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DAMPs-activated neutrophil extracellular trap exacerbates sterile inflammatory liver injury

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Abstract

Innate immunity plays a crucial role in the response to sterile inflammation such as liver ischemia/reperfusion (I/R) injury. The initiation of liver I/R injury results in the release of damage associated molecular patterns (DAMPs), which trigger innate immune and inflammatory cascade via pattern recognition receptors. Neutrophils are recruited to the liver after I/R and contribute to the organ damage, innate immune and inflammatory responses. Formation of neutrophil extracellular trap (NET) has been recently found in response to various stimuli. However, the role of NETs during liver I/R injury remains unknown. We show that NETs form in the sinusoids of ischemic liver lobes *in vivo*. This was associated with increased NET markers, serum level of myeloperoxidase (MPO)-DNA complexes and tissue level of citrullinated-histone H3 compared to control mice. Treatment with peptidyl-arginine-deiminase (PAD) 4 inhibitor or DNase I significantly protected hepatocytes and reduced inflammation after liver I/R as evidenced by inhibition of NET formation, indicating the pathophysiological role of NETs in liver I/R injury. *In vitro*, NETs increase hepatocyte death and induce Kupffer cells to release proinflammatory cytokines. DAMPs, such as HMGB1 and histones, released by injured hepatocytes stimulate NET formation through Toll-like receptor (TLR4)- and TLR9-MyD88 signaling pathways. After neutrophil depletion in mice, the adoptive transfer of TLR4 knockout (KO) or TLR9 KO neutrophils confers significant protection from liver I/R injury with significant decrease in NET formation. In addition, we found inhibition of NET formation by PAD4 inhibitor or DNase I reduces HMGB1 and histone-mediated liver I/R injury.

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Conclusion—DAMPs released during liver I/R promotes NET formation through TLRs signaling pathway. Development of NETs subsequently exacerbates organ damage and initiates inflammatory responses during liver I/R.

Keywords

Sterile inflammation; ischemia/reperfusion injury; danger associated molecular pattern molecules; neutrophils; neutrophils extracellular traps

Introduction

Sterile inflammation is a pathophysiologic immune response in the absence of pathogens that accompanies injury of numerous organs and varied etiologies (1). It is of particular significance to the liver where sterile inflammation contributes to alcoholic steatohepatitis, nonalcoholic steatohepatitis (NASH), drug-induced liver injury, and ischemia-reperfusion (I/R). Such sterile inflammation is a key process in liver I/R injury that unavoidably occurs as a consequence of liver surgery when hepatic blood supply is temporarily occluded to reduce blood loss in resection or as an inherent consequence of liver transplantation, trauma or hypovolemic shock. It results in an initial hepatocellular damage from the interruption of oxygen supply (ischemia), followed by a subsequent, rapid inflammatory response upon reperfusion (1, 2). The resultant damaging effects of these events determine the postoperative outcome. Despite decades of scientific and clinical interest, therapeutic options remain ineffective to prevent or treat liver I/R injury (3). More research is needed to identify the mechanisms by which liver I/R promotes an injurious sterile inflammatory response.

Following liver I/R, damaged hepatocytes have been shown to release nuclear damage associated molecular patterns (DAMPs), such as High Mobility Group Box-1 (HMGB1) protein and extracellular histones, which exacerbate the hepatic injury through activation of Toll-like receptor (TLR)4 and 9 receptors (4, 5). The resultant activation creates an inflammatory milieu that incites the influx of inflammatory cells, including neutrophils. Neutrophils are recruited to the site of liver injury shortly after ischemia begins, thus mediating the early responses to tissue injury. Neutrophil infiltration and accumulation in the ischemic liver lobe further contributes to the inflammation-associated damage by releasing reactive oxygen species, numerous inflammatory mediators, as well as various proteolytic enzymes (6).

In addition to the mechanisms described above, novel aspects of neutrophil biology may contribute to I/R-induced liver injury. Activated neutrophils have been recently described to form Neutrophil Extracellular Traps (NETs) in response to various pathogenic infections and DAMPs (7). NETs are extracellular scaffolds consisting of nuclear DNA studded with granule proteins, histones, and cytoplasmic anti-microbials. Peptidyl-arginine-deiminase-4 (PAD4) catalyzed citrullination of proteins, including histone H3, is a required step preceding chromatin decondensation and release (8). NETs have been described as a beneficial mechanism of host defense against pathogens, serving to capture and destroy bacteria, fungi, and protozoa *in vitro* and *in vivo* (9); however, NETs have recently been

implicated as harmful contributors in various sterile inflammatory conditions including atherosclerosis, venous thrombosis, lung injury, and tumor metastasis, among others (10, 11).

The role of DAMPs released following ischemic liver injury in activating neutrophils to form NETs and the role of NETs themselves in liver I/R remain unknown. Elucidating the mechanisms of NET formation in liver I/R will increase our understanding of the molecular pathophysiology of liver ischemic injury and provide significant insight into the mechanisms by which ischemic tissues notify the immune system of impending cell damage. We found in this study that neutrophils form NETs in the setting of liver I/R. NET formation is dependent on DAMPs, such as HMGB1 and histones, released from stressed hepatocytes and mediate NET formation through TLR4 and TLR9 signaling. Targeting NETs using DNase I or specific PAD4 inhibitors ameliorated the hepatic I/R-induced injury in mice. As liver resection or transplantation represent potential cures for patients with malignancies or end stage liver disease, liver protective therapeutic strategies using DNase I or PAD4 inhibitors could minimize liver I/R injury and improve clinical outcomes.

Materials and Methods

Animals

Male wild-type (WT C57BL/6) mice (8-12 weeks old) were purchased from Jackson ImmunoResearch Laboratories. TLR4 knockout (KO) and WT, TLR9^{CpG/CpG} mutant and WT, TLR4/TLR9 double KO and WT, MyD88^{-/-} and MyD88^{+/+} mice were provided by Dr. Timothy Billiar (University of Pittsburgh Medical Center, Pittsburgh, PA). LysM^{eGFP} knockin mice were provided by Dr. Thomas Graf. Animal protocols were approved by the Animal Care and Use Committee of the University of Pittsburgh, and the experiments were performed in adherence to National Institutes of Health guidelines for the use of laboratory animals.

Liver ischemia/reperfusion

A nonlethal model of segmental (70%) hepatic warm ischemia and reperfusion was used as previously described (12). Mice received intraperitoneal injections of histones (25 mg/kg, Sigma-Aldrich), recombinant HMGB1 (rHMGB1, 10 µg per mouse), DNase I (2.5 mg or 5 mg/kg, Roche), or PAD4 inhibitor YW3-56 (10 mg/kg), or YW4-03 (10 mg/kg) (13) immediately after ischemia, or PBS 1h prior to ischemia. Sham animals underwent anesthesia, laparotomy, and exposure of the portal triad without hepatic ischemia.

Neutrophil depletion, isolation and adoptive transfer

Mouse neutrophils were isolated from bone marrow of tibias and femurs as described previously (10). Neutrophils were sorted on a BD Aria Plus high-speed sorter after incubation with APC-conjugated anti-mouse Ly6G antibody and APC-Cy7 CD11b (BD Biosciences) (purity, >96%) (**Supplementary Fig. 1**). Neutrophil depletion was performed as described previously (14) with an intra-peritoneal injection of 500 µg anti-Ly6G antibody (1A8) (BioXCell) 24 and 2 hours before I/R. TLR9KO, TLR4 KO or WT freshly isolated neutrophils were injected into the spleens of WT mice just before I/R.

Quantification of NETs—To quantify NETs in cell culture supernatant and in mouse serum, a capture ELISA myeloperoxidase (MPO) associated with DNA was performed as described previously (15). For the capture antibody, Mouse MPO ELISA kit (Hycult biotech, HK210-01) was used according to the manufacturer's directions. A peroxidase-labeled anti-DNA mAb (component No.2, Cell Death ELISA^{PLUS}, Roche; Cat. No: 11774424001) was used. Serum nucleosome quantification was performed using Cell Death Kit (Roche). Free serum DNA levels were quantified with the PicoGreen assay kit (Invitrogen).

Isolation, culture, and treatment of hepatocytes and Kupffer cells (KCs)—Hepatocytes were isolated, plated (3×10^6 cells/plate) and stimulated with hypoxia as previously described (4). Supernatants from hypoxic or necrotic hepatocytes were harvested and used as conditioned media in subsequent co-culture assays. KCs were collected as previously described (16).

***In vitro* NET formation**—Neutrophils were plated to adhere in coated plates for 1-hour before stimulation for 4 hours with Phorbol 12-myristate 13-acetate (PMA, 100 nM, Sigma-Aldrich), or histones (5, 25 or 50 $\mu\text{g/mL}$) or rHMGB1 (0.2, 1 or 2 $\mu\text{g/mL}$, gifted by Dr. Timothy Billiar), or 2 mL hypoxic or necrotic media from hepatocytes.

Immunofluorescent staining—For immunofluorescence staining, liver sections were fixed, stained, and imaged using confocal microscopy as previously described (16). Liver tissue or neutrophils were incubated with the specific primary antibodies for Ly6G (1:100, BD Bioscience), citrullinated-histone H3 (Cit-H3 1:50; Abcam), or histone H2A.X (1:800, Abcam). All slides were scanned under the same conditions for magnification, exposure time, lamp intensity and camera gain. Confocal images were acquired using Olympus Fluoview 1000 microscope with a PlanApo N ($\times 40$ with and without a 2.5 digital zoom). Sequential scanning was applied for acquiring individual emission channels when multiple fluorophores were involved. The thickness of the sections were imaged by focusing on the top of the section, setting the Z-axis to 0, and then refocusing to the bottom of the section, an average of 14 sections were acquired. Data images were acquired using FV10-ASW software and imported into Imaris (BITPLANE) for 3D volume reconstruction.

Liver damage assessment—Serum alanine aminotransferase (sALT) levels were measured using the DRI-CHEM 4000 Chemistry Analyzer System (HESKA). The extent of parenchymal necrosis in the ischemic lobes was evaluated as previously described (17).

SYBR green qRT-PCR—mRNA of TNF- α , IL-6, MCP-1, CXCL-10, IL-1 β , and β -actin was quantified in triplicate by SYBR Green qRT-PCR as previously described (17).

Immunoblotting—Western blot assays were performed using whole cell lysates from either liver tissue or neutrophils, or media. Membranes were incubated overnight using the following antibodies: PAD4 (1:1000 Cell signaling), Cit-H3 (1:500 Abcam), and actin as an internal control.

Fluorescence 2-photon intravital microscopy and visualization of NET

formation—For multichannel 2-photon intravital microscopy, the mouse median lobe was gently positioned and visualized on a customized glass coverslip over the microscope objective (Olympus FluoView FV1000, Olympus) after the falciform ligament was dissected, as described previously (18). NET formation was visualized by labeling extracellular DNA with SYTOX Green (5 μ M, Invitrogen). Approximately 30-second-long stacks were repeatedly scanned up to 60 times for a maximum imaging time of 30 minutes per location. Up to 5 different locations per ischemic lobe were imaged. All acquired movies were analyzed using Imaris.

Flow cytometry analysis—Hepatocytes and non-parenchymal cells were obtained from ischemic liver lobes after warm liver I/R. Cell death of hepatocytes (propidium iodide (PI) staining (19)) and neutrophils infiltration (CD11b⁺Ly6G⁺) was analyzed by flow cytometry as previously described (16).

Statistical analysis—Results are expressed as mean standard deviation (SD). Group comparisons were performed using ANOVA and Student's t-test. A $p < 0.05$ was considered statistically significant.

RESULTS

Neutrophil extracellular traps are formed in vivo after liver I/R

It has been shown that significant infiltration and accumulation of neutrophils occurs in ischemic lobes after liver I/R and neutrophils promote the inflammatory response and subsequent injury (14, 16). To investigate whether infiltrated neutrophils form NETs after liver I/R, we first sought to measure NET formation in the serum of mice undergoing liver I/R. When neutrophils form NETs, chromatin DNA associated with other neutrophil proteins is released locally and in the circulation. By measuring MPO-DNA complexes, we ensure that the circulating nucleosomes are derived from NETs. We found that the quantity of MPO-DNA complexes was significantly increased in mice undergoing 1 hour ischemia and 6 hours of reperfusion compared to sham mice. Similarly, mice undergoing extended ischemia (1.5 hours) further increased production of MPO-DNA (**Fig. 1A**). Serum levels of free DNA and nucleosomes, surrogate markers for NET formation, were also significantly higher after I/R compared to sham surgery (**Supplementary Fig. 2A and 2B**). To confirm the formation of NETs within the liver, we performed immunofluorescence staining of the ischemic liver lobe sections. In the sinusoids of ischemic lobes, after 1 hour of ischemic injury followed by 6 hours of reperfusion, Ly6G positive cells co-localized with extracellular histone H2AX and positively-stained web-like DNA, indicating NET formation (**Fig. 1B**). In comparison, few intact Ly6G positive cells were observed in sham-treated animals. Additionally, 3D images of Ly6G and Cit-H3 double staining also confirmed NET formation in ischemic liver lobes (**Fig. 1C**). We further confirmed our findings using intravital 2-photon confocal microscopy. Consistent with others' findings (18), while the liver microstructure was intact with sham operation, it was obliterated after liver I/R (**Fig. 1D and Supplementary Video 1 and 2**). We observed in real time the expulsion of extracellular DNA stained with SYTOX green from crawling GFP-labeled neutrophils after

liver I/R, suggesting DNA released from neutrophils during liver sterile inflammatory injury. No DNA was released from GFP-labeled neutrophils within the liver sinusoids of sham-operated mice. Ischemic liver lobes also exhibited significantly higher levels of citrullinated-histone H3, another specific marker of NET formation, after liver I/R compared to sham liver isolates (**Fig. 1E**). Taken together, these results indicate increased NET formation after liver I/R; however, the role of NETs remains uncertain.

Inhibition of NET formation by PAD4 inhibitors or DNase I protects the liver from I/R injury in mice

We next sought to evaluate the role of NETs in the setting of liver I/R. To inhibit NETs, we treated mice with either specific inhibitors of PAD4 (YW3-56 or YW4-03), an essential enzyme for NET formation that catalyzes citrullination of histone H3, or DNase I that has similarly been shown to alter NET function by cleaving the DNA strands comprising the extracellular NET structure. Circulating MPO-DNA complexes were significantly reduced in the YW3-56, YW4-03, and DNase I-treated mice (**Fig. 2A**). Serum levels of free DNA and nucleosomes were also significantly lower after I/R in PAD4 inhibitors or DNase I-treated mice compared to control-treated I/R mice (**Supplementary Fig. 3A and 3B**). In addition, liver tissue from YW3-56, YW4-03 or DNase I-treated mice had significantly lower levels of citrullinated-histone H3 compared to vehicle-treated mice undergoing liver I/R (**Fig. 2B**). We found that either monotherapy with YW3-56, YW4-03 or DNase I significantly reduced liver damage as demonstrated by reduced serum ALT levels compared with mice receiving vehicle (**Fig. 2C**). Histologic evaluation of liver damage was consistent with sALT levels, with severe sinusoidal dilatation and confluent pericentral hepatocellular necrosis present in liver tissue from vehicle-treated mice but not from YW3-56, YW4-03 or DNase I-treated mice (**Fig. 2D and Supplementary Fig. 3C and 3D**). In sum, these data provide evidence that inhibition of NET formation prevents liver I/R injury.

NETs are cytotoxic toward hepatocytes and initiate inflammatory responses both in vitro and in vivo

To further investigate the mechanism by which NETs damage the liver during liver I/R, we examined whether NETs are cytotoxic to hepatocytes. Bone marrow derived neutrophils were stimulated with PMA to form NETs (7). By flow cytometry, we found that the conditioned media obtained from PMA-stimulated neutrophils significantly increased cell death of hepatocytes compared with media from un-stimulated neutrophils. Co-incubation with PAD4 inhibitor lowered this cytotoxicity (**Fig. 3A**). Liver resident Kupffer cells (KCs), the major population of NPCs, have been shown to recruit neutrophils by releasing chemoattractants during liver I/R (20). However, whether NETs activate KCs to contribute to inflammatory responses during liver I/R remains unknown. We cultured KCs with the conditioned media obtained from PMA-stimulated neutrophils or control media from un-stimulated neutrophils. Both inflammatory cytokines and chemokines significantly increased in KCs stimulated with NET-media compared with control media (**Fig. 3B**). *In vivo*, we observed a significant decrease in both hepatocyte cell death (**Fig. 3C**) and production of pro-inflammatory mediators in mice treated with various NET inhibitors compared with sham treatment (**Fig. 3D**). These findings indicate that NETs mediate liver I/R injury by directly damaging parenchymal cells and activating other inflammatory cells. Of note, both

treatments of PAD4 inhibitors and DNase I did not alter early infiltration of neutrophils at 3 hours of reperfusion (**Supplementary Fig. 4**), suggesting that although NET inhibition reduces the production of inflammatory markers and organ injury, the early recruitment of neutrophils to site of injury is intact.

Histones and HMGB1, acting as DAMPs, induce NET formation

Various infectious and noninfectious stimuli have been reported to induce NETs; however, stimuli responsible for NET formation during liver I/R injury remain unknown. We first sought to mimic the ischemic micro-environment of I/R injury *in vitro*. We isolated murine hepatocytes and exposed the cells in culture to either hypoxia or induced necrosis by incubation at 60°C. The conditioned media from these cultures was incubated with bone marrow derived neutrophils. Similar to the PMA-treated neutrophils (positive control), significantly higher levels of MPO-DNA complexes were observed in the neutrophil media after stimulation with conditioned hepatocyte media, compared to levels found after normal media stimulation (negative control) (**Fig. 4A**). Consistently, conditioned media from either hypoxic or necrotic hepatocytes induced significant NET formation as shown by immunofluorescent imaging compared to normal media (**Supplementary Fig. 5A**).

We have previously reported that HMGB1 and histones are the major DAMPs released from stressed hepatocytes during liver I/R to invoke an inflammatory response and subsequent liver damage (4, 12). Therefore, we next determined whether exogenous histones or HMGB1 in isolation could induce NET formation. Neutrophils treated with different concentrations of histones, HMGB1 or PMA demonstrated a dose-dependent increase in citrullination of histone H3 as demonstrated by western blot (**Fig. 4B**). Furthermore, as anticipated, treatment with the PAD4 inhibitor significantly reduced this, despite exogenous stimulation (**Supplementary Fig. 5B**). Immunofluorescence confocal microscopy confirmed NET formation after stimulation by exogenous histones or HMGB1, and absence of NETs in normal controls. YW3-56, YW4-03 or DNase I treatment abolished NET formation induced by exogenous histones or HMGB1 (**Fig. 4C**). Of note, DNase I treatment destroyed the NET structure while having no effect on citrullination of histone H3. For additional confirmation, we visualized by 2-photon intravital confocal microscopy NET formation in real time in response to treatment with DAMPs. Real-time video acquisition demonstrated a stable and robust SYTOX green release, indicating NET formation, beginning 1 hour after stimulation with DAMPs (**Fig. 4D** and **Supplementary video 3**).

Histones and HMGB1 induce NET formation through TLR9 or TLR4 signaling

We and others previously reported that histones and HMGB1 released during liver I/R contribute to proinflammatory immune responses and organ damage via TLR9 and TLR4 and the downstream signaling molecule MyD88 (4, 12, 21, 22). Therefore, we sought to determine whether histones and HMGB1 induce NET formation by activating neutrophil TLRs. TLR4 WT neutrophils stimulated with histones or HMGB1 exhibited increased citrullination of histone H3 compared to untreated WT neutrophils. We found a reduction in the citrullination of histone H3 in TLR4 KO neutrophils in response to both HMGB1 and histones, with a greater reduction observed in HMGB1 stimulated-neutrophils (**Fig. 5A and B**). In contrast, both HMGB1 and histones exhibited a decrease in citrullinated histone H3 in

TLR9 KO neutrophils with more of a reduction following histone stimulation. These results suggest that although both receptors recognize HMGB1 and histones, TLR4 is the dominant receptor for HMGB1 and TLR9 is the dominant receptor for histones to activate NET formation. This is in agreement with previous findings that HMGB1 can exert its effect by interacting with TLR4 or TLR9 (5, 23) and likewise histones can also bind to TLR9 or TLR4 (21, 22). To confirm these findings, we stimulated TLR4/TLR9 double KO neutrophils with either HMGB1 or histone. Neither induced any NET formation in TLR4/TLR9 double KO neutrophils (**Fig. 5A and B**). Immunofluorescence confocal microscopy also revealed fewer NETs in TLR9 KO and TLR4 KO neutrophils after stimulation with either histones or HMGB1 (**Fig. 5C**). Furthermore, we measured MPO-DNA complexes in the media of TLR9 KO or TLR4 KO neutrophils after stimulation with histones or HMGB1, and found a significant decrease compared to the media of WT neutrophils after stimulation (**Fig. 5D**). Collectively, these results suggest that DAMPs including extracellular histones and HMGB1 are capable of mediating NET formation *in vitro* through both TLR9 and TLR4 signaling pathways.

NET formation after liver I/R in mice is dependent on TLRs signaling

We next determined whether histone and HMGB1 induce NET formation through TLR4 or TLR9 *in vivo*. TLR4 mutant mice and their WT counterparts were administered either histone, HMGB1, or PBS after ischemia. After ischemia alone, protein levels of citrullinated histone H3 in liver isolates from TLR4 KO mice were significantly lower than in those from WT mice (**Fig. 6A**). Following ischemia with administration of HMGB1 or histone, TLR4 KO mice expressed significantly lower levels of citrullinated histone H3 than in WT (**Fig. 6A**). Likewise, TLR9 KO mice exposed to these conditions expressed significantly less citrullinated histone H3 than in WT (**Fig. 6B**). Consistent with these findings, citrullination of histone H3 was significantly lower in MyD88 KO mice exposed to these conditions compared to WT (**Fig. 6C**).

To further study whether NET formation during liver I/R is specifically dependent on TLR4 or TLR9 within neutrophils in response to DAMPs, we performed neutrophil depletion in WT mice by injection of anti-Ly6G monoclonal antibody. Neutrophils obtained from TLR4 KO or TLR9 KO mice were adoptively transferred into neutrophil-depleted WT mice that then underwent liver I/R. We found that adoptive transfer of either TLR4 KO or TLR9 KO neutrophils resulted in significantly fewer circulating MPO-DNA complexes and lower tissue levels of citrullinated-histone H3 compared to WT mice receiving adoptive transfer of WT neutrophils (**Fig. 6D**). These results demonstrate that NET formation after liver I/R is a result of TLR4 and TLR9 within neutrophils. Moreover, liver damage, indicated by sALT levels, was significantly reduced in the mice receiving adoptive transfer of either TLR4 KO or TLR9 KO neutrophils and correlated with decreased NET formation (**Fig. 6E, F and Supplemental Figure 6**), indicating a pathological role for NETs during liver I/R injury.

Inhibition of NET formation reduces HMGB1 and histone-mediated liver I/R injury

We have previously shown that exogenous HMGB1 or histones worsen liver I/R injury. In this study, we show that histones and HMGB1 activate NET formation in the liver after hepatic I/R. We next sought to examine whether NET formation mediates the deleterious

effect of exogenous DAMPs on liver I/R. Mice undergoing liver I/R were administrated the PAD4 inhibitor YW4-03 or DNase I, with or without the co-administration of exogenous DAMPs (histones or HMGB1). Either YW4-03 or DNase I resulted in significantly less NET formation after liver I/R irrespective of co-administration of DAMPs (**Fig. 7A and 7B**). Inhibition of NETs led to significantly less hepatic injury (**Fig. 7C and 7D, and Supplemental Fig. 7**). Importantly, this protective effect was more pronounced in exogenous histone- or HMGB1-treated mice, further supporting the importance of NETs in DAMPs-mediated liver I/R injury.

Discussion

The role of DAMPs in activating signaling through pattern-recognition receptors, such as TLRs, with the subsequent recruitment and activation of innate immune cells has been shown to be a key role in pathogenesis of I/R injury (24). TLR9 in neutrophils has been shown to boost production of pro-inflammatory cytokines and chemokines during liver I/R injury (14). Our previous findings demonstrated that both extracellular histones and HMGB1 directly exacerbate organ damage and initiate sterile inflammatory responses through TLR9 or TLR4 during liver I/R (4, 5). We have also shown that extracellular histones mediate inflammatory and innate immune responses through activating the nucleotide-binding domain, leucine-rich repeat containing protein 3 (NLRP3) inflammasome (16). Therefore, we hypothesized that histones and HMGB1 might activate NET formation and that NETs could contribute to inflammation and injury during liver I/R. We found in this study that following liver I/R there was an increase in formation of NETs within the liver, which correlated with liver injury. Inhibiting NET formation by specific PAD4 inhibitors or by degradation using DNase I significantly ameliorated the resultant liver injury. We also demonstrate a mechanism by which extracellular histones and HMGB1 activate neutrophils to form NETs through TLR9 and TLR4 (**Figure 8**).

Our study demonstrates that NET formation during liver I/R is detrimental; however, the exact mechanism by which NETs exacerbate liver I/R injury is not yet known. Histone and HMGB1, in addition to activating NETs, are also part of the extruded cellular contents during NET formation. The histone and HMGB1 in the NET fibers may themselves act as DAMPs and accentuate the inflammatory response as evidenced by the increase in the pro-inflammatory cytokines associated with NET formation. Histones and HMGB1 contained in NETs may also play a role in further recruiting and activating neutrophils to form NETs. In addition, other proteins in the NET web may promote inflammation and organ injury. For example, neutrophil elastase decreases the endothelial production of prostacyclin-2 through the inhibition of endothelial nitric oxide synthase activation and thereby contributes to the development of I/R-induced liver injury (25). Neutrophil elastase has also been shown to decrease IGF-I production through the inhibition of sensory neuron stimulation, which may lead to an increase of neutrophil accumulation and hepatic apoptosis through activation of caspase-3 in rats (26). Therefore, whether NETs directly trap and damage neighboring hepatocytes or increases the inflammatory response through histones, HMGB1, and other proteins in the NET structure deserves to be the focus of future investigations.

A number of DAMPs have been shown to recruit and activate neutrophils through TLR4 and TLR9 signaling in the setting of sterile inflammation. Heat shock protein (HSP) 72, S100A8 and S100A9 cause neutrophil recruitment, degranulation and cytokine production whereas hyaluronic acid released in the extracellular matrix acts to decrease neutrophil recruitment and activation through TLR4 (27). Mitochondrial DNA enhances neutrophil recruitment and oxidative burst through TLR9 (27). HMGB1 has been shown to activate NET formation in both LPS-induced lung injury (28) and myocardial infarction models (29). In addition, we show in this study that HMGB1 and histones, released from stressed hepatocytes, activate neutrophils to form NETs through TLR4 or TLR9 and exacerbate liver injury after I/R. Interestingly, HMGB1 has been recently shown to exhibit different isoforms which are important determinants of HMGB1 recognition and activity depending on the redox status of the thiol groups in HMGB1 (30). It is possible that HMGB1 is released in its reduced form from stressed hepatocytes and acts via the CXCL12-CXCR4 axis to recruit neutrophils as this redox form has been shown to be a chemoattractant. Similarly, HMGB1 in its oxidized disulfide form, required for recognition by TLR4 receptors, may be required in neutrophils to activate NADPH oxidase and subsequent formation of NETs. The role of the redox state of HMGB1 in NET formation is currently an active area of study in our laboratory.

Therapeutic interventions exploiting the mechanisms demonstrated in this study to prevent NET formation, specific PAD4 inhibitors and DNase I, might prove to be helpful to improve outcomes in the clinical setting after liver resection, transplantation, or liver shock. Both gene deletion and pharmacological inhibition of PAD4 significantly abrogates NET formation without affecting mouse viability (8, 31). By using genetic knockout mice or PAD inhibitor Cl-amidine treatment, inhibition of PAD4 has been recently shown to be protective in murine model of lupus (32), cardiac infarction (29) and deep vein thrombosis (33) by preventing NET formation. Consistently, our *in vivo* data also reveal that YW4-03, a newly synthesized specific PAD4 inhibitor (13) conferred a significant protection from liver I/R injury and could represent a potential novel therapeutic agent. In addition, DNase I, a nonspecific inhibitor of NETs through chromatin degradation, can also be useful in the protection against liver I/R injury as demonstrated by this study. Similar to PAD4 inhibitors, DNase I treatment has been shown to be protective in the setting of cerebral I/R transfusion related lung injury and deep vein thrombosis, all of which NETs have been shown to play a role in (34-36).

In conclusion, our study demonstrates that DAMPs released during liver I/R result in NET formation via TLR dependent pathways and subsequently exacerbate liver I/R. We suggest that targeting NETs by specific PAD4 inhibitors and DNase I could become a promising new approach in the treatment or prevention liver I/R injury in patients undergoing liver surgery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

DAMP	Damage associated molecular pattern
PAMP	Pathogen associated molecular pattern
PRR	pattern recognition receptor
TLR	Toll-Like Receptor
I/R	Ischemia/Reperfusion
HMGB1	High Mobility Group Box 1
NET	Neutrophil extracellular traps
PAD4	Peptidyl-arginine-deiminase 4
PMA	Phorbol 12-myristate 13-acetate
sALT	Serum alanine aminotransferase
Cit-H3	Citrullinated-histone H3
MPO	myeloperoxidase

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Figure 1.

Liver I/R induces NET formation. (A) NETs form after either 1 hour or 1.5 hour ischemia time followed by 6 hours reperfusion, or after 1 hour ischemia time followed by 1 hour, 3 hours, 6 hours, or 24 hours of reperfusion, as assessed by serum level of MPO-DNA complex. Results are expressed as the relative folds increase of MPO-DNA complex compared with sham; mean $\pm$ SE (n=6). *P<0.05 versus sham. (B) Representative immunofluorescence images by confocal microscopy of liver sections after 60 min ischemia and 6 h reperfusion in mice (magnification $\times 40$; n=6) with staining for Ly6G (red), nuclei

(blue), histone H2AX (green), and F-actin (gray). Arrows: released histone H2AX from neutrophils. (C) Three-dimensional reconstructed image of Cit-H3-positive cells associated with Ly6G antigen. Liver sections after 60 min of ischemia and 6 hours of reperfusion in mice (magnification $\times 40$; $n=6$) with staining for Ly6G (red), nuclei (blue), Cit-histone H3 (green), and F-actin (gray). (D) Representative 2-photon microscopy images reveal DNA expulsion from neutrophils in real-time after liver I/R with SYTOX Green staining in LysMeGFP knockin mice compared with sham LysMeGFP knockin mice. Green: SYTOX Green; red: neutrophil; blue: sinusoids. Arrows: released DNA from neutrophils. (E) Cit-histone H3 protein levels were determined by Western blot in sham-treated mice and mice that underwent 1 hour or 1.5 hour ischemia followed by 6 hours reperfusion, or 1 hour ischemia followed by 1 hour, 3 hours, 6 hours, and 24 hours of reperfusion. For Western blotting results, each lane represents a separate animal. The blots shown are representative of three experiments with similar results.

Figure 2.

Inhibition of NET formation by PAD4 inhibitors (YW3-56 or YW4-03) or DNase I protects the liver from I/R injury in mice. (A) Serum MPO-DNA complex levels were assessed in control-, YW3-56-, YW4-03-, or DNase I-treated mice after either sham laparotomy or 1 hour of ischemia and 6 hours of reperfusion. Data represent the mean $\pm$ SE ($n = 6$). * $P < 0.05$ vs. DMSO-treated group after liver I/R. ** $P < 0.05$ vs. control-treated group after liver I/R. (B) Cit-H3 protein levels were determined by Western blot in control-, YW3-56-, YW4-03-, or DNase I treated-mice after 1 hour of ischemia and 6 hours of reperfusion.

Hepatic protein lysates from ischemic lobes were obtained; each lane represents a separate animal. The blots shown are representative of three experiments with similar results. (C) Serum ALT levels were assessed in control-, YW3-56-, YW4-03-, or DNase I-treated mice after either sham laparotomy or 1 hour of ischemia and 6 hours of reperfusion. Data represent the mean $\pm$ SE (n = 6). *P < 0.05 vs. DMSO-treated group after liver I/R. **P < 0.05 vs. control-treated group after liver I/R. (D) Quantification of necrotic hepatocytes in H&Estained liver sections from control-, YW3-56-, YW4-03-, or DNase I-treated mice 6 hours after reperfusion.

*P < 0.05 vs. DMSO-treated group after liver I/R. **P < 0.05 vs. control-treated group after liver I/R.

Figure 3.

NETs damage hepatocytes and initiate inflammatory responses both in vitro and in vivo. (A) Cell damage, as measured by flow cytometry for propidium iodide (PI) staining, in normal primary hepatocytes cultured for 4 hours with media from PMA-stimulated neutrophils or from non-stimulated neutrophils. (B) mRNA levels of TNF- α , IL-6, IL-1 β and MCP-1, CXCL10 were determined in normal KCs cultured for 4 hours with media from PMA-stimulated neutrophils or from non-stimulated neutrophils. Results are expressed as the relative increase of mRNA expression compared with sham group. *P < 0.05 compared to

control neutrophil media group. (C) Cell damage, as measured by flow cytometry for PI staining, in hepatocytes obtained from control-, YW4-03-, or DNase I-treated mice after 1 hour of ischemia and 6 hours of reperfusion. (D) Tissue mRNA levels of TNF- α , IL-6, IL-1 β and MCP-1 were determined in control-, YW3-56-, YW4-03-, or DNase I-treated mice after 1 hour of ischemia and 6 hours of reperfusion. Results are expressed as the relative increase of mRNA expression compared with sham group. *P < 0.05 compared to control-treated liver I/R group. Data represent the mean $\pm$ SE and are representative of three experiments with similar results.

Figure 4.

Histones and HMGB1 induce NET formation. (A) Media level of MPO-DNA complex was assessed for NET formation after stimulation by negative control normal media, positive control PMA, conditioned media from necrotic hepatocytes, conditioned media from hypoxic hepatocytes for 4 hours in normal neutrophils. Results are expressed as the relative fold increase of MPO-DNA complex from three experiments compared with negative control. * $P < 0.05$ when compared with negative control. (B) Cithistone H3 protein levels were obtained from WT neutrophils stimulated with exogenous histones at dosages ranging

from 0 to 50 $\mu\text{g/mL}$ for 4 hours or with rHMGB1 at dosages ranging from 0 to 5 $\mu\text{g/mL}$ for 4 hours. (C) Immunofluorescent staining of NETs was assessed by confocal microscopy in neutrophils co-cultured with exogenous histones (25 $\mu\text{g/mL}$), or rHMGB1 (1 $\mu\text{g/mL}$) for 4 hours or PMA respectively, or co-stimulated with histones or HMGB1 together with YW3-56, YW4-03 or DNase I. Blue: nuclei; green: cit-histone H3; red: F-actin. (D) NETs were identified by 2-photon microscopy in the representative images of NETs generated 4 hours by stimulation of histones and HMGB1 with SYTOX Green staining. Yellow: SYTOX Green. Arrows: released DNA from neutrophils. Results shown are representative of three experiments with similar results.

Figure 5.

Histones and HMGB1 induce NET formation through TLR9 or TLR4 signaling. (A) Cit-H3 protein levels were determined by Western blot in WT control, TLR4 KO, TLR9 KO or TLR4/TLR9 double KO neutrophils stimulated with negative control PBS, or exogenous histones (25 $\mu\text{g/mL}$) for 4 hours. (B) Cit-H3 protein levels were determined by Western blot in WT control, TLR4 KO, TLR9 KO or TLR4/TLR9 double KO neutrophils stimulated with negative control PBS, or rHMGB1 (1 $\mu\text{g/mL}$) for 4 hours. (C) Immunofluorescent stain of NETs was assessed by confocal microscopy in TLR4 WT or TLR4 KO, TLR9 WT or TLR9

KO neutrophils co-cultured for 4 hours with exogenous histones or rHMGB1. Figure is representative of three experiments with similar results. Blue: nuclei; green: cit-histone H3; red: F-actin. (D) Media level of MPO-DNA complex was assessed as NETs formation after stimulation by negative control PBS, positive control PMA, exogenous histones (25 µg/mL), rHMGB1 (1 µg/mL) for 4 hours in WT, TLR4 KO, TLR9 KO neutrophils. Results are expressed as the relative fold increase of MPO-DNA complex from three experiments compared with negative control. * $P < 0.05$ when compared with sham. The blots shown are representative of three experiments with similar results.

Figure 6.

(A) Cit-H3 protein levels were determined by Western blot in PBS- or rHMGB1- or histone-treated TLR4 WT or TLR4 KO mice 6 hours after reperfusion. Hepatic protein lysates from ischemic lobes were obtained; each lane represents a separate animal. (B) Cit-H3 protein levels were determined by Western blot in PBS- or HMGB1- or histone-treated TLR9 WT or TLR9 KO mice 6 hours after reperfusion. Hepatic protein lysates from ischemic lobes were obtained; each lane represents a separate animal. (C) Cit-H3 protein levels were determined by Western blot in PBS- or histones-, or rHMGB1 treated MyD88 WT or

MyD88 KO mice 6 hours after reperfusion. Hepatic protein lysates from ischemic lobes were obtained; each lane represents a separate animal. (D) Serum MPO-DNA complex levels and Cit-H3 protein levels were assessed in neutrophil-depleted- WT mice, and neutrophil-depleted-WT mice with adoptive transfer of WT, TLR4 KO, or TLR9 KO neutrophils after either sham laparotomy or 1 h of ischemia and 6 h of reperfusion. Data represent the mean $\pm$ SE (n = 6 mice per group). *P < 0.05 vs. WT neutrophil adoptive transferred-WT group after liver I/R. (E) Serum ALT levels were assessed in neutrophil-depleted-WT mice, and neutrophil-depleted-WT mice with adoptive transfer of WT, TLR4 KO, or TLR9 KO neutrophils after either sham laparotomy or 1 hour of ischemia and 6 hours of reperfusion. Data represent the mean $\pm$ SE (n = 6 mice per group). *P < 0.05 vs. WT neutrophils adoptive transferred-WT group after liver I/R. (F) Quantification of necrotic hepatocytes in H&E-stained liver sections (Supplementary Figure 6) from neutrophil-depleted-WT mice, and neutrophil-depleted WT mice with adoptive transfer of WT, TLR4 KO, or TLR9 KO neutrophils after either sham laparotomy or 1 hour of ischemia and 6 hours of reperfusion. The graph is representative of liver sections from six mice per group. *P < 0.05 vs. WT neutrophils adoptive transferred-WT group after liver I/R. The blots shown are representative of three experiments with similar results.

Figure 7.

Inhibition of NET formation reduces HMGB1 and histone-mediated liver I/R injury. (A) Serum MPO-DNA complex levels were assessed in WT mice treated with control PBS, exogenous histones (20 mg/kg), rHMGB1 (10 μ g/mouse) co-treated with control DMSO or YW4-03; or WT mice treated with control PBS, exogenous histones (20 mg/kg), rHMGB1 (10 μ g/mouse) co-treated with control PBS or DNase I after 1 hour of ischemia and 6 hours of reperfusion. Data represent the mean $\pm$ SE (n = 6). *P < 0.05 vs. control treated group after liver I/R. (B) Cit-H3 levels were assessed in WT mice treated with control PBS,

exogenous histones (20 mg/kg), HMGB1 (10 µg/mouse) co-treated with control DMSO or YW4-03, or WT mice treated with control PBS, exogenous histones (20 mg/kg), HMGB1 (10 µg/mouse) co-treated with control PBS or DNase I after 1 hour of ischemia and 6 hours of reperfusion. Hepatic protein lysates from ischemic lobes were obtained; each lane represents a separate animal. The blots shown are representative of three experiments with similar results. (C) Serum ALT levels were assessed in WT mice treated with control PBS, exogenous histones (20 mg/kg), HMGB1 (10 µg/mouse) co-treated with control DMSO or YW4-03, or WT mice treated with control PBS, exogenous histones (20 mg/kg), HMGB1 (10 µg/mouse) co-treated with control PBS or DNase I after 1 hour of ischemia and 6 hours of reperfusion. Data represent the mean $\pm$ SE (n = 6 mice per group). *P < 0.05 vs. control treated group after liver I/R. (D) Quantification of necrotic hepatocytes in H&E-stained liver sections from WT mice treated with control PBS, exogenous histones (20 mg/kg), HMGB1 (10 µg/mouse) co- treated with control DMSO or YW4-03, or WT mice treated with control PBS, exogenous histones (20 mg/kg), HMGB1 (10 µg/mouse) co-treated with control PBS or DNase I after 1hour of ischemia and 6 hours of reperfusion. The graph is representative of liver sections from six mice per group. *P < 0.05 vs. control treated group after liver I/R.

Figure 8.

Schematic representation of DAMPs released from damaged hepatocytes inducing NET formation during liver I/R injury. During liver I/R injury, Histones and HMGB1 released from damaged hepatocytes function as DAMPs to promote PAD4 activation via Toll-like receptor (TLR4 and TLR9) signaling pathways, which subsequently activate NET formation. Development of NETs during liver I/R injury is detrimental, as NETs initiate pro-inflammatory responses.