Complement and the Alternative Pathway Play an Important Role in LPS/D-GalN-Induced Fulminant Hepatic Failure

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Abstract

Fulminant hepatic failure (FHF) is a clinically severe type of liver injury with an extremely high mortality rate. Although the pathological mechanisms of FHF are not well understood, evidence suggests that the complement system is involved in the pathogenesis of a variety of liver disorders. In the present study, to investigate the role of complement in FHF, we examined groups of mice following intraperitoneal injection of LPS/D-GalN: wild-type C57BL/6 mice, wild-type mice treated with a C3aR antagonist, C5aR monoclonal antibody (C5aRmAb) or CR2-Factor H (CR2-fH, an inhibitor of the alternative pathway), and C3 deficient mice (C3−/− mice). The animals were euthanized and samples analyzed at specific times after LPS/D-GalN injection. The results showed that intraperitoneal administration of LPS/D-GalN activated the complement pathway, as evidenced by the hepatic deposition of C3 and C5b-9 and elevated serum levels of the complement activation product C3a, the level of which was associated with the severity of the liver damage. C3a receptor (C3aR) and C5a receptor (C5aR) expression was also upregulated. Compared with wild-type mice, C3−/− mice survived significantly longer and displayed reduced liver inflammation and attenuated pathological damage following LPS/D-GalN injection. Similar levels of protection were seen in mice treated with C3aR antagonist, C5aRmAb or CR2-fH. These data indicate an important role for the C3a and C5a generated by the alternative pathway in LPS/D-GalN-induced FHF. The data further suggest that complement inhibition may be an effective strategy for the adjunctive treatment of fulminant hepatic failure.

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Introduction

Fulminant hepatic failure (FHF) is a severe clinical syndrome characterized by hepatic cell injury resulting from a variety of hepatic disease processes, leading to multiorgan failure [1,2]. Although the incidence of FHF is low, the associated mortality is extremely high and is always related to liver transplantation, viral infection and shock [3]. Bacterial lipopolysaccharide (LPS), the main pathogenic component of gram-negative bacteria, can cause systemic inflammatory response syndrome, which may lead to acute liver injury and multiorgan failure. D-galactosamine (D-GalN) increases the sensitivity of mice to LPS and augments the lethal effects of LPS [4,5]. Mouse models of LPS/D-GalN-induced hepatitis have been previously described [6,7]. It has been reported that tumor necrosis factor (TNF)-α-mediated hepatocyte apoptosis may be the cause of LPS-induced liver injury [8–10].

The complement system plays important roles in mediating both acquired and innate responses against microbial infection and in immune homeostatic processes, such as the removal of immune complexes and apoptotic cells [11]. Recent evidence from several studies has suggested that the complement system is involved in the pathogenesis of a variety of liver disorders, including liver fibrosis, viral hepatitis, alcoholic liver disease and hepatic ischemia/reperfusion injury (IRI) [12–16]. In these disease settings, complement activation products promote tissue inflammation and injury, particularly via the generation of the complement activation products C3a and C5a, which promote inflammation via direct and indirect mechanisms by interacting with their receptors [17–19].

Although complement activation has been reported in LPS-treated liver and lung tissues [20–22], little is known about the role of complement in FHF, especially during the early period of the disease. In this study, the role of complement in fulminant hepatic...
failure was systematically investigated using the LPS/D-GalN-induced FHF model. Our study further analyzed the important role of alternative pathway-generated C3a in LPS/D-GalN-induced FHF and suggested a promising strategy for the adjunctive clinical treatment of fulminant hepatic failure.

Materials and Methods

Ethics statement
All procedures involving animals were approved by the Laboratory Animal Center, State Key Laboratory of Pathogenesis and Biosecurity, Beijing Institute of Microbiology and Epidemiology IACUC’s (The permitted number is BIME 2009-15). The study of animals was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals.

Animals and materials
Wild-type (wt) female C57BL/6 and C3−/− female mice (B6.129S4-C3tm1Oj/J) 8 weeks of age and weighing 20–25 g were used in this study. Lipopolysaccharide (LPS; from Escherichia coli strain 0111:B4) and D-GalN were purchased from Sigma. All drugs were dissolved in pyrogen-free saline. The C3αR antagonist (SB 290157, #559410) was purchased from Calbiochem. Monoclonal antibody to mouse C3αR (HM1076) was purchased from HyClone Biotechnology B.V (HyClone Biotechnology, The Netherlands). CR2-H was prepared as previously described [23].

Treatment of mice with LPS/D-GalN
The mice were divided into the following groups: wt mice treated with LPS/D-GalN (wt group), C3−/− mice treated with LPS/D-GalN (C3−/− group), wt mice treated with LPS/D-GalN and C3αR antagonist (C3αR antagonist group), wt mice treated with LPS/D-GalN and C5αRmAb (C5αRmAb group), and wt mice treated with LPS/D-GalN and CR2-βH (CR2-βH group). Wild-type mice treated with saline were used as the control group. The treatment protocols were as follows. The C3αR antagonist (2 mg/kg, diluted in saline containing 0.5% DMSO) and C5αRmAb (600 μg/kg) was injected i.v. 45 min before and 45 min after the LPS/D-GalN injection. CR2-βH (40 mg/kg) dissolved in PBS was injected i.v. immediately after the LPS/D-GalN injection. The dose of C3αR antagonist and CR2-βH used was based on previously reported doses and were in line with doses used in various mouse models of inflammation and injury [24–26]. LPS/D-GalN was dissolved in 200 μl saline and injected i.p. at 2.5 μg/kg LPS and 300 mg/kg D-GalN. The animals were euthanized 0, 1, 4 and 8 hours after the LPS/D-GalN injection. Plasma or serum samples were collected for the analysis of C3α, liver enzymes and proinflammatory cytokines. Liver tissues were processed for histopathological analysis and immunostaining to evaluate the C3, C3αR, C5αR and C5α-9 deposition and the expression of C5αR mRNA and C5αR mRNA.

Histological analysis of liver damage
The liver tissue was fixed in 10% formalin at room temperature and subsequently embedded in paraffin. Liver sections from each animal were sliced and stained with Hematoxylin and eosin (H&E). The extent of the liver damage was assessed by two independent observers blinded to the treatment groups.

Measurement of serum C3α levels in mice
To assess whether complement was activated in the liver by LPS/D-GalN, plasma levels of the complement activation product C3α were measured using ELISA (BD Pharmingen). Briefly, a 96-well plate was coated with purified rat anti-mouse C3α antibody (BD Pharmingen, 558250) overnight at 4°C and then blocked with 10% FBS for 1 hour at room temperature. The samples were diluted 1:2 in blocking buffer and incubated for 2 hours. Bound C3α was detected using a biotinylated rat anti-mouse C3α antibody (BD Pharmingen, 558250) and avidin/horseradish peroxidase(BD Pharmingen, 554058). Reactive C3α levels were measured using the TMB substrate solution (BD Pharmingen 555214).

Immunostaining to assess complement deposition
Frozen liver sections were sliced and fixed in cold acetone. The sections were incubated overnight at 4°C with rat anti-mouse C3mAb (1:20 dilution, HyCult Biotechnology bv, Uden, Netherlands), rabbit anti-mouse C3αR polyclonal antibody (1:50 dilution, Santa Cruz Biotechnology), rabbit anti-mouse C5αR polyclonal antibody (1:80 dilution, Santa Cruz Biotechnology) and anti-C5b-9 polyclonal antibody (5 μg/ml, Calbiochem, San Diego, CA). Biotinylated IgG was then added, followed by an avidin-biotin-peroxidase conjugate (Beijing Zhongshan Biotechnology Co., Ltd.). Immunoreactivity was detected using DAB and by counterstaining with hematoxylin.

Biochemical evaluation of liver injury and proinflammatory cytokine expression in serum
Blood was collected at 0, 1, 4 and 8 hours. Serum samples were prepared as previously described [27] and stored at −80°C. The extent of liver injury was determined by measuring the concentrations of alanine aminotransferase (ALT) and proinflammatory cytokines (IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12) in the serum. ALT was measured using a Beckman CX5 Chemistry Analyzer. The cytokines were assayed using the BD™ CBA Mouse Inflammation Kit (Cat.No. 532964) according to the manufacturer’s instructions.

Detection of C3αR mRNA and C5αR mRNA by relative quantitative real-time PCR
Total RNA was isolated from the liver tissue using the RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. Any genomic DNA contamination was eliminated by treating the samples with RNase-free DNase (Promega). RT was performed with 1 μg of total RNA, using an RT kit (TaKaRa) according to the manufacturer’s instructions. After the reverse transcription step, which comprised incubations for 45 min at 48°C, 5 min at 99°C and 5 min at 5°C, C3αR mRNA and C5αR mRNA expression levels (normalized to GAPDH) were quantified using SYBR Green real-time PCR (Tiangen, China) with primers.
designed by PRIMERS software. The primers for C3aR were sense 5’-tctcactgaggcatctattcagtt-3’ and antisense 5’-attgccgtgctacgttctg-3’. The relative C3aR and C5aR expression data were analyzed using the 2-ΔΔCt method [20].

Survival analysis

In the survival study, an additional eight mice in each group (the wt, C3−/−, C3aR antagonist, C5aRmAb and CR2-fH groups) were monitored for 24 hours period after which all mice were euthanized. Any mice showing signs of severe distress such as weakness, lack of mobility, severe jaundice and presented morbid condition during this period were euthanized and recorded as end point in survival study. Mice in the event of severe distress were euthanized by overdose of pentobarbital (150 mg/kg, i.p.) followed by cervical dislocation.

Statistical analysis

The significance of differences between groups was analyzed using a one-way ANOVA and subjected to Tukey’s multiple comparisons test. Differences in cytokine and ALT levels between the groups at the indicated times were analyzed using a two-way ANOVA. Survival differences were analyzed using a Kaplan-Meier survival curve with a log-rank test. The data are represented as the mean±SEM. All the analyses were performed using GraphPad Prism software. P values lower than 0.05 were considered statistically significant.

Results

Complement activation in fulminant hepatic failure induced by LPS/D-GalN

C3 was deposited in the liver parenchyma of the LPS/D-GalN-treated mice, as detected by immunohistochemistry. A low level of C3 deposition was detected around the central vein (CV) at the 0-hour time point (Fig. 1A), whereas increased levels of C3 deposition were observed in the parenchyma, especially around the CV, 1 hour after the LPS/D-GalN injection (Fig. 1B), although no obvious pathologic injury was detected. The C3 deposition levels in the liver sections progressively increased over time after the LPS/D-GalN injection, reaching their highest value at 8 hours, primarily in the centrilobular regions (Fig. 1C and D).

Protein and gene expression levels of C3aR and C5aR were analyzed in liver tissue samples isolated at 0, 1, 4 and 8 hours post-LPS/D-GalN injection by immunohistochemistry and relative quantitative real-time RT-PCR. The results show that C3aR and C5aR expressed on non-parenchymal cells, especially kupffer cells in mice without LPS/D-GalN injection. However, the expression of C3aR and C5aR on both non-parenchymal cells and hepatocytes increased with time after LPS/D-GalN injection (Fig. 1E–L). C3aR mRNA and C5aR mRNA expression levels correlated with immunohistological data. C3aR mRNA increased 1 hour after the LPS/D-GalN injection, but we consistently found that C3aR mRNA levels returned to near control levels at 4 hours, and then increased again at 8 hours after the LPS/D-GalN injection (p<0.01) (Fig. 1M). In contrast, the C5aR expression linearly increased with time throughout the 8 hour period following the LPS/D-GalN injection (Fig. 1N). In addition, the plasma C3a levels were significantly elevated as early as 1 hour after the LPS/D-GalN injection and remained elevated for 8 hours (Fig. 1O). Collectively, the above data demonstrate significant complement activation in the liver post-LPS/D-GalN injection, and the extent of complement activation correlated with the severity of the liver injury.

C3 deficiency attenuates LPS/D-GalN-induced pathologic damage in the liver

C3 activation is the central step in the complement cascade; therefore, we investigated the effects of C3 deficiency on LPS/D-
GalN-induced liver injury. As shown in Fig. 2, the C3−/− mice displayed a significantly lower degree of liver injury after the LPS/D-GalN treatment compared with the wt mice. A detailed examination of whole livers and the H&E staining of mouse liver sections 8 hours after the LPS/D-GalN injection indicated a much lower degree of hemorrhage with less severe vascular congestion, hepatocellular damage and inflammatory cell infiltration in the C3−/− mice (Fig. 2G–J and L) compared to the wt mice (Fig. 2A–D and F). The deposition of C5b-9 was detected in wt mice but no C5b-9 deposition in C3−/− mice (Fig. 2E and K). The serum ALT concentrations were also significantly lower in the C3−/− mice compared with the wt mice from 4 to 8 hours after the LPS/D-GalN injection (Fig. 3A). The serum levels of TNF-α and IL-6 were significantly lower in the C3−/− mice than in the wt mice at 1 hour after the LPS/D-GalN injection, returning to baseline in both groups by 4 hours (Fig. 3B and C). There was no significant

Figure 3. The effects of C3 deficiency on inflammation and survival after LPS/D-GalN injection. (A) The response patterns to different ALT concentrations in C3−/− and wt mice (n=4-5). (B–D) The serum levels of proinflammatory cytokines after LPS/D-GalN injection. The concentrations of TNF-α and IL-6 in the C3−/− mice were lower than in the wt C57BL/6 mice at 1 hour, but the levels of these cytokines in both groups decreased to the same concentrations at later time points. There was no difference in MCP-1 between the groups (n = 4–5). (E) The C3−/− mice had prolonged life spans and an increased survival rate after LPS/D-GalN injection (n = 8). *** indicate p<0.001, in the comparison between the C3−/− and C57BL/6 groups. The means±SEM are shown. The results are representative of 3 separate experiments.
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Inhibition of C3aR signaling suppresses liver inflammation and attenuates the LPS-D/GalN-induced liver damage

As shown in Fig. 1, the mRNA expression of both C3aR and C5aR was upregulated following the LPS/D-GalN-induced liver injury. The complement activation products C3a and C5a are known candidates for mediating liver damage. Therefore, we investigated the effects of C3aR inhibition on liver inflammation and injury. A gross examination of livers and a microscopic analysis of liver sections from mice 8 hours after the LPS/D-GalN injection revealed the treatment with the C3aR antagonist markedly reduced the amount of liver hemorrhage and parenchymal damage compared with the saline group mice. This reduced injury was associated with less C3 deposition in the liver tissues of the C3aR antagonist-treated mice compared with the control group (Fig. 4C and F). Blocking the interaction of C3a with C3aR also reduced the C3aR mRNA and C5aR mRNA levels compared with the saline group and resulted in a more significant reduction in the C3aR mRNA compared with that of C5aR mRNA (Fig. 4G and H). Furthermore, the ALT levels were also significantly lower in the C3aR antagonist-treated mice compared with the saline group from 4 to 8 hours post-LPS/D-GalN injection (Fig. 4E). The serum levels of TNF-α, IL-6 and MCP-1 were reduced in the C3aR antagonist-treated mice at the early time points after LPS/D-GalN injection; however, 4 hours later, the MCP-1 level had increased in the C3aR antagonist-treated mice (Fig. 4J–L). Finally, C3aR blockage improved the survival rate of the LPS/D-GalN-injected mice (P<0.01, n = 8) (Fig. 4M).

Inhibition of C5aR signaling and targeted complement inhibition alleviates liver injury in LPS/D-GalN-injected mice

The effects of C5aR inhibition on liver inflammation and injury were also studied. The results were similar to that of C3aR antagonist treatment with attenuated liver hemorrhage and parenchymal damage, decreased C3 deposition compared with the control mice (Fig. 5A–F), and improved the survival rate (P<0.01, n = 8) (Fig. 5J). These results indicated an important role for C5a in mediating LPS/D-GalN-induced liver injury in mice. Furthermore, the role of the alternative complement pathway was investigated in our injury model using CR2-2H, a complement inhibitor that targets the sites of complement activation and specifically inhibits the alternative pathway [26]. Mice were treated with 0.8 mg CR2-2H i.v. immediately after LPS/D-GalN administration, with saline used as the control treatment. After 8 hours, the CR2-2H-treated mice displayed minimal evidence of focal hemorrhage and parenchymal damage compared with the control group (Fig. 5G and H). C3 deposition was also significantly reduced in the CR2-2H-treated mice compared with the control group (Fig. 5I). Almost 50% of the CR2-2H-treated mice survived for 24 hours, whereas all the control group mice died by 12 hours post-LPS/D-GalN injection (P<0.001, n = 8) (Fig. 5K). These data indicate a key role for the alternative complement pathway in causing liver injury in this model.

Discussion

Acute liver injury is a dramatic clinical syndrome with a high mortality rate and is characterized by sudden and severe damage to hepatic cells, leading to multigorgan failure [29,30]. Inflammatory cytokines, especially TNF-α, have been linked to the pathogenesis of hepatocyte apoptosis and liver injury [8–10]. Recent evidence has indicated a role for complement in the pathogenesis of a variety of liver diseases [13,31,32]. Immunohistochemistry analyses performed in patients with fulminant and acute hepatitis have shown that the membrane attack complex (MAC) is deposited around necrotic areas, indicating activation of the complement system and its involvement in the pathogenesis of liver injury [13]. In a hemorrhagic shock and tissue trauma (HS/T) mouse model, complement factor 3 deficiency or temporary C3 depletion by CVF led to both reduced transaminase levels and a blunted cytokine release, with a well-preserved hepatic structure [31]. In a previous study by Stephen Tomlinson [16] in an IRI mouse model, complement was found to play a key role in the enhanced susceptibility of steatotic livers to IRI. The results of these studies led us to hypothesize that the activation of complement or its activation products may mediate hepatic injury in FHF.

LPS is the main pathogenic factor of gram-negative bacteria and can cause systemic inflammatory response syndrome, which may lead to FHF and multigorgan failure. LPS/D-GalN-treated mice models are frequently used to study the pathogenesis of liver injury [8,9,10]. In this study, the data from the mouse model with FHF induced by LPS/D-GalN demonstrated that liver injury was associated with elevated serum C3a levels, extensive hepatic deposition of C3 and increased hepatic expression of C3aR mRNA and C5aR mRNA, along with elevated serum levels of proinflammatory cytokines and ALT. However, all of the measured parameters of inflammation and injury were significantly reduced by C3 deficiency, blockage of C3aR or inhibition of the alternative complement activation pathway. These data suggest an important role for complement activation, especially the alternative complement activation pathway, in the pathogenesis of LPS/D-GalN-induced liver injury.
Figure 5. Inhibition of C5aR signaling and targeted complement inhibition alleviates liver injury. (A–I) Both C5aRmAb and CR2-fH groups displayed reduced liver damage (A–B, D–E, G–H) and decreased C3 deposition (C, F, I) 8 hours after LPS/D-GalN injection. (J) The different response patterns of ALT concentration in the C3aR antagonist mice and the wt mice (n = 4–5). (J–K) Treatment with the C5aR antagonist or CR2-fH increased the survival rate of the mice after LPS/D-GalN injection (n = 8). ** and *** indicate p<0.01 and p<0.001, respectively, relative to the saline group. The means ± SEM are shown. Magnification of the H&E and immunohistochemically stained images: ×200. The results are representative of 3 separate experiments. doi:10.1371/journal.pone.0026838.g005
The anaphylatoxins C3a and C5a are proinflammatory polypeptides generated by the activation of the complement cascade. Both C3a and C5a have multifunctional roles in liver inflammation and regeneration via interactions with their cognate receptors, C3aR and C5aR, respectively. C5aR has been shown to be constitutively expressed in Kupffer cells and stellate cells under normal conditions, but its expression is increased in hepatocytes in response to inflammatory cytokines [33,34,35,36]. To identify the potential roles of complement activation products in the production of inflammatory cytokines in mice challenged with LPS/D-GalN, we investigated the effect of C3aR or C5aR blockage respectively on LPS/D-GalN-induced liver inflammation. Blocking the binding of C3a and C5a to their receptors protected mice against liver injury apparently. Furthermore, C3aR antagonist reduced the levels of the inflammatory cytokines TNF-α and IL-6, which suggests that the induction of inflammatory cytokines in our model was dependent on complement. These data are in agreement with previous in vitro findings [37,38] that C3a and C5a enhanced the release of IL-6 and TNF-α in a dose-dependent and PGE2-independent manner and that C5aR antagonist treatment after partial hepatic ischemia and reperfusion significantly decreased serum and tissue TNF-α level and attenuated liver histopathology [39].

Although C3a is generally a less potent mediator than C5a, the serum concentration of C3 is 10 times higher than that of C5 [40]. C3a also regulates vasodilatation, increases the permeability of small blood vessels, and induces contraction of smooth muscles. Besides, C3a triggers oxidative burst in macrophages, neutrophils and eosinophils [41,42,43], and regulates the synthesis of IL-6 and TNF-α from B cells and monocytes [37]. Studies have found that blockade or knockout of C3aR effectively attenuated tissue damage [44,45,46,47] and C3aR−/− mice are more susceptible than wt mice to an i.v. challenge with LPS [48]. In a rat model of ARDS, C3a had profound systemic hemodynamic and systemic cytokine effects, whereas, C3a exerted direct intra-lung modulation of neutrophil infiltration and cytokine production [49]. In our previous study on acute lung injury induced by paraquat in a mouse model, C3aR inhibition significantly attenuated lung injury and increased the survival rate after paraquat administration [25]. Collectively, the blockade of C3a receptor in this study is capable of offering a significant survival benefit by decreasing the inflammatory response. Also, we found that the blockade of both C3a and C5a signals had similar level of protection (data not shown).

Along with the inflammatory function of complement activation, its potential role in liver regeneration has been recently investigated [50,51]. He S et al. [52] found that in a combined model of IRI and PHx, both C3 deficiency and high-dose CR2-Crry, an inhibitor of C3 activation, produced severe hepatic injury and high mortality, whereas low-dose CR2-Crry was protective and strengthened hepatic proliferative responses. Taken together, these data confirm the multiple roles of complement activation in liver diseases and indicate that complement activation may have an important role in the pathogenesis of liver injury in our mouse model of FHF induced by LPS-D/GalN.

In our model, increased C3aR mRNA expression displayed a two-wave pattern, whereas C5aR mRNA expression increased progressively following LPS/D-GalN injection, suggesting that C3a/C3aR and C5a/C5aR signaling may have different functions at different stages of liver injury progression. The first phase of complement activation may be related to the priming of liver cells shortly after LPS-D/GalN injection [36]. Our results show that, compared with the C3aR expression trend, the C3aR antagonist significantly downregulated C5aR expression following LPS/D-GalN injection, which indicates that C5a may also contribute to LPS-induced liver injury. C5a has generally been regarded as a more potent proinflammatory mediator than C3a. Previous studies have shown that C5a stimulated the synthesis of proinflammatory cytokines and enhanced the transcription of type II acute phase protein α2-macroglobulin in the liver [34,35]. Therefore, our data showing a sharply decreased expression of C5aR further indicate the crucial function of C5a in mediating liver damage in LPS-D/GalN-induced FHF.

In summary, we demonstrated an essential role for complement activation, especially the alternative activation pathway, in the pathogenesis of LPS-D/GalN-induced liver injury. The interaction of anaphylatoxins with their receptors plays a role in the hepatic damage in this model. Inhibiting complement activation represents a potential therapeutic approach for the adjunctive treatment of LPS-induced fulminant hepatic failure.

**Author Contributions**

Conceived and designed the experiments: SS ZD YZ. Performed the experiments: SS YG GZ XZ JL JH HY YC HS FQ GX FY YW. Analyzed the data: SS YG GZ YZ. Wrote the paper: SS ST YZ.

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