Gut-derived Lipopolysaccharide Promotes T Cell-mediated Hepatitis in Mice through Toll-like Receptor 4

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Abbreviations

HCC, hepatocellular carcinoma; LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; Con A, Concanavalin A; IL-12, Interleukin 12; IFN-γ, Interferon γ; IL-4, Interleukin 4; GATA-3, GATA binding protein 3; T-bet, T-box transcription factor expressed in T cells; TLR, Toll-like receptor; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CFSE, Carboxyfluorescein Diacetate Succinimidylester; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; IL-2, Interleukin 2; IL-6, Interleukin 6; TNF-α, tumor necrosis factor-α.
Abstract

Robust clinical and epidemiologic data support the role of inflammation as a key player in hepatocellular carcinoma (HCC) development. Our previous data showed that gut-derived LPS promote HCC development by activating Toll-like receptor 4 expressed on myeloid-derived cells. However, the effects of gut-derived LPS on other types of liver injury models are yet to be studied. The purpose of the current study was to determine the importance of gut-derived LPS and TLR4 signaling in a T-cell mediated hepatitis-- Con A induced hepatitis model, which mimic the viral hepatitis. Reduction of endotoxin using antibiotics regimen or genetic ablation of Toll like receptor 4 (TLR4) in mice significantly alleviate Con A-induced liver injury by inhibiting the infiltration of T lymphocyte into the liver and the activation of CD4+ T lymphocytes as well as the production of Th1 cytokines; in contrast, exogenous LPS can promote Con A-induced hepatitis and CD4+ T cells activation in vivo and in vitro. Reconstitution of TLR4-expressing myeloid cells in TLR4-deficient mice restored Con A-induced liver injury and inflammation, indicating the major cell target of LPS. In addition, TLR4 may positively regulate the target hepatocellular apoptosis via the perforin/granzyme B pathway. These data suggest that gut-derived LPS and TLR4 play important positive roles in Con A-induced hepatitis and modulation of the gut microbiota may represent a new avenue for therapeutic intervention to treat acute hepatitis induced by hepatitis virus infection thus to prevent hepatocellular carcinoma.
Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of death from malignancy worldwide, and hepatitis B and C and associated liver cirrhosis represent major risk factors for HCC development. T cell response has a vital role during hepatitis virus infection. Myriad data support the role of inflammation as a key player in HCC development(1). The inflammation can be triggered by tissue damage, infection and toxin. Our previous data showed that gut-derived LPS promote the inflammation during HCC development by activating Toll-like receptor 4 expressed on myeloid-derived cells(2). However, the effects of gut-derived LPS and Toll-like receptor 4 (TLR4) on other types of liver injury models are yet to be studied.

Con A-induced hepatitis is a well-known experimental murine model with significantly elevated levels of transaminase as well as severe liver injury that can mimic many kinds of human T cell-mediated hepatitis, such as virus hepatitis and autoimmune hepatitis (3-5). In Con A-mediated hepatitis model, activated T cells release a series of cytokines. Different cytokines can be categorized as T helper 1 (Th1) and T helper 2 (Th2) associated cytokines by their biological function. Studies have illustrated that Th1 associated cytokines such as IL-12 and IFN-γ are dominant factors inducing the development of Th1 cells (6). Conversely, IL-4 production directs the development of a Th2 response (7). Th1/Th2 differentiation of T cells is largely dependent on the balance of GATA-3 (8) and T-bet (9). The balance of Th1 and Th2 associated cytokines exerts a crucial role in normal immunoregulation and neoplastic outcome.

Emerging evidence suggests a strong interaction between the gut microbiota and human...
liver diseases (10;11). Receiving ∼70% of its blood supply from the intestine through the portal circulation, the liver is the first line of defense against gut-derived antigens (11). In order to cope with these latent systemic pathogens the liver contains a large number of resident immune cells such as macrophages, natural killer cells, dendritic cells, T and B lymphocytes (12). When normal liver physiology is disrupted and inflammatory cells are activated, gut-derived factors likely augment or exacerbate certain liver diseases, leading to enhanced tissue damage and propagation of inflammation via activation of TLR-signaling pathways (13). The critical role of gut-derived endotoxin as a cofactor in acute and chronic liver diseases, both experimental and clinical, was already established more than 30-40 years ago (14). Our previous study also demonstrated that the endotoxin/TLR4 regulates the survival and proliferation of hepatocytes and their pre-neoplastic derivatives during chemically induced hepatocarcinogenesis (2).

Toll like receptors (TLRs) recognize pathogen-associated molecular patterns and activate signaling pathways that lead to the induction of innate immune and inflammatory genes (15). In addition, there is accumulating evidence that TLRs contribute significantly to the activation of adaptive immune responses such as dendritic cell maturation and T- and B-cell responses, specially, the TH1-dependent immune responses (16). TLR4 is an important member of TLRs, which could sense endotoxin and activate transcription factors that initiate innate immunity. TLR4 was also expressed on T lymphocyte, playing a vital role in adaptive immunity. Recent studies have demonstrated the contribution of TLR4 signaling to the trapping of CD8\(^+\) T cells within the murine liver (17). Moreover, other studies indicate that direct interactions of gut-derived antigens signaling through TLR4 on CD4\(^+\) T cells contributes to their regulatory
development and function (18).

In the current study, we report that sustained LPS accumulation exacerbate Con A-induced hepatitis in mice. Both reduction of LPS using antibiotics regimen or genetic ablation of TLR4 can suppress the liver injury markedly. This attenuated hepatitis primarily results from the reduced infiltration of T lymphocytes and suppressed activation of CD4$^+$ T cells as well as decreased levels of Th1 cytokine production. In contrast, exogenous LPS challenge can significantly aggravate Con A-induced liver injury and promote activation of CD4$^+$ T lymphocytes as well as secretion of Th1-associated cytokines. Reconstitution of TLR4-expressing myeloid cells in TLR4-deficient mice restored Con A-induced hepatic injury, indicating that myeloid cells are the major targets of LPS in this model. In addition, perforin/granzyme B mechanism is also involved in the protection of TLR4 ablation. These results suggest that preventing circulating endotoxin accumulation could have a beneficial impact on liver function for patients with T cell-mediated hepatitis such as viral hepatitis thus to prevent the development of HCC.
Materials and Methods

Animals

Male wild type (wt; C57BL/10ScSnJ), and TLR4 deficient (TLR4-/-; C57BL/10ScNJ) mice were obtained from the Model Animal Research Center of Nanjing University, Nanjing, China. Male C57BL/6 mice (6-8 weeks old, weighing 18-22 g) and NOD/SCID mice were obtained from the Shanghai Experimental Center, Chinese Science Academy, Shanghai, and maintained at an animal facility under pathogen-free conditions. All animals received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the NIH (publication 86-23 revised 1985). For detailed information related to animal experiments, see the Supporting Experimental Procedures.

Flow cytometry analysis.

Hepatic mononuclear cells or splenic cells were isolated and then labeled with anti-CD3-PE/CY7, anti-CD4-FITC mAb and anti-CD69-APC mAb (BioLegend, San Diego, CA). Stained cells were assessed on a Moflo-XDP system (Beckman Coulter, Fullerton, CA) equipped with Summit 5.1 Software. When assessing the activation of CD4⁺ T cells, CD4 positive subpopulation was first gated and the expression of CD69 in this population was determined. The results were shown as the proportion of CD4⁺69⁺ cells in total CD4⁺ cells. For determination of proliferation of T cells, the isolated splenic cells were stimulated with Con A or purified anti-mouse CD3ε (BioLegend) in the presence of CFSE (Molecular Probes) for the indicated period and then determined by flow cytometry.

Isolation and Culture of CD4⁺ cells
Complete RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM l-glutamine, 100U/ml penicillin and 100 μg/ml streptomycin was used throughout the experiments. Spleen-derived CD4⁺ T cells were negatively selected by using the CD4⁺ T cell Isolation Kit (Stemcell Technologies, Vancouver, Canada). CD4⁺ T cells were stimulated with 5 μg/ml Con A or 200ng/ml LPS or both. 20 hours later, the cells were collected and stained with indicated antibodies for flow cytometry analysis.

Statistics

Data are expressed as means ± SE. Differences were analyzed by the Student t test, and P values < 0.05 were considered significant.
Results

Depletion of gut-derived LPS alleviates Con A-induced liver injury in TLR4 intact mice

Our previous study has shown that gut-derived endotoxin can promote DEN-induced liver injury and hepatocarcinogenesis (2), so we conjecture that gut-derived endotoxin may also impose a serious impact on other types of liver injury. To address this question, we chose the widely-used Con A-induced hepatitis animal model, which mimics many kinds of human T cell-mediated hepatitis. As shown in the Fig.1A, the serum levels of LPS were markedly elevated 6, 20 hours after Con A administration accompanied with severe liver inflammation and tissue injury, suggesting that serum endotoxin may be a critical cofactor in Con A-induced liver injury.

We next investigated whether circulating LPS exacerbates Con A-induced hepatitis. Mice were treated with a cocktail of nonabsorbable broad-spectrum antibiotics for four weeks (19) and then injected with Con A. Mice receiving this cocktail regimen showed reduced serum levels of LPS (Fig.1B) and significant decrease in serum ALT and AST levels (Fig.1C), indicating reduced liver damage in the antibiotics-treated mice than controls. This was confirmed by H&E and TUNEL staining, which showed reduced apoptosis and necrosis in the liver of antibiotics-treated mice (Fig.1D, E). Reduced apoptosis was further confirmed by processing the pro-caspase-3 and PARP to the cleaved active form (Fig.1F). Moreover, compared with control mice, the expression of anti-apoptotic protein Mcl-1 was higher, while the pro-apoptotic protein Bax was lower in antibiotics-treated mice (Fig.1F). Both Erk1 and Erk2 have important roles in regulating cell proliferation and cell differentiation (20). We found that the phosphorylation of Erk1/2 was significantly higher in antibiotics-treated mice.
(Fig.1G) at 1 and 3 hours following Con A injection. These findings indicate that deletion of gut-derived LPS can protect the mouse from Con A-induced hepatitis. However, in the TLR4 (innate receptor of LPS)-deficient mice, deletion of gut-derived LPS had no protective roles in Con A-induced hepatitis, as there was no significant difference in serum ALT levels between the antibiotics treated and untreated group (Fig.1H).

In conclusion, these data definitely indicated that the gut-derived LPS promote Con A induced hepatitis through TLR4 and may regulate the apoptotic pathway in the parenchymal cells.

**Depletion of gut-derived LPS suppresses T cell infiltration and CD4⁺ T cell activation**

Next we analyzed the key pathways that were involved in Con A-induced injury. T cells especially the CD4⁺ T cells have been shown to play essential roles in the Con A-induced liver injury(3). We isolated liver mononuclear cells from the two groups and determined the percentage of CD3⁺ cells (T cells). As shown in Fig.2A, after administration of Con A, the percentage of CD3⁺ cells was significantly lower in the antibiotics-treated mice compared with the control group, indicating that depletion of gut-derived LPS suppresses T cell infiltration to liver. The activation of CD4⁺ T lymphocytes was determined by flow-cytometric analysis of the early activation marker CD69. As shown in Fig.2B, administration of Con A led to a significant increase in percentage of activated CD4⁺ cells (CD69⁺CD4⁺ positive cells in whole CD4⁺ subpopulation) in both groups, but it was lower in antibiotics group compared with control group both at 6 hours (52.55% VS 45.31%) and 20 hours (53.78% VS 45.48%). These findings suggest that activation of CD4⁺ T cells is suppressed by depletion of gut-derived LPS.
Deletion of gut-derived LPS suppresses secretion of Th1-associated cytokines

Immune cells activated by Con A synthesize and secrete a series of inflammatory cytokines and these cytokines are vital components of Con A-induced liver injury (3;4). Using ELISA, expression of key T helper 1 cytokines such as TNF-α, IFN-γ, IL-12, IL-2 and IL-6, as well as T helper 2–associated cytokines IL-10 in the two groups were assessed. Fig.2C shows that there is no difference in baseline of Th1-associated cytokines and Th2-associated cytokines at 0 hour; but at 3, 6 and 20 hours after Con A administration, the levels of Th1 cytokines in antibiotics-treated mice were much than in controls. Interestingly, Th2–associated cytokines IL-10 were much higher in antibiotics-treated mice than control mice (Fig.2D). These data suggest that LPS may augment the Th1 response while inhibit the Th2 response to promote Con A-induced liver injury.

Exogenous LPS significantly aggravates Con A-induced liver injury

To further evaluate the contribution of LPS to the pathogenesis of Con A-induced liver injury, mice were co-injected with LPS and a low dose of Con A (10mg/kg) or Con A alone. As shown in Fig.3A, the ALT levels of the LPS + Con A-injected group were much higher than that of Con A group. Meanwhile, the proportion of activated CD4^+ cells in mice treated with LPS+Con A were higher than the mice injected with Con A or exogenous LPS(80.75% VS 62.04% VS 5.30%)(Fig3B). The Th1 cytokines such as IL-2, TNF-α, IFN-γ and IL-12 were also elevated in the LPS+Con A group (Fig.3C). To investigate the possible mechanisms of these findings, we evaluated the expression of T-bet (an important regulator of Th1 differentiation) by quantitative PCR. As shown in Fig.3D, the expression of T-bet was significantly increased in Con A+LPS-treated mice compared with Con A-treated mice 3
hours after injection. These data suggested that LPS can promote Con A-induced hepatitis via promoting CD4^+ T cells activation and Th1 cytokine production.

**TLR4 deficiency greatly attenuates Con A-induced liver injury**

LPS exerts its effects primarily through its innate receptor TLR4, we then assessed the effect of TLR4 deficiency on Con A-induced hepatitis. Wild-type (wt) mice and TLR4 knockout (TLR4^−/−) mice were injected with Con A intravenously and sacrificed 0, 6 and 20 hours thereafter. Analysis of liver histopathology revealed blunted inflammation and tissue injury in TLR4^−/− mice compared with wt mice (Fig.4A). This was confirmed by serum ALT and AST levels (Fig.4B). TUNEL staining showed that more apoptotic cells were present in wt livers compared to livers from TLR4^−/− mice (Fig.4C). This was further confirmed by the activation of caspase-3 and PARP (cleavage form of caspase 3 and PARP) (Fig.4D). Moreover, as shown in Fig.4E, TLR4 deficiency protected mice from a lethal dose of Con A: 66.7% of TLR4 wt mice died 9 hours after Con A injection, whereas 87.5% of TLR4^−/− mice survived within 24 hours.

Besides, similar to the finding in the antibiotics-treated mice, Mcl-1 was much higher whereas Bax was much lower in TLR4^−/− mice at 6 and 20 hours after Con A injection; the phosphorylation of Erk1/2 was significantly higher in TLR4^−/− mice (Fig.4F) at 1 and 3 hours following Con A injection. Taken together, these data demonstrate that TLR4 deficiency protect the mouse from Con A-induced hepatitis.

**Deletion of TLR4 suppresses T cell infiltration and CD4^+ T cell activation following Con A administration**

To further verify TLR4-dependent steps to aggravate Con A-induced hepatitis, we
isolated liver mononuclear cells from wt and TLR4−/− mice following Con A injection and assessed the percentage of T cells and CD4+ T cell activation by flow-cytometric analysis. As shown in Fig.5A, the percentage of CD3+ cells (T cells) was significantly elevated in wt mice compared with TLR4−/− mice after Con A injection. The activation of CD4+ T cells was also increased in wt mice compared with TLR4−/− mice both at 6 (77.45% VS 66.77%) and 20 hour (60.00% VS 30.69%) (Fig.5B). To rule out the possibility of the influence of TLR4 in cell proliferation, splenic cells isolated from wt and TLR4−/− mice were labeled with CFSE and incubated in the presence or absence of Con A or anti-CD3. After 0, 24 or 48 hours, splenic cell division was shown by CFSE dilution (Fig.5C), there are no marked differences in splenic cell proliferation between wt and TLR4−/− mice. These data suggested that TLR4 may promote CD4+ T cells activation directly, other than promote T cell proliferation.

Next we ask whether LPS can promote T cell activation directly. Isolated splenic cells from wt and TLR4−/− mice were stimulated with 1ug/ml Con A or 200ng/ml LPS or both. 20 hours later, the expression of CD69 was determined by flow-cytometric analysis. As shown in Fig.5D, the proportion of activated wt splenic cells stimulated with Con A and exogenous LPS was higher than that of the cells stimulated with Con A (77.09% VS 66.86%). Conversely, in TLR4−/− cells, although it was all lower than that in the wt cells, there was no obvious difference in the proportion of activated CD4+ cell between the two different treatment (Con A+LPS 62.35% VS Con A 61.70%).

As T cell response requires the antigen-presenting cells (APC), to rule out the probability that TLR4 expressed in APCs affects the CD4+ T cell activation, we isolated CD4+ T cells and performed the stimulation experiment mentioned above. Similar data were observed in the
enriched CD4^+ T cell pool, in which more than 95% of splenic cells were positive for CD4 (Fig.5E). These data indicated that LPS alone have little effect to activate T lymphocytes, but it could promote Con A-induced CD4^+ T lymphocytes activation effectively, suggesting LPS may have a direct role in the T cell activation.

**TLR4 ablation in bone marrow-derived cells alleviate Con A-induced liver injury**

As TLR4 is expressed both on myeloid-derived cells and parenchymal cells in the liver, the difference of liver injury between wt and TLR4^/- mice might be caused by a bone marrow-derived cell or a resident liver cell such as hepatocytes, endothelial or stellate cells. To rule out these possibilities, we performed bone marrow transplantation experiments in the TLR4 knockout and wild type mice. Five weeks later, the chimeric mice were injected with Con A and the liver injury and the activation of T cells in the liver was determined. As shown in Fig.6A and 6B, both the Con A-induced liver injury and CD4^+ T cell activation were associated with bone marrow type: the chimeric mice with TLR4^/- marrow [TLR4^/-/TLR4^/- (TLR4^/- bone marrow→TLR4^/- mice) and TLR4^/-/wt (TLR4^/- bone marrow→wt mice) ] showed milder liver injury and attenuated CD4^+ T cell activation. These data suggested that TLR4 expressed on myeloid cells played crucial roles in Con A-induced liver injury. To elucidate the role of TLR4 expressed on T cells in Con A-induced hepatitis directly, we performed T cell transfer experiment using the NOD/SCID mice (T cell, B cell and NK cell deficient) according to the literature (21). As shown in Fig.6C, adoptive transfer of wt T cells resulted in exacerbated Con A-induced liver damage in recipient mice compared with in mice transferred with TLR4^/- T cells. These data demonstrated that TLR4 expressed on T cells played key roles in Con A-induced hepatitis.
**TLR4 deletion significantly reduces the production of Th1 cytokines**

With ELISA, expression of T helper 1 cytokines such as TNF-α, IFN-γ, IL-12, IL-2 and IL-6, as well as T helper 2–associated cytokines IL-10, was assessed at 0, 3, 6 and 20 hours after Con A injection. Fig. 6D shows that there is no difference in baseline of Th1-associated cytokines and Th2-associated cytokines before Con A injection; however, after Con A administration, wt mice exhibited much higher levels of Th1 cytokines compared with TLR4−/− mice. Interestingly, Th2–associated cytokine IL-10 were much higher in TLR4−/− mice in contrast to wt mice (Fig.6D).

We also evaluated the expression of T-bet by quantitative PCR. As shown in Fig.6E, T-bet expression showed no difference between wt and TLR4−/− animals before Con A administration; however, 1 and 3 hours after Con A injection, expression of T-bet was significantly increased in wt mice compared with TLR4−/− mice.

Taken together, our results demonstrate a TLR4-related enhancement of key cytokines of the Th1 response, indicating that unusually prolonged exposure to these cytokines might exacerbate T cell-mediated hepatitis. However, TLR4 deletion could significantly increase the production of IL-10 which can lighten Con A-induced liver injury.

Previous studies have identified that significant increases in pro-inflammatory cytokine expression aggravate the induction of apoptosis (22) via enhancing T lymphocyte cytotoxicity. Apoptosis induced by cytotoxic T lymphocyte has been proposed as a mechanism of tissue damage (23). An important pathway in T lymphocyte mediated cytotoxicity is the granule exocytosis of perforin/granzymes (24-26). To determine the role of granzyme B and perforin in Con A-induced liver injury, we measured their expression by qPCR. Our results
demonstrate that the expression of perforin was much higher in wt mice compared to the TLR4−/− mice (Fig.6F). Furthermore, the levels of granzyme B were significantly higher in wt mice than in TLR4−/− mice particularly 20 hours post Con A injection (Fig.6F), indicating that the perforin/ granzyme B pathway is a possible mechanism of augmenting Con A-induced liver injury by TLR4.
Discussion

Because of the anatomic links to the gut, there is a potential interrelation between the gut antigens, gut microbiota products (specifically endotoxin) and liver diseases. Although liver is constantly exposed to microbial products from the enteric microflora, no obvious inflammation occurs in the healthy liver. However, when normal liver physiology is disrupted and inflammatory cells are activated, gut-derived factors likely augment or exacerbate certain liver diseases leading to enhanced tissue damage and propagation of inflammation. Activation of innate immunity, specifically TLR4 signaling, has emerged as a central component of the liver’s inflammatory response to gut bacteria under pathologic conditions (13;27). Abundant data demonstrate that TLR4 ligand, endotoxin, is elevated in experimental models of hepatic fibrosis (28) and patients with chronic liver diseases (29;30). We have now observed that serum endotoxin levels were elevated in an immune-mediated liver injury model. This endotoxin may result from: (1) decreased clearance of LPS: hepatocyte damage and reticuloendothelial system (mainly the Kupffer cells) dysfunction induced by Con A; (2) increased gut permeability: the detoxification ability of liver is reduced because of the liver damage. Indeed, gut barrier dysfunction leading to elevated intestinal permeability is also considered the main cause of endotoxemia in alcoholic liver disease (31) and has an important role in LPS/GalN-induced fulminant hepatitis (32); (3) bacteria overgrowth and translocation.

Con A is a potent T cell mitogen with tropism for the liver. In mice, Con A induces a severe liver injury that is a model of human T cell-mediated hepatitis, which is dependent on the activation of CD4\(^+\) lymphocytes(3), and requires Th1 cytokines such as IL-2, IFN-\(\gamma\) and TNF-\(\alpha\). It is reported that deficiency of Toll signaling resulted in failure to generate
Th1-dependent immune responses (16), and TLR4 also play vital roles in the function of CD4\(^+\) and CD8\(^+\) T cells (17;18). Using antibiotics to reduce the LPS levels and TLR4 knockout mice, we demonstrated that gut-derived LPS can promote Con A-induced liver injury in TLR4-dependent manner. Our data suggest that this effect is mediated partly by activation of CD4\(^+\) T cells: both depletion of LPS and TLR4 ablation can suppress the infiltration of T lymphocytes into liver and activation of CD4\(^+\) T cells \textit{in vivo}, while exogenous LPS exacerbates Con A-induced liver injury and promote activation of CD4\(^+\) T lymphocytes induced by Con A. As LPS augments ConA-induced wt CD4\(^+\) T cell activation and the activation of TLR4\(^{−/−}\) CD4\(^+\) T cells stimulated with Con A \textit{in vitro} is suppressed, it is reasonable to speculate that LPS has a direct role in T cell activation through TLR4, may be a costimulatory role, just like TLR2 (33). The BMT experiment further indicated that TLR4 expressed on myeloid cells other than the resident liver cells plays crucial roles in the Con A induced liver injury. Moreover, the result of T cell transfer experiment also demonstrated that TLR4 expressed on T cells played key roles in Con A-induced hepatitis. Therefore, even though T cell is not the only effective cell type originated from bone marrow involved in Con A-induced hepatitis (3;34), their activation augmented by gut-derived LPS through Toll-like receptor 4 plays vital role in this hepatitis model. However, the more detailed mechanisms need to be further investigated.

Our previous study on TLR4 and HCC used the carcinogen diethylnitrosamine (DEN) to induce hepatitis and showed that TLR4\(^{−/−}\) mice had aggravated liver injury after DEN treatment. However, in the current work, TLR4 knockout showed decreased liver injury induced by Con A. The reasons for this difference may be in terms of the acute liver damage
induced by DEN, TLR4 activation (by LPS, et al) on hepatocytes could protect them from apoptosis by induction of anti-apoptotic proteins, such as Bcl-xL and A20. TLR4 deficiency therefore ablated the protective effects of both LPS and endogenous ligands on hepatocytes, thus leading to more severe liver damage. However, in the Con A model, the BMT experiment showed that TLR4 expressed on myeloid cells played major roles in liver damage. On the other hand, Con A is a more potent inducer of liver damage than DEN: Con A-hepatitis is a fulminant hepatitis, the ConA treated mice usually died with several hours; while the DEN was a milder liver poison, and does not lead to death at the dosage we used (100mg/kg).

T lymphocyte-associated cytokine production has previously been shown to be critical for the injury response. Th1 and Th2 cytokines are antagonistic in activity and their balance and regulation is crucial for the immune response (6;7). However, in pathologic conditions such as allergic and autoimmune diseases, polarized Th1 and Th2 responses are found and believed to be critical to the outcome of these conditions (8). Here we found that exogenous LPS promotes Th1 cytokine production induced by Con A through TLR4, whereas reduction of LPS using antibiotics or the absence of TLR4 reinforces the expression of Th2 cytokines. T-bet is a powerful transcription factor involved in Th1 development (9). Our results suggested that LPS promote Con A-induced expression of T-bet through TLR4. The Th1 cytokines, particularly TNF-α, are important cytotoxic mediators. It’s conceivable that the elevated Th1 associated cytokines promote Con A-induced cellular immunity and aggravate liver tissue damage. Moreover, T lymphocyte cytotoxicity has been proposed as mechanism of apoptosis (25;26). An important pathway in T lymphocyte-mediated cytotoxicity is the granule exocytosis of perforin/granzymes (26). In the current study, we found that TLR4
regulates perforin/granzyme B expression, indicating the important role of TLR4 in hepatocytes apoptosis through enhancing T lymphocyte cytotoxicity.

It has been demonstrated that immune cells contribute to liver tumorigenesis. CD4$^+$ or CD8$^+$ T cells expressing inflammatory cytokines as well as cytolytic proteins contribute to hepatocyte death, tissue remodeling, and transformation, finally leading to HCC (35;36). Compared with other causes of cirrhosis, immune-mediated hepatitis is associated with a higher risk of developing HCC. Here we present a new feature of LPS/TLR4 signaling in T cells using the Con A-induced hepatitis model: gut-derived LPS have a direct role in promoting T lymphocyte activation through TLR4. Our data suggest that modulation of the gut microbiota and lessening the gut translocation of endotoxin may represent a new avenue for therapeutic intervention to treat or prevent immune-mediated hepatitis. Therefore, it will be interesting to determine whether the regimen with anti-endotoxin effects will prove beneficial in preventing or delaying T cell-mediated hepatitis and hepatitis-induced HCC.
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Figure Legends

Fig1. Antibiotics treatment suppresses Con A-induced liver injury and apoptosis in TLR4 intact mice. (A) The serum LPS concentration at the indicated time after Con A injection in the untreated mice. *, P<0.05; ***, P<0.001, 0h vs 20h. (B) The serum LPS concentration at the indicated time after Con A injection in the antibiotics-treated mice and untreated mice. (C) ALT and AST levels in mice of antibiotics-treated and control group. (D) H&E staining of livers from Con A-injected mice with or without antibiotics treatment. (E) TUNEL assay of liver sections from antibiotics-treated and control mice 0, 6, or 20 hours after Con A administration. (F) Antibiotics-treated and controls were treated with Con A. At the indicated times the livers were removed and lysed for western blot with antibodies against the indicated proteins. (G) Expression of phospho-Erk1/2 in antibiotics-treated and control mice 0, 1 or 3 hours after Con A treatment. (H) ALT levels in TLR4⁻/⁻ mice of antibiotics-treated and untreated groups after Con A administration. *, P<0.05; **, P<0.01.

Fig2. Deletion of gut-derived LPS suppresses T cell infiltration, activation of CD4⁺ T lymphocyte and Th1 associated cytokines production. (A) Liver mononuclear cells were isolated from antibiotics-treated and control mice 0, 6, 20 hours after Con A administration and percentage of CD3⁺ cells were determined by flow cytometry. (B) The percentage of activated CD4⁺ T cells were determined by dual staining with CD4 and the activation marker CD69 followed by flow cytometric analysis. Percentages represent the proportion of CD4⁺CD69⁺ cells in whole CD4⁺ cells subpopulations. (C and D) Expression of Th1–associated cytokines (C) TNF-α, IFN-γ, IL-6, IL-2 and IL-12 and (D) Th2-associated cytokines IL-10 in the serum of antibiotics-treated mice and controls. ***, P<0.001; **, P<0.01; *, P < 0.05.

Fig3. Exogenous LPS aggravates Con A-induced liver injury. (A) Mice were injected with Con A+LPS or Con A alone. At the indicated times, serum ALT levels were determined. (B and C) The activation of CD4⁺ T cells (B) and serum levels of Th1–associated cytokines IL-2, TNF-α, IFN-γ and IL-12 (C) in mice treated with Con A+LPS or Con A alone. (D) Expression of T-bet mRNA in livers of Con A-administrated mice treated with or without LPS. *, P<0.05; **, P<0.01.
**Fig4. Loss of TLR4 greatly attenuates Con A-induced liver injury in mice.** (A) Histological analysis of liver sections from wt or TLR4−/− mice at the indicated times post Con A injection. n=3 per time point per group. (B) Serum ALT and AST levels in mice of different genotypes were determined at the indicated times after Con A injection. *, P<0.05. (C) TUNEL assay of liver sections from wt and TLR4−/− mice. (D) Wt and TLR4−/− mice were treated with Con A. At the indicated times the livers were removed and lysed for western blot with antibodies against the indicated proteins. (E) Wt and TLR4−/− mice were injected with lethal dose of Con A (25mg/kg). The survival time of treated mice were monitored for 24 hours. (F) Expression of Mcl-1, Bax and phospho-Erk1/2 in the liver lysates of wt and TLR4−/− mice at At the indicated times after Con A treatment was assessed.

**Fig5. Deficiency in TLR4 does not affect T cell proliferation but inhibit CD4+ T cell activation following Con A injection in vivo and in vitro.** (A) Liver mononuclear cells were isolated from wt and TLR4−/− mice 0, 6, 20 hours post Con A administration and percentage of CD3+ cells were determined by flow cytometry. ***, P<0.001. (B) The percentage of activated CD4+ T cells were determined by dual staining with CD4 and the activation marker CD69 followed by flow cytometric analysis. (C) Splenic cells isolated from TLR4 wt and TLR4−/− mice were stained with CFSE and cultured in the presence of Con A or anti-CD3 antibody. After 0, 24, 48 hours of culture, cells were analyzed by FACS for CFSE fluorescence. No significant differences were observed in splenic cell proliferation following Con A or anti-CD3 antibody treatment between wt and TLR4−/− mice. (D) Isolated splenic cells from wt and TLR4−/− mice were stimulated with Con A or LPS or Con A+LPS for 20 hours and stained for CD4 and CD69. Percentages represent the proportion of CD4+CD69+ in whole CD4+ subpopulation. (E) Enriched spleen-derived CD4+ T cells were stimulated with Con A or LPS or Con A+LPS for 20 hours, CD4 and CD69 were detected by FACS. Percentages represent the proportion of CD4+CD69+ in whole CD4+ subpopulation.

**Fig6. TLR4 expressed on bone marrow-derived cells promotes Con A-induced liver injury.** (A) Bone marrow-transplanted chimeric mice were treated with Con A, and the serum ALT was measured at indicated times. n=6-8, ***, P<0.001, *, P<0.05. (B) Liver mononuclear cells were isolated from chimeric mice at the indicated times post Con A administration. The activation of CD4+ T cells were determined by the flow cytometry. (C) NOD/SCID mice with transferred T cells were treated with Con A, and the serum ALT was
measured 6 hours later. n=6-8, *, P<0.05. (D) The expression of Th1–associated cytokines IL-2, TNF-α, IFN-γ, IL-12, IL-6 and Th2-associated cytokines IL-10 were determined by ELISA. n=6-8, *, P < 0.05; **, P<0.01; ***, P<0.001. (E) Expression of T-bet mRNA in wt and TLR4−/− liver. (F) Expression of perforin and granzyme B mRNA in wt and TLR4−/− liver 0, 6 or 20 hours after Con A treatment.*, P < 0.05; **, P<0.01; ***, P<0.001.
Figure 1

A

B

C

D

E

F

G

H
**Figure 3**

A

![Graph showing cytokine levels over time](image)

B

**CD69 positive cell**

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6 hours post injection (in vivo)

C

**Th1-associated cytokine**

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D

![Graph showing cell counts](image)
Figure 4

A

B

C

D

E

F

GAPDH

ERK1/2

p-ERK1/2

Bax

Figure 4

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Gut-derived Lipopolysaccharide Promotes T Cell-mediated Hepatitis in Mice through Toll-like Receptor 4

Yan Lin, Lexing Yu, Hexin Yan, et al.