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Circulating Histones Exacerbate Inflammation in Mice With Acute Liver Failure

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ABSTRACT

Circulating histones are a newly recognized mediator implicated in various inflammatory diseases. It is likely that the release of histones, from dying hepatocytes or inflammatory leukocytes, into the circulation initiates and amplifies inflammation during the course of acute liver failure (ALF). In this study, we investigated a putative pathogenic role of circulating histones in a murine model of ALF induced by D-galactosamine (GalN) plus lipopolysaccharide (LPS). Hepatic function and histological indexes, myeloperoxidase (MPO) activity, hepatocyte apoptosis and the levels of circulating histone were measured in GalN/LPS-treated mice. GalN/LPS caused severe liver damage and a notable increase in plasma concentration of circulating histones. To further assess the role of circulating histones in our model, we administered exogenous histones and anti-histone H4 antibody. Notably, exogenous histones aggravated GalN/LPS-induced hepatotoxicity, whereas anti-histone antibody significantly protected mice. Circulating histones may serve as both a functional marker of ALF activity and as an inflammatory mediator contributing to the progression of ALF. Blockade of circulating histones shows potent protective effects, suggesting a potential therapeutic strategy for ALF. J. Cell. Biochem. 114: 2384–2391, 2013. © 2013 Wiley Periodicals, Inc.

KEY WORDS: CIRCULATING HISTONE; ACUTE LIVER FAILURE; INFLAMMATION; APOPTOSIS

Acute liver failure (ALF), or fulminant hepatitis, is a serious clinical syndrome that manifests as sudden and severe hepatic injury, including progressive and massive hepatocellular necrosis, jaundice, coagulopathy, and hepatic encephalopathy [Polson and Lee, 2005; Lee, 2008; Adebayo et al., 2012]. Over time, ALF can lead to multiple organ failure and eventually death. Despite recent therapeutic advances, the etiology of ALF remains poorly understood [Lee, 2008]; in particular, the mechanisms that trigger multiple organ

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dysfunction syndrome (MODS) and contribute to the propagation of ALF are unclear [Rolando et al., 2000; Lee, 2008]. It is notable that in most cases of ALF, the onset occurs abruptly from a relatively stable condition, most likely due to activation of an acute systemic inflammatory response. There is accumulating evidence that systemic inflammatory responses syndrome (SIRS) is closely associated with MODS progression and a poor prognosis of ALF [Rolando et al., 2000; Henson, 2005]. In addition to ALF, SIRS occurs in a variety of medical conditions such as sepsis, trauma, ischemia–reperfusion and autoimmunity, ultimately contributing to serious clinical sequelae [Abu-Amara et al., 2010; Zhang et al., 2010]. It is known that macrophages and other cells of the immune system are involved in the initiation of SIRS, as evidenced by activation of both pro- and anti-inflammatory cascades of the innate immune system [Antoniades et al., 2008]. However, the factors that activate these cells are largely unknown.

Recently, circulating/extracellular histones have been recognized as a pivotal mediator of lethal systemic inflammatory diseases, both infectious and noninfectious, including sepsis and acute ischemia–reperfusion organ injuries [Xu et al., 2009, 2011; Huang et al., 2011; Allam et al., 2012]. Increased concentrations of circulating histone are observed during acute inflammatory states and appear to contribute to organ dysfunction and mortality. For example, Xu et al. identified extracellular histones as a major mediator of fatal sepsis. Extracellular histone release amplified inflammation and led to fatality in three experimental mouse models with sepsis [Xu et al., 2009]. Of note, ALF shares some similarities with sepsis regarding the features of systemic inflammation, progression to multiple organ dysfunction, and functional immunoparesis [Antoniades et al., 2008]. We speculated that circulating histones might be a similarly critical mediator of systemic inflammation and contribute to the onset and progression of ALF. We tested this hypothesis by using a murine model with D-galactosamine (GalN) plus lipopolysaccharide (LPS)-induced ALF. We demonstrate that histones were increased in the peripheral circulation of mice challenged with GalN/LPS and that this increase correlated well with the severity of liver damage. Exogenous histones aggravated the lethality of GalN/LPS in mice, whereas blocking circulating histones with a histone H4 mAb protected mice from fatal outcomes.

MATERIALS AND METHODS

REAGENTS

GalN, LPS and calf thymus histones were obtained from Sigma–Aldrich (St. Louis, MO). Mouse mAb to histone H4 (L64C1) was from Cell Signaling Technology, Inc. (USA). Mouse anti-histone H4 mAb was prepared following a previously described method [Monestier et al., 1993].

ANIMAL MODEL AND EXPERIMENTAL DESIGN

We used 8-week-old male C57BL/6 mice obtained from Capital Medical University Animal Breeding Unit. Mice were housed in a room maintained at a constant temperature ($25 \pm 2°C$) and humidity ($50 \pm 10\%$), with free access to food and water and subjected to a 12-h light/dark cycle. The mice were fasted overnight prior to the experiments. All the animal studies were performed under protocols approved by the Animal Research Committee of Capital Medical University, Beijing, P.R. China.

In the first experiment, mice were given a lethal dose of GalN (700 mg/kg) plus LPS (40 μg/kg) by intraperitoneal injection to induce ALF. In the second experiment, exogenous histones (20 mg/kg) or PBS were injected intravenously to mice immediately after an intraperitoneal injection of a sublethal dose of GalN (300 mg/kg)/LPS (15 μg/kg). In the third experiment, mice received anti-histone H4 mAb (20 mg/kg) or control IgG intravenously immediately after a lethal dose of GalN/LPS injection. All mice were sacrificed at predetermined time points (0–12 h) to collect blood and liver tissues for subsequent analysis. Mouse blood was collected by retro-orbital bleeding into a tube containing the anti-coagulant and centrifuged to separate plasma and stored at −80°C. The liver was rapidly excised and placed in fixative for histological assessment.

LIVER DAMAGE ASSESSMENT

Plasma levels of alanine aminotransferase (ALT), a classic marker of hepatic damage, were measured by using an automated multi-parametric analyzer (AU 5400, Olympus, Japan), according to an automated procedure. Liver tissues were fixed in 10% neutral formalin solution for 2–4 h, washed with PBS several times, embedded in paraffin wax and cut into 5 μm thick sections. Liver sections were stained with hematoxylin and eosin (H&E) using a standard protocol, and analyzed by light microscopy.

MEASUREMENT OF CIRCULATING HISTONES

Given that nucleosomes are complexes composed of histones and DNA, measuring the concentrations of nucleosomes allows for the relative quantification of histones [Holdenrieder et al., 2001; Holdenrieder and Stieber, 2009]. We first measured the concentrations of nucleosomes in the plasma of mice using a cell death detection ELISA kit (Roche Applied Science, USA). Xu et al. [2009, 2011] reported that histone H4 plays a central role in mediating cytotoxicity in contrast to other histones. Thus, we conducted additional measurements of the presence of histone H4 in the plasma of mice using western blot analysis. Briefly, 5 μl of plasma was mixed with 4× loading buffer and loaded onto a 12% polyacrylamide-SDS gel. After electrophoretic separation, proteins were transferred onto PVDF membrane. The membrane was blocked with TBST containing 5% BSA, followed by incubation with mouse mAb to histone H4 (1:500, Cell Signaling). The membrane was then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000). Antibody binding was visualized with an ECL chemiluminescence system (Pierce Biotech, Inc., USA) and short exposure of the membrane to X-ray films.

QUANTIFICATION OF MPO IN THE LIVER OF MICE

Myeloperoxidase (MPO) activity, an index of neutrophil, monocyte/macrophage infiltration, was measured in mouse liver tissue homogenates using a commercial kit (BioVision, CA, USA). The samples were evaluated spectrophotometrically at 412 nm and expressed as units per milligram of tissue (U/mg).

MEASUREMENT OF HEPATOCYTE APOPTOSIS

Apoptotic hepatocytes were primarily detected by terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL)

staining using a commercial kit (R&D Systems, Inc., USA). Briefly, following deparaffinization and rehydration, 5 μm liver sections were incubated in proteinase K in PBS (10 μg/ml) for 30 min. After washing with PBS, these sections were incubated with TUNEL reaction mixture for 60 min at 37°C. Following converter-POD addition and diaminobenzidine (DAB) substrate reaction, these sections were examined using light microscopy. To quantify the occurrence of cellular apoptosis, we measured activation of caspase-3 in the livers of mice. Liver homogenates were prepared in lysis buffer containing 100 mM HEPES, pH 7.4, 20% glycerol, 10 mM dithiothreitol, and cocktail protease inhibitors and analyzed using a colorimetric caspase-3 assay kit (Chemicon Int., Inc., USA) according to the manufacturer's instructions.

DETERMINATION OF MOUSE TNF-α LEVELS

Plasma samples were analyzed for mouse TNF-α levels using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Inc., USA), according to the manufacturer's instructions.

STATISTICAL ANALYSIS

Data were expressed as the mean ± standard deviation (SD) and were analyzed using Student's *t*-test. The Wilcoxon signed-rank test was used to compare data that did not satisfy the requirements for Student's *t*-test. The results were considered statistically significant when $P < 0.05$. For survival analysis, the log-rank Mantel–Cox test was applied.

RESULTS

GALN/LPS CAUSED SEVERE LIVER DAMAGE AND INCREASED THE RELEASE OF CIRCULATING HISTONES IN MICE

In this study, a lethal dose of GalN/LPS was given to induce ALF in mice. All mice died within 9–12 h after GalN/LPS injection and severe hepatic damage was observed, demonstrated by increased plasma ALT levels (Fig. 1A), as well as typical histopathological manifestations of liver failure including severe hepatocyte necrosis or apoptosis, inflammatory cell infiltration, fatty degeneration and hemorrhage (Fig. 1B). There was no abnormal appearance or histological alterations in the livers of control mice.

Following GalN/LPS challenge, the concentrations of circulating nucleosomes, which is representative of the relative histone levels, were increased significantly in a time-dependent manner. The time-dependent increase in circulating nucleosome correlated with plasma ALT levels, a characteristic for acute liver damage (Fig. 1C). Western blot analysis confirmed that histone H4 was present in the plasma of mice for >12 h

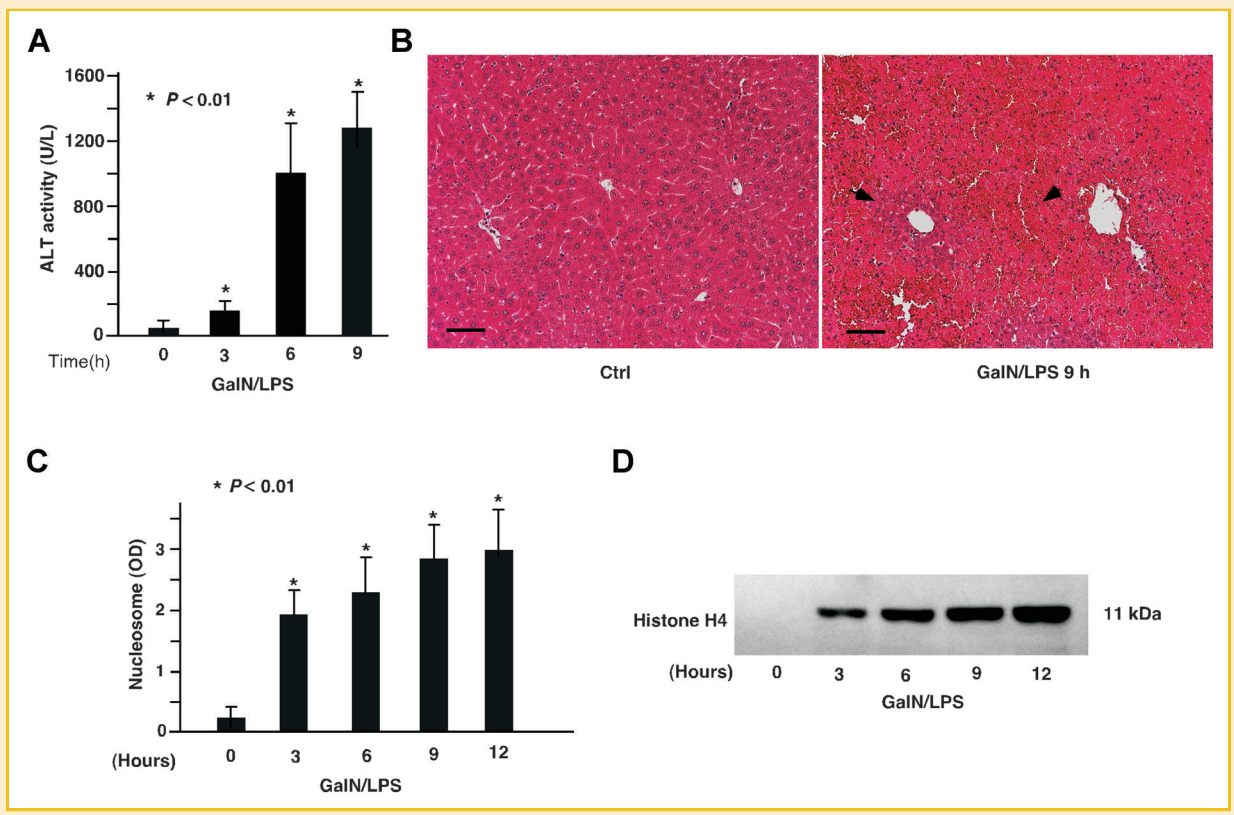

Figure 1. GalN/LPS caused acute liver injury and a significant release of circulating histones in mice. A: Plasma ALT levels were notably increased in a time-dependent manner following GalN/LPS injection to mice (mean ± SD, n = 4–5 mice/group). $^*P < 0.01$ versus the controls. B: H&E-stained sections of liver damage at 9 h after GalN/LPS administration. Arrowheads indicate hemorrhage, inflammatory infiltrates. Scale bars: 100 μm. Data are representative of at least three experiments. C: The levels of nucleosomes in the plasma of GalN/LPS-treated mice were increased remarkably in a time-dependent manner (mean ± SD, n = 4–5 mice/group). The pattern was directional with changes of ALT levels. $^*P < 0.01$ versus the controls. D: Western blot analysis indicated that histone H4 was present in the plasma of GalN/LPS-treated mice for >12 h. The blots are representative of at least three independent experiments.

(Fig. 1D). Additionally, there were other histones, including H2A, H2B, and H3, that were released simultaneously (Supplementary Fig. 1).

CIRCULATING HISTONES COME FROM DYING HEPATOCYTES AND NEUTROPHILS

It has been suggested that circulating histones are mostly derived from apoptotic or necrotic cells [Xu et al., 2009; Allam et al., 2012]. Additionally, neutrophil extracellular traps may be another source of extracellular histones [Papayannopoulos et al., 2010]. Thus, we examined hepatocyte apoptosis by a combination of TUNEL staining and a caspase-3 activity assay in the liver of GalN/LPS-treated mice. TUNEL staining, which may be used to locate and visualize apoptotic cells, showed a considerable increase of apoptotic cells in the liver of GalN/LPS-treated mice (Fig. 2A, B). Likewise, caspase-3, the key mediator implicated in the downstream of apoptotic events, was prominently initiated in the liver of GalN/LPS-treated mice (Fig. 2C).

Neutrophil infiltration was determined by measuring MPO activity in the liver of GalN/LPS-treated mice. GalN/LPS administration notably increased MPO activity in the liver tissues of mice, indicating significant infiltration of neutrophils or macrophages (Fig. 2D). Taken together, these results suggest that the high levels of circulating histones are a result of an increase in apoptotic hepatocytes and neutrophil infiltration in response to GalN/LPS intoxication in mice.

CIRCULATING HISTONES EXACERBATED ACUTE LIVER DAMAGE IN MICE

To further determine the detrimental role of circulating histones in acute liver injury, we administered exogenous histones to GalN/LPS-treated mice. It has been reported that extracellular histones are highly cytotoxic towards endothelium cells, induce intravascular thrombosis, and can cause death when administered at high doses (75 mg/kg). Furthermore, sublethal concentrations of histones (50 mg/kg) cause platelet aggregation and thrombocytopenia in mice [Xu et al., 2009; Ammollo et al., 2011]. In this study, we administered low doses of exogenous histones (20 mg/kg) and GalN/LPS that, when given separately, resulted in no obvious damage in mice. However, we found that a combined administration of non-lethal exogenous histones and GalN/LPS significantly increased the death rate of mice (Fig. 3A) and exacerbated the inflammation of liver in mice, as evidenced by the increased plasma ALT activity and the aggravated histological alterations (Fig. 3B, C). In addition, the levels of TNF-α in the plasma of the mice treated with both histones and GalN/LPS were much higher than in mice treated only with exogenous histones or GalN/LPS (Fig. 4). We conclude that circulating histones originating from cell death or neutrophil infiltration are major contributors to liver failure in mice following GalN/LPS treatment.

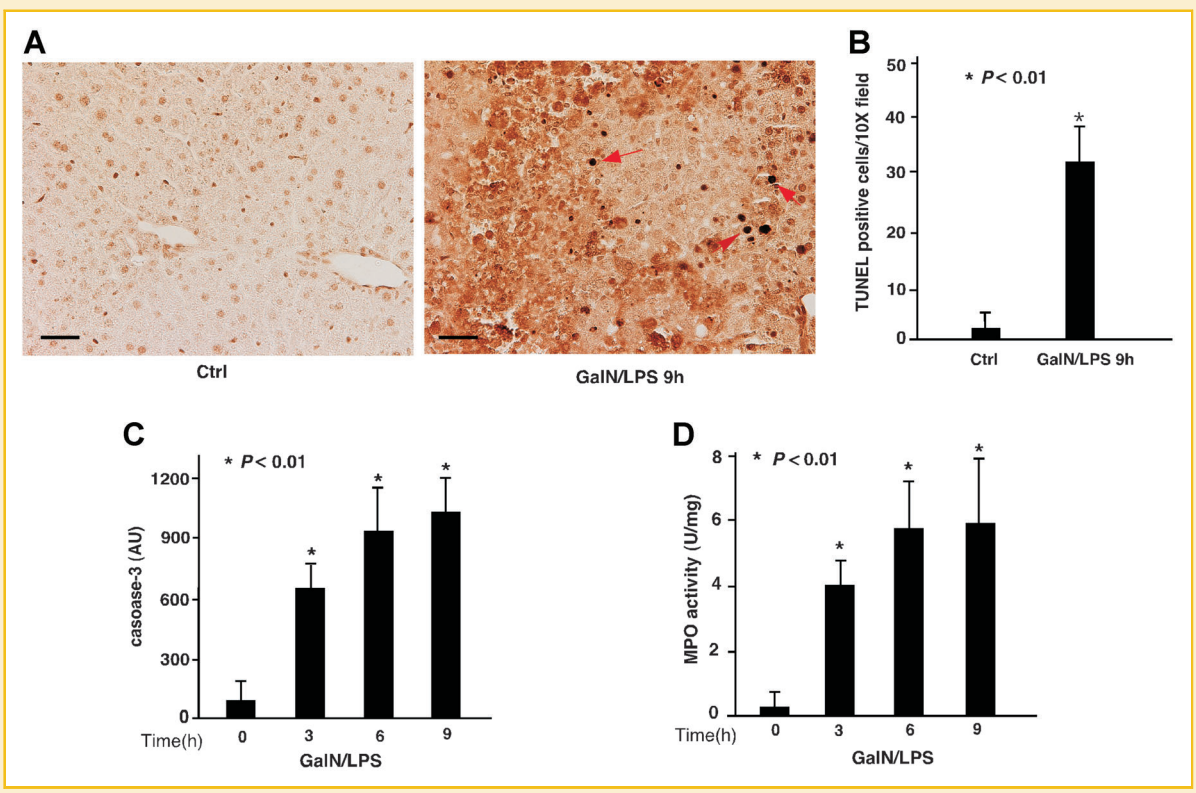

Figure 2. Circulating histones were likely derived from apoptotic hepatocyte and infiltrated neutrophils. A: Hepatocyte apoptosis was visualized by TUNEL staining. The representative images indicated that TUNEL-positive cells were increased remarkably in GalN/LPS-treated mice at 9 h. Arrowheads indicate apoptotic cells. Scale bars: 100 μm. B: Quantification of apoptotic cells via TUNEL staining (mean ± SD, n = 4–5 mice/group). $^{*}P < 0.01$ versus the controls. C: The activity of caspase-3 in the livers of GalN/LPS-treated mice was notably increased in a time-dependent manner (mean ± SD, n = 4–5 mice/group). $^{*}P < 0.01$ versus the controls. D: The levels of MPO activity were determined in the liver tissues of mice. It showed that MPO activity was elevated remarkably following GalN/LPS administration. $^{*}P < 0.01$ versus the controls.

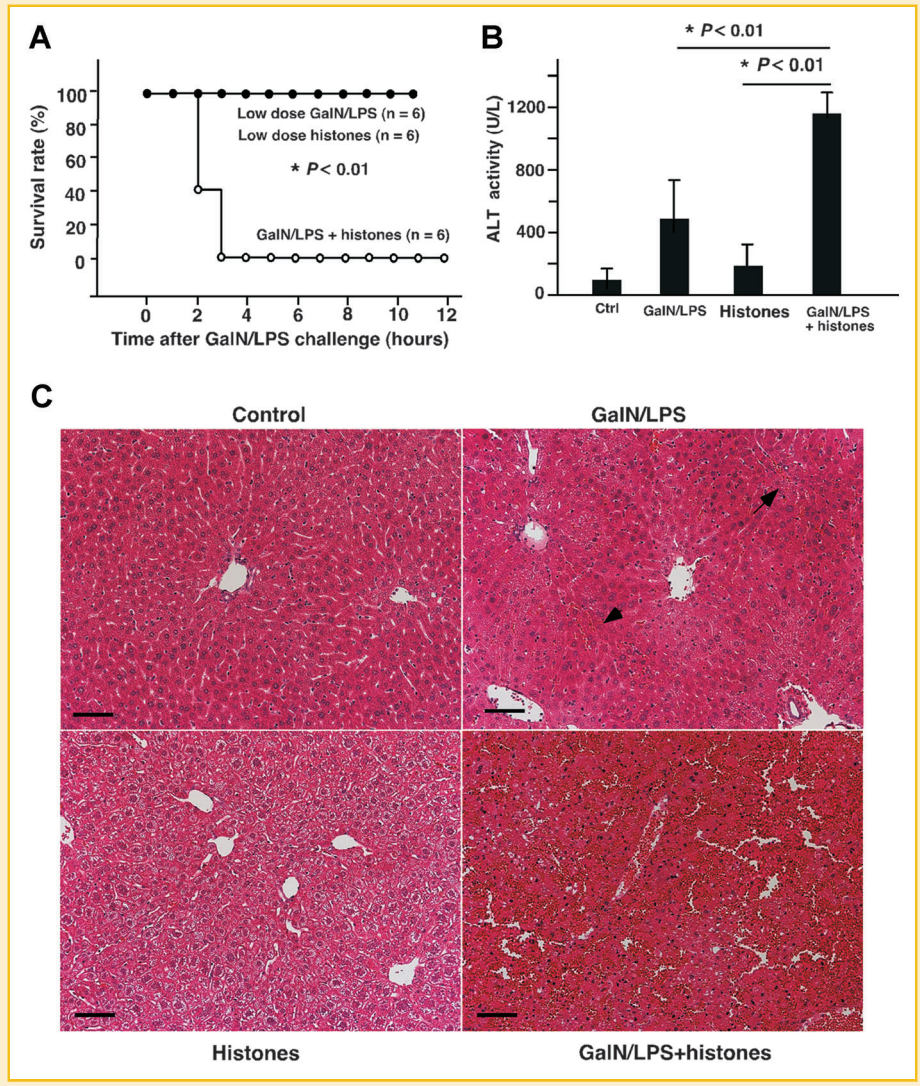

Figure 3. Increased levels of circulating histones exacerbated acute liver damage. A: Both GalN/LPS and exogenous histones at low doses caused no deaths in mice. However, GalN/LPS plus exogenous histones significantly decreased the survival rate of mice. All mice in the histones + GalN/LPS group died within 12 h ($^{*}P < 0.01$ vs. GalN/LPS- or histones-treated group). B: Plasma ALT levels were notably increased in exogenous histones + GalN/LPS groupas compared with GalN/LPS- or histones-treated mice (mean ± SD, n = 6 mice/group $^{*}P < 0.01$). C: H&E-stained sections of liver tissues at 6 h after administration of low dose of GalN/LPS, histones or GalN/LPS plus histones. Only mild degeneration of hepatocytes and inflammatory infiltrates were observed in low dose GalN/LPS-treated mice, whereas no obvious liver damage in histones-treated mice. There were very severe hemorrhage, necrosis and inflammatory cells infiltration in GalN/LPS plus histones-treated mice. Scale bars: 100 μm.

HISTONE H4 BLOCKADE ALLEVIATED ACUTE LIVER FAILURE

We sought to assess the pathological role of histones by blocking histone H4. Histone H4 blockade by antibody was previously used to evaluate the functional contribution of extracellular histones in mouse models of endotoxemia and acute kidney injury [Xu et al., 2009; Allam et al., 2012]. In this study, we first showed that anti-histone H4 antibody recognizes H4 only, and has no cross-reactivity with other histones (Supplementary Fig. 2). Then, we injected mice intravenously with this neutralizing antibody (20 mg/kg) or control IgG immediately after administration of a lethal dose of GalN/LPS. Anti-histone H4 antibody significantly improved the survival rate of GalN/LPS-treated mice (Fig. 5A). The antibody also alleviated acute liver damage, as demonstrated by reduced plasma ALT levels at 6 h after GalN/LPS injection, and improved the histological scores in the livers of mice (Fig. 5B, C). Additionally, the plasma TNF-α levels were remarkably reduced by the histone blockade, suggesting that the inhibition of TNF-α may play an important role in anti-histone-related hepato-protection (Fig. 5D).

DISCUSSION

Histones are essential components of eukaryotic nucleosomes. Histone H2A, H2B, H3, and H4 build a core octamer that is wrapped with a 146 bp long double-stranded DNA segment to form a nucleosome. Histone H1 supports the nucleosome to build chromatin strands and chromosomes [Holdenrieder et al., 2001; Holdenrieder and Stieber, 2009; De Meyer et al., 2012]. Histones in the nucleus play important roles in the regulation of DNA repair, gene transcription,

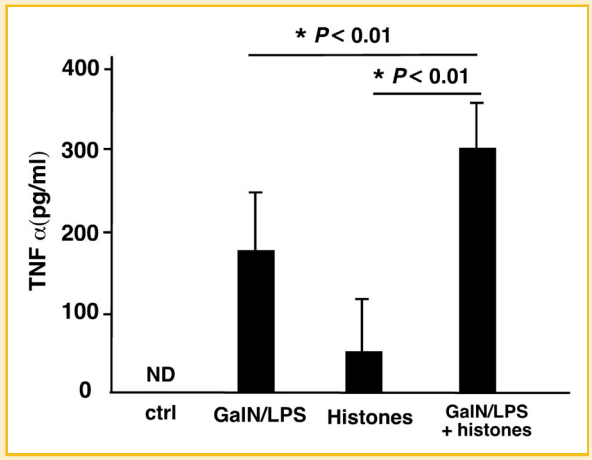

Figure 4. The plasma TNF-α levels were notably increased in exogenous histones + GalN/LPS treated group as compared with GalN/LPS- or histones-treated mice (mean ± SD, n = 6 mice/group $^{*}P < 0.01$). ND, not detected.

and chromatin remodeling [Fuchs et al., 2011; Shukla et al., 2011]. Traditionally, research has focused on the post-translational modifications to histones, such as acetylation and methylation, which are believed to be associated with a variety of pathophysiological conditions including carcinogenesis [Murata et al., 2007; Shukla et al., 2011]. However, in some cases, histones in the form of nucleosome fragments can be detected in the cytoplasm and in the extracellular milieu (e.g., peripheral circulation). Increased concentrations of nucleosomes are observed in the circulation of patients with myocardical infarction, stroke, infections, trauma, cancer and autoimmune diseases [Zeerleder et al., 2003; Holdenrieder and Stieber, 2009]. Additionally, a connection between nucleosome levels and disease severity has been established. Circulating nucleosomes may act as a predictor for some critical diseases [Zeerleder et al., 2003]. It has been suggested that nucleosomes are liberated from dying cells or secreted by activated inflammatory cells such as neutrophils in the form of "extracellular traps" [Holdenrieder et al., 2001; Semeraro et al., 2011]. Indeed, the release of nucleosomes by inflammatory cells is part of an antimicrobial defense during bacterial infection, with histones combining microbicidal with prothrombotic properties to fight invading microbes and maintain hemostasis [Kawasaki and Iwamuro, 2008; Holdenrieder and Stieber, 2009; Huang et al., 2011]. However, the extracellular roles of nucleosomes, especially histones, are not well studied. The distinction between the actual function of extracellular histone as mere bystanders or as active mediators in disease remains unclear.

Recent studies report that circulating histones may function as damage-associated molecular pattern (DAMP) molecules similar to HMBG1, contributing to tissue injury, organ inflammation and subsequent malfunction [Xu et al., 2009; Zhang et al., 2010; Huang

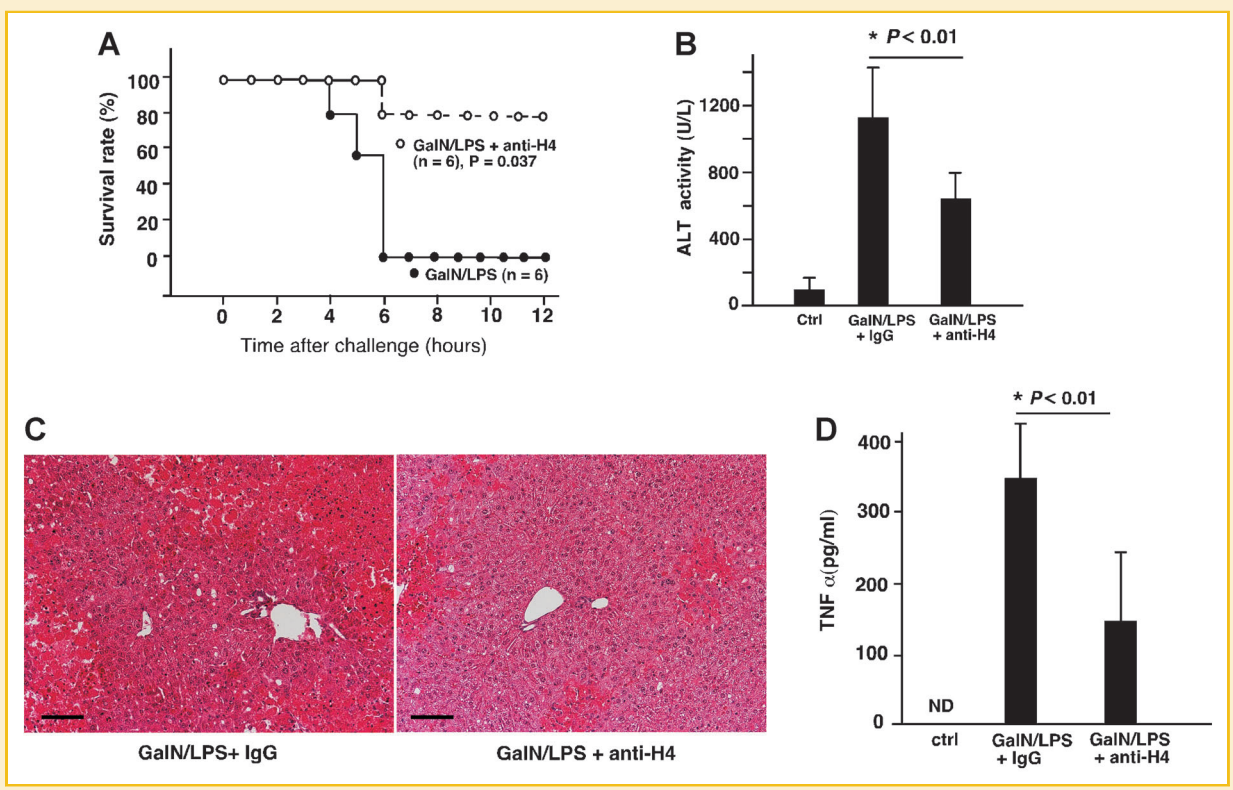

Figure 5. Histone H4 neutralization alleviated acute liver failure. A: Anti-histone H4 antibody administration significantly improved the survival rate of GalN/LPS-treated mice $^{*}P < 0.01$. B: The plasma levels of ALT were decreased in the histone antibody treated group as compared with the GalN/LPS-treated group ($^{*}P < 0.01$). C: The representative H&E stained sections indicate an improvement in the livers of histone antibody + GalN/LPS-treated mice at 6 h. Scale bars: 100 μm. D: The plasma TNF-α levels were decreased in histone antibody + GalN/LPS-treated mice as compared with GalN/LPS-treated group ($^{*}P < 0.01$). ND, not detected.

et al., 2011]. Xu et al. [2009] for the first time revealed that extracellular histones act as a key mediator of cell damage and organ dysfunction during the hyperinflammatory reaction such as sepsis. Extracellular histones increased markedly in response to sepsis and induced endothelial cytotoxicity and triggered an inflammatory and thrombotic response in vivo, and eventually led to MODS and death. Direct injection of histones into mice resulted in death with pathologic features similar to those found in sepsis [Xu et al., 2009]. Of note, the pathogenicity of histones can be reversed by proteolytic inactivation by activated protein C (APC) or by anti-histone antibody neutralization [Xu et al., 2009]. Huang et al. studied the role of circulating histones in sterile inflammatory liver injury and indicated that endogenous histones derived from necrotic hepatocytes following hepatic ischemia/reperfusion (I/R) injury serve as a crucial link between initial damage and activation of inflammation [Huang et al., 2011]. Moreover, Allam et al. revealed that circulating histones from dying renal cells aggravated acute kidney injury in mice and that neutralization of histones using a mAb significantly protected against injury. In addition to these functions, Fuchs et al. [2011] found that circulating histones could activate platelets directly and lead to thrombocytopenia in vivo, which was likely another important mechanism contributing to organ malfunction. All of these findings indicate a strikingly novel role of circulating histones associated with inflammation and organ damage.

Because ALF and sepsis share some similarities such as SIRS and MODS in the progression of diseases [Antoniades et al., 2008], we speculated that circulating histone might play a similarly pathological role in the initiation and aggravation of liver failure. To test this hypothesis, we investigated the role of circulating histones in mice with GalN/LPS-induced ALF. Our study showed that GalN/LPS treatment induced severe liver failure in mice and significantly increased concentrations of circulating histones. The concentrations of circulated histones correlated with the severity of liver damage. The released circulating histones included H2A, H2B, H3, and H4, which may still be complexed into a form of nucleosome. To elucidate the source of circulating histone in the plasma of mice, we first quantified markers of inflammation, namely MPO in the liver of GalN/LPS-treated mice. MPO is a heme protein stored in granules of neutrophils and monocytes, and thus is a classic marker for neutrophil or macrophage infiltration [Malle et al., 2007; Papayannopoulos et al., 2010]. MPO activity was increased remarkably during the course of liver failure, indicating significant activation of neutrophils or macrophages. Meanwhile, we used TUNEL staining to locate and quantify apoptotic hepatocytes in the mouse livers. There was an extensive and significant amount of hepatocyte apoptosis following GalN/LPS administration to mice. To strengthen these results, we also measured the activity of caspase-3, a key protein in the apoptotic pathway associated with both death receptor- and mitochondrion-dependent apoptosis [Zhang et al., 2003]. Consistent with the TUNEL staining, caspase-3 activity was increased prominently in the livers of GalN/LPS-treated mice. We thus concluded that the elevated levels of circulating histones were likely derived from apoptotic hepatocytes or neutrophil infiltration in our model.

We further examined whether histones released into the circulation had adverse effects on the liver. To avoid direct cellular toxicity, a nonlethal dose of histones was administered together with a nonlethal dose of GalN/LPS. We found that exogenous histones, even at a low dose, significantly aggravated GalN/LPS-induced hepatotoxicity in mice, which is consistent with previous studies [Xu et al., 2011]. Although the released histones include H2A, H2B, H3, and H4, only histone H4 exhibits major toxicity. The toxicity from H4 was much stronger than the other histones based on the studies by Xu et al. Therefore, we used anti-histone H4 antibody to neutralize circulating histones [Xu et al., 2009, 2011]. Neutralization of histone H4 provided a potent protective effect against liver failure caused by GalN/LPS. TNF-α levels in the plasma of mice were inhibited following a blockade of circulating histone, which might be an important mechanism by which the neutralization of circulating histones, mainly H4, exerts its protective effects.

Taken together, circulating histones, mainly H4, not only act as a marker for indicating disease activity but also play an active role in the initiation and aggravation of liver damage. Indeed, circulating histone is potentially a novel therapeutic target in a variety of inflammatory and chemical toxin-mediated diseases. Inactivation of circulating histone by pharmacologic interventions such as anti-histone neutralizing antibody may become a clinically promising strategy to minimize organ damage or mortality related to ALF. In contrast to other drugs acting on histones such as APC, neutralizing antibodies against histones may have more promising clinical potential in the treatment of ALF patients. These antibodies circumvent the risk of hemorrhaging that can be caused by APC therapy.

CONCLUSIONS

Our data clearly show that circulating histones correlate with the severity of disease and likely contribute to liver injury by stimulating systemic inflammation. Additionally, blocking circulating histones has potential as a clinical therapy for ALF.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Fig. S1. Identification of circulating histones in the plasma of GalN/LPS-treated mice. A: The plasma of GalN/LPS-treated mice collected at different time point was separated inpolyacrylamide-SDS gel and stained with Coomassie Blue. B: The plasma was subjected to SDS-PAGE and western blotting for histone H2A, H2B, and H3, respectively.

Fig. S2. Assessment of specificity of anti-histone H4 mAb. A: 20 μg calf thymus histones were subjected to SDS-PAGE and Coomassie Blue staining. It clearly showed five types of histones. B: Exogenous histones were subjected to SDS–PAGE and Western blotting for histone H4. It indicated that anti-histone H4 mAb had no cross-reactivity with other histones.