NLRP3 is dispensable for D-galactosamine/Lipopolysaccharide-induced fulminant hepatitis

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Research

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Abstract

Background: The NOD-like receptor family protein 3 (NLRP3) inflammasome is involved in the progression of liver inflammation. NLRP3 inactivation has been shown to protect mice against multiple types of experimental liver injury. However, it is unclear whether NLRP3 contributes to D-Galactosamine (DGalN) plus lipopolysaccharide (LPS) induced fatal hepatitis. This study aims to examine the function of NLRP3 inflammasome in DGalN/LPS induced acute liver failure.

Method: The expression of inflammasomes was detected by quantitative PCR. Wild type (WT) mice and NLRP3 knockout mice were given an intraperitoneal injection of DGalN plus LPS. Liver damage was examined at 6 h post DGalN/LPS administration by histological analysis and serum aminotransferases measurement. Hepatocyte apoptosis and pyroptosis was detected with TUNEL assay, immunohistochemistry (IHC) staining, and western blot. Hepatic macrophages ($F4/80^+$) and neutrophils ($Ly6G^+$) were analyzed by IHC staining. The proinflammatory cytokines levels in serum and liver were examined using cytometric bead array and quantitative PCR. Hepatic IL1β level was further detected by western blot.

Result: The hepatic NLRP3 activity was upregulated in WT mice treated with DGalN/LPS. *Nlrp3*$^{-/-}$ and WT mice showed similar mortality against lethal dose of DGalN/LPS. Serum aminotransferases levels and liver necrosis area of *Nlrp3*$^{-/-}$ mice did not differ from that in WT mice after DGalN/LPS injection. The numbers of liver TUNEL positive cells and cleaved caspase3 positive hepatocytes were comparable between *Nlrp3*$^{-/-}$ and WT mice. The hepatic $GSDMD^{Nterm}$ peptide production also showed no difference between *Nlrp3*$^{-/-}$ and WT mice. Mice treated with DGalN/LPS displayed significantly increased numbers of intrahepatic $F4/80^+$ cells and $Ly6G^+$ cells, but the cell numbers in *Nlrp3*$^{-/-}$ mice livers were similar to those in WT mice. Consistently, serum and hepatic TNFα, IL6, and MCP-1 levels were similar between *Nlrp3*$^{-/-}$ and WT mice upon DGalN/LPS administration, but serum IL1β and hepatic mature IL1β level in *Nlrp3*$^{-/-}$ mice was reduced.

Conclusions: NLRP3 ablation does not protect mice from DGalN/LPS induced acute liver failure, nor affect hepatocyte apoptosis and pyroptosis. Deficiency of NLRP3 has a limited effect on intrahepatic inflammatory response induced by DGalN/LPS treatment. NLRP3 inflammasome does not appear to be a major contributor to DGalN/LPS induced fatal hepatitis.

Background

Sepsis is a clinical syndrome of life-threatening organ dysfunction caused by a dysregulated host response to infection [1]. As an important immune organ, the liver is essential for host survival in sepsis, which is responsible for clearing bacteria and endotoxin and regulating immune defense during infection [2, 3]. However, the liver is also damaged during sepsis by pathogens, microbial products, or proinflammatory mediators; in turn, the injured liver further acts as the main source of shock-related soluble mediators (e.g. TNFα) which induce systemic inflammatory response syndrome (SIRS) and

multiple organ dysfunction syndromes (MODS) [2, 3]. Patients with preexisting liver injury are much more susceptible to sepsis than healthy individuals. The increase of pathogens or toxins in the liver resulted from infection is one of the most common causes of acute hepatic failure in patients with underlying liver diseases [4]. Liver damages either before or after sepsis have important effects on clinical outcomes and the severity of this disease.

NLRP3 acts as a cytosolic pattern recognition receptor which is sensitive to unusually many and diverse stimuli [5]. Once activated, NLRP3 recruits ASC and caspase1 in order to form the NLRP3 inflammasome, resulting in the maturation of several proinflammatory cytokines, including IL18 and IL1β, and leading to pyroptotic cell death [5, 6]. As a key constituent of the natural immune system, NLRP3 inflammasomes can promote host immune defense against pathogenic infections [7]. Nevertheless, overactivation of NLRP3 inflammasomes triggers a wild variety of human diseases progression [8]. NLRP3 inflammasome has been shown to be present in sinusoidal endothelial cells, hepatocytes, hepatic stellate cells, and Kupffer cells [9]. Increasing numbers of research have uncovered the direct role of NLRP3 inflammasome activation in various acute and chronic liver diseases [9–11]. Global *Nlrp3* knock-in mice that constitutively express activated NLRP3 show marked pyroptotic cell death of hepatocyte as well as severe liver inflammation and hepatic fibrosis [12]. In contrast, deficiency of NLRP3 protects mice against multiple types of experimental liver injury, including acetaminophen (APAP)-induced acute liver failure (ALF), carbon tetrachloride and thioacetamide (TAA)-leaded liver fibrosis, alcohol-induced liver injury, and diet-induced non-alcoholic steatohepatitis [13–17]. However, controversial findings of the role and function of NLRP3 in these experimental models of liver injury have also been reported [18–21]. For example, Williams et al demonstrated that mice lacking each component of the NLRP3 inflammasome (caspase1, ASC, and NLRP3) had similar liver injury as wild-type (WT) animals in APAP-induced ALF model [18]. Lipopolysaccharide (LPS), a glycolipid constituent of the outer membrane of gram-negative bacteria, intraperitoneal administration alone induces SIRS, endotoxic shock, and death, but does not trigger liver injury. In contrast, low doses of LPS challenge combined with hepatocyte specific transcriptional inhibitor d-galactosamine (DGalN) does not lead to septic shock, but to ALF [22, 23]. It has been frequently used as an animal model that closely represents ALF in clinic [23, 24]. In this model, LPS induced TNFα production by Kupffer cells (KCs) is demonstrated to play critical roles in the etiopathogenesis of ALF [25–28]. In addition, several studies showed that IL1β also contributes to the amplification of hepatocytes death and hepatic inflammation [29, 30]. However, type I IL-1R knockout mice showed no difference in sensitivity to the challenge of DGalN plus LPS [31]. Therefore, the functional role and molecular mechanism of NLRP3 inflammasome in ALF require further study.

Given the enhanced expression of IL1β and component of NLRP3 inflammasome in liver after low doses LPS injection in DGalN pretreated mice [32], this experimental model seemed to be particularly suitable for testing the role of NLRP3 in ALF. Herein, we studied the role of NLRP3 inflammasome in DGalN plus LPS induced ALF using NLRP3 knockout (*Nlrp3*$^{-/-}$) mice. Unexpectedly, we found that mice deficient in NLRP3 showed very similar responses to DGalN/LPS treatment compared with WT mice, which suggests NLRP3 inflammasome has no relevant impact on DGalN plus LPS induced liver failure.

Methods

Mice and fatal hepatitis model

Nlrp3$^{-/-}$ mice were purchased from Dr. Tao Li, and maintained in Animal Center. Animal Center provided SPF rooms with *ad libitum* diet. Male mice between 6 and 8 weeks were used to establish the model of fatal hepatitis. Liver injury was typically induced by 20 mg/mouse DGalN (Sigma, G0500) injection, followed by intraperitoneal injection of 0.2 μg/mouse LPS (Sigma, L6529). For survival analysis, a lethal dose of DGalN (20 mg/mouse) plus 0.3 μg/mouse LPS was injected. In our experiments, mice were compared to the weight-, and age-matched WT littermates systematically.

Histological analysis

Liver tissues were excised at indicated times after DGalN/LPS injection. Liver tissues were cut and then fixed with paraformaldehyde (4%). After embedded in paraffin, the sections were performed with H&E staining for morphological analysis. The necrosis was expressed as a percentage of necrotic areas of ×200 magnification per slide. The pictures of liver sections were acquired using Nikon Digital Sight DS-U3 camera. Scale bar, 50 μm. Image analysis procedures were performed with IPP v6.0 software (Media Cybernetics, Inc).

Quantitative PCR

TRIzol reagent was used to extract liver total RNA. Then hepatic RNA was reverse transcribed into cDNA. Quantitative PCR was performed using LightCycler 480 system. Relative gene expression was evaluated by ΔCT method. Primer Bank was used for genes specific primers design. Primers were listed in Table S1 in Supplementary Information.

Immunohistochemistry staining

The immune cell infiltration in mice livers was determined by immunohistochemistry staining (IHC) with CD11b, F4/80, or Ly6G antibodies. IHC staining with a cleaved caspase3 antibody was used to check the caspase3 activity. Positive cells were calculated in 3 fields per slide under light microscopy (×200 or ×400). Scale bar, 50 μm. Antibodies used for IHC staining were listed in Table S2.

TUNEL assay

The analysis of hepatocyte apoptosis was carried out with the TUNEL assay using a commercial kit (Roche, 11684817910). Nuclei were counterstained with hematoxylin. TUNEL positive cells were analyzed from randomly selected 3 fields of ×200 magnification for each sample. Scale bar, 50 μm.

Detection of aminotransferases and cytokines

Serum aminotransferases were measured at Beijing CIC Clinical Laboratory (Beijing, China). Mouse TNF, IL6, IL1β, and MCP-1 Flex Set were used to detect serum and hepatic cytokines following the standard

protocol. We use BCA Assay Kit to determine liver total protein quantification (Thermo Fisher Scientific, 23227).

Western blot

Total liver protein was picked up using PBS buffer containing proteinase inhibitor and Triton X100. After quantitative detection, protein extracts were denatured in Laemmli buffer. Proteins were then separated using SDS PAGE, and followed by transferring to the PVDF membrane. Then the membrane was incubated with primary and secondary antibodies, and the blot was detected using chemiluminescent reagents. Antibodies' information was provided in Table S2.

Statistics. Statistics were calculated with GraphPad Prism. Results were shown as means ± SEM. We use the Kolmogorov-Smirnov test to examine the distribution of variables. To check the significance of differences between the two groups, a standard two-tailed unpaired Student's *t*-test was used. Log-rank (Mantel-Cox) test was used for survival analysis. *P*-value <0.05 was considered statistically significant. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

Results

The activity of inflammasomes is increased after DGalN plus LPS challenge

To characterize the involvement of inflammasomes in DGalN plus LPS induced liver damage, the hepatic expressions of inflammasomes including NLRP-1, 2, 3, 6, 10, 12, AIM2, and NLRC4 were examined by quantitative PCR. Results exhibited that DGalN/LPS treatment upregulated the mRNA levels of hepatic NLRP2, NLRP3, and AIM2 significantly (NLRP1 not detected) (Fig. 1a). The hepatic expression and activity of caspase1 were increased remarkably after DGalN/LPS injection (Fig. 1b). Moreover, the results showed a marked rise of serum IL1β and IL18 levels in DGalN/LPS treated mice (Fig. 1c). The hepatic mRNA levels of IL1β and IL18 also increased significantly upon DGalN/LPS administration (Fig. 1d). Consistently, the expressions of NLRP3, pro-IL1β, mature IL1β, and IL18 were notably upregulated after DGalN/LPS injection (Fig. 1e). These data indicate that inflammasomes, especially NLRP3 inflammasome, are activated during DGalN/LPS challenge.

Role of NLRP3 inflammasome in DGalN/LPS caused fatal hepatitis

To address the importance of NLRP3 in DGalN/LPS caused hepatitis, we established a mouse model of DGalN/LPS-driven ALF by using *Nlrp3*$^{-/-}$ mice. Our results showed that a lethal dose of DGalN/LPS treatment caused 90% mortality in WT mice and 100% mortality in *Nlrp3*$^{-/-}$ mice within 9 h (Fig. 2a). Accordingly, representative pictures showed comparable severity of liver damage between WT and *Nlrp3*$^{-/-}$ mice challenged with a sublethal dose of DGalN/LPS (Fig. 2b). Moreover, WT and *Nlrp3*$^{-/-}$ mice had similar levels of serum aminotransferases after DGalN/LPS administration (Fig. 2c). Histological analysis exhibited that the extent of liver damage in *Nlrp3*$^{-/-}$ mice was not significantly different from control animals at 6 h after DGalN/LPS injection (Fig. 2d), characterized by similar areas of necrosis (Fig.

2e). Our data indicate that NLRP3 ablation does not protect mice from DGalN plus LPS caused fatal hepatitis.

NLRP3 deficiency has no effect on hepatocyte apoptosis and pyroptosis induced by DGalN/LPS

We next investigated whether NLRP3 deficiency affects hepatocyte apoptosis and pyroptosis induced by DGalN/LPS using TUNEL assay, IHC staining, and western blot assay. DGalN/LPS treatment induced a remarkable increase of TUNEL positive cells in both WT and *Nlrp3*$^{-/-}$ mice livers; however, no significant differences were observed between these two groups (Fig. 3a-b). Consistently, IHC analysis of hepatic cleaved caspase3 showed that the number of positively stained hepatocytes did not differ between *Nlrp3*$^{-/-}$ and WT mice (Fig. 3c-d). Additionally, both WT and *Nlrp3*$^{-/-}$ mice displayed similar levels of increased hepatocyte pyroptosis after DGalN/LPS administration, as assessed by hepatic GSDMD^{Nterm} peptide production (a marker of pyroptosis) (Fig. 3e). These data suggest that NLRP3 deficiency does not appear to affect the hepatocyte apoptosis and pyroptosis induced by DGalN/LPS.

NLRP3 inactivation has no significant effect on hepatic infiltration of proinflammatory cells in DGalN/LPS treated mice

DGalN/LPS-induced acute liver injury was associated with liver infiltration with remarkable immune cells. Our results showed that deficiency of NLRP3 does not affect the numbers of CD11b^{+} cells (Fig. 4a-b), macrophages (F4/80^{+}) (Fig. 4c-d), and neutrophils (Ly6G^{+}) (Fig. 4e-f) in mice livers under physiological conditions, as assessed by IHC staining. We found that WT mice livers displayed significantly increased numbers of CD11b^{+} cells (Fig. 4a-b), F4/80^{+} cells (Fig. 4c-d), and Ly6G^{+} cells (Fig. 4e-f) after DGalN/LPS treatment, but the numbers of these cells were comparable with those in *Nlrp3*$^{-/-}$ mice. These data indicate that NLRP3 does not play a major role in DGalN/LPS induced intrahepatic proinflammatory cell infiltration.

Nlrp3$^{-/-}$ mice have similar TNFα, but reduced IL1β levels compared with WT mice after DGalN/LPS treatment

We next examined whether NLRP3 ablation affects proinflammatory cytokines production in DGalN/LPS treated mice. The serum TNFα, IL6, and MCP-1 levels were remarkably increased in WT mice upon DGalN plus LPS treatment, but the levels in *Nlrp3*$^{-/-}$ mice were similar to those in WT mice (Fig. 5a). Consistently, no significant differences were observed in hepatic TNFα, IL6, and MCP-1 levels between *Nlrp3*$^{-/-}$ and WT mice after DGalN/LPS administration (Fig. 5b). Quantitative PCR also showed similar mRNA levels of TNFα, IL6, and MCP-1 between two groups (Fig. 5c). The increased mRNA level of IL1β also has no difference between *Nlrp3*$^{-/-}$ and WT mice liver after DGalN/LPS treatment (Fig. 5d). In contrast, the hepatic mature IL1β was elevated in two genotype mice after DGalN/LPS injection, but the increase was suppressed in *Nlrp3*$^{-/-}$ mice (Fig. 5e). Consistently, DGalN/LPS treatment induced rapid increases of serum IL1β levels in WT mice, however, the rise was significantly blunted in NLRP3 deficient mice (Fig. 5f). Nevertheless, in contrast to LPS treated alone, DGalN plus LPS administration displayed a

much milder reduction of IL1β in NLRP3 deficient mice [33]. The incomplete blockage of IL1β production in *Nlrp3*$^{-/-}$ mice suggested that there might be NLRP3-independent pathways to produce IL1β after DGalN/LPS treatment. As predicted, NLRP2 and NLRP12 mRNA levels were markedly upregulated in the liver of NLRP3 deficient mice after DGalN plus LPS challenge (Fig. 5g). Collectively, the results indicate a limited role of NLRP3 in regulating DGalN/LPS leaded proinflammatory cytokines production.

Discussion

The expression of NLRP3 is increased in viral- and drug-induced fulminant hepatitis [34], but its actual role in these pathological conditions has not been fully studied. In this paper, we have performed a detailed analysis of liver injury in *Nlrp3*$^{-/-}$ mice in DGalN plus LPS challenge conditions. We demonstrated that *Nlrp3*$^{-/-}$ and WT mice had similar survival rates and liver injury after DGalN/LPS challenge, as assessed by measuring serum aminotransferase, liver necrotic area, and hepatocyte apoptosis/pyroptosis. These results are in good agreement with previous findings that mice deficient for NLRP3 had unaltered susceptibility to APAP-induced fulminant hepatitis [18], and suggest that NLRP3 inflammasome does not appear to be a critical factor in DGalN/LPS induced ALF.

NLRP3 deficiency had no obvious effect on the hepatic infiltration of neutrophils and macrophages, as well as the production of TNFα, IL6, and MCP-1, indicate that DGalN/LPS induced hepatic inflammatory response was unaffected by NLRP3 deficiency. Proinflammatory cytokines, especially TNFα, are considered as the key driving forces to cause liver injury in DGalN/LPS induced fulminant hepatitis model [35]. Our results, therefore, suggest that NLRP3 deficiency does not alleviate the overall cytokine production disorder in DGalN/LPS induced ALF. These findings are at variance with the previous reports that inflammasome-independent proinflammatory cytokines such as TNFα and MCP-1 were decreased in steatotic liver injury in *Nlrp3*$^{-/-}$ mice [36], and suggest that NLRP3 deficiency may not affect proinflammatory M1 macrophage polarization as occurred with LPS caused inflammation in steatotic livers [37].

The deficiency of NLRP3 selectively decreased the inflammasome-dependent cytokine IL1β in serum from mice treated with DGalN/LPS. Previous studies have shown that mice deficient in IL1β, IL18, or type I IL-1 receptor (IL-1R1) had unaltered susceptibility to the lethal effects of LPS in the DGalN model compared to WT mice [31, 38, 39], which indicates IL1β, as well as IL18, is not a major contributor to the pathogenesis of DGalN/LPS leaded liver injury. However, some results showed the contradictions with the viewpoints mentioned above. DGalN/LPS induced ALF was significantly attenuated in mice with hepatocyte-specific deletion of IL1-R1 [40]. Besides, blocking IL-1 signaling by treatment with the recombinant human IL-1 receptor antagonist (IL-1ra) *anakinra* alleviated DGalN/LPS leaded liver injury [30, 40]. Moreover, the NLRP3 inhibitor MCC950 has also shown to protect mice from chemically-induced liver injury [41, 42]. Here, we found that although NLRP3 deficiency decreased serum and hepatic IL1β levels in DGalN/LPS treated mice, the liver injury is not improved, suggesting that IL1β may have a limited role in DGalN/LPS induced liver injury. Strikingly, compared with WT mice, *Nlrp3*$^{-/-}$ mice response to

DGalN/LPS only reduced serum IL1β concentration by about 50%, which was significantly higher than that of mice treated with LPS alone in *Nlrp3*$^{-/-}$ mice [33]. These data suggest that there might be NLRP3 independent pathways to produce IL1β and IL18 in DGalN/LPS model. This speculation is supported by the previous demonstration that IL1β and IL18 processes are also mediated by other inflammasomes [43]. Our results showed that NLRP2 and AIM2 were upregulated in the liver of DGalN/LPS treated WT mice. Especially, the hepatic mRNA levels of NLRP2 and NLRP12 were remarkably promoted in *Nlrp3*$^{-/-}$ mice challenged with DGalN/LPS. Therefore, the NLRP3 deficiency caused IL1β reduction may be partially compensated by other inflammasomes such as NLRP2 and NLRP12. The roles of NLRP2 and NLRP12 in ALF are worthy of further studies.

Conclusion

Our study revealed that deficiency of NLRP3 has no protective effects against LPS induced liver injury and mortality in DGalN sensitized mice, which indicates a limited role of NLRP3 inflammasome in DGalN/LPS induced acute liver failure.

Declarations

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Authors' contributions

WZ conducted the experiments. GMR, SST, TW, YTL collected and analyzed the data. HC, YQZ, MY, CHG, CYL gave technical or material support. GMR contributed to the study design. WZ, RHY, and XMY drafted the manuscript. RHY and XMY obtained funding and supervised the project. All authors read and approved the final manuscript.

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Availability of data and materials

All materials are commercially available, and the datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Beijing Institute of Lifeomics (NO. IACUC-DWZX-2020-568).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

ALF: Acute liver failure; APAP: Acetamino; CBA: Cytometric bead array; DGalN: D-Galactosamine; H&E: Hematoxylin and eosin; IHC: Immunohistochemistry; LPS: Lipopolysaccharide; NLRP3: NOD-like receptor family protein 3; SEM: Standard error of the mean; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; WT: Wild type

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Figures

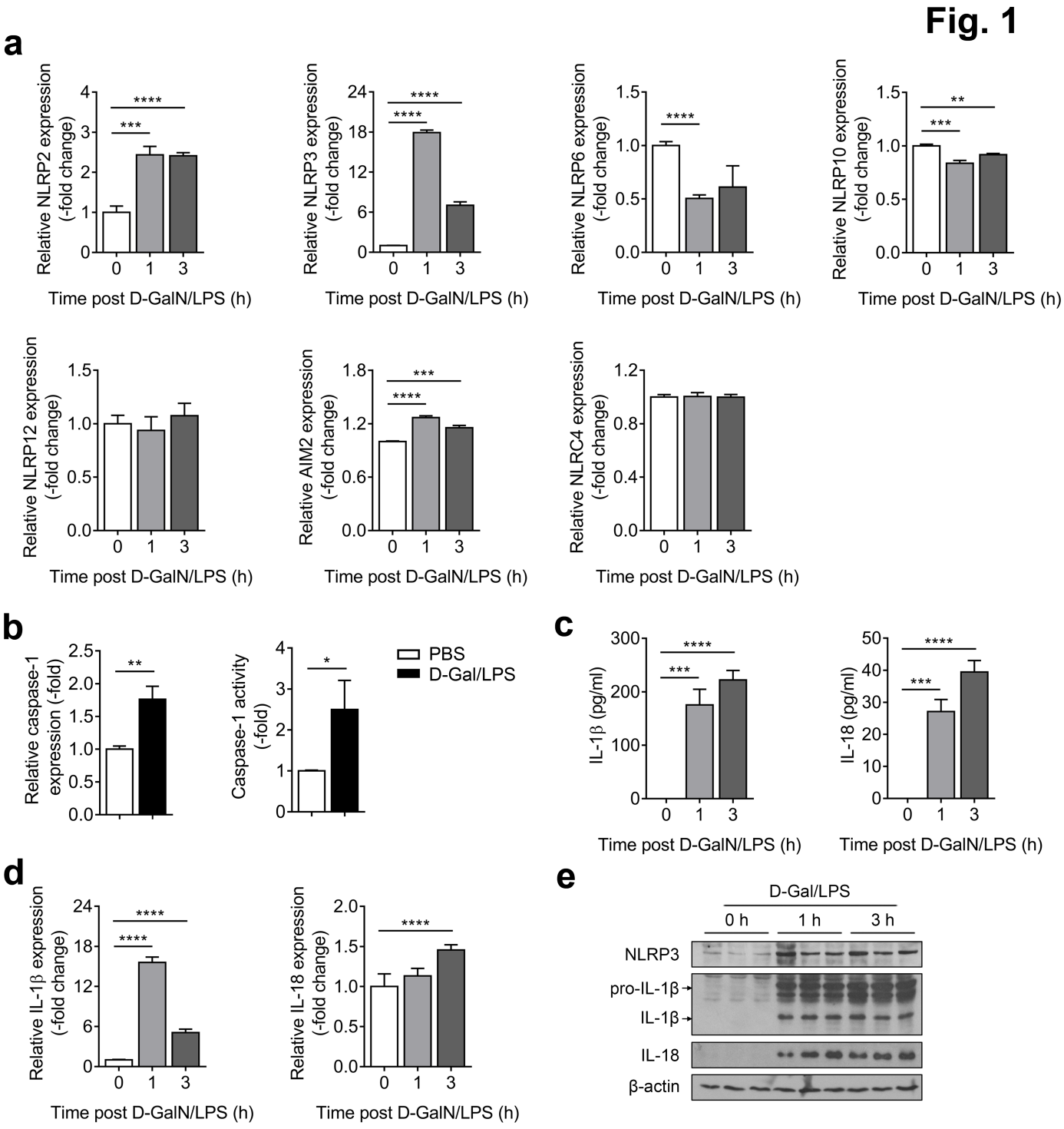

Figure 1

The activity of inflammasomes is increased after DGalN plus LPS challenge. WT mice were challenged with DGalN/LPS for indicated times (n = 3-5). a. The hepatic mRNA expression of inflammasomes. b. Liver caspase1 mRNA level and caspase1 activity. c. Serum IL1β and IL18 levels. d. The hepatic mRNA levels of IL1β and IL18. e. Western blot assay of liver NLRP3, pro-IL1β, mature IL1β, and IL18.

Fig. 2

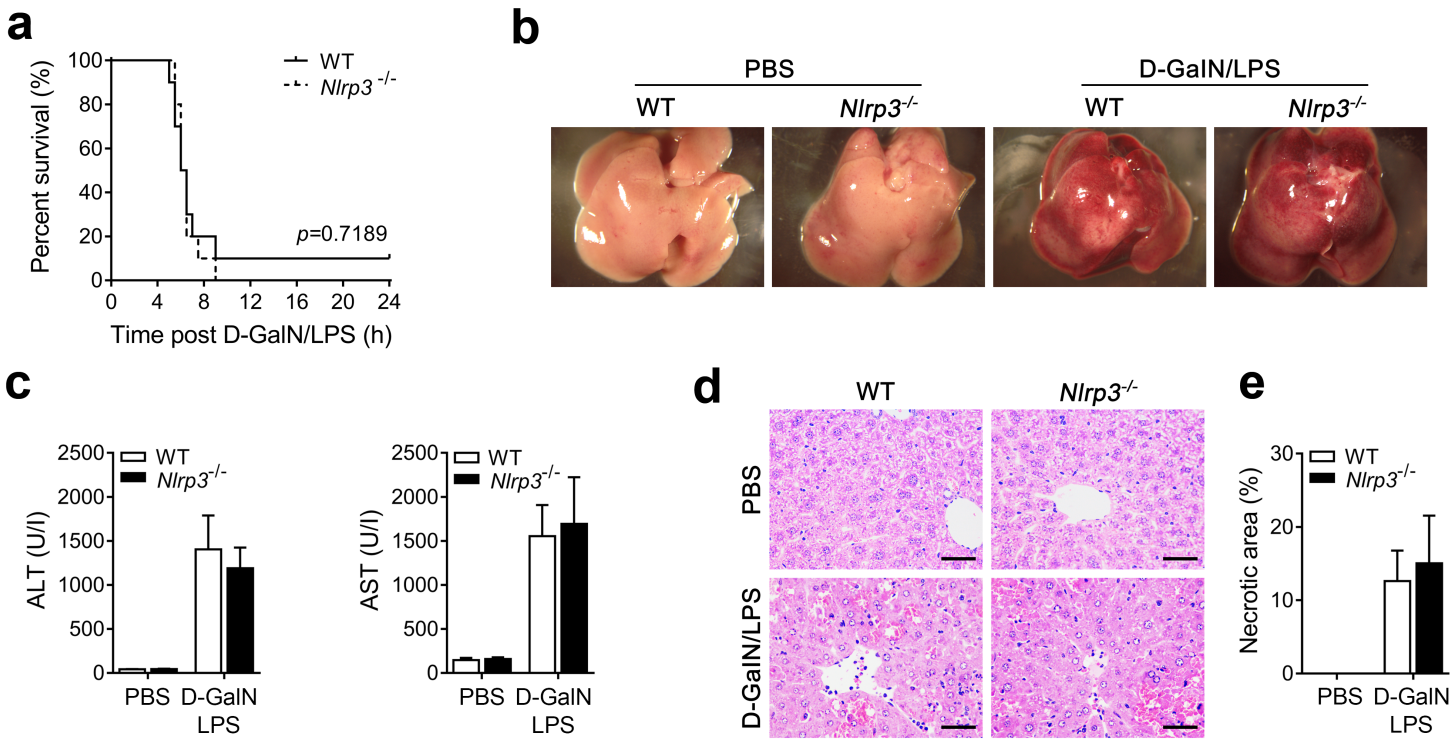

Figure 2

Role of NLRP3 inflammasome in DGalN/LPS induced fatal hepatitis. a. Survival curve for WT and Nlrp3−/− mice challenged with DGalN (20 mg/mouse) plus LPS (0.3 μg/mouse) (n = 10). b-e. WT and Nlrp3−/− mice were injected by a sublethal dose of DGalN/LPS or PBS for 6 h (n = 6-8). b. Representative pictures of liver were shown. Liver damage was assessed by serum aminotransferases (c), H&E staining (d), and percentage of hepatic necrotic area (e). The necrotic area was shown as a percentage of the total field area.

Fig. 3

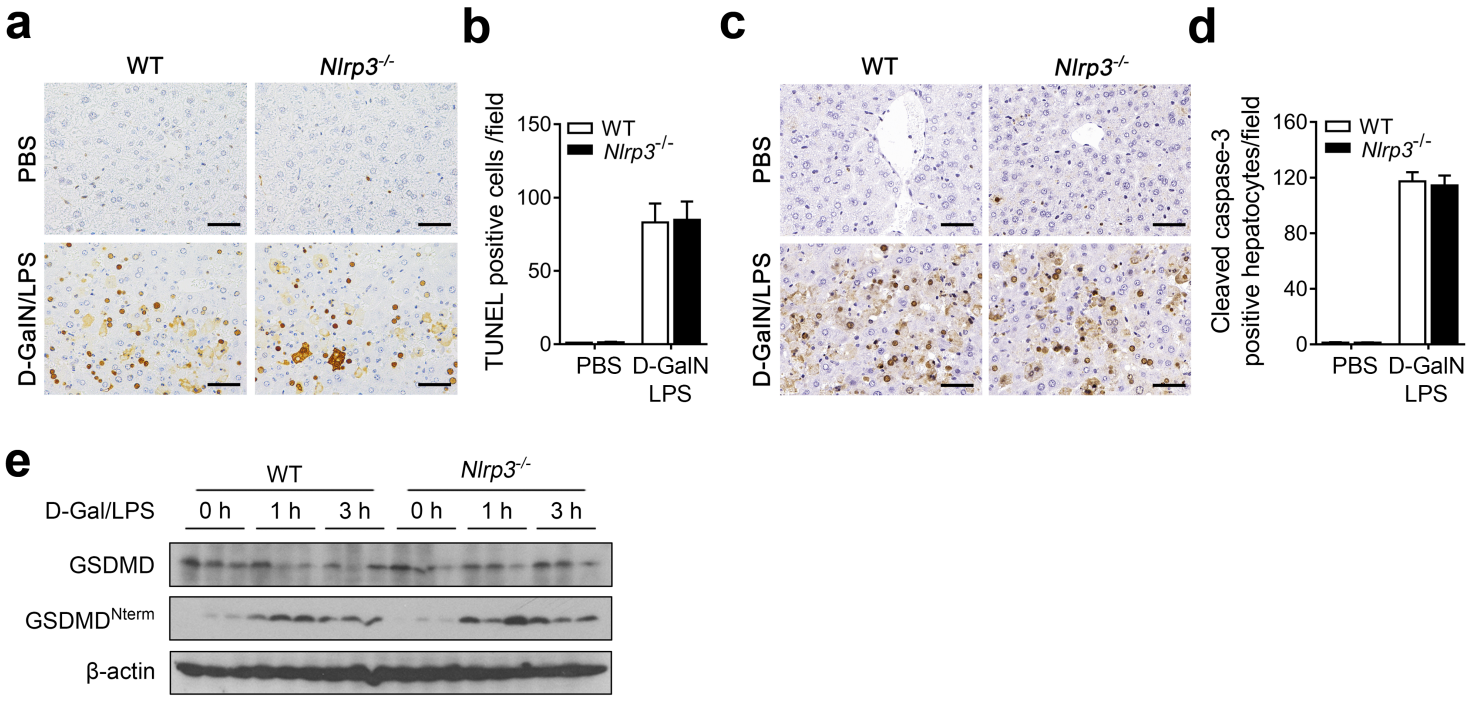

Figure 3

NLRP3 deficiency has no effect on hepatocyte apoptosis and pyroptosis induced by DGalN/LPS. WT and Nlrp3−/− mice were injected with PBS or DGalN/LPS for 6 h (n = 6-7). Representative pictures of TUNEL stained hepatic sections (a) and TUNEL positive cells per field were counted (b). Liver IHC analysis of cleaved caspase3 (c) and caspase3 positive stained hepatocytes in each field were counted (d). e. Western blot assay of hepatic GSDMD and GSDMDNterm peptide after DGalN/LPS treatment for indicated times.

Fig. 4

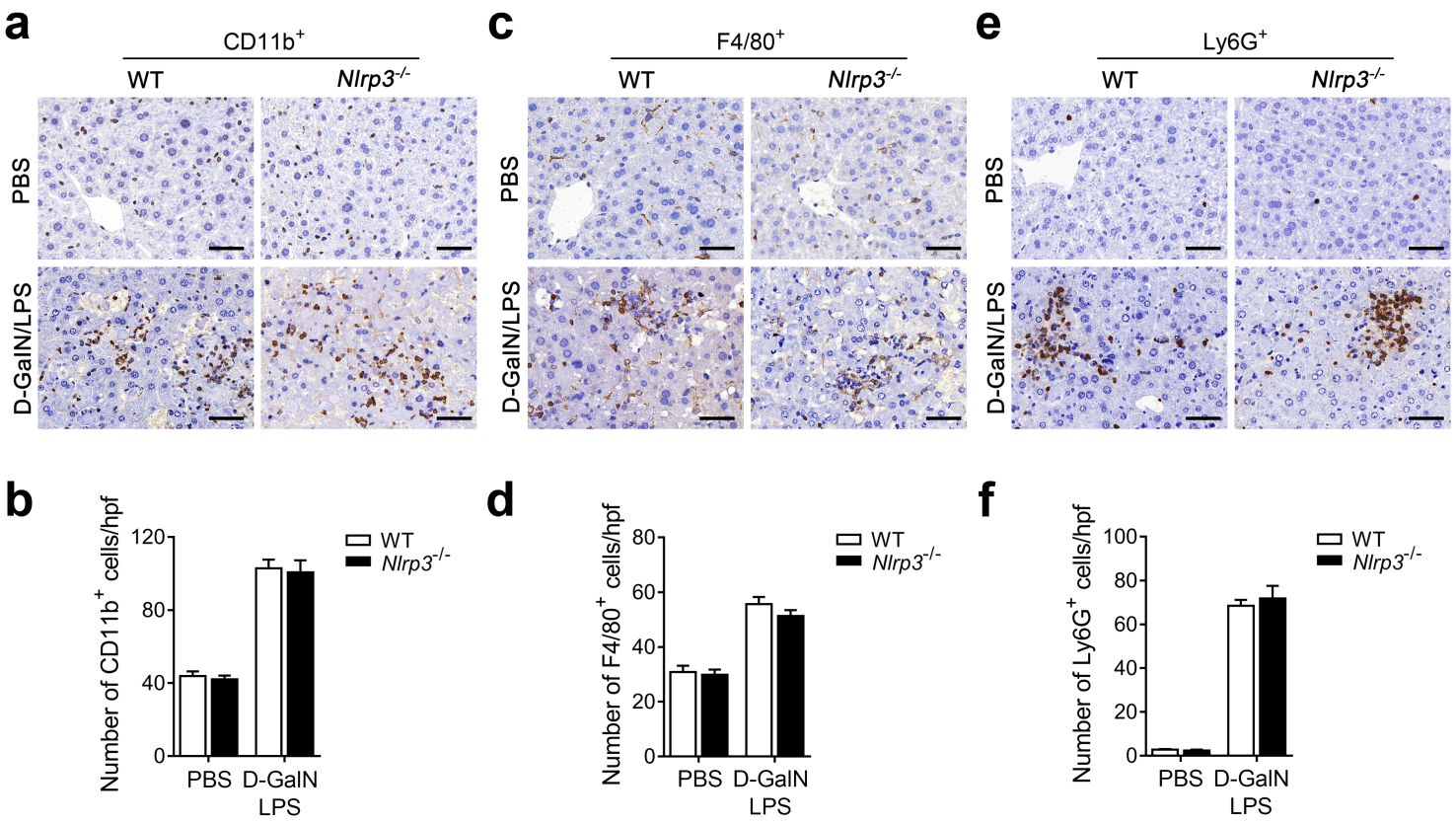

Figure 4

NLRP3 inactivation has no significant effect on hepatic infiltration of proinflammatory cells in DGalN/LPS treated mice. WT and Nlrp3−/− mice were injected with PBS or DGalN/LPS for 6 h (n = 5-6). IHC of CD11b+ (a, b), F4/80+ (c, d), and Ly6G+ cells (e, f). Representative IHC pictures were shown and positive cells per high-power field (hpf, × 400) were counted.

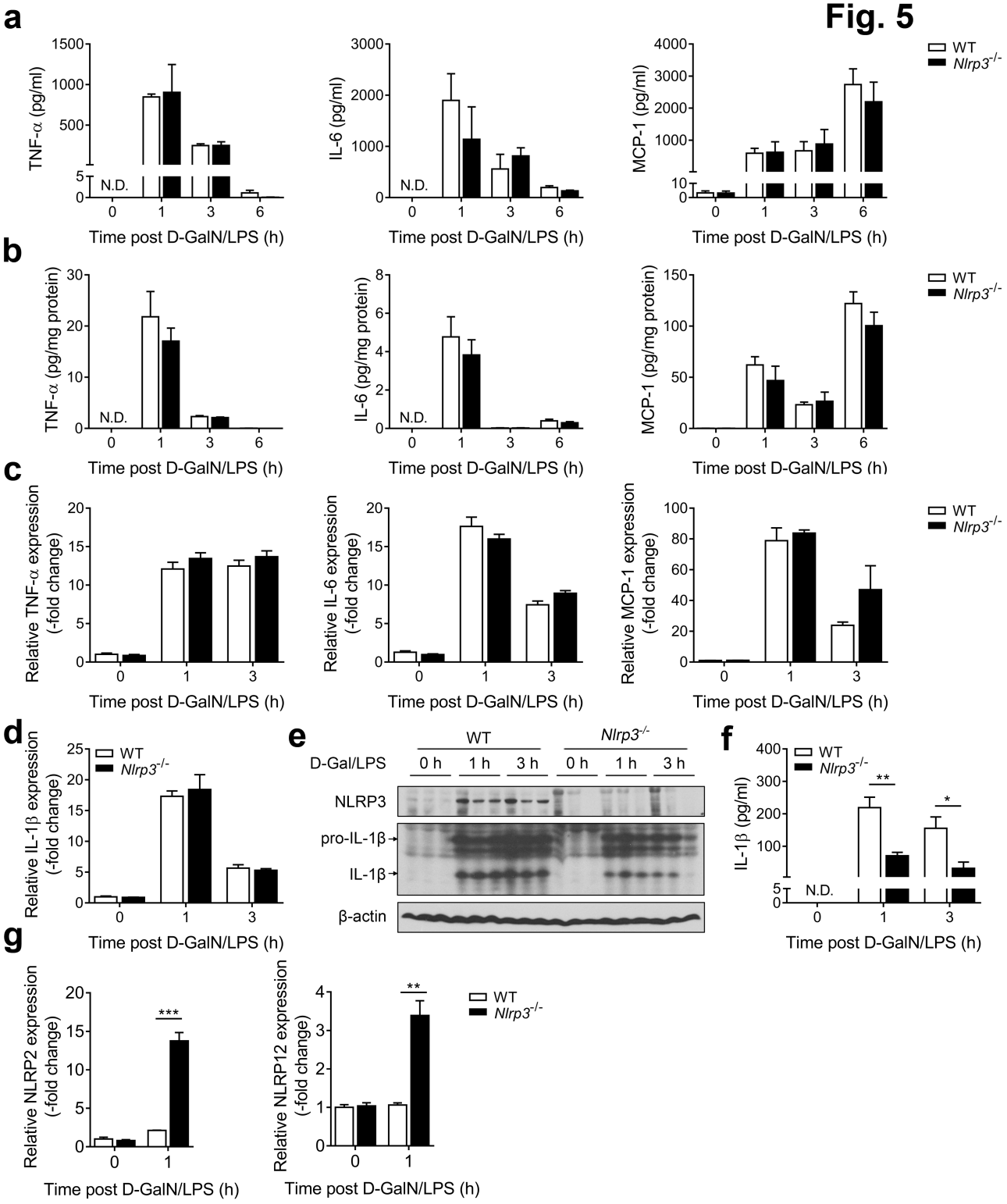

Figure 5

Nlrp3−/− mice have similar TNFa, but reduced IL1β levels compared with WT mice after DGalN/LPS treatment. WT and Nlrp3−/− mice were administrated with DGalN/LPS for the specified times (n = 4-8). a. Serum TNFα, IL6, and MCP-1 levels. b. Levels of hepatic TNFα, IL6, and MCP-1. c. Liver TNFα, IL6, and MCP-1 mRNA levels. d. Liver IL1β mRNA levels. e. Western blot assay of hepatic NLRP3, pro-IL1β, and mature IL1β. f. Serum IL1β level. g. Hepatic mRNA levels of NLRP2 and NLRP12. N.D., not detected.

Supplementary Files

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