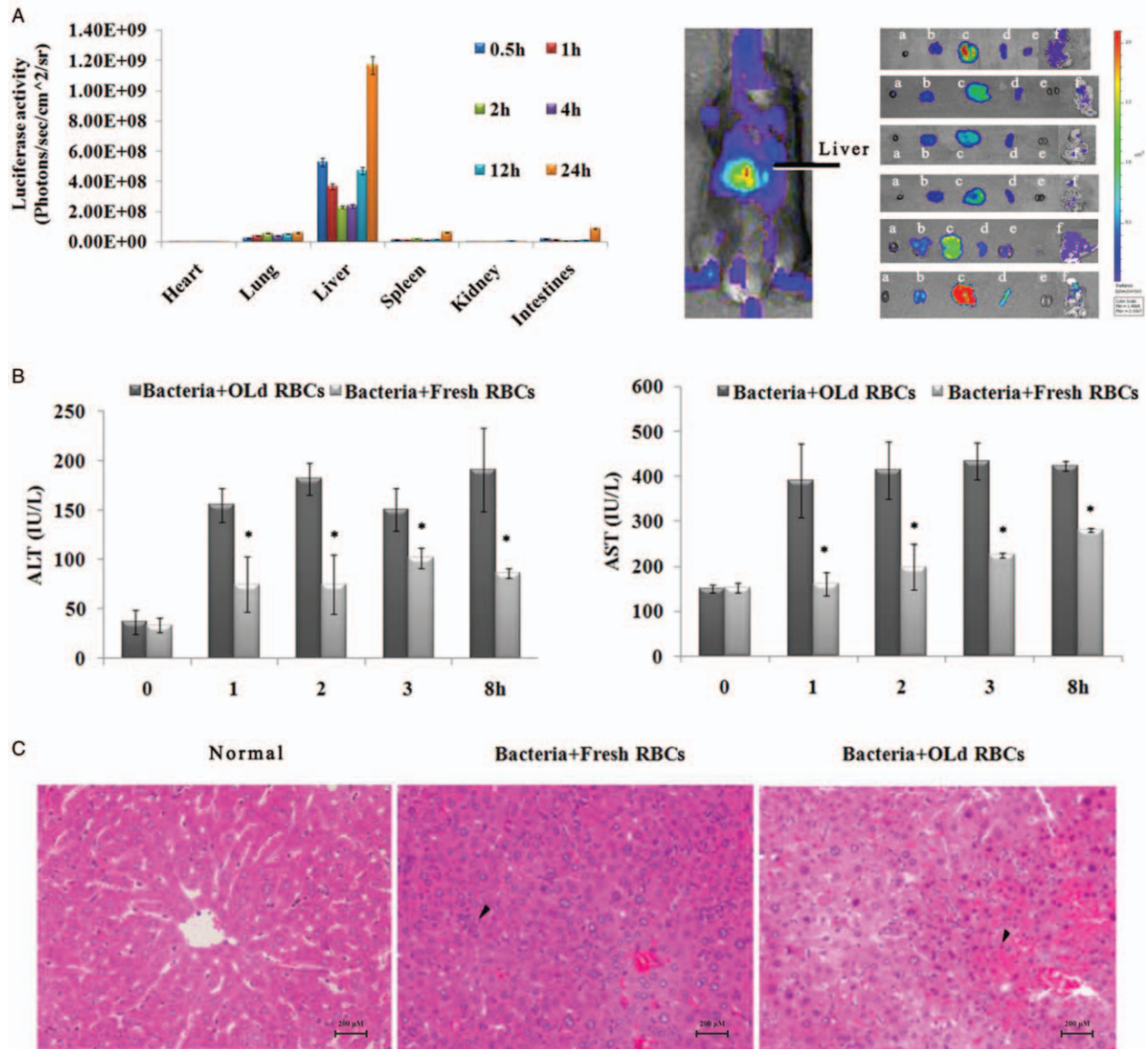


FIG. 2. BLI of C57BL/6 mice infected i.v. with *P aeruginosa* and increased liver injury induced by transfusion with stored RBCs. Infected organs were imaged individually after dissection (A). Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels are shown (B). Histological examination of tissue damage through HE staining of mouse liver sections from mice is shown (C). \* $P < 0.05$  ( $n = 5$ /group).

treated groups, both at 2 and 4 h after RBC injection. This result indicated that the injection of stored RBCs led to dramatic Kupffer cell fluctuations in the livers of mice infected with *P aeruginosa*, which was consistent with previous data showing that the liver recruits transient macrophages (tMΦs) after a stressful erythrocyte challenge (20). As shown in Figure 5B, the transcript levels of Arginase 1 (Arg1), mannose receptor C type 2 (Mrc2), and CD163 were similar between the stored-RBC-treated and fresh-RBC-treated groups; however, tissue samples from the stored-RBC-treated group had higher inducible nitric oxide synthase (iNOS), TNF- $\alpha$ , and MCP1 mRNA levels compared with those in the fresh-RBC-treated group ( $P < 0.05$ ). These data were consistent with our *in vitro* data and revealed, for the first time, that the *in vivo* transfusion of stored RBCs could significantly enhance the polarization of liver-resident Kupffer cells toward an M1 phenotype, which is

responsible for the enhanced proinflammatory response mediated by transfused, stored RBCs.

#### Stored RBCs induced hepatic apoptosis by disturbing the dynamic balance between the NF- $\kappa$ B and JNK pathways

Figure 6A shows that luciferase induction in the NF- $\kappa$ B-luc mouse models was detectable at 2 h and increased gradually at 4 and 8 h post-transfusion with fresh RBCs. EMSA analysis documented concordant results, as evidenced by reduced signaling at 8 h post-transfusion (Fig. 6B). These data indicated that NF- $\kappa$ B activation could be inhibited by the transfusion of stored RBCs.

Our data showed that luciferase activities significantly increased in mice treated with stored RBCs (Fig. 6C) compared with that observed in mice treated with fresh RBCs, indicating that stored RBCs enhanced hepatocyte apoptosis in mice. This

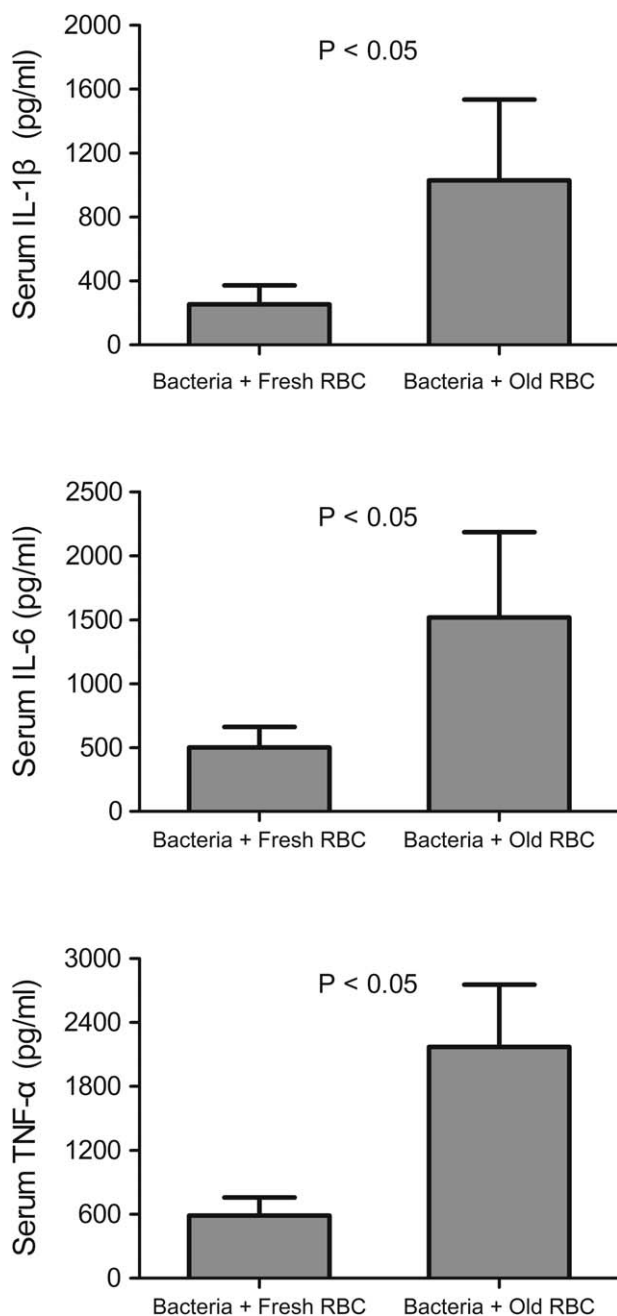


FIG. 3. **Transfusion of stored RBCs synergizes with inflammatory responses to bacteria.** The plasma cytokines TNF- $\alpha$ , IL-6, and IL-1 $\beta$  were measured. The results shown are representative of two independent experiments. \* $P < 0.05$  ( $n = 10$ /group). IL-6 indicates interleukin 6; TNF, tumor necrosis factor.

result was confirmed by western blotting to detect phosphorylated JNK proteins and activated caspase-3 proteins (Fig. 6D). These data indicated that the JNK and caspase pathways were enhanced by the transfusion of stored RBCs. Stored RBCs therefore induce hepatic injury by disturbing the dynamic balance between the NF- $\kappa$ B and JNK pathways (Fig. 7).

## DISCUSSION

*P aeruginosa* infection has a high mortality rate and often leads to disseminated infection, which can result in bacteremia

and septic shock (27). Data from observational studies have indicated that prolonged RBC storage before transfusion increases mortality, serious infections, and multi-organ failure (28). However, the inflammatory pathogenic effects induced by transfusing stored RBCs on sepsis are not fully understood.

Since liver injury promotes the progression of sepsis and affects clinical outcomes, we studied the central role of the liver in exacerbating infection following transfusion with older RBCs. In this study, we showed that the transfusion of stored RBCs enhanced sepsis-induced liver injury *in vivo*, and liver injury exacerbated the severity of sepsis and decreased survival in *P aeruginosa*-infected mice.

Macrophages are believed to be responsible for producing inflammatory cytokines during early sepsis and for mediating sepsis-induced liver injury (25, 29). However, the mechanisms underlying macrophage polarization and activation within the liver after transfusing old RBCs during sepsis remain unclear.

Most studies investigating the crosstalk between stored RBC transfusion and macrophages have focused on peripheral blood populations rather than on intrahepatic populations. The results from early studies have suggested that transfusions of old RBCs resulted in iron delivery to the mononuclear phagocyte system, followed by the polarization of macrophages toward the classical M1 macrophage-activation pathway and a proinflammatory cytokine response (30). This impaired modulatory activity in macrophages supported their potential contribution to liver injury. Until recently, it was reported that the liver is a primary organ that supports rapid erythrocyte removal and iron recycling (20).

Regarding the important role that Kupffer cells play in erythrophagocytosis, we suspected that Kupffer cells might contribute to older RBC-induced exacerbation of infection. In this study, we discovered a critical role of Kupffer cells in oversensitive liver injury after stored-RBC transfusion in *P aeruginosa*-infected mice. We found that the injection of stored RBCs led to increased Kupffer cells in the liver infected with *P aeruginosa*, which was consistent with previous data showing that tM $\Phi$ s appeared in the liver after a stressful erythrocyte challenge. Upon transfusion with stored RBCs, the activated-M1 phenotype of Kupffer cells increased and enhanced pro-inflammatory cytokine production was observed in the liver. Pro-inflammatory cytokines, such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , promote liver destruction and acute liver injury, as shown by increased AST and ALT levels in serum. Our results confirmed the data obtained from peripheral blood populations (30) and suggested a novel mechanism whereby stored RBCs contribute to sepsis progression, which may be exploited for therapeutic interventions.

A growing number of studies have implicated cytokine-dependent pathways in the development of liver injury, chronic liver disease, hepatic inflammation, and liver fibrogenesis (31). Cytokines can activate specific intracellular pathways in hepatocytes that influence cell fate in different manners, e.g., proapoptotic signals via the caspase cascade, as well as survival pathways, such as the NF- $\kappa$ B pathway. We confirmed that transfusion with stored RBCs in *P aeruginosa*-infected mice elicited enhanced JNK activation and inhibited NF- $\kappa$ B activation, demonstrating that stored RBCs transfusion disrupted the balance between cell survival and cell death in the liver.

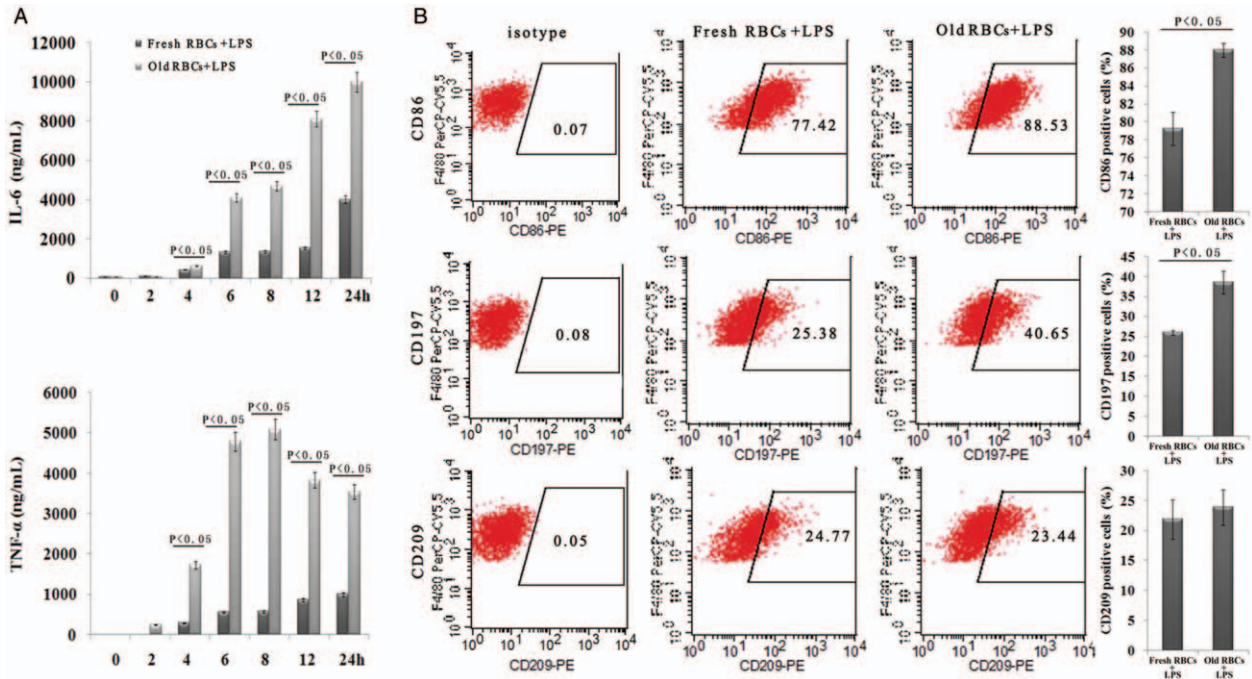


FIG. 4. **Stored RBCs regulate cytokine secretion and macrophage polarization.** A, IL-6 and TNF- $\alpha$  cytokines were quantified in culture supernatants by ELISA. BMDMs were harvested and analyzed by flow-cytometric (B). \* $P < 0.05$  ( $n = 5$ /group). BMDMs indicates bone marrow-derived macrophages; IL-6, interleukin 6; TNF, tumor necrosis factor.

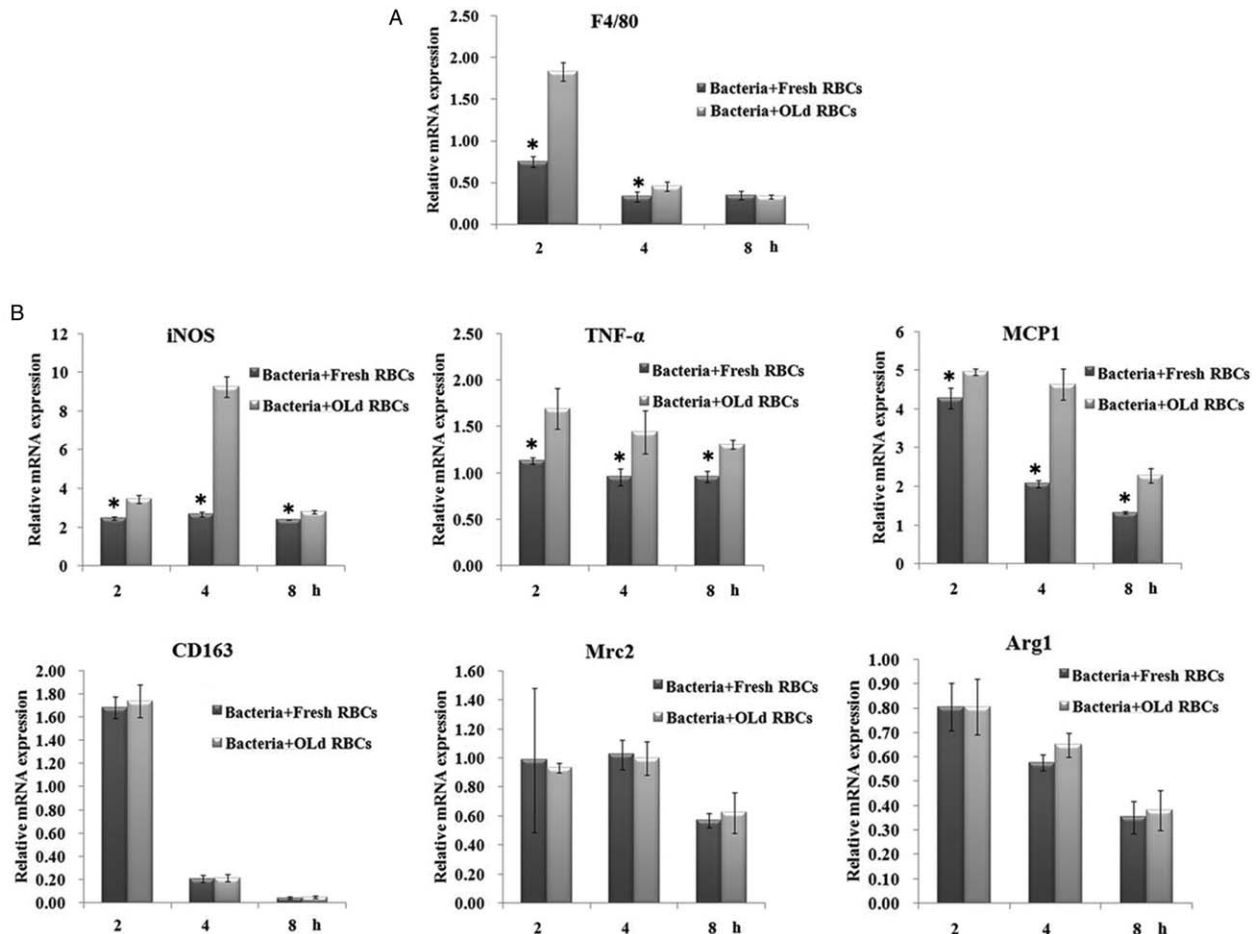


FIG. 5. **RT-PCR analysis of hepatic F4/80 (A), and M1 and M2 genes (B) in *Paeruginosa*-infected mice transfused with stored RBCs or fresh RBCs at 2, 4, and 8 h after treatment.** \* $P < 0.05$  ( $n = 5$ /group).

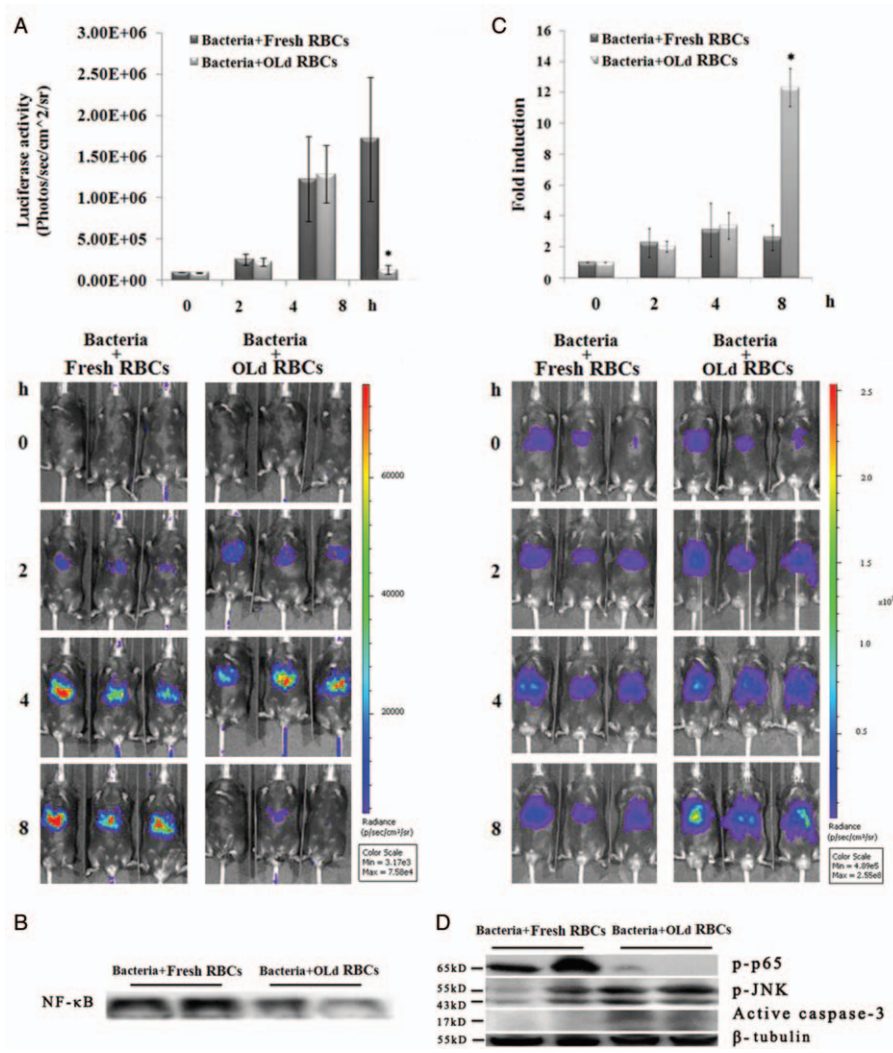


FIG. 6. **Stored-RBC-induced hepatic apoptosis was dependent on activation of the NF-κB and JNK signaling pathways.** A, Dynamic NF-κB activation detected by BLI in *P aeruginosa*-infected mice transfused with RBCs. B, NF-κB activation induced by RBCs in *P aeruginosa*-infected mice in hepatocytes at 8 h post-treatment, as confirmed by EMSA. C, Monitoring the *in vivo* activation of caspase-3 upon treatment with RBCs in *P aeruginosa*-infected mice. D, Representative western blot analysis of p-65, p-JNKs, and activated caspase-3 proteins. \**P* < 0.05 (n = 5/group). NF-κB indicates nuclear factor-kappaB.

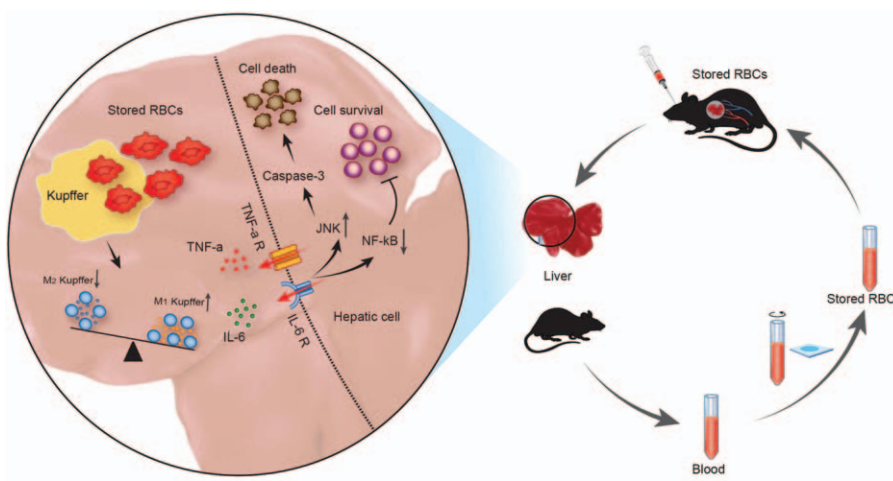


FIG. 7. **Mechanisms schematics of stored RBCs effect on liver injury.** Stored RBCs transfusion aggravates the liver injury induced by bacterial infection, associating with increased activation of M1-polarized Kupffer cells, which produce many inflammatory cytokines.

Bioluminescence imaging has been proven to be a highly sensitive method for monitoring gene expression in transgenic mice harboring the luciferase reporter (32). The noninvasive nature of this technology enables convenient longitudinal studies, which leads to the generation of statistically preferable data sets with fewer animals (33, 34).

Mice RBCs may not store as well as human RBCs. At the same time, although we tried to mimic standard human sepsis, differences in clinical practice remain. Findings in this mice model should be extended to human transfusions with caution. Nonetheless, the findings obtained in the mouse model may be confirmed by randomized prospective human studies.

The limitation of our study is that mice were not anemic before transfusion, as is the case in most transfused patients. Future studies will aim at potentially susceptible mice models, such as those with hemoglobinopathies (i.e., sickle cell anemia and beta thalassemia) and acutely ill neonatal mice that mimic as closely as possible standard human intensive care.

In conclusion, the results of our study provide a new understanding of the mechanism whereby transfusion with older RBCs exacerbates gram-negative infection and explains how stored RBCs influence the pathogenesis of sepsis-associated hepatic injury and liver-mediated immune responses to sepsis. The understanding of this mechanism will be helpful in providing guidance toward making transfusions safer.

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