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Natural killer T cells play a necessary role in modulating of immune-mediated liver injury by gut microbiota

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Gut microbiota are implicated in many liver diseases. Concanavalin A (ConA)-induced hepatitis is a well-characterized murine model of fulminant immunological hepatic injury. Oral administration of pathogenic bacteria or gentamycin to the mice before ConA injection, liver injury and lymphocyte distribution in liver and intestine were assessed. Our data show that administration of pathogenic bacteria exacerbated the liver damage. There was more downregulation of activation-induced natural killer T (NKT) cells in the liver of pathogenic bacteria-treated ConA groups. Also, there was a negative correlation between the numbers of hepatic NKT cells and liver injury in our experiments. Moreover, intestinal dendritic cells (DCs) were increased in pathogenic bacteria–treated ConA groups. The activation of DCs in Peyer's patches and the liver was similar to the intestine. However, depletion of gut gram-negative bacteria alleviated ConA-induced liver injury, through suppressed hepatic NKT cells activation and DCs homing in liver and intestine. *In vitro* experiments revealed that DCs promoted NKT cell cytotoxicity against hepatocyte following stimulation with pathogenic bacteria. Our study suggests that increased intestinal pathogenic bacteria facilitate immune-mediated liver injury, which may be due to the activation of NKT cells that mediated by intestinal bacterial antigens activated DCs.

Hepatitis, commonly induced by virus infection, autoimmune diseases, or alcohol abuse, can lead to liver fibrosis, cirrhosis, and carcinoma. Concanavalin A (ConA)-induced hepatitis is a well-characterized model of fulminant immunological hepatitis. Previous studies have shown that the role of natural killer T (NKT) cells was critical in the process of ConA-induced hepatic injury[1]. In addition, NKT cell activation by ConA leads to a rapid reduction in NKT cell numbers due to profound downregulation of the NKT cell receptor[2]. Liver plays a major role in metabolism and detoxification, it constantly exposed to microbial products from the enteric microflora and liver can metabolize the gut-derived toxins; however, this ability is impaired when liver is injured. Many studies have reported that structural and microbiota disorders of the intestine are closely related to liver fibrosis[3,4] and hepatocellular carcinoma (HCC)[5]. These studies have indicated that the intestinal microbiota might play an important role in the pathogenesis of liver disease.

Large numbers of microorganisms inhabit the gut symbiotically and are crucial for regulating intestinal motility, intestinal barrier homeostasis, and nutrient absorption[6]. A balanced composition of gut microflora confers a diversity of health benefits; however, dysbacteriosis of the intestinal microflora leads to altering immune responses and results in enhanced disease susceptibility[7–9]. Breakdown of the gut microflora homeostasis might induce an inappropriate immune response, resulting in acute and chronic inflammatory liver diseases[10]. A recent report demonstrated that intestinal dysbacteriosis induced intestinal inflammation, thereby promoting the release of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) by intestinal cells, which might contribute to the development of chronic inflammation in HCC patients[11]. In mice with non-alcoholic fatty liver disease (NAFLD), dysbacteriosis induced TNF-α overexpression plays a pathogenic role in NAFLD progressing to fibrosis[12]. Elevated TNF-α production can directly induce hepatocyte necrosis, but also activate T lymphocytes, dendritic cells (DCs), NK cells and Kupffer cells simultaneously. In addition,

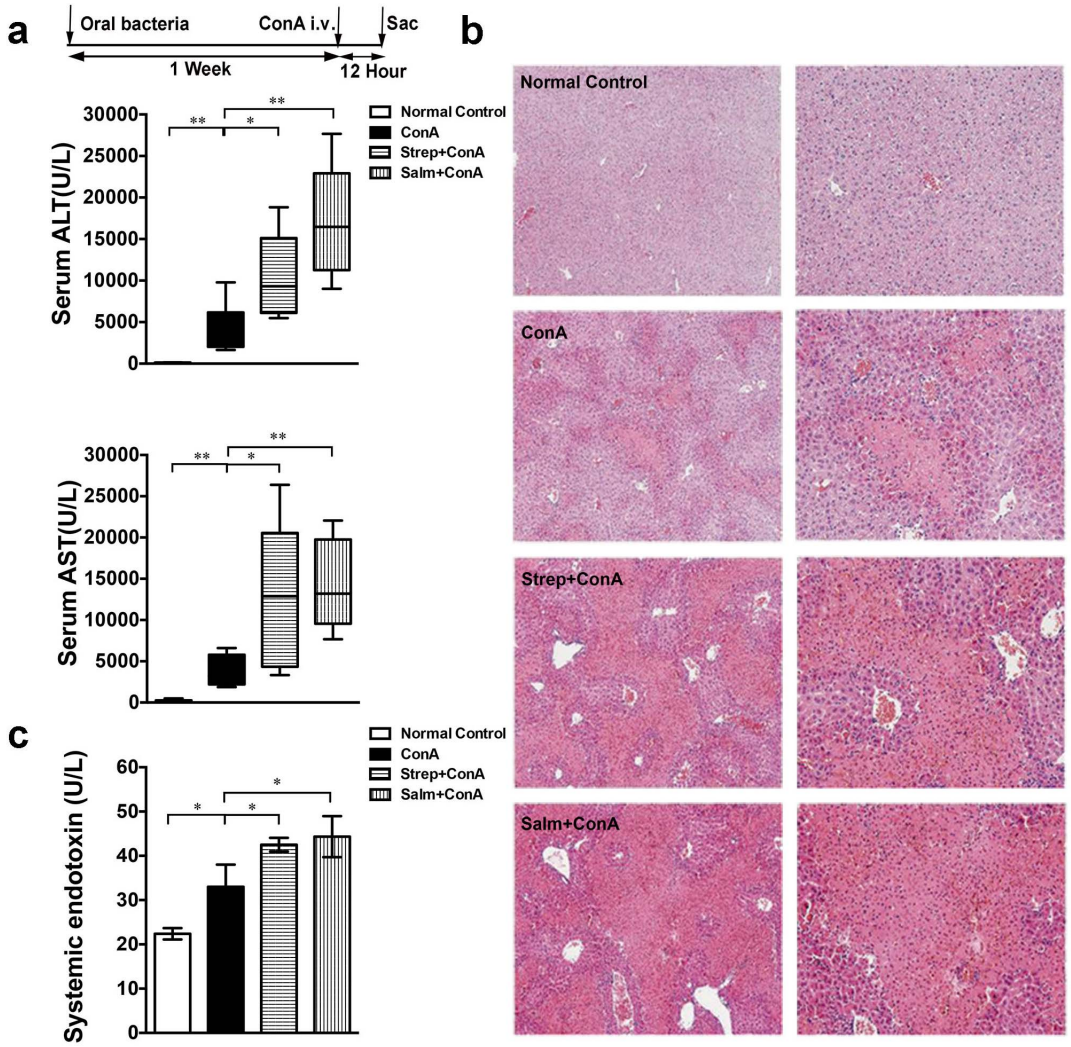

Figure 1 | **Exogenous pathogenic bacteria exacerbated ConA-induced liver injury.** Mice received gavage with *Streptococcus* or *Salmonella* (5×10^8 CFU/g) followed by injection of ConA (10 mg/kg), and sacrificed 12 h later (n = 8). (a) ALT and AST levels. (b) Hepatic H&E sections and representative images, Left: 40× magnification, right: 200× magnification. (c) Systemic endotoxin concentrations. Data presented are the means ± SD. *p< 0.05, **p< 0.01, one-way ANOVA.

dysbacteriosis can lead to endotoxin accumulation in the portal vein, which promotes fibrosis and HCC via activation of toll-like receptor four[13].

However, the correlation between intestinal microbial alteration and immunological hepatic injury, particularly the influence of intestinal microbial alteration on immune cell activation and migration in the intestine and liver, remains obscure. Thus, we investigated whether changes of the gut microflora affect liver inflammation, and studied the relevant immune mechanism of liver inflammation influenced by the microbial variation.

Results

Pathogenic bacteria exacerbated ConA- induced liver injury. Previously, it was reported that depletion of the host microflora affects HCC[13], therefore we conjectured that gut-derived bacteria might have a serious impact on liver injury. We administered *Salmonella* (gram-negative, G^-) and *Streptococcus* (gram-positive, G^+) to the mice for one week prior to ConA injection, as expected, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were higher in mice treated with *Salmonella* or *Streptococcus* before ConA injection than the mice that received ConA only (ConA group) (Fig. 1a). Consistent with the ALT levels, histological examination showed diffuse and massive degenerative liver alterations after ConA injection, while the necrosis and lymphocyte infiltration in the Salm + ConA and Strep + ConA groups were more severe (Fig. 1b). In addition, *Salmonella* and *Streptococcus* to the mice for one week prior to PBS injection did not cause marked liver injury, which suggested that pathogenic bacteria did not cause significant liver damage independently (Supplementary Figure S1a–c). Mice were also treated with common intestinal bacteria, *E.coli* (G^-) and *Lactobacillus* (G^+) before ConA injection to further investigate the effect of different bacteria. And we found such intestinal non-pathogenic bacteria treatment prior to Con A injection did not aggravate the liver injury (Supplementary Fig S1d–g).

Endotoxin is a crucial factor in bacteria-promoted liver injury[5]. We examined the level of systemic endotoxin, which was significantly increased after ConA injection. Importantly, serum endotoxin levels were increased markedly in Salm + ConA and Strep + ConA groups compared to the ConA group (Fig. 1c).

Pathogenic bacteria promoted activation-induced NKT cell downregulation. To investigate how gut-derived bacteria influenced ConA-induced liver injury, we analyzed the liver infiltrating cells. NKT cell activation after stimulation with anti-CD3 or a

specific ligand, α-GC, results in rapid NKT cell downregulation in mice[14]. In addition, NKT cell activation leads to a rapid reduction in NKT cell numbers in ConA-induced hepatitis due to profound downregulation of NKT cell receptor[15]. Figure 2a shows that ConA led to significant reduction of the percentage of NKT ($NK1.1^+$, TCR-β^+) cells in the liver, i.e., NKT cell activated, and this was much more severe in the mice with pathogenic bacteria-treated ConA groups. The result also showed a similar change in the percentage of invariant natural killer T (iNKT, CD1d tetramer$^+$ TCR-β^+) cells. In addition, the percentage of DCs ($CD11c^+$) was markedly increased following ConA administration. Moreover, the proportion of hepatic DCs was notably increased in the Salm + ConA and Strep + ConA groups compared with the ConA group, indicating that administration of pathogenic bacteria promoted the Con A-induced DC augmentation and NKT cell activation. There was no difference in T ($NK1.1^-$, TCR-β^+) cells, NK ($NK1.1^+$, TCR-β^-) cells and the expression of CD44 on T cells between the pathogenic bacteria-treated ConA groups and the ConA group (Fig 2a). The absolute number of the lymphocyte was also measured, the infiltrated lymphocytes in the liver were increased after Con A injection, moreover the absolute numbers of NKT cells and DCs were in line with their percentages, and the absolute numbers of NK and T cells were not significantly increased after pathogenic bacteria administration (Supplementary Fig S2a). To investigate the significance of hepatic NKT cells activation in liver injury, we examined the association of the hepatic NKT cell downregulation with liver injury. We found a negative correlation between the percentage of hepatic NKT cells and ALT level in ConA-induced hepatitis (Normal Control, ConA, *Salmonella* + ConA, *Streptococcus* + ConA groups) (Fig. 2b). However, there was no statistically significant correlation between splenic NKT cell frequency and ALT (Supplementary Fig S2b). Furthermore, IFN-γ production in NKT cells was increased after ConA treatment, which was also markedly higher in the Salm + ConA and Strep + ConA groups compared to the ConA group (Fig. 2c). However, there was no significant difference in IFN-γ production in T cells after pathogenic administration (Supplementary Fig S2c). The presenting antigens $CD1d^+$APCs, mainly including DCs, promote NKT cell activation. These data suggest that liver DCs and NKT cells may play important roles in gut microflora-related ConA-induced liver injury.

The activated immune cells, following the ConA injection, synthesized and secreted a series of inflammatory cytokines that have a vital effect on ConA-induced liver injury. In the pathogenic bacteria-treated ConA groups, TNF-α and IL-12 levels were significantly higher than that in the ConA group (Fig. 2d). However, there was no significant difference in IL-4 levels between the pathogenic bacteria-treated ConA groups and the ConA group.

Intestinal DCs may influence ConA-induced liver injury. To explore the effect of gut microbiota on immune cells, we next investigated the change in the intestines following oral pathogenic bacteria. Figure 3a shows that, compared with the normal control, the intestinal mucosa microvilli were shorter and there was edema following ConA injection, moreover, the intestines of mice that received the administration of *Streptococcus* or *Salmonella* with ConA treatment were more severely damaged, characterized by disappearance of the mucosa, thinned seromuscular layer, ruptured microvilli, and lymphocyte infiltration; however, the difference between the two pathogenic bacteria treatments was not significant. Interestingly, there was a marked increase in the percentage of intestinal DCs and NK cells after ConA administration. Meanwhile, the percentage of DCs was markedly increased in Strep + ConA or Salm + ConA groups compared to the ConA group (Fig. 3b). Nevertheless, the percent of γδT cells, the main T cell subset in the intestine[16], was similar between the mice with or without pathogenic bacteria treatment prior to Con A injection. There was also no significant difference between the other cells in the four groups (Fig. 3b, Supplementary Fig S3a). IL-12 levels were significantly increased after ConA injection. Compared to the ConA group, the levels of inflammatory cytokines such as TNF-α and IL-12 in the intestinal tissue were significantly elevated following *Salmonella* or *Streptococcus* with ConA treatment. However, there was no significant difference on IL-4 production in the pathogenic bacteria-treated ConA groups compared to the ConA group (Fig. 3c).

As the immune sensors of the intestine[17], the distribution of mononuclear cells from Peyer's Patches (PPs) were detected. There was no distinction between the percentage of T lymphocytes and the expression of CD44 on T cells in all four groups, while the percentage of DCs was higher in the pathogenic bacteria-treated ConA groups compared to the ConA group (Fig. 3d). The change of DCs was similar to the intestine.

Depletion of gram-negative gut-derived bacteria alleviated ConA-induced liver injury and suppressed NKT activation and DCs homing in liver and intestine. To verify the mechanism of intestinal pathogenic bacteria in liver injury, we treated mice with gentamycin, which is bactericidal mainly for gram-negative (G^-) organisms in the gut, for one week before ConA injection. Figure 4a shows that ALT and AST levels in gentamycin-treated mice (Genta + ConA group) were significantly lower than that in ConA group. Also, there was only focal and mild liver injury (Fig. 4a) as well as significantly reduced endotoxin level (Fig. 4b) in Genta + ConA mice in consistent with the ALT levels.

Then, we analyzed the liver infiltrating cells. The percentage of NKT cells in the liver was significantly upregulated in the Genta + ConA group compared to the ConA group. Moreover, the proportion of DCs was notably decreased in the Genta + ConA group compared with the ConA group, but we detected with no marked change in other cells (Fig. 4c). Besides, TNF-α and IL-12 levels were significantly lower in the Genta + ConA group (Fig. 4d). The results indicated that depletion of intestinal G^- bacteria suppressed DC augmentation and NKT cell activation.

We next investigated the change in the intestines following the depletion of G^- bacteria and ConA treatment. Figure 4e shows that the shorter, edema intestinal mucosa microvilli were remitted following gentamycin treatment and ConA injection. Interestingly, the marked increase in the percentage of intestinal DCs after ConA administration was obviously inhibited in the Genta + ConA group (Fig. 4f). There was also no significant difference between the other cells between the two groups (Fig. 4f). IL-12 levels were significantly lower in the Genta + ConA group than ConA group. In contrast, there was no significant difference in TNF-α level in the two groups (Fig. 4g). The change in the percentage of DCs in PPs was the same as that in the intestine (Fig. 4h).

Changes in intestinal microbiota after pathogenic bacteria and ConA treatment. To investigate the effect of intestinal microbial alteration on the gut-liver axis, we measured the bacterial population in stool obtained from the cecum in the five groups. Table 1 lists the major differences in the numbers of 16S rRNA gene copies of the predominant bacterial groups in the five groups. Compared with the normal controls, there were higher 16S rRNA gene copy numbers for *Enterobacteriaceae* and fewer copy numbers for *Bifidobacterium* and *Lactobacillus*in in the ConA group. The copy numbers for *Enterobacteriaceae* and *Bacteroides-Prevotella* were significantly increased in the pathogenic bacteria-treated ConA groups but reduced in the Genta + ConA group compared to the ConA group, whereas *Bifidobacterium* and *Lactobacillus* were both decreased in the pathogenic bacteria-treated ConA groups but increased in the Genta + ConA group (Table 1).

NKT cells play a critical role in the process that pathogenic bacteria aggravate the Con A-induced liver injury. To further

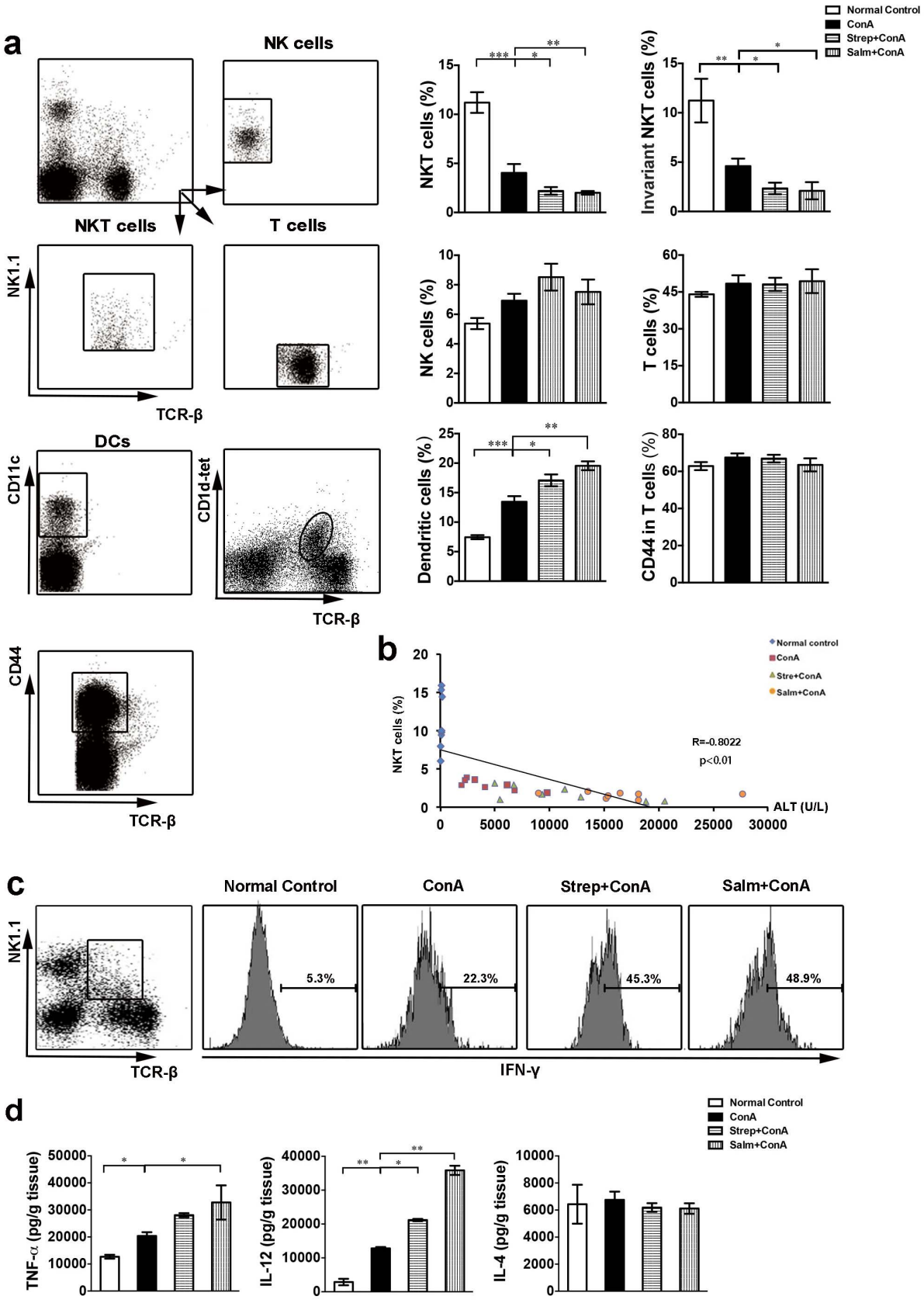

Figure 2 | Exogenous pathogenic bacteria promoted DC augmentation and NKT cell activation. (n = 8). (a) Liver-infiltrating leukocytes were prepared from the four groups; the percentage of DCs NKT, iNKT, NK, T, and CD44^{+}T cells were determined using flow cytometry. (b) The percentage of NKT cells in liver was correlated with serum ALT level in ConA-induced liver injury. (c) Intracellular IFN-γ of hepatic NKT cells was detected by flow cytometry. (d) ELISA of TNF-α, IL-12, and IL-4 levels in hepatic tissue from the four groups. Data presented are the means ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, one-way ANOVA.

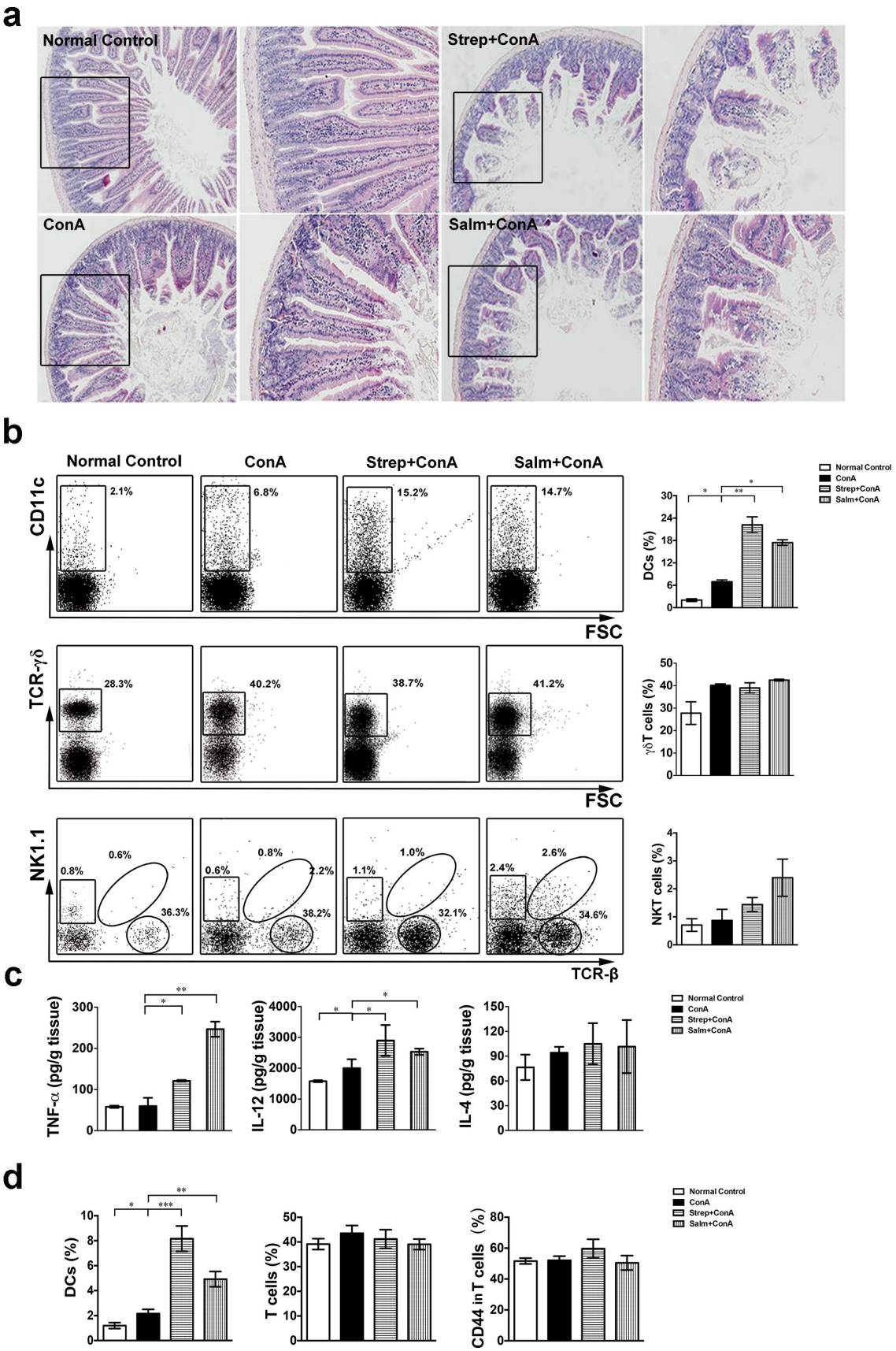

Figure 3 | **Exogenous pathogenic bacteria exacerbated intestinal tract damage.** (n = 8). (a) H&E sections and representative images of morphological structures of intestine. Left: 40× magnification, right: insets depicting the intestinal tract at 200× magnification. (b) The ratio of γδ T, DC, NKT, and NK cells in the intestine was detected by flow cytometric analysis. (c) ELISA measurement of TNF-α, IL-12, and IL-4 levels. (d) Lymphocytes were isolated from PPs. The percentages of DCs, T and $CD44^+$ T cells were assessed after pathogenic bacteria with ConA administration. Data presented are the means ± SD. *$P < 0.05$, **$P < 0.01$, ***$p < 0.001$, one-way ANOVA.

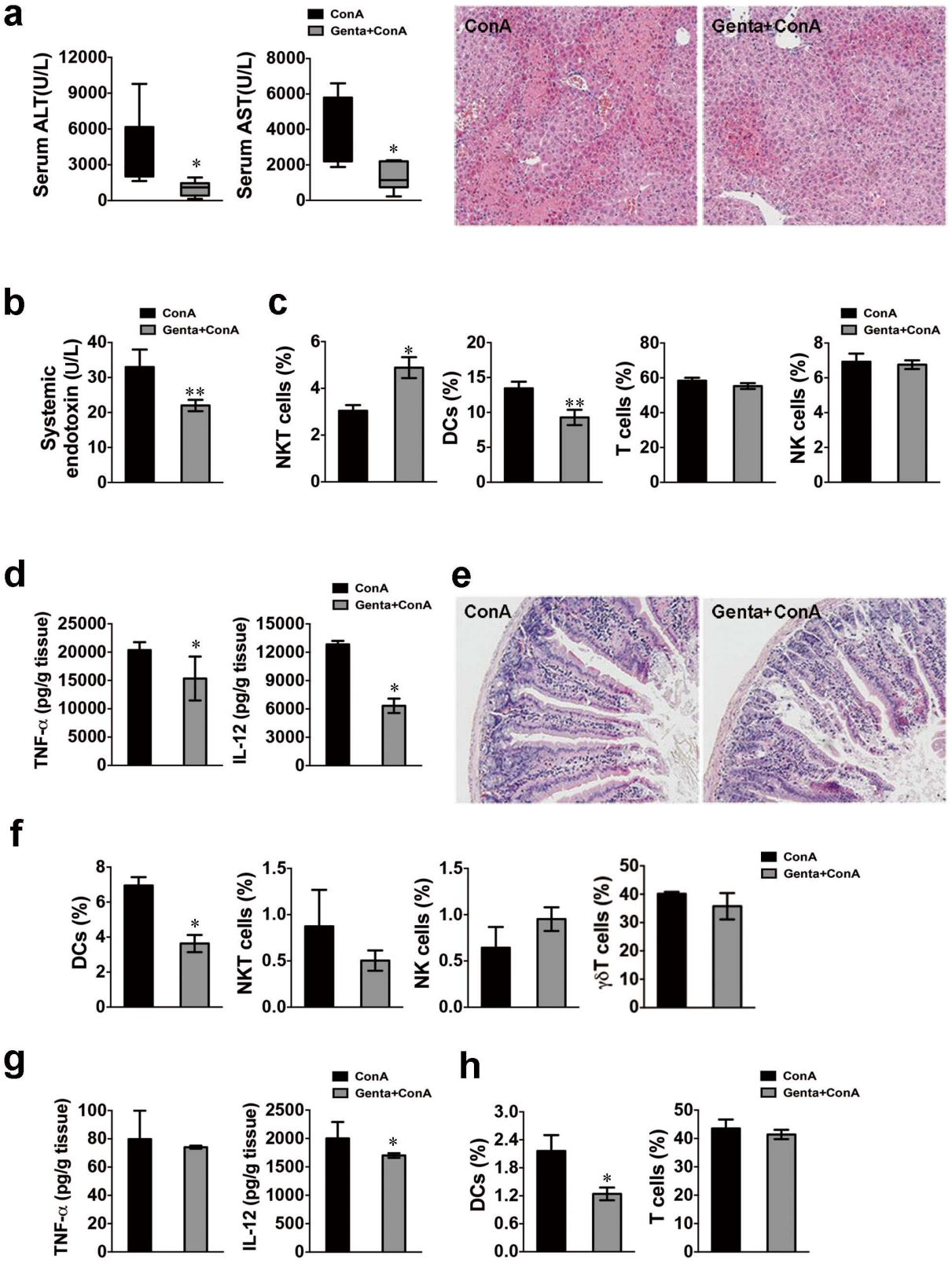

Figure 4 | Deletion of gut G⁻ bacteria alleviated ConA-induced liver and intestine damage. Treated mice received oral gentamycin (1×10^4 U/g) for one week followed by injection of ConA (10 mg/kg) and were sacrificed 12 h after ConA administration (n = 8). (a) The ALT, AST levels and representative hepatic H&E images (200×). (b) Systemic endotoxin levels. (c) The percentage of hepatic DCs and NKT, NK and T cells was determined by flow cytometry. (d) ELISA of TNF-α and IL-12 expression in hepatic tissue. (e) Representative intestinal H&E images (200×). (f) Intestinal γδ T, DC, NKT, and NK cell ratios were determined using flow cytometric analysis. (g) ELISA assessment of the intestinal TNF-α and IL-12 expression. (h) Lymphocytes were isolated from PPs, The percentages of DCs and T cells were assessed after gentamycin and ConA administration. Data presented are the means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, Mann-Whitney U-test.

Table 1 | Fecal microbes and virulence factors with high prevalence in five group subjects

	Normal	ConA	Gentamycin+ConA	*Streptococcus* +ConA	*Salmonella*+ConA
Bacteroides–Prevotella	9.14 ± 0.17	9.04 ± 0.14	5.09 ± 0.27*	10.17 + 0.08***	10.17 + 0.08***
Bifidobacteria	9.44 ± 0.22	8.63 ± 0.16#	9.63 ± 0.18*	7.02 + 0.13***	6.75 + 0.41**
Enterobacteriaceae	6.44 ± 0.17	7.56 ± 0.25#	4.95 ± 0.15***	11.33 + 0.42**	9.24 + 0.21**
Lactobacillus	9.24 ± 0.29	7.14 ± 0.23##	9.17 ± 0.10**	4.72 + 0.46***	5.31 + 0.42**

Data were reported as the average estimate of Logarithms of fecal PCR target genetic operons copy numbers present in 1 g of feces (wet weight), and its detection limits is less than 5×10^3copies/g (n=8). Data are represented as means ± SD. #, in comparison to normal group; *, in comparison to Con A group. # or*, $P < 0.05$; ## or**, $P < 0.01$; ### or***, $P < 0.001$, one-way ANOVA.

confirm the role of NKT cells in pathogenic bacteria aggravating the Con A-induced liver injury, wild type (WT) and NKT cell-deficient CD1d$^{-/-}$ mice received *Salmonella* treatment before Con A. Figure 5a showed that ALT and AST levels in CD1d$^{-/-}$ mice were significantly lower than WT mice, but the percentages of T, NK and DC cells were similar between WT mice and CD1d$^{-/-}$ mice (Fig. 5b). In addition, IL-12 level was not significantly different between WT mice and CD1d$^{-/-}$ mice (Fig. 5c). Besides, we treated the WT mice with anti-IL-12 antibody before ConA injection. The liver injury was markedly reduced in the anti-IL-12 antibody treated mice (Fig. 5d), moreover, the downregulation of NKT cells (Fig. 5e) and IFN-γ production by NKT cells (Fig. 5f) were ameliorated. In addition, IL-12 neutralization antibody did not influence IFN-γ production in T cell (Supplementary Fig S3b). Collectively, the NKT cells play a critical role in the mechanism of pathogenic bacteria aggravate the Con A-induced liver injury, and the activation of NKT cells was partly depend on IL-12, mainly produced by DCs.

Pathogenic bacteria enhanced NKT cell cytotoxicity against hepatocytes via DC-mediated antigen presentation. To investigate the mechanism that pathogenic bacteria aggravated the ConA-induced liver injury, we tested whether pathogenic bacterial antigens would increase NKT cell cytotoxic activity. We analyzed the effects of pathogenic bacteria on NKT cell cytotoxic activity against a murine hepatocyte cell line (TLR2). The cytotoxicity of NKT cells stimulated with DCs in the presence of inactivated *Streptococcus* or *Salmonella* was significantly increased, which suggested that pathogenic bacterial antigens improve the cytotoxicity of NKT cells (Fig. 6a). We measured the TNF-α production by NK cells, NKT cells, and T cells in pathogenic bacteria treated groups. We found that TNF-α production by NKT cells was significantly higher than NK cells and T cells (Supplementary Fig S3c), and TNF-α secretion in the supernatant incubated with pathogenic bacteria was markedly higher than without bacteria treated (Fig. 6a). TNF-α production in NKT cells was increased after ConA treatment, which was also markedly higher in the Salm + ConA and Strep + ConA groups compared to the ConA group, while TNF-α produced by NK and T cells was not significantly changed. (Supplementary Fig S3c) Furthermore, TNF-α neutralization antibody inhibited the cytotoxicity of NKT cells against hepatocytes (Fig. 6b). In consequence, the cytotoxicity of NKT cell against hepatocytes may depend on TNF-α.

Bacterial Translocation after liver injury. The number of translocated bacteria to the liver was significantly increased in the ConA group compared to the normal controls (Table 2). Compared to the ConA group, the number of translocated bacteria was significantly reduced in the Genta + ConA group, but increased in the pathogenic bacteria-treated ConA groups (Table 2). The change in the percentage of translocated bacteria to the mesenteric lymph nodes was consistent with the liver. These data imply that bacteria translocated to the liver might supply antigens for hepatic NKT cell activation.

Discussion

The liver receives portal blood which enriched with nutrients absorbed by the intestine and is exposed to gut-derived factors, including bacterial endotoxins and cytokines[11,18]. Intestinal dysbacteriosis can lead to the accumulation of liver damage in fibrosis and HCC[5,19,20]. Excessive numbers of *Salmonella* promote the development of liver damage[21]; in addition, the ratio of *Streptococcaceae* in gut microflora is markedly increased in patients with cirrhosis[22]. Thus, we studied the influence of intestinal dysbacteriosis on ConA-induced liver injury via gavage with pathogenic bacteria prior to ConA administration. Intestinal microbiota play a crucial role in the process of liver damage, however, whether intestinal microbiota modulate the liver damage involved immune cell is still unknown.

We found that administration of exogenous pathogenic bacteria aggravated liver damage. Previous studies have shown that NKT cells play a critical role in the process of ConA-induced hepatic injury[1,23]. In addition, hepatic NKT cell activation in ConA-induced hepatitis was characterized by rapid NKT cell receptor downregulation. NKT cells are specialized T cells of the immune system that express markers of the NK cell lineage, such as NK1.1, which recognize glycolipid antigens, presented by the MHC class I-related protein CD1d. In mice, these cells are sometimes referred to invariant NKT (*i*NKT) cells[24]. It was reported that the microbiota regulated susceptibility to hepatic injury by Fas/FasL pathway[9], and NKT mediated hepatic damage greatly involved to Fas/FasL pathway[1], these suggested the influence of microbiota on liver injury was associated with NKT cells. Our results showed the downregulation of both NKT and iNKT cells were more pronounced in the pathogenic bacteria-treated ConA group. In contrast, the depletion of gut G^- bacteria by gentamycin alleviated the ConA-induced liver injury and was accompanied by reduced liver immune cell infiltration, and NKT cell downregulation was impaired in these mice. Therefore, we speculated that NKT cells might play an important role in the impact of intestinal microbial alteration on immunological hepatic injury. Moreover, NKT cell activation is associated with DCs. In this study, intestinal microbial alteration led to variation of intestinal DC distribution. Compared to DCs in the ConA group, there were more DCs in the pathogenic bacteria-treated ConA groups, which was similar to the trend of DCs in the PPs and liver, whereas intestinal DCs in the gentamycin-treated ConA group were decreased. This indicates that intestinal microbial alteration might contribute to the activation of gut-derived DCs, and then induce the activation of hepatic DCs, consequently leading to hepatic NKT cell activation and liver injury. The *in vitro* experiments determined that DCs, stimulated with pathogenic bacteria, promoted NKT cell cytotoxicity against liver parenchyma cells. These results suggest that intestinal pathogenic bacteria aggravate liver damage may originate from DCs activation, which subsequently augment hepatic NKT cell cytotoxicity against liver parenchyma cells.

As powerful professional APCs, DCs are the center of immune response regulation and have strong migration ability[25,26]. Intestinal DCs can also induce helper T (Th) cell differentiation into Th1 or Th2 cells, which secrete IFN-γ or IL-4, IL-10, and IL-13[27,28]. As the key initiators and regulators of mucosal adaptive immune responses, DCs also play an important role in maintaining intestinal homeostasis, particularly the response to intestinal bacterial antigens[29,30]. When we studied the number and function of DCs, we found that pathogenic bacteria significantly increased the proportion of DCs in

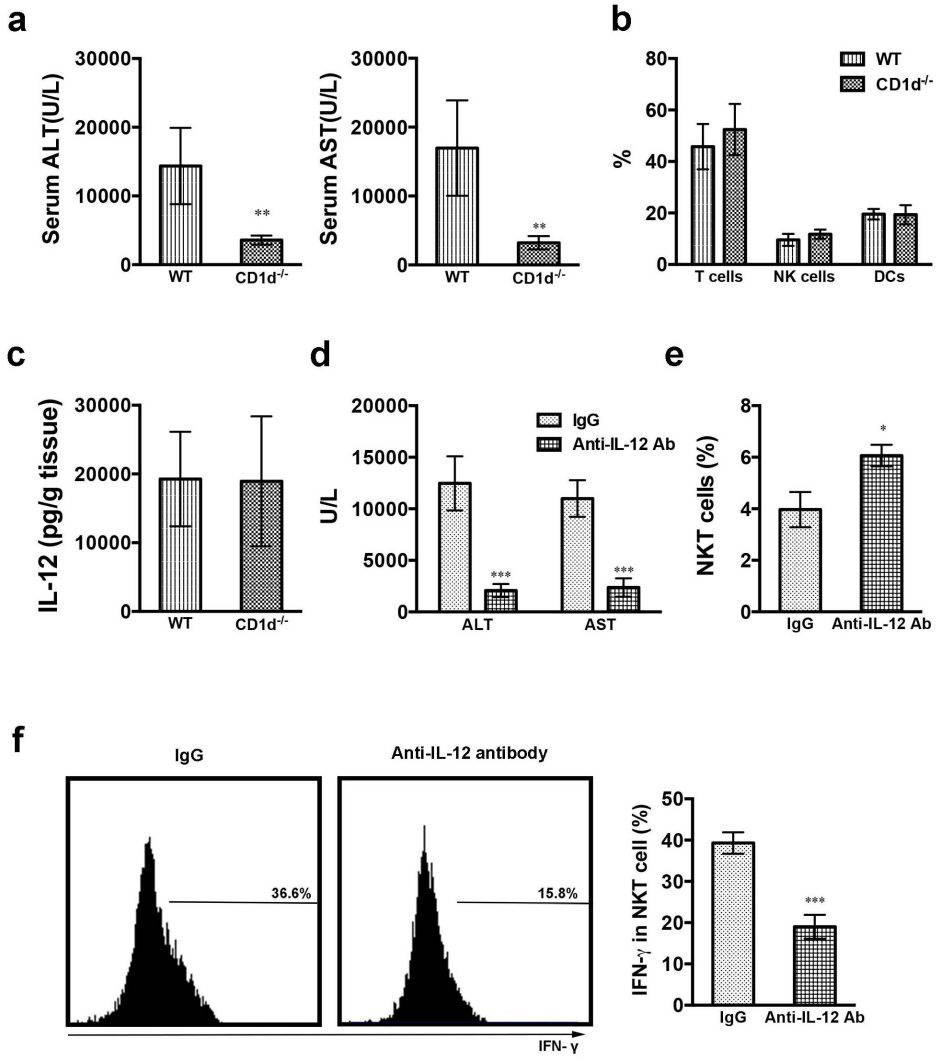

Figure 5 | NKT cells was critical in the pathogenic bacteria aggravate the Con A-induced liver injury. (n = 6). (a–c) $CD1d^{-/-}$ and WT were gavaged with *Salmonella* (5×10^8 CFU/mouse) for one week prior to Con A (10 mg/kg) injection, and sacrifice 12 h later. (a) Serum ALT and AST. (b) hepatic T cells, NK cells and DCs. (c) hepatic IL-12 level were showed. (d–f) Two hours before Con A injection, wild type mice received anti-IL-12 neutralizing antibody (250 μg). (d) ALT and AST levels. (e) The percentage of hepatic NKT cells. (f)The intracellular IFN-γlevel of hepatic NKT cells. Data presented are the means ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, Mann-Whitney U-test.

the intestine and liver. Compared to the ConA group, the tendency for variation of the hepatic DCs was similar to that of the intestine following the depletion of G^- bacteria or pathogenic bacteria treatment, suggesting the importance of DCs in the gut-liver axis. DCs may migrate from the gut to the liver, but the pathway is unclear. In rats that had undergone liver transplantation, DCs migrated to the spleen and PPs[31]. Therefore, we detected lymphocyte distribution in the spleen (Supplementary Fig. S4, 5) and PPs. The change of splenic DCs was not consistent with the intestinal and hepatic DCs. Interestingly, the trend of DCs in the PPs was very similar to the intestine and liver in ConA-induced liver injury, suggesting that DCs may first migrate to PPs, and then to liver. IL-12 derived from DCs induces Th1 differentiation and produces IFN-γ, exacerbating liver damage[32]. The change trend of the IL-12 level was consistent with the DCs, supporting the changes of DCs in the liver.

Previous research has reported obvious downregulation of NKT cells following ConA administration[1,23]. In the immune response process where dysbacteriosis influences the severity of hepatitis, we found that the severity of liver damage was related to NKT cell activation. Previous studies have found that NKT cells recognized glycolipids from pathogenic bacteria during microbial infections through antigens presented by glycolipid-activated DCs[33,34]. *In vitro* experiments revealed that NKT cells activated by DCs stimulated with pathogenic bacteria had greater cytotoxicity against liver parenchyma cells than cells that had not been stimulated with bacteria. Moreover, NK cells were activated following treatment with exogenous pathogenic bacteria, indicating that the exogenous pathogenic bacteria aggravated the inflammation. However, NK cell deletion does not inhibit the development of ConA-induced hepatic injury[35], suggesting that NK cell is not critical in ConA-induced hepatic injury. Besides, Lin et al. found that the gut-derived endotoxin promotes the liver injury through TLR-4, mediated by T cell activation[36]. NKT cell was an unconventional T cell subset, thus in the study of Lin et al. T cells might also include the NKT cells.

Quantitative 16S rRNA analysis of intestinal bacteria in the feces revealed intestinal dysbacteriosis. *Salmonella* or *Streptococcus* administration decreased the copy numbers of *Bifidobacterium* and *Lactobacillus,* but increased *Enterobacteriaceae* and *Bacteroides-Prevotella*. Although *Salmonella* is gram-negative and *Streptococcus* is gram-positive, serum endotoxin levels were increased in both *Salmonella* and *Streptococcus* treated mice. The increased endotoxin levels might be associated with increased gut permeability and more

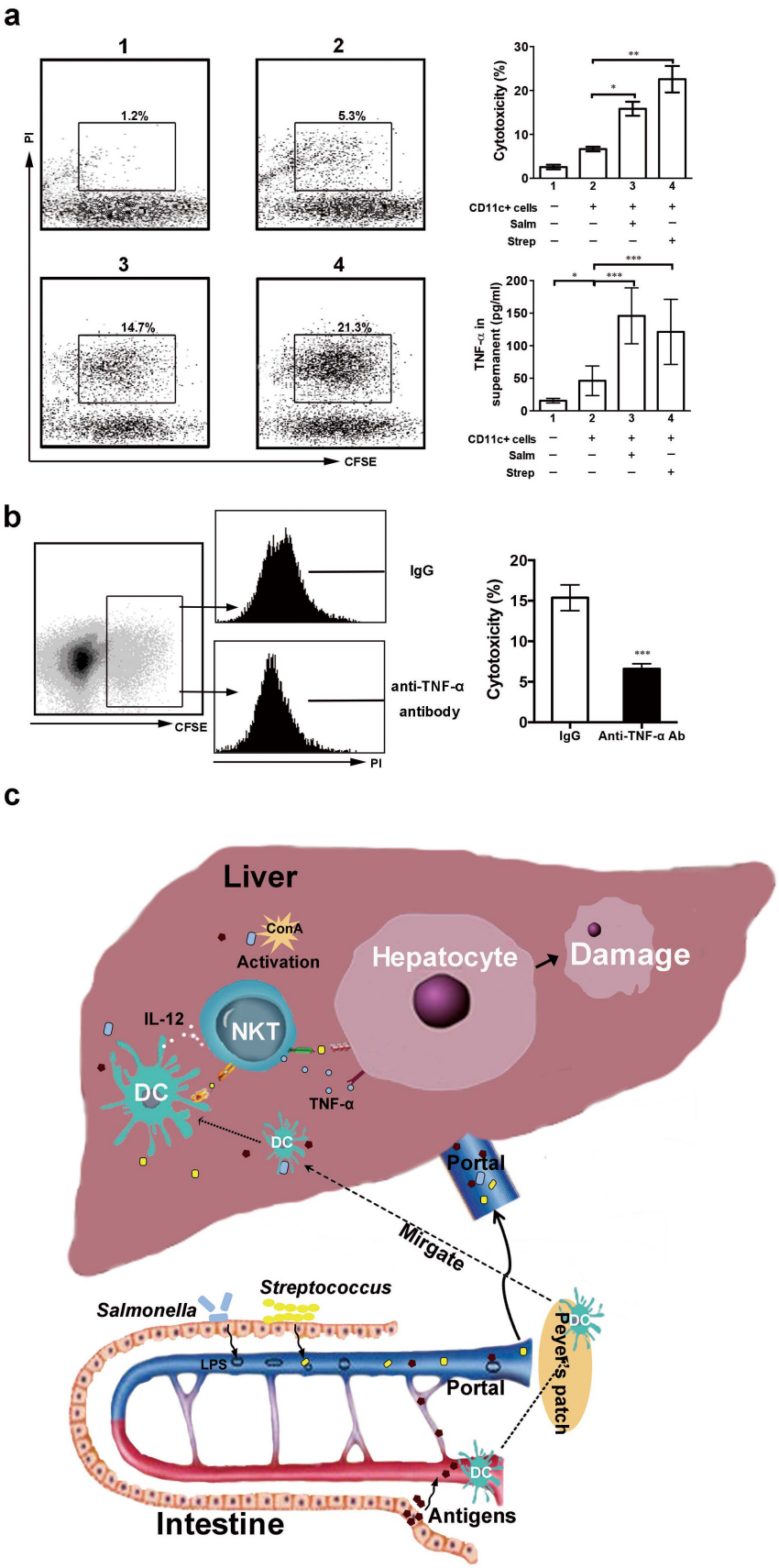

Figure 6 | **Pathogenic bacterial antigens enhanced NKT cell cytotoxicity via DC activation *in vitro*.** (a) NKT cells from the liver were stimulated for 48 h *in vitro*. Cytotoxic assay with target TLR2 cells denoted as the PI-positive rate as analyzed by flow cytometry. The TNF-α level in the supernatant was detected by ELISA. (b) NKT cell cytotoxicity was detected after anti-TNF-α antibody neutralization, co-cultured with $CD11c^+$ cells and *Salmonella*. (c) Schematic representation of immune cell activation in ConA-induced hepatic injury controlled by intestinal microbiota. Data presented are the means ± SD. *P < 0.05 **P < 0.01, one-way ANOVA.

Table 2 | Bacterial translocation to the liver after liver injury

GROUP	liver	MLN
Normal	12.5% ± 5.10%	21.9% ± 8.27%
ConA	21.9% ± 5.98%	34.4% ± 4.64%
Genta + ConA	0	12.5% ± 3.95%
Strep + ConA	56.3% ± 11.9%*	87.5% ± 19.5%**
Salm + ConA	71.9% ± 6.01%**	90.6% ± 24.2%**

Data reported are incidence of bacterial translocation per group. (n=8)
Data presented are the means ± SD. *compared to ConA group. *$P < 0.05$, **$P < 0.01$, one-way ANOVA.

Enterobacteriaceae and *Bacteroides-Prevotella*. In conclusion, during the course of ConA-induced hepatitis, intestinal pathogenic bacterial antigens induced DC activation in the intestine, and intestinal DCs may migrated into the liver, passing through the PPs, which could led to NKT cell activation in the liver. Another pathway might be that the pathogenic bacteria increased gut permeability, thus large amounts of bacterial antigen from the intestine entered the liver, subsequently activated the hepatic DCs and in turn result in the activation of NKT cells. Upon activation, NKT cells caused liver injury (Fig. 6c). Our data suggest that modulation of the gut microbiota can affect liver injury via DCs induced NKT cells activation. Capitalizing on the preponderant intestinal flora and DC activation may represent a new avenue for treating or preventing immune-mediated hepatitis.

Methods

All experimental procedures were carried out in accordance with the 1996 National Institutes of Health Guide for the Care and use of Laboratory Animals and the experimental procedures were approved by the Local Committee of Animal Use and Protection.

Mice. C57BL/6 female mice were purchased from the Zhejiang Chinese Medical University Medical Experiment Animal Research Center (Hangzhou, China). $CD1d^{-/-}$ female mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and bred and maintained at University of Science and Technology of China, Hefei, China. All mice were maintained under specific pathogen-free conditions and used six to eight weeks old.

Mice were under daily intragastric administration with a dosage of pathogenic bacteria or gentamycin (Genta). Group A Streptococcus and Salmonella enteritidis was 5×10^8 colony-forming units [CFU]/200 μl/mouse, and gentamycin (Genta) was 2×10^4 U/200 μl/mouse. Such treatment was once per day and maintained for a week prior to ConA administration (n=8). Group A Streptococcus and Salmonella enteritidis were isolated from a patient and maintained in our laboratory as previously described[21,37]. Streptococcus was grown in brain heart infusion broth (Oxoid, Basingstoke, UK) supplemented with 5% (v/v) defibrinated goat blood; Salmonella was grown on a blood agar base (bioMérieux, Durham, North Carolina, USA) at 37°C overnight, then harvested and diluted in phosphate-buffered saline (PBS). ConA was purchased from Vector Laboratories (Burlingame, California, USA). Intravenous injections of Con A (10 mg/kg) were administered via the tail vein 12 h before examination. The normal group mice were treated with 200 μl of PBS by daily gavage or tail vein injection 12 h before examination. To block IL-12, 250 μg purified anti–IL-12 MAb (Biolegend San Diego, California, USA) or isotype-control rat IgG were injected intravenously to the mice 2 hour before ConA treatment.

Measurement of liver injury. Serum alanine aminotransferase (ALT) and aspartate transaminase (AST) levels were measured using a standard clinical automated analyzer (SRL, Tokyo, Japan). Livers were fixed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) and examined.

Intracellular cytokine staining. Cells (1×10^6/ml) from the liver were stimulated for 4 h with 50 ng/ml PMA and 500 ng/ml ionomycin (Sigma-Aldrich). Cells were harvested, washed, stained with anti-NK1.1-PE mAb anti-T cell receptor (TCR)-β-FITC mAb Surface-stained cells and fixed by Cytofix/CytopermFixation/Permeabilization Kit (BD, Bioscience). Permeabilized cells were incubated for 30 min at room temperature with anti-IFN-γ-APC-mAb or anti-TNF-α-APC-mAb. Stained cells were washed twice in permeabilization buffer and resuspended in PBS supplemented with 0.3% w/v BSA and 0.1% w/v sodium azide. The number of cytokine-expressing NKT cells per 10^5 NKT cells was determined by flow cytometry.

Flow cytometry analysis. Hepatic and intestinal mononuclear cells or splenic cells were isolated and labeled with anti-CD11c-PE mAb, anti-NK1.1-PE mAb, anti-CD44-FITC mAb, anti-T cell receptor (TCR)-β-FITC mAb, anti-TCR-β-Allophycocyanin (APC) mAb, anti-TCR-γδ-APC mAb, anti-IFN-γ-APC-mAb (all from eBioscience, San Diego, California, USA), anti-TNF-α-APC-mAb (Biolegend San Diego, California, USA). Stained cells were assessed using a FC500 System (Beckman Coulter, Brea, California, USA) equipped with Summit 5.1 software. For the cytotoxic assay, target cells were labeled in advance with carboxyfluorescein diacetate succinimidylester (CFSE; Molecular Probes, Eugene, Oregon, USA) as indicated and analyzed using flow cytometry.

Cytotoxic assay. NKT cells and DCs ($CD11c^+$) were isolated from the liver and spleen, respectively, using a FACS Aria III instrument (Becton Dickinson); the purity of the sorted cells was > 95% DCs were first cultured with inactivated *Streptococcus* or *Salmonella* at a 1 : 10 ratio. Bacterial sonicates were generated by adding 70% ethanol then washing the bacteria thrice with PBS, followed by resuspension in PBS at a concentration equivalent to 10^9 CFU/mL before use. NKT cells were cultured overnight with recombinant IL-2 stimulus in the presence of 100-Gy irradiated DCs. The target cells (TLR2 hepatocyte cell line; RIKEN Cell Bank, Tsukuba, Japan) were labeled in advance with CFSE (Molecular Probes) as previously described. The cells were harvested and seeded at the indicated effector/target cell (E/T) ratio of 10 : 1. To block TNF-α, 20 ng/ml purified TNF-α MAb (Biolegend San Diego, California, USA) or isotype-control rat IgG were incubated with TLR2 for 6 h before cytotoxic assay. NKT cell cytotoxic activity was assayed using flow cytometry.

Isolation and quantitative PCR of intestinal microbiota DNA. DNA was extracted from feces and bacterial precipitates using a Qiagen Stool Kit (Hilden, Germany) and a modified cell lysis protocol. Qualitative procedures and the method for detecting bacterial population were performed as described previously[38]. And the copy number of target DNA was calculated by comparison with serially diluting standards, (10^1 to 10^8 copies of plasmid DNA containing the respective amplicon for each set of primers) running on the same plate. Bacterial quantity was expressed as $\log_{10}$ bacteria per gram of stool. The specific primers used were as follows: *Bacteroides-Prevotella*: 5′-GAAGGTCCCCCACATTG-3′ (sense),
5′-CAATCGGAGTTCTTCGTG-3′ (anti-sense);
Bifidobacteria: 5′-GGGTGGTAATGCCGGATG-3′ (sense),
5′-TAAGCCATGGACTTTCACACC-3′ (anti-sense);
Enterobacteriacea: 5′-CATTGACGTTACCCGCAGAAGAAGC-3′ (sense),
5′-CTCTACGAGACTCAAGCTTGC-3′ (anti-sense);
Lactobacillus: 5′-AGCAGTAGGGAATCTTCCA-3′ (sense),
5′-ATTTCACCGCTACACATG-3′ (anti-sense)[39].

Endotoxin analysis. Blood samples (100 μL) were centrifuged at 3000 g for 10 min at room temperature to separate the serum for analysis; the amount of endotoxin in the separated serum was determined using a quantitative chromogenic Limulus amebocyte lysate assay as instructed by the manufacturer (Eihua Medical, Shanghai, China).

Bacterial translocation. Liver and mesenteric lymph node samples were weighed and placed in a sterile glass homogenizer. The homogenate (50 μL) was then placed on a blood agar base and an anaerobic blood agar base (bioMérieux) within 30 min of sample collection and incubated at 37°C for 24 h. The number of CFU per plate was counted and corrected for the original weight of the sample as described previously.

Enzyme-linked immunosorbent assay. TNF-α, IL-4, and IL-12 (p70) levels were measured using mouse enzyme-linked immunosorbent assay (ELISA) Ready-Set-Go Kits (eBioscience) as specified by the manufacturer. Cytokine content in the liver, spleen, and intestine extracts was expressed as amounts per gram of tissue.

Statistics. Comparisons between groups were made using Mann-Whitney U test, or ANOVA tests of GraphPad Prism software for two or multigroup comparisons (GraphPad Software, Inc.). $P < 0.05$ were considered significant. NS, no significance.

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Author contributions

H.D. designed the experiments; J.C., Y.W., G.C., Y.D., Y.Z. and C.L. performed the experiments; Y.W. and J.H. analyzed the data; L.L., T.U., R.X. and L.B. provided technical and material support. H.D., J.C. and Y.W. wrote the manuscript; all authors reviewed the manuscript.

Additional information

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