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Synergistic effects of antibodies against high-mobility group box 1 and tumor necrosis factor-α antibodies on D-(+)-galactosamine hydrochloride/lipopolysaccharide-induced acute liver failure

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High-mobility group box 1 (HMGB1) protein is released into the serum after tissue damage, and serves as a warning signal to enhance the inflammatory response. Acute liver injury is one of the diseases that starts with tissue damage and ends with systemic inflammation. We used D-(+)-galactosamine hydrochloride (D-GalN)/lipopolysaccharide (LPS)-treated mice as an acute liver injury model to explore the functions of HMGB1 in more detail. HMGB1 is released into the serum at a very early stage of D-GalN/LPS-induced acute liver injury. It upregulates the expression of tumor necrosis factor-α (TNF-α), interleukin-6, inducible nitric oxide synthase, and tissue factor. TNF-α and HMGB1 form a positive feedback loop to amplify the downstream signals. mAbs against HMGB1 and TNF-α have synergistic effects in protecting mice from D-GalN/LPS-induced acute liver failure. The results suggest that HMGB1 is a key mediator in D-GalN/LPS-induced acute liver injury. Tissue damage and cell necrosis shortly after administration of D-GalN and LPS lead to early HMGB1 release, and HMGB1 acts synergistically with TNF-α to promote pathological processes in acute liver failure.

Introduction

Acute liver failure (ALF) is a disease that involves rapid deterioration of liver function, which leads to multiorgan failure, coagulation dysfunction, and even death. Several mechanisms are responsible for ALF, such as virus infection, drug or toxin intake, and metabolic disorder [1]. However, the pathophysiology of ALF remains poorly understood, and thus warrants more extensive studies. Because of its increased intestinal permeability and weakened monocyte–macrophage clearance function, ALF has been reported to result in systemic inflammation in both clinical trials and animal research [2,3], as represented by a significant increase in the levels of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6 [4,5].

TNF-α is considered to be the most important mediator in the liver injury response. It is involved in hepatitis [6–11], fulminant liver failure [4], and alcoholic liver injury [12,13]. TNF-α expression has been reported to be upregulated and correlated with the level of tissue damage in most models of liver injury [14–17]. Inhibition of TNF-α activity with soluble TNF-α receptors or by knocking out TNF-α or its receptor has been proven to protect the morphology

Abbreviations
ALF, acute liver failure; ALT, alanine aminotransferase; D-GalN, D-(+)-galactosamine hydrochloride; H&E, hematoxylin–eosin. HMGB1, high-mobility group box 1; IL, interleukin; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; siRNA, small interfering RNA (siRNA); TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α.

of the liver, and therefore to be beneficial for mouse survival [18–20]. The importance of TNF-α in hepatic inflammation lies in the fact that it is the first-wave cytokine that leads to the cascades of proinflammatory cytokine upregulation [21]. In a standard lipopolysaccharide (LPS)-induced sepsis model, the serum TNF-α and IL-1 levels reach their peaks at 2 and 4–6 h after LPS administration, respectively [22,23]. In a subsequent phase of the inflammatory response, substantial tissue damage occurs, and high-mobility group box 1 (HMGB1) protein can be detected in serum [24].

HMGB1 is thought to be one of the prototypes of the damage-associated molecular pattern [25,26], and it sends warning signals to the host when released into the extracellular space. HMGB1 can be secreted by monocytes or tissue macrophages in response to proinflammatory stimuli [27], and be passively released during necrosis or, in some cases, apoptosis [28,29]. It exerts different functions when interacting with cellular receptors of different cells. For example, it promotes inflammatory cell recruitment when binding to endothelial cells, the epithelial-to-mesenchymal transition and wound healing when binding to epithelial cells, and pathogen recognition and tissue repair when binding to immune cells [30]. However, the most prominent function of HMGB1 lies in its ability to stimulate a wave of proinflammatory cytokines. By doing so, it forms an amplification loop whereby more inflammation leads to more tissue damage, and more tissue damage leads to more inflammation in return, which will eventually lead to sepsis and even death. This represents a model of systematic inflammation caused by LPS in which inflammation occurs prior to tissue damage, and most symptoms of the sepsis syndrome can only be observed at least 16–24 h after LPS administration. In this model, TNF-α and HMGB1 could be described as an 'early effector' and a 'late effector', respectively. However, in the case of ALF, such as fulminant hepatitis, inflammation and tissue damage occur simultaneously, and the pathological processes develop quite rapidly, even causing mortality within 12 h. In spite of these data, how HMGB1 functions in this scenario remains unclear. Diet restriction prior to acetaminophen intoxication in mice will deplete the basic hepatic ATP stores needed for apoptotic cell death in stressed hepatocytes, and the lack of energy will cause necrotic rather than apoptotic hepatic cell death. Necrosis generates HMGB1 redox isoforms driving inflammation, in contrast to the noninflammatory, terminally oxidized HMGB1 formed during apoptosis [31]. HMGB1 was found to be correlated with alanine aminotransferase (ALT) activity and prothrombin time during clinical acetaminophen hepatotoxicity [32]. Besides inflammatory cell recruitment and its relationship with physiological indices, HMGB1 could also stimulate proinflammatory cytokine synthesis, and purified recombinant HMGB1 significantly stimulated the release of TNF-α in human monocyte cultures and in the serum of Balb/c mice [33]. Yang *et al.* found that Toll-like receptor 4 (TLR4) was required for HMGB1-dependent activation of macrophage TNF-α release, and that the cysteine at position 106 within the B box mediated TLR4-dependent activation of macrophage TNF-α release [34]. HMGB1 contains three conserved redox-sensitive cysteines: Cys23, Cys45, and Cys106. Cys106 thiol and the Cys23–Cys45 disulfide bond are required for HMGB1 to induce TNF-α production in macrophages [35].

In a rat model of ALF in which D-(+)-galactosamine hydrochloride (D-GalN) was administered into the penile vein, Takano *et al.* [36] found a sustained increase in plasma ALT, lactate dehydrogenase (LDH) and HMGB1 levels within 48 h. Moreover, polyclonal antibody against HMGB1 significantly reduced plasma ALT, LDH, TNF-α and IL-6 levels, and exhibited protective effects on the survival of rats. Given such evidence, in the present study we used D-GalN to cause liver injury and a small amount of LPS to simulate accidental infection. The experimental mice died within 12 h under these conditions, which, to some extent, resemble fulminant hepatitis in humans. As we tried to explore a more detailed scenario, we unexpectedly found that HMGB1 was released at a very early stage of D-GalN/LPS-induced ALF, and that this occurred upstream of TNF-α, triggering a cascade of proinflammatory cytokine expression. In other words, HMGB1 played a primary role in the pathological progress of ALF in our model. On the basis of these findings, we explored a therapeutic strategy for ALF according to the synergistic effects of mAbs against TNF-α and HMGB1.

Results

D-GalN and LPS induce HMGB1 and TNF-α, respectively

Intraperitoneal injection of D-GalN (500 $mg \cdot kg^{-1}$) or LPS (10 $\mu g \cdot kg^{-1}$) alone did not cause death in the experimental mice; however, their simultaneous administration resulted in a very high lethal rate within 12 h (Fig. 1A). Combined injection of the two substances also caused serum ALT and morphological changes (Fig. S1). The results suggest that D-GalN and LPS have synergistic effects on pathological development.

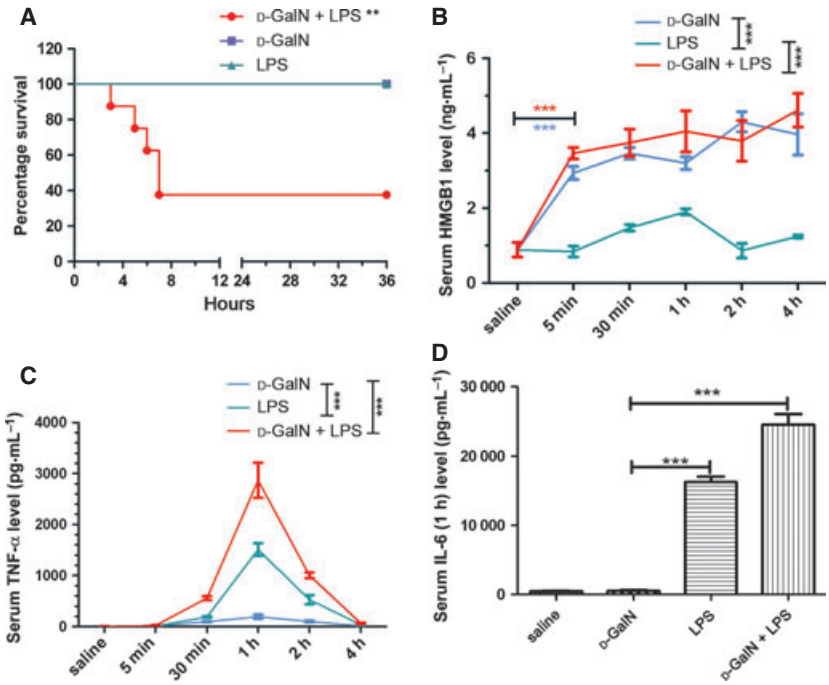

Fig. 1. D-GalN and LPS induce ALF. (A) Intraperitoneal injection of D-GalN (500 mg·kg^{-1}) and LPS (10 μg·kg^{-1}) resulted in lethal effects on mice in 12 h (each group contained eight mice). The data shown in (A) are from an experiment repeated four times. (B) D-GalN induced HMGB1 release in 5 min (n = 6). (C, D) LPS induced TNF-α and IL-6 secretion (n = 6; serum IL-6 level was assayed at 1 h after injection). The data shown in (B), (C) and (D) are from experiments repeated three times. *P < 0.05; **P < 0.01; ***P < 0.001.

The mice were intraperitoneally injected with D-GalN (500 mg·kg^{-1}), LPS (10 μg·kg^{-1}), or D-GalN combined with LPS, after which blood from the eye socket was collected 5 min to 4 h after injection, and sera were isolated from whole blood for ELISA. (We did not perform any assay beyond the 4-h time point, as the mice began to die within 4 h.) As shown in Fig. 1B,C, HMGB1 was released as early as within 5 min after injection of D-GalN rather than LPS. In contrast, the serum TNF-α level notably increased after treatment with LPS rather than D-GalN, as did the serum IL-6 level (Fig. 1D). The kinetics of TNF-α were observed as a pulse, and it reached a peak at 1 h; on the other hand, the kinetics of HMGB1 were detected as a pattern of sustained growth. In both kinetic curves, combined injection of D-GalN and LPS caused stronger responses. These results suggest that D-GalN and LPS have synergistic effects on pathological development, and that these may be mediated by HMGB1 and TNF-α, respectively.

Activation loop between HMGB1 and TNF-α

As the dynamic changes in HMGB1 and TNF-α showed, HMGB1 was released even earlier than TNF-α in pathological processes, and combined injection of D-GalN and LPS generated more effective responses than treatment with either D-GalN or LPS alone. These data indicate that regulatory relationships between HMGB1 and TNF-α exist. Neutralizing mAb targeting HMGB1 or TNF-α was used to block HMGB1 or TNF-α functions in ALF induced by D-GalN (500 mg·kg^{-1}) and LPS (10 μg·kg^{-1}) to verify the regulatory relationships. Blood from the eye socket was collected at 1 h, and sera were isolated from whole blood for ELISA. We found that the mAb against HMGB1 significantly reduced D-GalN/LPS-induced TNF-α upregulation (Fig. 2A). Similarly, the mAb against TNF-α reduced D-GalN/LPS-induced HMGB1 release (Fig. 2B). These results suggest that HMGB1 and TNF-α form an activation loop to enhance their serum levels reciprocally.

HMGB1 plays an important role in ALF pathological development

The mASb against HMGB1 not only suppressed D-GalN/LPS-induced TNF-α upregulation, but also brought D-GalN/LPS-induced upregulated levels of ALT back to the normal range (Fig. 3A), suggesting that HMGB1 plays an important role in liver damage. After 6 h of D-GalN (500 mg·kg^{-1}) and LPS (10 μg·kg^{-1}) administration, the liver of each mouse was dissected and observed with the naked eye. We found that D-GalN and LPS treatment led to obvious pathological changes in the liver. Dark purple regions and symptoms of edema were observed, along with disseminated bleeding spots. By contrast, the livers of the mice treated with mAb against HMGB1 did not show the above-described symptoms, and looked simi-

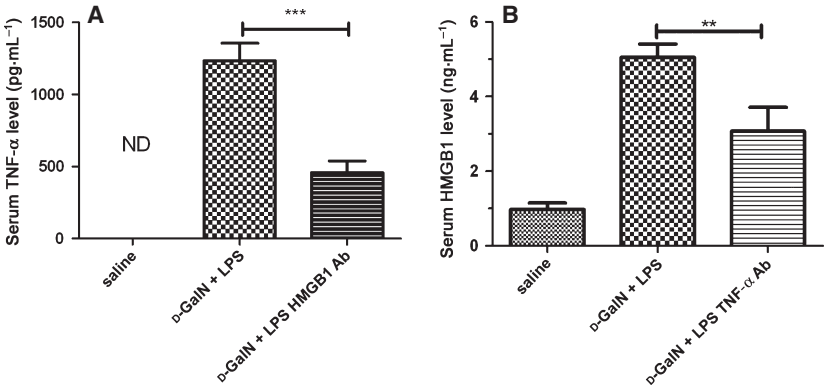

Fig. 2. Regulatory relationships between HMGB1 and TNF-α. (A) Treatment with antibody against HMGB1 significantly reduced the D-GalN/LPS-induced TNF-α level. (B) Treatment with antibody against TNF-α notably reduced D-GalN/LPS-induced HMGB1 release. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. $n = 6$. The data shown in (A) and (B) are from experiments repeated three times. Ab, antibody; ND, not detected.

lar to those of the normal mice (Fig. 3N). The hematoxylin–eosin (H&E)-stained sliced liver sections showed massive hepatocyte death and severe hyperemia in the D-GalN/LPS-treated mice, whereas treatment with mAb against HMGB1 protected the mice from these morphological changes (Fig. 3C).

The protective effect of the mAb against HMGB1 was confirmed with small interfering RNAs (siRNAs) designed to knock down HMGB1 expression. siRNA targeting HMGB1 was delivered to the mice by hydrodynamic injection. Seventy-two hours later, the mice were intraperitoneally injected with D-GalN and LPS; their survival rates were recorded during the next 36 h. As compared with negative control siRNA, siRNA targeting HMGB1 significantly improved the survival of mice (Fig. 3D). Furthermore, siRNA targeting HMGB1 suppressed TNF-α and IL-6 levels in the D-GalN/LPS-induced ALF mice (Fig. 4A,B), providing direct and conclusive evidence that HMGB1 plays a pivotal role in the pathological development of ALF.

HMGB1 and TNF-α mediate inducible nitric oxide synthase (iNOS) and tissue factor expression

iNOS has been reported to be a downstream effector of proinflammatory cytokines, whereas HMGB1 has been shown to induce iNOS upregulation in a rat model of acute lung injury [37]. We used a real-time PCR assay to monitor iNOS mRNA levels in mouse liver tissue. Mice were intraperitoneally injected with neutralizing mAb against HMGB1 ($2.5\ mg{\cdot}kg^{-1}$) or mAb against TNF-α ($2.5\ mg{\cdot}kg^{-1}$) simultaneously with D-GalN ($500\ mg{\cdot}kg^{-1}$) and LPS ($10\ \mu g{\cdot}kg^{-1}$). After 4 h, the mice were killed, and their total RNA was extracted from liver tissue homogenates with Trizol. mRNA was reverse-transcribed, and iNOS-specific primers were used in the real-time PCR assay. We found that iNOS mRNA increased by 15-fold in the D-GalN/LPS-treated group as compared with the saline-treated group, and that both mAb against HMGB1 and mAb against TNF-α could reduce D-GalN/LPS-induced iNOS upregulation (Fig. 4C).

The massive bleeding observed in the liver of the mice suggested pathological changes in the coagulation process. On the basis of a previous report that HMGB1 upregulated tissue factor in endothelial cells [38], we monitored tissue factor mRNA levels with a real-time PCR assay. Mice were intraperitoneally injected with neutralizing mAb against HMGB1 ($2.5\ mg{\cdot}kg^{-1}$) or mAb against TNF-α ($2.5\ mg{\cdot}kg^{-1}$) simultaneously with D-GalN ($500\ mg{\cdot}kg^{-1}$) and LPS ($10\ \mu g{\cdot}kg^{-1}$). After 30 min, the mice were killed, and total RNA was extracted from liver tissue homogenates with Trizol. mRNA was reverse-transcribed, and tissue factor-specific primers were used in the real-time PCR assay. As expected, D-GalN and LPS administration induced a notable increase in the tissue factor mRNA level within 30 min, and both mAb against HMGB1 and mAb against TNF-α reduced D-GalN/LPS-induced tissue factor upregulation (Fig. 4D).

Synergistic effects of mAbs against HMGB1 and TNF-α

Antibody against Myc was used as a control, and it provided no protection to ALF mice (Fig. S2). By contrast, both mAb against HMGB1 and mAb against TNF-α ($2.5\ mg{\cdot}kg^{-1}$, the lowest dose of mAb against HMGB1/TNF-α that can provide 100% protection against ALF; Fig. S3) protected the mice from D-GalN/LPS-induced lethal effects (Fig. 5A,B). Even more strikingly, the collaboration between mAb against TNF-α and mAb against HMGB1 resulted in lower concentrations of the two antibodies providing full protection in the mice. As shown in Fig. 5C, $25\ \mu g{\cdot}kg^{-1}$ mAb against TNF-α or mAb against HMGB1 (equal to a 1% antibody dose, as in Fig. 5A, B) was injected alone or simultaneously with D-GalN

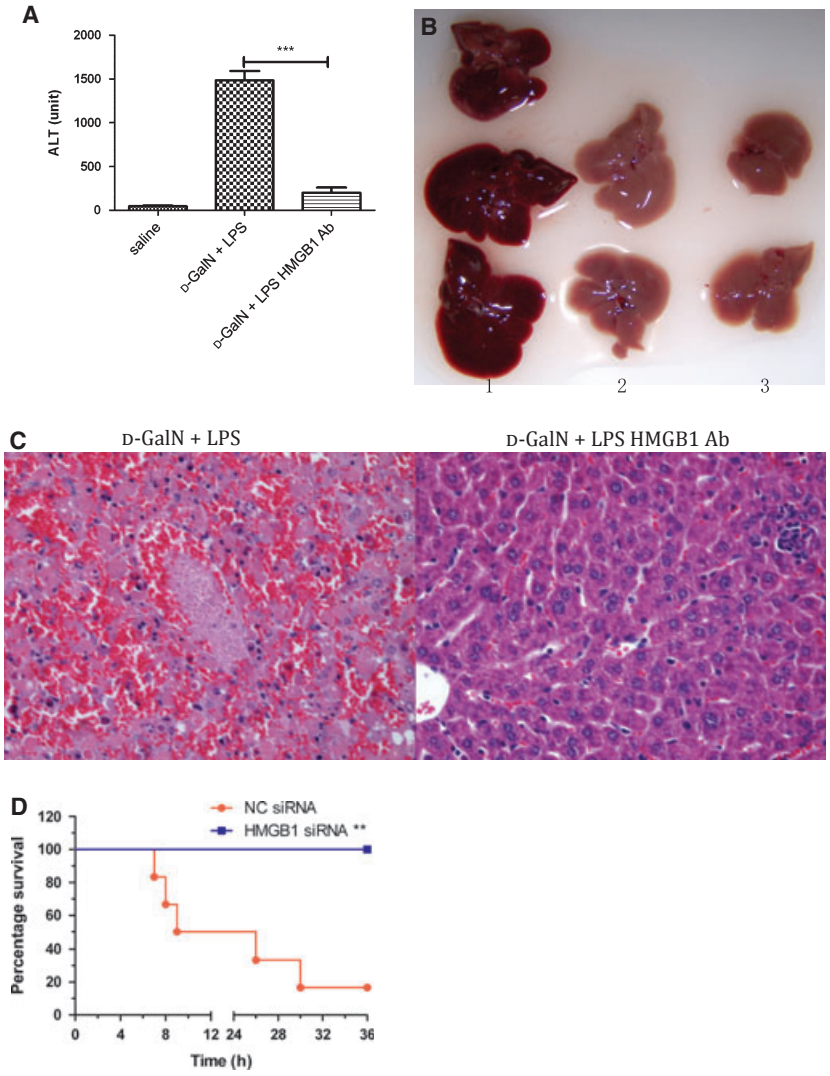

Fig. 3. HMGB1 plays an important role in the pathological development of ALF. (A) Mice were intraperitoneally injected with antibody against HMGB1 (2.5 mg·kg^{-1}) simultaneously with D-GalN (500 mg·kg^{-1}) and LPS (10 μg·kg^{-1}), blood from the eye socket was collected after 4 h of D-GalN and LPS administration, and serum was obtained for ALT assay. Antibody against HMGB1 notably reduced D-GalN/LPS-induced ALT upregulation ($n = 6$). The data shown in (A) are from an experiment repeated three times. (B) Mice were killed after 6 h of intraperitoneal injection of saline (lane 1) or antibody against HMGB1 (2.5 mg·kg^{-1}; lane 2) simultaneously with D-GalN (500 mg·kg^{-1}) and LPS (10 μg·kg^{-1}) (lane 3 shows liver samples of normal mice), and HMGB1 effectively removed D-GalN/LPS-induced edema and disseminated bleeding spots (each group contained two to three mice). The data shown in (B) are from an experiment repeated five times. (C) Mice were killed after 6 h of intraperitoneal injection with antibody against HMGB1 (2.5 mg·kg^{-1}) or saline simultaneously with D-GalN (500 mg·kg^{-1}) and LPS (10 μg·kg^{-1}). On the basis of the H&E-stained sliced liver sections (×200), there were mass mortality of hepatocytes and a wide range of hyperemia in the D-GalN/LPS-induced ALF mice, whereas these morphological changes were not seen after treatment with antibody against HMGB1 (each group contained six mice, and we obtained similar results; representative data are shown). The data shown in (C) are from an experiment repeated three times. (D) Delivery of siRNA targeting HMGB1 by hydrodynamic injection protected the mice from D-GalN/LPS-induced lethal effects ($n = 8$). The data shown in (D) are from an experiment repeated three times. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Ab, antibody; NC, negative control.

and LPS administration. Single antibody treatment gave a 50–60% survival rate in 12 h, whereas injection of the two antibodies together gave full protection against D-GalN/LPS-induced death (Fig. 5C).

The combined treatment with mAb against TNF-α and mAb against HMGB1 could even maintain effective protection when they were used several hours after the administration of D-GalN and LPS. The mice were

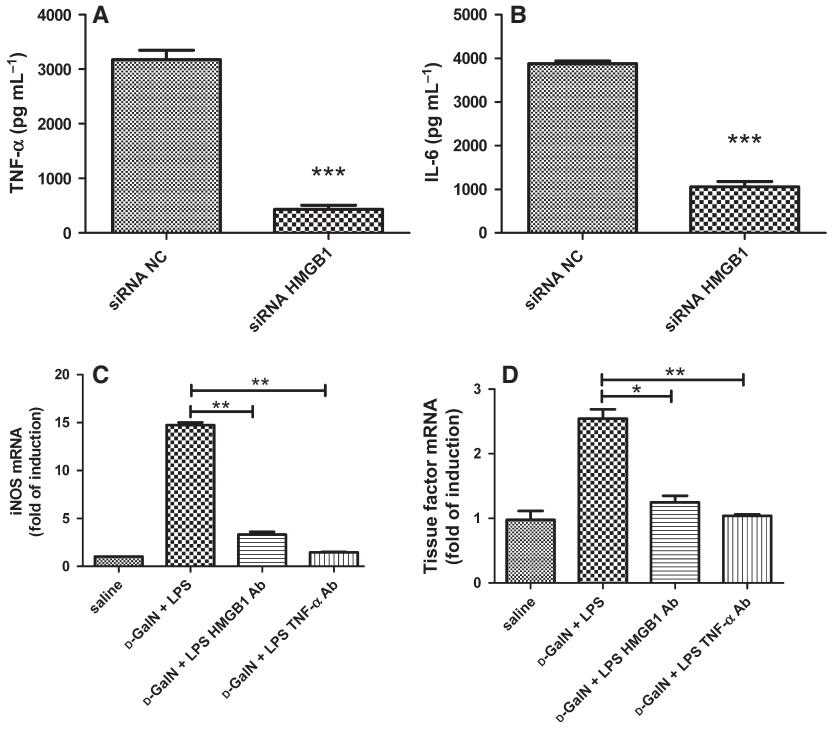

Fig. 4. HMGB1 and TNF-α mediate downstream cytokine expression. (A, B) Synthetic siRNA was delivered *in vivo* with a hydrodynamic injection method; 72 h later, the mice were intraperitoneally injected with D-GalN (500 $mg \cdot kg^{-1}$) and LPS (10 $\mu g \cdot kg^{-1}$), blood from the eye socket was collected after 1 h of D-GalN and LPS administration, and serum was obtained for ALT assay. Treatment with siRNA targeting HMGB1 significantly reduced D-GalN/LPS-induced TNF-α and IL-6 levels. (C) Both antibody against HMGB1 and antibody against TNF-α reduced the D-GalN/LPS-induced iNOS mRNA level. (D) Both antibody against HMGB1 and antibody against TNF-α reduced the D-GalN/LPS-induced tissue factor mRNA level. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. $n = 6$. The data shown in (A–D) are from experiments repeated three times. Ab, antibody; NC, negative control.

intraperitoneally injected with neutralizing mAb against HMGB1 or TNF-α 3 h after D-GalN (500 $mg \cdot kg^{-1}$) and LPS (10 $\mu g \cdot kg^{-1}$) administration, and neither mAb against HMGB1 nor mAb against TNF-α (25 $\mu g \cdot kg^{-1}$) alone provided effective protection against ALF. However, combining the two antibodies at the same dosage achieved full protection against D-GalN/LPS-induced death (Fig. 5D). Moreover, such combined antibody treatment gave 50% protection even 4.5 h after D-GalN and LPS administration (Fig. 5E).

Discussion

Several hypotheses, including the inhibition of mRNA or protein synthesis, have been proposed to explain the sensitizing effects of D-GalN. However, none of them could explain the molecular mechanism underlying the synergistic effects of D-GalN and LPS. In one study, the rat plasma HMGB1 level increased 48 h after treatment with D-GalN [36]. During the same period, the plasma LDH level, an indicator of cell necrosis [38], also increased [36]. We observed similar phenomena in our model of ALF, albeit within a much shorter time scale. In our experiments, serum HMGB1 could be detected at a very early stage of ALF, i.e. as early as 5 min after D-GalN and LPS administration. The increase in serum HMGB1 level was synchronous with the increase in serum LDH level (Fig. S4). How could HMGB1 be released so quickly? The reported functions of D-GalN (e.g. on RNA or protein synthesis inhibition) do not include causing cell necrosis in such a short period. However, it has been reported that oxygen consumption in the liver and hepatocyte metabolism can reach very high levels shortly after D-GalN administration [39]. As a consequence, we reasoned that that high oxygen consumption may result from excessive anaerobic metabolism in hepatocytes, which will produce a myriad of lactate compounds and protons, with the latter triggering calcium influx (through acid-sensing ion channels) and mitochondrial permeability transition, whose changes are likely to generate further membrane damage and, ultimately, hepatocyte necrosis [40]. We investigated whether D-GalN leads to cell necrosis and HMGB1 release. The synergistic effects between D-GalN and LPS could then be explained by the activation loop between HMGB1 and TNF-α.

Our data suggest that HMGB1 is crucial in the pathological process of ALF. HMGB1 could be detected from serum as early as within 5 min, even earlier than TNF-α, which is generally acknowledged as a first-wave proinflammatory cytokine. More importantly, HMGB1 regulates TNF-α expression, which, in turn, regulates later-stage effectors, such as iNOS and tissue factor. These findings place HMGB1 at the most upstream point of the signaling cascade, highlighting its pivotal role in D-GalN/LPS-induced

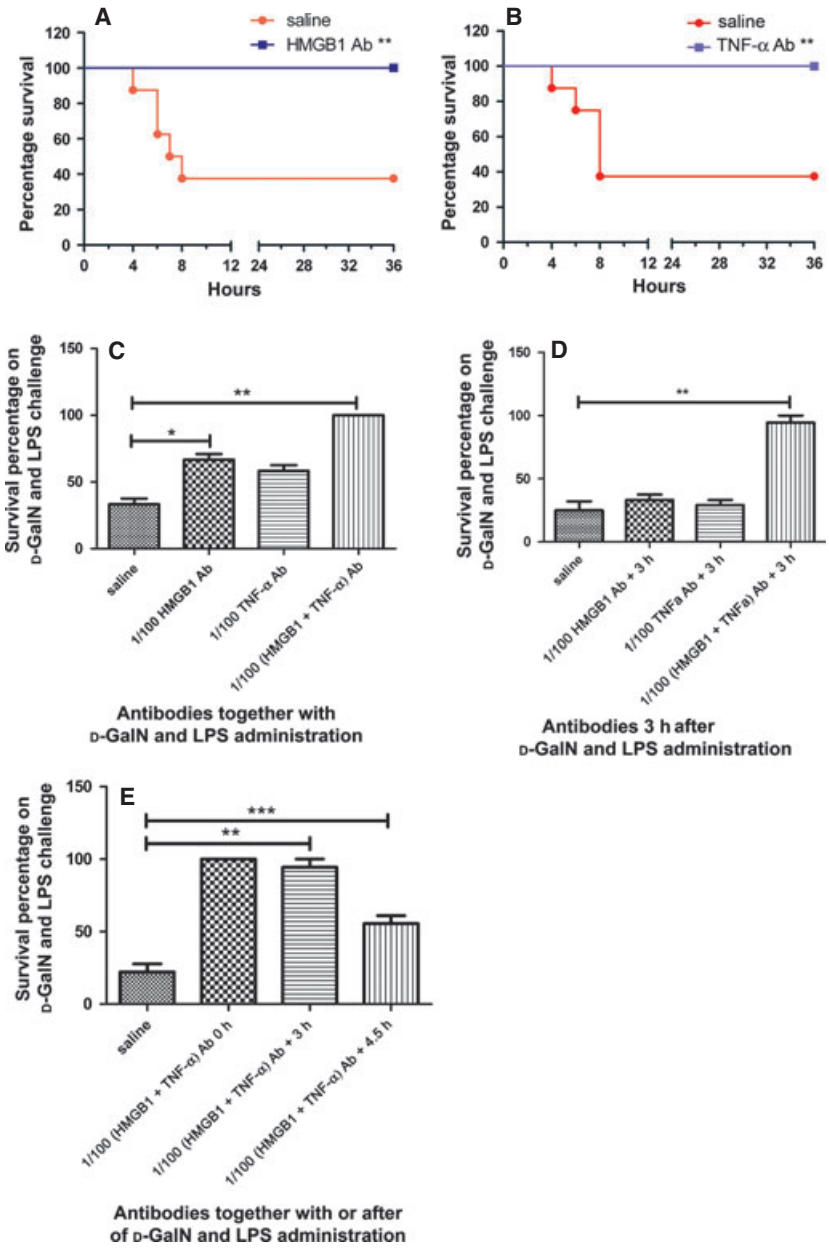

Fig. 5. Synergistic effects of antibodies against HMGB1 and TNF-α. (A, B) Mice were intraperitoneally injected with antibody against HMGB1 (2.5 mg·kg^{-1}) or antibody against TNF-α (2.5 mg·kg^{-1}) simultaneously with D-GalN and LPS. Both antibodies protected the mice from death. (C) A total of 25 μg·kg^{-1} of antibody against HMGB1 or antibody against TNF-α provided only a 60% survival rate for ALF; however, antibodies against HMGB1 and TNF-α applied in combination (25 μg·kg^{-1} each) fully protected against ALF. (D) Combination of the two antibodies (25 μg·kg^{-1} each) offered full protection against ALF, even at 3 h postadministration. (E) Combination of the two antibodies (25 μg·kg^{-1} each) provided partial protection against ALF even at 4.5 h postadministration. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Each group had six to eight mice. Survival rates were recorded 12 h after D-GalN and LPS administration in (C–E). The data shown in (A–E) are from experiments repeated three times.

ALF. Indeed, neutralizing antibodies and siRNA targeting HMGB1 significantly improved the survival rate of D-GalN/LPS-treated mice. It was reported that immunohistochemical analysis showed strong and clear staining of HMGB1 in the nuclei of hepatocytes before induction of ALF [36], and that Kupffer cells in the liver released an array of proinflammatory mediators, including TNF-α, upon activation [41]. We reasoned that the increased HMGB1 and TNF-α levels in serum most likely originated from the liver. The hydrodynamic injection of siRNA targets mostly hepatocytes, and our siRNA experiments effectively reduced lethality in mice. This could be seen as indirect evidence that hepatocytes are the source of HMGB1.

The primary goal of this research was to investigate a therapeutic strategy for ALF. Our results suggest that neither D-GalN nor LPS individually caused lethality in mice, but that collaboration between them resulted in an extremely high death rate in < 8 h. With D-GalN treatment, a large amount of HMGB1 could be detected in serum, although there was no change in the TNF-α or IL-6 level, whereas LPS treatment resulted in the opposite effect. These observations are consistent with the notion that D-GalN triggers liver injury, whereas LPS induces inflammatory responses.

The pathogenetic scenario of this study was based on the fact that D-Gal induces immediate hepatocyte necrosis (systemic HMGB1 levels already increased within 5 min after exposure). This HMGB1 from necrotic cells is passively released in its reduced form (i.e. the state of the nuclear HMGB1), in which all the three cysteines have thiol groups and are capable of strong chemotactic activity against neutrophils and monocytes, but not capable of activating the cells. However, administered LPS will activate the cells and induce the release of a proinflammatory cytokine cascade from these recruited inflammatory cells. In this newly generated oxidative environment, the released HMGB1 will become mildly oxidized to form a disulfide bond in the A box, thereby enabling the HMGB1 to become a dangerous TLR4 ligand [35,42,43]. On the basis of this, we have reason to believe that combining antibodies against HMGB1 and TNF-α will provide better therapeutic effects than using only one antibody. Indeed, we could significantly reduce the dosage of antibodies when antibodies against HMGB1 and TNF-α were used simultaneously. Moreover, delayed administration of antibodies against HMGB1 and TNF-α at 3 h or even at 4.5 h quite effectively protected against lethality. As damage and the inflammation process occurred very early and exacerbated the conditions very rapidly in D-GalN/LPS-induced ALF, how can antibodies targeting early mediators (e.g. HMGB1 and TNF-α) of ALF given at a relatively late stage achieve such excellent protective effects? On the one hand, the HMGB1 released early will be in reduced form, attracting granulocytes and monocytes but unable to stimulate the TLR4–MD2 receptor complex, because there is no Cys23–Cys45 linkage, which is required for TLR4–MD2 binding. However, LPS will somehow later generate an oxidative milieu, in which the extracellular reduced HMGB1 will be gradually oxidized to yield a disulfide bridge in the A box, converting it into a dangerous TLR4–MD2 ligand. The kinetics of this critical process are presently unknown, but it is possible that it may take several hours to accomplish. The addition of antibody against HMGB1 after 3 h may possibly neutralize the reduced HMGB1 before it becomes oxidized. On the other hand, research has shown that HMGB1-induced TNF-α release occurs in a biphasic pattern, with an early peak at 3 h after the addition of HMGB1, followed by another peak after 8–10 h [33]. On the basis of these data, we speculated that there could be another TNF-α peak after 4 h of D-GalN and LPS stimulation. (However, as the mice began to die at 4 h, we could not assay the TNF-α level beyond this time point.) The addition of antibody against TNF-α after 3 h may have blocked the delayed release of TNF-α.

The liver dissected from the dead mice showed signs of edema, whereas H&E staining of sliced liver tissue sections showed massive death of hepatocytes, disintegration of the vascular endothelium, and blood infiltration in tissues. These observations suggest that ALF could cause severe coagulation disorders. Tissue factor is regulated by HMGB1 [44] (see also Fig. 4D) and TNF-α. Tissue injury releases tissue factor into the serum, leading to the activation of an exogenous pathway of coagulation, and possibly triggering coagulation dysfunction, such as disseminated intravascular coagulation. This may provide an explanation for the hematoma in the livers of D-GalN/LPS-treated mice.

Experimental procedures

Reagents and antibodies

Neutralizing mAbs against HMGB1 and TNF-α were produced in our laboratory. We have previously demonstrated the blocking activities of both mAb against HMGB1 and mAb against TNF-α [45]. mAb 3E8 against HMGB1 (K_d = 1.3 nM) and mAb 9C6 against TNF-α (K_d = 1.4 nM) were used in the present study. Streptavidin–horseradish peroxidase and TMB (3,3′,5,5′-tetramethylbenzidine) were purchased from eBioscience (San Diego, CA, USA), as were TNF-α and IL-6 ELISA kits. The QuantiTect SYBR Green PCR Kit was purchased from Qiagen (Venlo, The Netherlands).

ELISA

TNF-α and IL-6 in serum were measured by ELISA according to the manufacturer's instructions (eBioscience). Rabbit polyclonal antibody against HMGB1 was used as a capture antibody, and mAb 3B1 against HMGB1 with a biotin label was used as a detection antibody. mAb 3B1 showed high avidity (K_d = 7.8 nM) [45], and it has been used in a very interesting study on HMGB1 and liver necrosis published in 2009 [46]. Recombinant HMGB1 (AB box) protein was used as a standard.

Delivery of siRNA into mice by injection

Female C57BL/6 mice (6–8 weeks of age and weighing 18–20 g) were purchased from Vital River (Beijing, China). The mice were kept in a temperature-controlled room (22 °C) with a 12-h light/12-h dark cycle, and provided with a standard pellet diet and water. Synthetic siRNA was delivered *in vivo* by use of a hydrodynamic injection method, in which 15 μg of siRNA dissolved in 2 mL of saline was injected into the tail vein over a period of 5–8 s. Four pairs of siRNA were used for hydrodynamic injection. Two pairs (573 and 764) reduced the HMGB1 mRNA level in liver

tissue effectively and protected mice from death (Fig. S5). siRNA-764 was selected for the experiments. The following siRNA oligonucleotide sequences were used: 356, 5′-CU CGUUAUGAAAGAGAAAUTT-3′; 573, 5′-GCAGCCCU AUGAGAAGAAATT-3′; 764, 5′-AGACGAAGAUGAAG AAGAATT-3′; and 1573, 5′-GUGGGACUAUUAGGAU CAATT-3′.

Induction of ALF

D-GalN (500 $mg \cdot kg^{-1}$; Sigma, St Louis, MO, USA) and LPS (10 $\mu g \cdot kg^{-1}$; Sigma) dissolved in saline were delivered *in vivo* by intraperitoneal injection. The survival curve was observed for the next 72 h.

Histopathology and immunohistochemistry

Liver specimens fixed in 10% neutral buffered formalin were embedded in paraffin, sectioned at a thickness of 5 μm, and stained with H&E for light microscopic examination.

Biochemical detection of ALT and LDH

Serum ALT and LDH levels were determined with an automatic biochemical analyzer (Hitachi, Tokyo, Japan) in the clinical laboratory of the 306th Hospital of People's Liberation Army (Beijing, China).

Preparation of RNA and real-time PCR

Following the manufacturer's protocol, total RNA was isolated from mouse liver tissue with Trizol (Invitrogen, Carlsbad, CA, USA). To measure the relative amounts of selected gene transcripts, we reverse-transcribed isolated RNA (2 μg per sample) with oligo-dT primer, using Moloney murine leukemia virus reverse transcriptase (Promega, Fitchburg, WI, USA).

Forty-five cycles of PCR were conducted. PCR programs were run on a Roter Gene RG6000 (Qiagen), and the results were analyzed. The following primer sequences were used: mouse glyceraldehyde-3-phosphate dehydrogenase – 5′-ATCAACGACCCCTTCATTGACC-3′ and 5′-CCAGTA GACTCCACGACATACTCAGC-3′; mouse iNOS – 5′-CC AACCGGAGAAGGACT-3′ and 5′-GGAGGGTGGTGC GGCTGGAC-3′; mouse tissue factor – 5′-GGTACATT CCTCACCCTGCG-3′ and 5′-CCTTCTTCCACATCAAT CGA-3′; and mouse HMGB1 – 5′-CCTACTTGGCAAAG CAAGGA-3′ and 5′-CGGTTAAAGGAGAGTCCTCG-3′.

Statistical analysis

All experiments were repeated at least three times, and representative data are shown. Data were expressed as mean ± standard error of the mean. The Mantel–Cox log-rank test was applied in survival curves. Student's *t*-test or one-way ANOVA/two-way ANOVA was applied in real-time PCR assays and ELISAs. $P \leq 0.05$ was considered to be statistically significant.

Ethical considerations

All experiments were conducted in conformity with NIH guidelines (NIH Pub. No. 85-23, revised 1996) and approved by the Animal Care and Research Advisory Committee of the Chinese Academy of Sciences.

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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. D-GalN and LPS induce ALF.

Fig. S2. Antibody against HMGB1 provides specific protection to ALF mice.

Fig. S3. Antibodies against HMGB1 and TNF-α protect mice from D-GalN/LPS-induced ALF in a dose-dependent manner.

Fig. S4. Increases in serum LDH level.

Fig. S5. siRNA targeting HMGB1 decreases expression of HMGB1 mRNA and protects mice from ALF.