Caspase-1 Is Hepatoprotective during Trauma and Hemorrhagic Shock by Reducing Liver Injury and Inflammation

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Adaptive immune responses are induced in liver after major stresses such as hemorrhagic shock (HS) and trauma. There is emerging evidence that the inflammasome, the multiprotein platform that induces caspase-1 activation and promotes interleukin (IL)-1β and IL-18 processing, is activated in response to cellular oxidative stress, such as after hypoxia, ischemia and HS. Additionally, damage-associated molecular patterns, such as those released after injury, have been shown to activate the inflammasome and caspase-1 through the NOD-like receptor (NLR) NLRP3. However, the role of the inflammasome in organ injury after HS and trauma is unknown. We therefore investigated inflammatory responses and end-organ injury in wild-type (WT) and caspase-1$^{-/-}$ mice in our model of HS with bilateral femur fracture (HS/BFF). We found that caspase-1$^{-/-}$ mice had higher levels of systemic inflammatory cytokines than WT mice. This result corresponded to higher levels of liver damage, cell death and neutrophil influx in caspase-1$^{-/-}$ liver compared with WT, although there was no difference in lung damage between experimental groups. To determine if hepatoprotection also depended on NLRP3, we subjected NLRP3$^{-/-}$ mice to HS/BFF, but found inflammatory responses and liver damage in these mice was similar to WT. Hepatoprotection was also not due to caspase-1–dependent cytokines, IL-1β and IL-18. Altogether, these data suggest that caspase-1 is hepatoprotective, in part through regulation of cell death pathways in the liver after major trauma, and that caspase-1 activation after HS/BFF does not depend on NLRP3. These findings may have implications for the treatment of trauma patients and may lead to progress in prevention or treatment of multiple organ failure (MOF).

INTRODUCTION

Trauma accounts for a major proportion of deaths worldwide (1). Among the causes of trauma-associated morbidity and mortality is a dysfunctional systemic immune response to severe injury, seen early as exaggerated systemic inflammatory response syndrome and late as a sustained counterregulatory antiinflammatory response (2). An overwhelming posttraumatic systemic inflammatory response can lead to organ dysfunction or failure in the setting of tissue injury and systemic hypoperfusion. This result is thought to occur through the release of reactive oxygen species, chemokines and proinflammatory cytokines such as interleukin (IL)-6, IL-12 and IL-18 by macrophages and endothelial cells and subsequent activation and localization of polymorphonuclear neutrophils in organs remote from the site of injury (3,4).

We (5,6) and others (7) have shown that the initial inflammatory response after hemorrhagic shock (HS) and peripheral tissue trauma is driven by the activation of Toll-like receptor-4 signaling by damage-associated molecular pattern (DAMP) molecules, such as high mobility group box-1 (HMGB1). DAMPs, such as extracellular matrix components and reactive oxygen species released after HS and trauma, have also been shown to activate other pattern recognition receptors, NOD-like receptors (NLRs), leading to inflammasome activation and subsequent activation and cleavage of caspase-1 (8). Caspase-1 activation is required for the proteolytic maturation of cytokines known to be involved in the injury response, namely IL-1β and IL-18, as well as other roles in inflammation and cell death pathways that may also influence the host response to ischemia and injury. Caspase-1 also plays a role in pyroptosis (caspase-1–dependent cell death) during infection (9), as well as a role in the regulation of both glucose and lipid metabolism, and cell survival (10,11). Many of

these functions of caspase-1 have been shown to be independent of the production of IL-1β and IL-18 through experiments involving double knockout mice in a model of sepsis (12).

Given this growing body of evidence indicating a diverse, multifaceted role for caspase-1 in inflammation, we hypothesized that it would play a significant role in the setting of trauma/posttraumatic inflammation. Therefore, we subjected wild-type (WT) and caspase-1$^{-/-}$ mice to HS with bilateral femur fracture (HS/BFF) with the hypothesis that caspase-1 deficiency would attenuate the proinflammatory response with decreased levels of inflammatory cytokines, therefore decreasing remote organ damage after severe trauma. However, in contradiction to our hypothesis, we found an increased proinflammatory cytokine profile, increased hepatocellular death and cellular damage in caspase-1$^{-/-}$ mice compared with WT mice after HS/BFF. Our data therefore suggest that caspase-1 balances the posttraumatic inflammatory response and is an important component of hepatocellular survival. We also show that caspase-1 activation after severe trauma is unlikely to occur through activation of the NLRP3 inflammasome, since we did not see increased inflammation or increased liver damage in NLRP3$^{-/-}$ mice after HS/BFF compared with WT mice. These findings shed new light on the role of caspase-1 in the setting of severe trauma and may lead to new therapeutic approaches for severely injured patients who are prone to multiple organ failure.

MATERIALS AND METHODS

Animal Care

All experimental protocols were approved by the Institutional Animal Use and Care Committee of the University of Pittsburgh. Experimental procedures were carried out in accordance with all regulations regarding the care and use of experimental animals, as published by the National Institutes of Health. Male C57BL/6 (WT) mice (Charles River Laboratories International, Wilmington, MA,

USA), caspase-1$^{-/-}$ mice (a gift from Richard Flavell, Yale University, New Haven, CT, USA [13]), and NLRP3 (NALP3, pyrin-1)$^{-/-}$ mice (Millennium Pharmaceuticals, Boston, MA, USA) aged 7–11 weeks, weighing 21–30 g, were used in experiments. Additionally, IL-18$^{-/-}$, IL-18R$^{-/-}$ and IL-1R1$^{-/-}$ mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Animals were allowed access to rodent chow and water *ad libitum*.

Genotyping of Caspase-1$^{-/-}$ Mice

Mice were genotyped by reverse transcriptase–polymerase chain reaction (PCR) of digested tail tissue using the following primers: *caspase-1* forward: GAGACATATAAGGGAGAAGGG; *caspase-1* reverse: ATGGCACACCACAGA TATCGG; and *caspase-1* neo: TGCTA AAGCGCATGCTCCAGACTG. PCR conditions used were as follows: 94°C for 3 min; then 35 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 30 s; and then held at 72°C for 5 min before cooling to 4°C until run on 2% agarose gel. Bands were visualized using ethidium bromide. WT mice were identified by a single band at 500 bp. Caspase-1$^{-/-}$ mice were identified by a single band at 300 bp. Heterozygous mice had both bands visible.

Experimental Groups

Mice from each mouse strain were assigned to three groups: control (no manipulation, n = 2–3 per strain), sham (femoral artery cannulation only, n = 3–5 per strain) and HS/BFF (1.5 h HS + 4.5 h fluid resuscitation and bilateral femur fracture, n = 4–6 per strain). HS/BFF group mice were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg; Ovation Pharmaceuticals, Deerfield, IL, USA) and inhaled isoflurane (Abbott Labs, Chicago, IL, USA). After bilateral groin dissections, both femoral arteries were cannulated using PE-10 tubing flushed with 2 units of heparin per animal (Pharmacia & Upjohn, Kalamazoo, MI, USA). Bilateral femur fractures were manually induced using hemostats. Baseline mean arterial pres-

sure was determined with the catheter connected to a blood pressure transducer (Micro-Med, Tustin, CA, USA). Then, using the second catheter, hemorrhage was induced to a mean arterial pressure of 25 mmHg for 1.5 h followed by fluid resuscitation with Ringer's solution (3× the volume of shed blood) through the catheter. Mice were kept under anesthesia for an additional 4.5 h. Sham group mice underwent initial cannulation procedures and anesthesia only. Control mice were sacrificed without any procedures performed to obtain physiological baseline levels. All animals were sacrificed after a total time course of 6 h.

Reagents

Antibodies for Western blot analysis: caspase-1 was from Upstate (Millipore, Billerica, MA, USA); β-actin was from Biovision (San Francisco, CA, USA); and caspase 8, 9 and 12 and cleaved poly(ADP-ribose) polymerase (PARP) were from Cell Signaling Technology (Danvers, MA, USA). Receptor interacting protein-1 (RIP-1) was from LifeSpan Biosciences (Seattle, WA, USA), and Bcl2 was from Abcam (Cambridge, MA, USA). Western blot analysis was performed as previously published (14). Western gel images were quantified by densitometry using Image J software (National Institutes of Health, Bethesda, MD, USA).

Blood and Tissue Collection and Plasma Analysis

Anesthetized mice were euthanized by cardiac puncture and blood withdrawal. Immediately after cardiac puncture, the liver and the lungs were harvested and snap-frozen in liquid nitrogen and then stored at –80°C. The collected heparinized blood samples were centrifuged at 2,300*g* for 10 min, and plasma was aliquoted and stored at –80°C. Immediately, one sample was used for quantification of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (Dry-Chem Veterinary Chemistry Analyzer, HESKA, Loveland, CO, USA; slides from Fujifilm Corporation, Asaka-shi Saitama, Japan). Plasma cytokine levels were determined
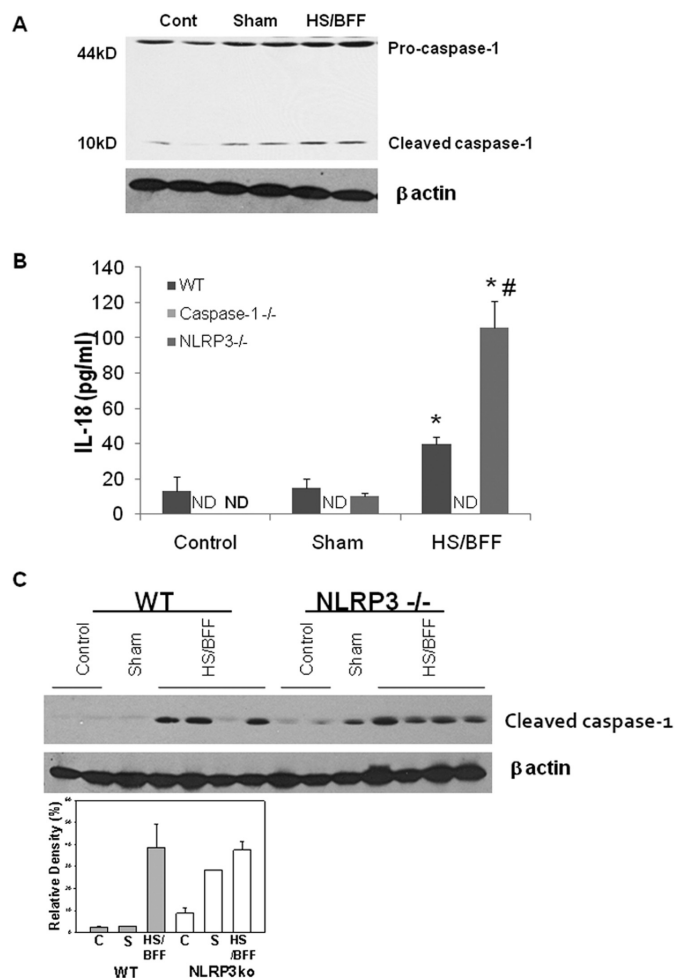
Figure 1. Caspase-1 is activated after HS/BFF. (A) Western blot images showing caspase-1 is activated (cleaved) in liver of WT (C57BL/6) mice at 6 h after HS/BFF. (B) Caspase-1$^{-/-}$ mice did not express IL18 at baseline or after HS/BFF, but NLRP3$^{-/-}$ mice expressed higher levels of IL18 than WT mice after HS/BFF measured by ELISA. (C) Western blot images with densitometry showing caspase-1 is activated (cleaved) in liver of NLRP3$^{-/-}$ mice at 6 h after HS/BFF. n = 3–6/group. Data show mean ± SEM. *$P < 0.05$, control versus HS/BFF; #$P < 0.05$ NLRP3$^{-/-}$ HS/BFF versus WT HS/BFF. C, control; ND, not detected; S, sham.

either by enzyme-linked immunosorbent assay (ELISA) for IL-18 (MBL, Naka-ku, Japan), IL-6 and IL-10 (R&D, Minneapolis, MN) or using Luminex™ multiplexing bead array platform (MiraiBio, Alameda, CA, USA) using a mouse cytokine bead sets for IL-1α, IL-1β, IL-6, IL-10, IL-12, keratinocyte-derived chemokine (KC) and monocyte chemoattractant protein (MCP)-1 (Invitrogen, San Diego, CA, USA).

Lung Myeloperoxidase Activity

Myeloperoxidase (MPO) activity was determined by ELISA of tissue lysates from lungs. The assay was performed according to the manufacturer's instructions (Mouse MPO ELISA kit; Cell Sciences, Canton, MA, USA). Briefly, 10 mg frozen lung tissue was homogenized in lysis buffer with a Tissue Tearor™ machine (Biospec Products, Bartlesville, OK, USA). After centrifugation at 1,500g for 15 min, supernatants were collected and diluted five-fold. Subsequently, MPO activity of each sample was determined and then standardized to its respective protein concentration (BCA Protein Assay Kit; Pierce, Rockford, IL, USA).

Immunofluorescence and Confocal Microscopy

Portions of harvested livers were fixed in 2% paraformaldehyde for 2 h followed by cryopreservation. Apoptotic cells in liver sections were identified using terminal deoxynucleotidyl-transferase dUTP nick end-labeling (TUNEL) staining following the manufacturer's protocol (Promega Corporation, Madison, WI). Nuclei were counterstained with Hoechst nuclear stain (Invitrogen). TUNEL-positive cells were imaged using a Nikon microscope (Nikon, Melville, NY, USA) and quantitated using a Metamorph™ image acquisition and analysis system (Universal Imaging, West Chester, PA, USA). TUNEL-positive cells from six random fields per section were counted blindly and expressed as a percentage of the total cell number for those fields. Immunofluorescence was used to determine the number of Ly-6G-positive neutrophils in liver sections. Liver sections (5 μm) were incubated with 2% bovine serum albumin (BSA) in PBS for 1 h, followed by five washes with PBS + 0.5% BSA (PBB). The samples were then incubated with rat Ly-6G primary antibody (1:100; BD Pharmingen, San Diego, CA, USA) for 1 h at 37°C. Samples were washed 5× with PBB followed by incubation in Cy3 secondary antibody diluted in PBB (1:1,000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Samples were washed 3× with PBB, followed by a single wash with PBS before a 30-s incubation with Hoechst nuclear stain. Nuclear stain was removed and samples were washed with PBS before being cover-slipped using Gelvatol (23 g polyvinyl alcohol 2000, 50 mL glycerol, 0.1% sodium azide to 100 mL PBS). Positively stained cells in six random fields were imaged on a Fluoview 1000 confocal scanning microscope (Olympus, Melville, NY, USA). Imaging conditions were maintained at identical settings with original gating performed using the negative control (no primary antibody).
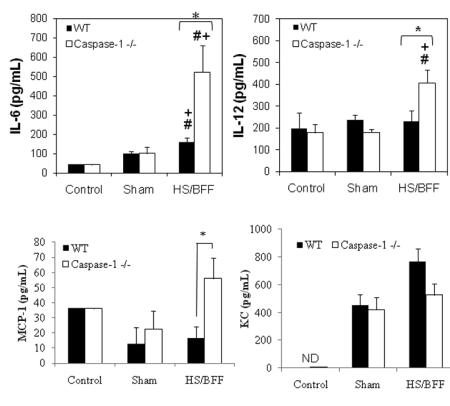
Figure 2. Increased proinflammatory cytokine and chemokine levels in caspase-1$^{-/-}$ mice after HS/BFF. IL6, IL12 and MCP-1 are all significantly increased in caspase-1$^{-/-}$ mice after HS/BFF compared with WT. KC increases in both WT and caspase-1$^{-/-}$ mice after HS/BFF, but is not different between the mouse strains. n = 3–6/group; data show mean ± SEM; *$P < 0.05$ WT versus caspase-1$^{-/-}$ HS/BFF; #$P < 0.05$ versus control; +$P < 0.05$ versus sham. ND, not detected.

Statistical Analysis

Statistical analysis was performed by analysis of variance (with *post hoc* testing according to the Student-Newman-Keuls method) and Student *t* test procedures using Sigmaplot 11 software (Systat Software, San Jose, CA, USA). Data are presented as mean ± standard error of mean (SEM) with differences being accepted as statistically significant if the *P* value was <0.05.

RESULTS

Caspase-1 Is Activated during HS/BFF in Mice

To assess the role of the inflammasome and caspase-1 in the inflammatory response and end-organ injury that occurs after trauma, we compared the responses of WT, caspase-1$^{-/-}$ and NLRP3$^{-/-}$ mice subjected to HS/BFF. Caspase-1 was activated (cleaved) in livers of WT mice at 6 h after HS/BFF (Figure 1A). The lack of functional caspase-1 in caspase-1$^{-/-}$ mice was confirmed by measuring circulating IL-18 levels in unmanipulated control mice, sham-treated mice (femoral artery

cannulation with anesthesia alone) and mice subjected to HS/BFF. As shown in Figure 1B, IL-18 was detectable at baseline in WT mice and also in sham-treated WT mice at low levels. IL-18 levels were significantly increased in WT mice at 6 h after HS/BFF compared with control or sham mice (Figure 1B). As expected, caspase-1$^{-/-}$ mice did not express any detectable IL-18 even after HS/BFF. We also investigated IL-18 levels in NLRP3$^{-/-}$ mice after HS/BFF, to determine whether this is the main inflammasome activated during trauma. Interestingly, IL-18 levels in NLRP3$^{-/-}$ mice were significantly higher than in WT mice (Figure 1B), and there was similar activation of caspase-1 in NLRP3$^{-/-}$ liver after HS/BFF compared with WT (Figure 1C). These data provide evidence for activation of caspase-1 within the initial 6 h after HS/BFF, but also suggest that NLRP3 is not the main inflammasome involved in this activation. Although IL-1β is also processed by caspase-1, this cytokine has not been proven as a reliable marker for caspase-1 activation, since there are multiple other pathways that can cleave pro–IL-1β to the active form (15). We detected low levels of IL-1β in plasma of both WT (21.2 ± 4.6 pg/mL) and caspase-1$^{-/-}$ (23.9 ± 12.1 pg/mL) mice at 6 h after HS/BFF.

Caspase-1$^{-/-}$ Mice Produce Increased Levels of Inflammatory Cytokines after Trauma

We next assessed the magnitude of the systemic inflammatory response by measuring circulating levels of several cytokines and chemokines known to be elevated after trauma. As expected, levels of IL-6, IL-12, KC and MCP-1 were all increased after HS/BFF in WT mice, with the greatest increases in IL-6 and KC (Figure 2). Unexpectedly, even greater increases in IL-6, IL-12 and MCP-1 were seen in caspase-1$^{-/-}$ mice after HS/BFF (Figure 2), with no differences between WT and NLRP3$^{-/-}$ circulating cytokine levels (data not shown). These data suggest that the absence of caspase-1 leads to a greater initial systemic inflammatory re-

sponse that does not depend on NLRP3 inflammasome activation. Interestingly, KC levels were not different between WT and caspase-1$^{-/-}$ mice after HS/BFF at 6 h.

Caspase-1$^{-/-}$ Mice Have Worse Organ Damage after Severe Trauma Compared with WT Mice

As markers of liver damage, we measured plasma levels of AST and ALT in WT, caspase-1$^{-/-}$ and NLRP3$^{-/-}$ mice after HS/BFF. AST and ALT levels were significantly increased in both WT and caspase-1$^{-/-}$ mice after HS/BFF compared with baseline levels (Figure 3A). However, the levels of both AST and ALT were significantly higher in plasma from caspase-1$^{-/-}$ mice compared with WT mice (Figure 3A). Again, there was no difference in AST and ALT levels in NLRP3$^{-/-}$ mice compared with WT mice at 6 h after HS/BFF (Figure 3A). These data further suggest that caspase-1 activation independent of NLRP3 inflammasome is protective during trauma, with increased liver damage corresponding well with increased cytokine levels in caspase-1$^{-/-}$ mice.

To further investigate the extent of increased liver damage in caspase-1$^{-/-}$ mice after HS/BFF, we assessed necrosis in the liver by hematoxylin and eosin (H&E) staining of liver sections as well as by RIP-1 expression in liver tissue. We found evidence of increased necrotic areas of liver in caspase-1$^{-/-}$ mice compared with WT mice by H&E staining, but again NLRP3$^{-/-}$ liver showed similar necrosis to WT liver at 6 h after HS/BFF (Figure 3B). Necrosis was mainly centrilobular and much more extensive in caspase-1$^{-/-}$ mice (Figure 3B). We also detected increased expression of RIP-1, a marker of necrosis, in caspase-1$^{-/-}$ whole liver lysates by Western blot compared with WT mice (Figure 3C). Even sham injury resulted in increased RIP-1 levels in caspase-1$^{-/-}$ liver compared with WT. Altogether, these data show evidence of increased cell death in the liver of caspase-1$^{-/-}$ mice after HS/BFF.

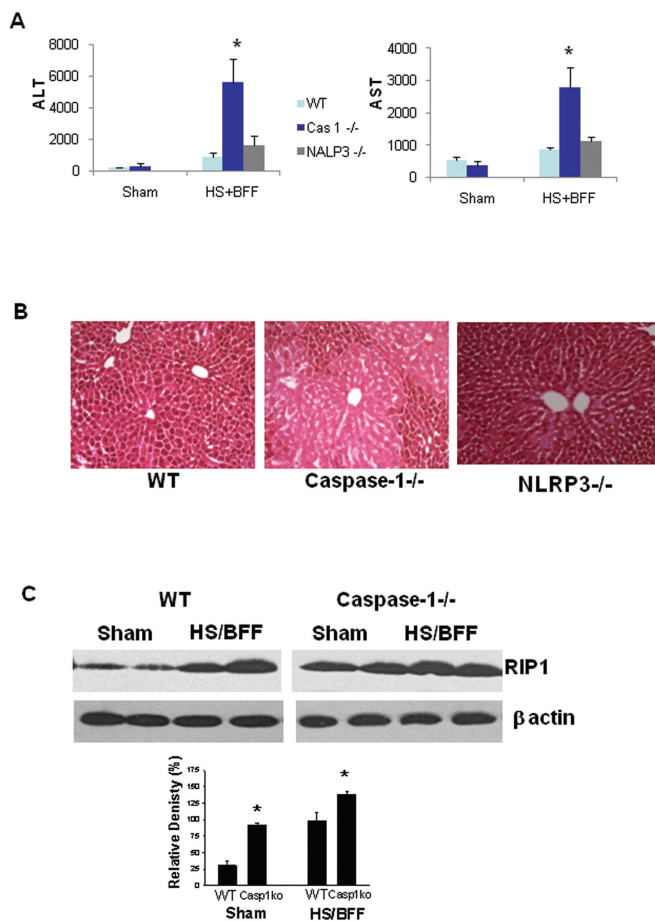Caspase-1-dependent cytokines, IL-1β and IL-18, have been shown in some

Figure 4. No difference in liver damage after HS/BFF in IL18ko, IL18Rko or IL1R1ko mice compared with WT (C57BL/6). AST and ALT levels in plasma at 6 h after HS/BFF are shown. n = 3 or 6/group. Data show mean ± SEM.

Figure 3. Increased remote organ damage after HS/BFF in caspase-1$^{-/-}$ mice. (A) ALT (*left*) and AST (*right*) in WT, caspase-1$^{-/-}$ and NLRP3$^{-/-}$ mice after HS/BFF. (B) H&E staining of liver from WT, caspase-1$^{-/-}$ and NLRP3$^{-/-}$ mice at 6 h after HS/BFF. (C) Western blot images and densitometry showing RIP-1 levels in whole liver cell lysates from WT and caspase-1$^{-/-}$ mice. n = 3–6/group. Data show mean ± SEM. *$P < 0.05$ WT versus caspase-1$^{-/-}$ HS/BFF.

studies of inflammation to be protective in certain models. We therefore wanted to determine whether a lack of IL-18 or IL-1β signaling was responsible for the increased liver damage seen in caspase-1$^{-/-}$ mice after HS/BFF. We used commercially available IL-18ko, IL-1R1ko and IL-18Rko mice and subjected them to HS/BFF similarly to caspase-1$^{-/-}$ mice. There were no statistical differences in either AST or ALT levels between these strains of mice and WT (C57BL/6) mice either after sham injury or after HS/BFF (Figure 4). IL-1R1ko mice trended toward lower levels of AST and ALT than WT or IL-18 signaling-deficient mice, but this was not statistically significant. These data suggest that neither IL-1β nor

IL-18 signaling is protective during HS/BFF.

Increased Neutrophil Influx in Caspase-1$^{-/-}$ Liver Compared with WT

We also determined neutrophil influx into both lung and liver as further markers of organ inflammation (Figure 5). Liver Ly-6G levels were significantly increased in caspase-1$^{-/-}$ mice compared with WT mice after HS/BFF (Figure 5B). However, pulmonary MPO levels were similar between WT and caspase-1$^{-/-}$ mice despite being significantly increased compared with sham and baseline (Figure 5A). These data may suggest caspase-1 regulates proinflammatory pathways preferentially in the liver, or that at the
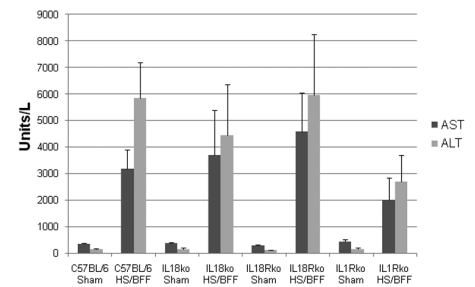
time point measured, any early or late differences in neutrophil accumulation in the lung may not be present at 6 h.

Caspase-1$^{-/-}$ Mice Have Increased Apoptosis in Liver after Trauma Compared with WT Mice

As caspase-1$^{-/-}$ mice showed increased proinflammatory cytokine production and also increased indication of liver damage after trauma compared with WT mice, we wanted to determine whether these differences were secondary to changes in apoptosis in the liver. Caspase-1 is known to have regulatory effects on apoptosis, as well as regulating another form of inflammatory cell death, pyroptosis (16). Both apoptosis and pyroptosis increase TUNEL staining in cells. We therefore determined the number of TUNEL-positive cells in liver sections from WT and caspase-1$^{-/-}$ mice after sham surgery and after HS/BFF, compared with unmanipulated controls. TUNEL staining increased significantly in livers of both WT and caspase-1$^{-/-}$ mice after sham surgery alone compared with controls (Figure 6A). Levels of TUNEL staining were significantly increased again in caspase-1$^{-/-}$ mice after HS/BFF, but this increase did not occur in WT mice after HS/BFF (Figures 6A, B).

These data suggest an increase in apoptosis in caspase-1$^{-/-}$ mice after HS/BFF because pyroptosis is considered to be caspase-1 and IL-1β dependent (16).
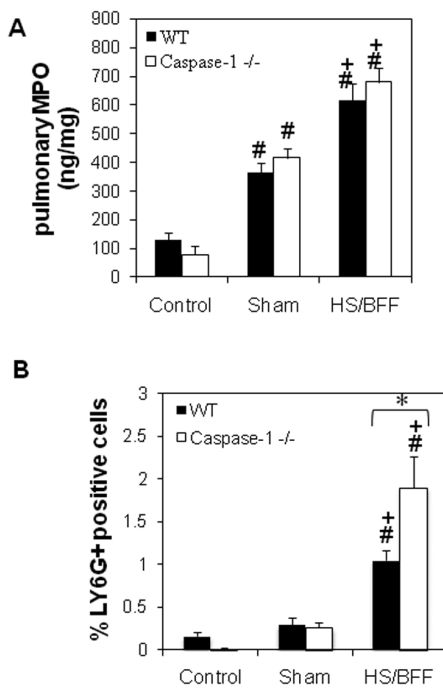
Figure 5. Increased liver neutrophils in caspase-1^{-/-} mice after HS/BFF. (A) Pulmonary MPO level in WT and caspase-1^{-/-} mice after HS/BFF. (B) Quantification of LY6G (neutrophil marker) immunohistochemical staining in liver sections of WT and caspase-1^{-/-} mice after HS/BFF. n = 3–6/group. Data show mean ± SEM. *$P < 0.05$ WT versus caspase-1^{-/-} HS/BFF; #$P < 0.05$ versus control; +$P < 0.05$ versus sham.

We therefore investigated levels of other apoptosis markers in liver of caspase-1^{-/-} and WT mice by Western blot. There were increased levels of proapoptotic caspase-12 and cleaved PARP (cPARP) at baseline in caspase-1^{-/-} liver compared with WT (Figures 6C, D). Although neither caspase-12 nor cPARP levels increased in caspase-1^{-/-} mice after HS/BFF, levels remained statistically significantly higher in caspase-1^{-/-} liver than in WT after HS/BFF. Caspase-8 and caspase-9 levels were significantly higher at baseline in WT liver compared with caspase-1^{-/-} liver (Figures 6C, D). Caspase-8 levels significantly increased in caspase-1^{-/-} liver after both sham injury and HS/BFF and were then significantly higher than corresponding WT levels. WT levels of caspase-9 decreased signifi-
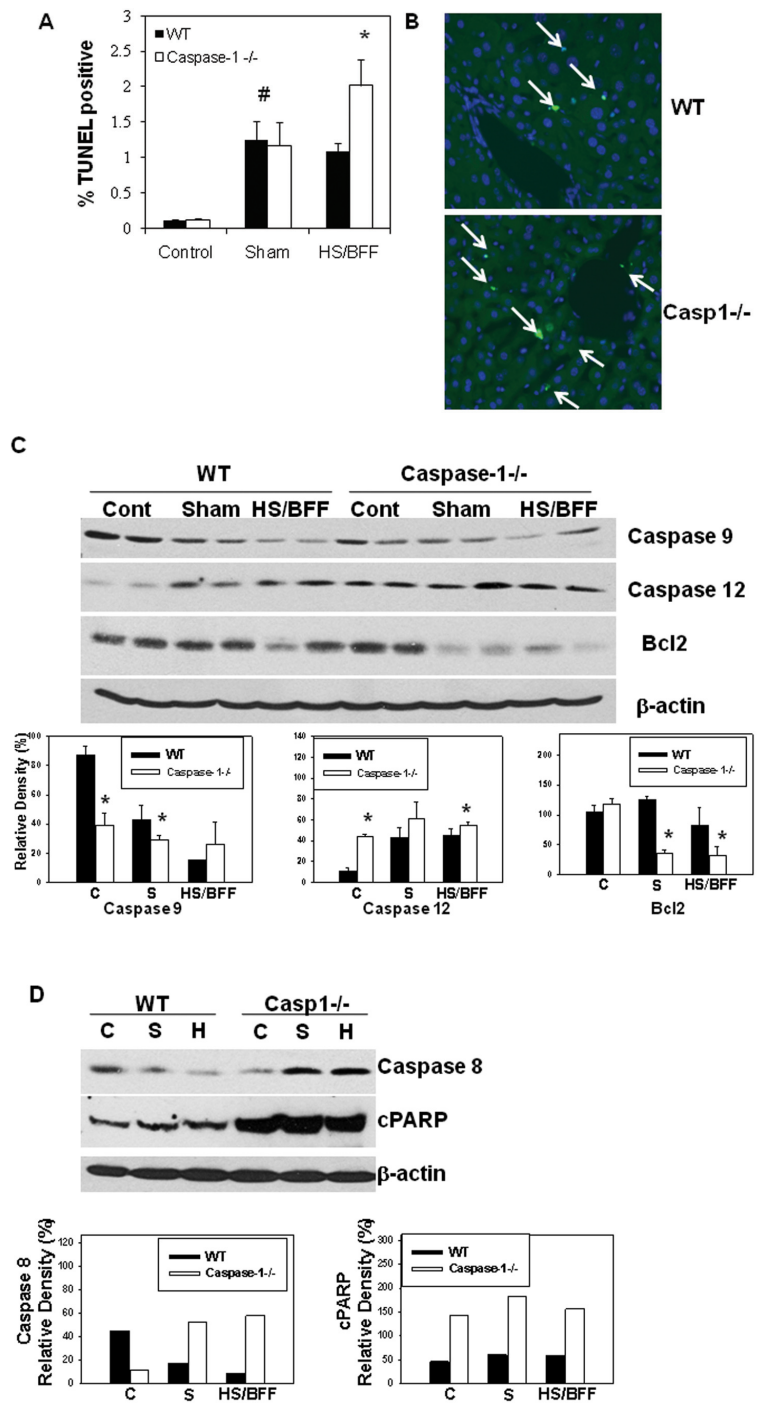

Figure 6. Increased liver apoptosis of caspase-1^{-/-} mice compared with WT after HS/BFF. (A) Percentage of TUNEL-positive hepatocytes per high-power vision field in WT and caspase-1^{-/-} mice after HS/BFF. (B) Representative confocal microscopy images of liver sections showing TUNEL staining (green fluorescence, marked with white arrows) in WT and caspase-1^{-/-} mice after HS/BFF. Western blot images with densitometry analysis showing levels of caspase-9, caspase-12 and Bcl2 (C) and caspase-8 and cPARP (D) in whole-cell liver lysates of WT and caspase-1^{-/-} mice. C, control; S, sham. Images representative of three separate Western blots. n = 3–6/group. Data show mean ± SEM. *$P < 0.05$ WT versus caspase-1^{-/-}; #$P < 0.05$ versus control.

cantly after HS/BFF, but caspase-9 levels in caspase-1$^{-/-}$ liver remained unchanged.

Changes in the antiapoptotic protein, Bcl2, were also seen in caspase-1$^{-/-}$ liver. At baseline, Bcl2 levels were similar between WT and caspase-1$^{-/-}$ mice (Figure 6C). However, Bcl2 levels were significantly decreased in caspase-1$^{-/-}$ liver after HS/BFF and even after minor sham injury. However, Bcl2 levels did not change in WT liver. Altogether these data show an increase in overall levels of proapoptotic proteins and a concomitant decrease in the antiapoptotic protein, Bcl2. Therefore, these findings support a regulatory role for caspase-1 in cell death pathways in the liver, which may result in hepatoprotection after HS/BFF.

DISCUSSION

Posttraumatic systemic inflammation plays a pivotal role in the development of subsequent remote organ damage (17,18). We have shown in this study that activation of caspase-1 after severe trauma may be protective in the liver and that this protection may be partly through the regulation of cell-death pathways. Caspase-1 activation (cleavage) is known to occur through the formation of the inflammasome, a signaling platform involving NLRs and apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) (19). Cleavage of caspase-1 then classically leads to the cleavage of pro-IL-1β, and pro-IL-18 with the subsequent release of active proinflammatory cytokines into systemic circulation (18,20,21). Blocking inflammasome formation and activation may therefore be a useful strategy in modifying the inflammatory response, especially in diseases where IL-1β plays a main role in inflammation, including during the inflammatory response to trauma (22,23).

We therefore expected that in mice lacking the main inflammasome effector protein, caspase-1, there would also be a reduction in the level of the inflammatory response associated with a severe trauma model that includes bilateral femur fracture and tissue hypoxia sec-

ondary to HS. Indeed, animal studies using pharmaceutical inhibition of caspase-1 or using caspase-1-deficient mice have shown decreases in inflammation in several models of infection or local inflammation/tissue irritation, including renal ischemia-reperfusion injury (24), acute pancreatitis (25), acute lung injury (26) and myocardial infarction (27). However, in contrast to these findings, we showed an increase in liver damage in mice deficient in caspase-1 in the setting of severe trauma, suggesting that, in this model, caspase-1 is protective, similar to data shown in a model of septic shock (12).

Caspase-1 is most well known for being the enzyme responsible for the activation and release of IL-1β and IL-18. However, hepatocytes (the main liver cell type) do not produce much of either cytokine when stressed or hypoxic. This result suggests that caspase-1 may play a different role in hepatocytes compared with activation of caspase-1 in immune cells, such as Kupffer cells. Caspase-1 is known to have many potential protein cleavage targets within a cell, including those related to cell-signaling pathways and cell metabolism (11,28). Our data provide evidence of the role of caspase-1 in the liver to protect cells from stress. If caspase-1 is absent, cells are more sensitive to stress-related inflammation and have increased susceptibility to cell death, particularly by apoptosis. This information is potentially important when thinking about new therapies to restrict inflammation after trauma and shows that we still have further to go in our understanding of the regulation of immune responses to trauma and injury.

One of our main findings is the increase in the extent of organ damage in caspase-1$^{-/-}$ mice in our trauma model compared with WT mice. This effect was seen mainly in the liver at the time point we investigated, with increased AST and ALT as well as an increase in the recruitment of neutrophils to the liver in caspase-1$^{-/-}$ mice. The liver is susceptible to damage secondary to increases in circulating proinflammatory cytokines,

such as the increases seen in IL-6 and IL-12 in caspase-1$^{-/-}$ mice after HS/BFF. Reducing cytokine-induced liver damage may therefore be one mechanism through which caspase-1 is protective in our model. However, it is also unclear at present if the higher cytokine levels in the caspase-1$^{-/-}$ mice contribute to the increased liver damage, or if higher cytokine levels are the result of greater organ injury.

Another important finding from our study is that activation of caspase-1 does not seem to occur primarily through the NLRP3 inflammasome. There are many NLRPs that have been identified as being expressed in different cell types, but at present, only a handful have been shown to form an inflammasome and activate caspase-1, including NLRP1, NLRP3, absent in melanoma-2 (AIM2) and ice protease activating factor (IPAF) (29). Our findings are particularly interesting because, to date, almost all DAMPs, including reactive oxygen species, activate the NLRP3 inflammasome. However, AIM2 may also be important in our model, since it has been shown to be activated by DNA, which can be released as a result of tissue damage and subsequent cell death. It is also possible that DAMPs can activate another inflammasome, such as NLRP1, or lead the formation of the ASC inflammasome without the need for an NLR, as has been shown in some infection models (30,31). Further studies are needed to fully identify the main activators of caspase-1 after trauma.

We have shown evidence that caspase-1 deficiency may also predispose to an increased tendency to the induction of apoptosis during trauma. Caspase-1 has been shown to regulate apoptosis pathways through binding to antiapoptotic proteins, Bcl2 and BclXL (32). We show here that caspase-1$^{-/-}$ mice significantly downregulate liver expression of Bcl2 after trauma, and this may have profound effects on apoptosis. A recent report also identified another regulator of apoptosis, PARP, as a cleavage target for caspase-1 itself (28). Our results support an effect of absence of caspase-1 leading

to increased baseline cPARP formation. Cleavage of PARP even at baseline in caspase-$1^{-/-}$ liver suggests that caspase-1 liver may be more susceptible to induction of apoptosis under stressed conditions.

Apoptosis is only one way a cell can die, and there are many survival pathways that regulate the induction of apoptosis and cell death overall. Accordingly, the list of substrates for caspase-1 is increasing, providing the potential for caspase-1 to have a hand in regulating multiple cell death and cell survival pathways that may be differentially activated in different models. We are continuing to explore the role of caspase-1-dependent cell activation further in HS and trauma to fully characterize these pathways. Improving our knowledge of the exact role and interactions of caspase-1 may provide new therapeutic options for the prevention of organ injury secondary to trauma.

DISCLOSURE
The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

REFERENCES
1. Krug E, Sharma G, Lozano R. (2000) The global burden of injuries. *Am. J. Public Health.* 90:523–6.
2. Dewar D, Moore F, Moore E, Balogh Z. (2009) Postinjury multiple organ failure. *Injury.* 40:912–8.
3. Flohé S, Flohé S, Schade F. (2008) Invited review: deterioration of the immune system after trauma: signals and cellular mechanisms. *Innate Immun.* 14:333–44.
4. Hildebrand F, Pape H, Krettek C. (2005) The importance of cytokines in the posttraumatic inflammatory reaction [in German]. *Unfallchirurg.* 108:793–4, 796–803.
5. Prince J, *et al.* (2006) Toll-like receptor-4 signaling mediates hepatic injury and systemic inflammation in hemorrhagic shock. *J. Am. Coll. Surg.* 202: 407–14.
6. Yang R, *et al.* (2006) Anti-HMGB1 neutralizing antibody ameliorates gut barrier dysfunction and improves survival after hemorrhagic shock. *Mol. Med.* 12:105–14.
7. DeMarco R, Fink M, Lotze M. (2005) Monocytes promote natural killer cell interferon gamma production in response to the endogenous danger signal HMGB1. *Mol. Immunol.* 42:433–44.
8. Zhou R, Tardivel A, Thorens B, Choi I, Tschopp J. (2010) Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nat. Immunol.* 11:136–40.
9. Brennan M, Cookson B. (2000) Salmonella induces macrophage death by caspase-1-dependent necrosis. *Mol. Microbiol.* 38:31–40.
10. Gurcel L, Abrami L, Girardin S, Tschopp J, van der Goot F. (2006) Caspase-1 activation of lipid metabolic pathways in response to bacterial pore-forming toxins promotes cell survival. *Cell.* 126:1135–45.
11. Shao W, Yeretssian G, Doiron K, Hussain S, Saleh M. (2007) The caspase-1 digestome identifies the glycolysis pathway as a target during infection and septic shock. *J. Biol. Chem.* 282:36321–9.
12. Sarkar A, *et al.* (2006) Caspase-1 regulates Escherichia coli sepsis and splenic B cell apoptosis independently of interleukin-1beta and interleukin-18. *Am. J. Respir. Crit. Care Med.* 174:1003–10.
13. Kuida K, *et al.* (1995) Altered cytokine export and apoptosis in mice deficient in interleukin-1beta converting enzyme. *Science.* 267:2000–3.
14. Scott M, Billiar T. (2008) Beta2-integrin-induced p38 MAPK activation is a key mediator in the CD14/TLR4/MD2-dependent uptake of lipopolysaccharide by hepatocytes. *J. Biol. Chem.* 283:29433–46.
15. Fantuzzi G, *et al.* (1997) Response to local inflammation of IL-1 beta converting enzyme deficient mice. *J. Immunol.* 158:1818–24.
16. Bergsbaken T, Fink S, Cookson B. (2009) Pyroptosis: host cell death and inflammation. *Nat. Rev. Microbiol.* 7:99–109.
17. Kaczorowski D, Mollen K, Edmonds R, Billiar T. (2008) Early events in the recognition of danger signals after tissue injury. *J. Leukoc. Biol.* 83:546–52.
18. Zedler S, Faist E. (2006) The impact of endogenous triggers on trauma-associated inflammation. *Curr. Opin. Crit. Care.* 12:595–601.
19. Latz E. (2010) The inflammasomes: mechanisms of activation and function. *Curr. Opin. Immunol.* 28:28–33.
20. Martinon F. (2008) Detection of immune danger signals by NALP3. *J. Leukoc. Biol.* 83:507–11.
21. Yu H, Finlay B. (2008) The Caspase-1 inflammasome: a pilot of innate immune responses. *Cell Host Microbe.* 4:198–208.
22. Geddes K, Magalhães J, Girardin S. (2009) Unleashing the therapeutic potential of NOD-like receptors. *Nat. Rev. Drug Discov.* 8:465–79.
23. Le G, Abbenante G. (2005) Inhibitors of TACE and caspase-1 as anti-inflammatory drugs. *Curr. Med. Chem.* 12:2963–77.
24. Chatterjee P, *et al.* (2004) Differential effects of caspase inhibitors on the renal dysfunction and injury caused by ischemia–reperfusion of the rat kidney. *Eur. J. Pharmacol.* 503:173–83.
25. Paszkowski A, Rau B, Mayer J, Möller P, Beger H. (2002) Therapeutic application of caspase 1/interleukin-1beta-converting enzyme inhibitor decreases the death rate in severe acute experimental pancreatitis. *Ann. Surg.* 235:68–76.
26. Zhang X, Zhu R, Xu W, Wan H, Lu H. (2007) Therapeutic effects of caspase-1 inhibitors on acute lung injury in experimental severe acute pancreatitis. *World J. Gastroenterol.* 13:623–7.
27. Frantz S, *et al.* (2003) Targeted deletion of caspase-1 reduces early mortality and left ventricular dilatation following myocardial infarction. *J. Mol. Cell Cardiol.* 35:685–94.
28. Shen J, *et al.* (2010) Caspase-1 recognizes extended cleavage sites in its natural substrates. *Atherosclerosis.* 210:422–9.
29. Bauernfeind F, *et al.* (2010) Inflammasomes: current understanding and open questions. *Cell. Mol. Life Sci.* 2010 Oct 31. [Epub ahead of print]
30. Lamkanfi M, Kanneganti T, Franchi L, Núñez G. (2007) Caspase-1 inflammasomes in infection and inflammation. *J. Leukoc. Biol.* 82:220–5.
31. Suzuki T, *et al.* (2007) Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in Shigella-infected macrophages. *PLoS Pathog.* 3:e111.
32. Clem R, *et al.* (1998) Modulation of cell death by Bcl-XL through caspase interaction. *Proc. Natl. Acad. Sci. U. S. A.* 95:554–9.