TLR-9 Activation Aggravates Concanavalin A-Induced Hepatitis via Promoting Accumulation and Activation of Liver CD4+ NKT Cells

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Increasing evidence suggests that TLRs are involved in the pathogenesis of liver diseases; however, the underlying mechanisms remain obscure. In this study, we found that treatment with CpG-oligodeoxynucleotide (ODN) promoted the accumulation and activation of murine hepatic NKT cells. Additional experiments showed that CpG-ODN preferred to act on CD4+ NKT cells, while having less effect on CD4− NKT cells. The effect of CpG-ODN on liver NKT cells depended on the presence of Kupffer cells and IL-12. Meanwhile, CpG-ODN pretreatment aggravated liver injury and promoted the production of inflammatory cytokines in a Con A-induced fulminant hepatitis model via TLR9 activation. Collectively, our data demonstrate that TLR9 stimulation prefers to promote the accumulation and activation of hepatic CD4+ NKT cells and suggest that TLR9 signaling might be involved in the pathogenesis of human hepatitis. The Journal of Immunology, 2009, 182: 3768–3774.

Liver diseases, including viral hepatitis, alcoholic liver disease, autoimmune hepatitis, and primary biliary cirrhosis, afflict >10% of the world population, but the immunopathogenesis of liver diseases remains largely undefined. TLRs recognize a diverse range of pathogen-associated molecular patterns and play a critical role in antimicrobial host defense (1, 2); however, increasing evidence suggests the involvement of TLRs in the pathogenesis of human diseases, such as autoimmune diabetes, inflammatory bowel diseases, multiple sclerosis, or systemic lupus erythematosus (3, 4). In liver, TLRs are widely expressed on Kupffer cells, hepatocytes, hepatic stellate cells, biliary epithelial cells, sinusoidal endothelial cells, and hepatic dendritic cells (DCs) (5–10). Also, the liver is constantly exposed to gut-derived bacterial products, suggesting that bacterial products and TLRs might be involved in liver diseases. Indeed, under pathological conditions, TLRs activate liver inflammatory signaling pathways and are actively involved in the pathophysiology of a large number of hepatic diseases (11). For example, in alcoholic liver disease, the activation of Kupffer cells depends on TLR4, because TLR4-mutated C3H/HeJ mice display strongly reduced levels of proinflammatory mediators in the liver (12). In liver fibrosis, LPS induces hepatic stellate cells to produce IL-8 and MCP-1 and activates transcription factor NF-κB and c-Jun through TLR4, indicating that LPS exerts direct effects on hepatic stellate cells during fibrogenesis (6). Ischemia-reperfusion-induced hepatic inflammation and hepatocellular damage are almost completely prevented in TLR4-deficient mice (13). Although TLR-mediated signals play an important role in the pathophysiology of a number of hepatic diseases, the underlying cellular and molecular mechanisms remain to be further investigated.

TLR9 recognizes bacterial and viral DNA and synthetic oligodeoxynucleotides (ODNs)3 containing unmethylated CG dinucleotides. CpG-ODN may activate the immune cells such as DCs, B cells and macrophages to produce Th1 cytokines (14). Kupffer cells are specialized macrophages located in the liver and the largest tissue resident macrophage population in the body. Kupffer cells can be activated by various bacterial stimuli and produce IL-12 and other cytokines (TNF-α, IL-1β, IL-18 etc.), which are partly responsible for the activation of liver mononuclear cells (MNcs) (IFN-γ production and cytotoxicity) (15, 16). In this study, we demonstrate that the TLR9 ligand-primed liver is more sensitive to Con A-induced acute liver injury, and we investigate the underlying mechanisms of Kupffer cell dependence.

Materials and Methods

Animals
Male C57BL/6 (B6) mice (6–8 wk old, weighing 20–24 g) were obtained from Shanghai Experimental Center, Chinese Science Academy (Shanghai, China) and maintained at an animal facility under pathogen-free conditions. TLR9 gene-deficient (TLR9−/−) mice on a B6 background were provided by Dr. S. Akira (Osaka University, Osaka, Japan). All mice used were 6 to 8 wk of age. The handling of mice and experimental procedures were conducted in accordance with experimental animal guidelines.

Reagents
Con A (type IV) was purchased from Sigma-Aldrich and dissolved in pyrogen-free PBS at 1 mg/ml. Synthetically produced mouse CpG-ODN with the sequence 5′-TCCATGACGTTCCTGACGTT-3′ and ODN control, which does not contain a CpG motif, with the sequence 5′-TCCATGACGTTCCTGAGCCT-3′, were obtained from Shanghai...

3 Abbreviations used in this paper: ODN, oligodeoxynucleotide; ALT, alanine aminotransferase; DC, dendritic cell; DN, double negative; FasL, Fas ligand; MNC, mononuclear cell.

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Sangon Biotechnology and separately dissolved in pyrogen-free PBS at 50 μg/ml. The mAbs used in this study included the following: anti-FcR, FITC-conjugated anti-NK1.1, anti-IgG2a, anti-TLR9, anti-CD4, and anti-CD8α; PE-conjugated anti-CD69, anti-Fas ligand (FasL), anti-IFN-γ, anti-IL-4, anti-NK1.1, anti-CD11c, and anti-F4/80; PE-Cy5-conjugated anti-CD3ε and anti-CD4; and allophycocyanin-conjugated anti-CD3 (Bioscience).

**Assay for serum transaminase levels**

To assay for serum alanine aminotransferase (ALT) levels, mice were anesthetized with ether and bled from the eye. Serum (50 μl) was mixed with 0.5 ml of ALT assay solution (Shanghai Rongsheng) and then measured in a spectrophotometer following the supplier’s protocol.

**H&E staining**

For histological analysis, liver tissue was fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections of 5-μm thickness were affixed to slides, deparaffinized, and stained with H&E to determine morphologic changes.

**Measurement of serum cytokine levels**

The serum samples were kept at −20°C until they were ready for cytokine measurement. Levels of IFN-γ, IL-4, TNF-α, and IL-12 were measured using commercially available ELISA kits from R&D Systems.

**Flow cytometric analysis**

After blocking with anti-FcR, cells were stained in darkness at 4°C for 30 min with the indicated mAbs and then washed and finally examined by flow cytometer (FACSCalibur; BD Biosciences). The data were analyzed with WinMDI 2.8 software. For the intracellular cytokine assay, after labeling the surface Ags the cells were fixed and permeabilized using a Cytofix/Cytoperm Plus kit (BD Pharmingen) and then stained with the indicated mAbs.

**BrdU staining**

Mice received a 200-μl (1.8 mg/ml) i.p. injection of the thymidine analog BrdU and were given BrdU (0.8 mg/ml) in drinking water. Surface staining was done as usual. Cells were then fixed, permeabilized, treated with DNase1, and stained with allophycocyanin-conjugated anti-BrdU according to the manufacturer’s procedures (BrdU flow kit from BD Pharmingen).

**Preparation of liver mononuclear cells**

Liver MNCs were prepared as described previously (17). Briefly, liver tissues were pressed through a 200-gauge stainless steel mesh. After one washing, the cells were resuspended in 40% Percoll (Invitrogen) solution, gently overlaid onto 70% Percoll solution, and centrifuged at 2,400 rpm for 30 min at room temperature. The interface cells between the Percoll solutions were aspirated and washed twice with RPMI 1640 medium containing 5% FCS.

**Isolation of hepatocytes**

Mice were anesthetized and the portal vein was cannulated under aseptic conditions. The liver was subsequently perfused with EGTA solution (5.4 mM KCl, 0.44 mM KH2PO4, 140 mM NaCl, 0.34 mM Na2HPO4, 0.5 mM EGTA, 25 mM tricine (pH 7.2)) and DMEM (Invitrogen) and digested with 0.075% collagenase solution. The isolated hepatocytes were cultured in Shanghai Experimental Center, Chinese Science Academy) in rat tail collagen (Sigma-Aldrich) coated plates for 24 h, then cultured in serum-free DMEM (18).

**Isolation of Kupffer cells**

Kupffer cells were isolated using a two-step collagenase perfusion method as described. Solution B was added with 0.1% pronase E and 20 μg/ml DNase. After perfusion, livers were excised and shaken in DMEM (Invitrogen) at 100 rpm for 20 min. The suspension was then centrifuged at 50 × g for 1 min at 4°C three times. The resulting suspension was then washed twice at 50 × g for 8 min at 4°C. The collected cells were gently layered on a double Percoll gradient (20% and 50%) and centrifuged at 800 × g for 15 min. The interface cells between the Percoll solutions were aspirated and applied to MACS to purify Kupffer cells using FITC-conjugated anti-F4/80 mAb.

**Purification of NK cells, NKT cells, CD4+ and CD4− NKT cells, and DCs**

Hepatic MNCs were stained with FITC-anti-CD4, PE-anti-NK1.1, or anti-CD11c mAb and PE-CY5-anti-CD3 mAb at 4°C for 30 min and then washed three times. Subsequently, the stained hepatic MNCs were automatically sorted by flow cytometry (BD Biosciences) in PBS with a total volume of 1 ml per 1 × 107 cells. The separated cells had ≥95% purity. NK cells, NKT cells, and DCs were gated on NK1.1+CD3−, NK1.1+CD3+, and CD11c−CD3+ populations, respectively. CD4+ and CD4− NKT cells were gated on NK1.1+CD3−CD4− and NK1.1+CD3+CD4+ populations.

**Cytotoxicity assay**

The cytolytic activities of hepatic MNCs, purified NK cells, and NKT cells and sorted CD4+ and CD4− NKT cells against murine hepatocytes were tested using a 4-h aspartate aminotransferase release assay according to the existing protocol (18). Each experiment was performed at various E:T ratios using triplicate samples.

**Neutralization of serum IL-12**

Anti-IL-12 mAb (CRL-2357; American Type Culture Collection) was obtained from a partially purified hybridoma culture supernatant by ammonium sulfate precipitation. Mice were given three injections of the indicated mAb (50 μg/mouse) i.p. on days 2, 1, and 0 before subsequent injections. This protocol resulted in a 90% decrease.

**Depletion of Kupffer cells**

Macrophages were eliminated in mice by the injection of GdCl3, (gadolinium chloride) purchased from Sigma-Aldrich. Macrophages were depleted in vivo 24 h after mAb injections by i.v. injections of GdCl3 (10 mg/kg body weight).

**Statistical analysis**

Results were analyzed using the Student’s t test or ANOVA where appropriate. All data in this study were expressed as the mean ± SEM. Values of p < 0.05 were considered significant.

**Results**

**The expression of TLR9 in subsets of liver MNCs**

To investigate the role of TLR9 in the modulation of immune responses in liver, we first examined TLR9 expression by flow cytometry in subsets of liver MNCs before or after CpG-ODN treatment. As shown in Fig. 1, TLR9 was widely expressed in various subsets of liver MNCs, including NK cells, T cells, DCs, Kupffer cells, and NKT cells. Moreover, the expression of TLR9 in Kupffer cells and NKT cells was up-regulated 12 h post-CpG-ODN treatment and returned to normal after 24 h.

**Treatment with CpG-ODN promotes the accumulation of liver CD4+ NKT cells**

To further investigate the effect of TLR9 activation on liver MNCs, CpG-ODN was administrated to C57BL/6 mice by i.v. injection. The liver MNCs were isolated and subjected to flow cytometry analysis. The results showed that the proportion and number of NKT cells in the liver were increased 12 h post-CpG-ODN injection, whereas other liver MNCs subsets, such as T cells and NK cells, were not influenced (Fig. 2, A and B). The expanded NKT cells could be explained by recruitment from other organs or division from resident hepatic NKT cells, and division was determined by BrdU incorporation experiment. The results showed that the liver NKT cells had almost no proliferation in a 12-h period, whereas CD4+CD8− double negative (DN) thymocytes had a significant proliferation (Fig. 2C), suggesting that CpG-ODN treatment promotes NKT cell recruitment to the liver. As the liver NKT cells mainly consist of CD4+ and CD4− (DN and CD8−) NKT cells, we then evaluated the change of these subgroups of NKT cells after CpG-ODN treatment. CpG-ODN treatment only increased the percentage and number of CD4+ NKT cells in the
liver but had no effect on DN or CD8\(^+\) NKT cells (Fig. 2, D and E). Taken together, these results indicate that TLR9 activation promotes the accumulation of liver CD4\(^+\) NKT cells.

**Treatment with CpG-ODN activates liver CD4\(^+\) NKT cells**

We also examined the effect of TLR9 activation on the function of liver MNCs. As shown in Fig. 3A, CpG-ODN treatment could

**FIGURE 1.** TLR9 expression in various subsets of liver MNCs. C57BL/6 mice were treated with CpG-ODN (10 \( \mu \)g/mouse) for 12 and 24 h, and then the liver MNCs were isolated and determined by flow cytometry. NK cells, T cells, and NKT cells were gated on NK1.1\(^+\)CD3\(^-\), NK1.1\(^+\)CD3\(^+\), and NK1.1\(^+\)CD3\(^+\) populations, respectively. Kupffer cells (KC) were gated on a F4/80\(^+\) population. DCs were gated on a CD11c\(^+\)CD3\(^-\) population. Gray-filled histograms represent isotype Ab group, the black-lined empty histograms represent the CpG-ODN-treated 0-h group, the green-lined histograms represent the 12-h group, and the red-lined histograms represent the 24-h group. Bar graphs show the mean fluorescence intensity (MFI) \( \pm SD \) of TLR9 expression in various hepatic MNCs (n = 3).

**FIGURE 2.** CpG-ODN promotes the accumulation of hepatic CD4\(^+\) NKT cells. A and B, C57BL/6 mice were treated with CpG-ODN (10 \( \mu \)g/mouse). At various time points, the lymphocyte populations of liver and spleen were analyzed by flow cytometry using anti-NK1.1 and anti-CD3 Abs (A), and the hepatic MNCs (T, NKT, and NK cells) were counted (B), respectively. C, C57BL/6 mice were injected i.v. with CpG-ODN and i.p. with BrdU, and BrdU was added in drinking water for 12 h. BrdU incorporation was measured for hepatic NKT cells (NK1.1\(^+\)CD3\(^-\)) and thymocytes (CD4\(^-\)CD8\(^-\) DN) from the CpG-ODN group, the BrdU group, and the CpG-ODN plus BrdU (CpG-ODN+BrdU) group, respectively. Numbers indicated the percentages of BrdU\(^+\) cells. L NKT, Liver NKT cells; T DN, CD4\(^+\)CD8\(^-\) thymocytes. D and E, C57BL/6 mice were treated with CpG-ODN (10 \( \mu \)g/mouse). After various time points, the hepatic NKT cells (NK1.1\(^+\)CD3\(^-\)) were analyzed for expression of CD4 and CD8 (D), and the hepatic CD4\(^+\), CD8\(^+\) and CD4\(^+\)CD8\(^-\) (DN) NKT cells were counted (E), respectively. Values are shown as means \( \pm SD \) (n = 5).

**FIGURE 3.** CpG-ODN treatment enhances the cytotoxicity of hepatic CD4\(^+\) NKT cells against primary hepatocytes. C57BL/6 mice were given ODN control or CpG-ODN (10 \( \mu \)g/mouse) for 12 h. A, Hepatic MNCs were isolated and the cytotoxicity was tested against that of freshly isolated hepatocytes from C57BL/6 mice at the indicated E:T cell ratio. B, NKT and NK cells were sorted from hepatic MNCs, and their cytotoxicities against primary hepatocytes from C57BL/6 mice were tested at a 10:1 cell ratio (E:T). C, Hepatic CD4\(^+\) and CD4\(^-\) NKT cells were sorted, and their cytotoxicities against primary hepatocytes from C57BL/6 mice were tested at a 10:1 cell ratio (E:T). NKT and NK cells were gated on NK1.1\(^+\)CD3\(^-\) and NK1.1\(^+\)CD3\(^+\) populations, respectively. The results shown are representative of three experiments.
significantly enhance the cytotoxicity of liver MNCs against primary hepatocytes. Additional experiments showed that CpG-ODN treatment enhanced the cytotoxicity of liver NKT cells, especially increasing the cytotoxicity of CD4+ NKT cells while having little effect on CD4− NKT cells (Fig. 3, B and C). We also examined the expression of FasL, IFN-γ, and IL-4 by hepatic CD4+ or CD4− NKT cells after CpG-ODN treatment. As shown in Fig. 4, although CpG-ODN had little effect on CD4− NKT cells, it preferred to increase the percentage and number of FasL+, IFN-γ+, and IL-4+ CD4+ NKT cells. These results strongly suggest that CpG-ODN treatment prefers to activate liver CD4+ NKT cells.

**The effect of CpG-ODN on liver NKT cells depends on Kupffer cells**

We then investigated the mechanisms underlying the effect of CpG-ODN on liver NKT cells. As shown in Fig. 5A, CpG-ODN alone had no effect on the cytotoxicity of NKT cells against primary hepatocytes but could enhance the cytotoxicity significantly in the presence of Kupffer cells. Liver DCs could not increase their cytotoxicity. To further confirm the role of Kupffer cells, we depleted Kupffer cells using GdCl3 before CpG-ODN treatment. The results showed that Kupffer cell depletion could impair the increase of liver NKT cells induced by CpG-ODN.
CpG-ODN (Fig. 5, C and D). We also found that CpG-ODN treatment could promote Kupffer cells to produce IL-12 and that neutralization of IL-12 before CpG-ODN treatment could impair the increase of liver NKT cells (Fig. 5, B–D). These findings suggested that the effect of Kupffer cells on hepatic NKT cells might be mediated by IL-12. Moreover, depletion of Kupffer cells or neutralization of IL-12 prevented the activation of liver NKT cells induced by CpG-ODN (Fig. 5E). These data demonstrate that the effect of CpG-ODN on liver NKT cells depends on Kupffer cells.

TREATMENT WITH CPG-ODN AGGRAVATES CON A-INDUCED HEPATITIS

NKT cells, Kupffer cells, and IL-12 are important in Con A-induced hepatitis. Kupffer cell depletion, NKT cell deficiency, and IL-12 neutralization can prevent Con A-induced hepatitis (19–21). Because CpG-ODN treatment could increase the number and promote the activation of liver NKT cells, we then investigated whether CpG-ODN treatment could regulate the progression of hepatitis induced by Con A. C57BL/6 mice were pretreated with CpG-ODN 12 h before challenge with Con A, and then the serum and liver tissue samples were collected at various time points after Con A injection for evaluation of ALT levels and H&E staining. The results showed that treatment with CpG-ODN could increase Con A-induced elevation of serum ALT levels (Fig. 6A) and pathological liver injury (Fig. 6B). We also found that CpG-ODN treatment increased the serum concentrations of IFN-γ, IL-4, and TNF-α, which play critical role in Con A-induced hepatitis after Con A treatment (Fig. 6C). To confirm the role of TLR9 activation in the effect of CpG-ODN on hepatitis, we also performed the experiments on TLR9 knockout mice. As shown in Fig. 6D, the effect of CpG-ODN was impaired in TLR9 knockout mice significantly.

Discussion

Because TLRs are widely expressed in liver and liver is constantly activated by gut-derived bacterial products, TLR signaling has been suggested to participate in the progression of liver disease. In this study, we provide evidences that TLR signaling aggravates immune-mediated liver damage by demonstrating that pretreatment with CpG-ODN can greatly aggravate a low dose of Con A-induced, T cell-mediated liver injury.

Our results demonstrate that TLR9 stimulation promotes the accumulation and activation of hepatic NKT cells. After treatment with CpG-ODN, the proportion and number of CD4+ NKT cells are significantly increased, and the function of these cells is greatly enhanced. Because the hepatic NKT cells could not divide within 12 h, these increased NKT cells should be recruited from other organs. Investigators have realized that the liver is an organ with predominant innate immunity and that liver lymphocytes are abundant in innate immune cells such as...
NK, NKT, and γδ T cells (22, 23), but the mechanisms of how this microenvironment with predominant innate immunity is formed remain unclear. Our finding might provide a clue for this phenomenon; liver anatomically receives blood from the gut through the portal vein, which is rich in bacterial products including various ligands for TLRs, and the persistent activation of TLR signaling might be a possible reason for the abundance of innate immune cells in the liver. Indeed, our previous results have shown that treatment with polyinosinic-polycytidylic acid, a ligand for TLR3, also induced the recruitment of NK cells into liver (24).

Kupffer cells account for ~80% of the total population of fixed tissue macrophages in the body and are responsible for the elimination of insoluble waste by phagocytosis. Kupffer cells also have been shown to express TLRs, including TLR2, TLR4, and TLR9 (5, 25, 26). Upon TLR4 stimulation, Kupffer cells produce TNF-α, IL-1β, IL-6, IL-12, IL-18, and several chemokines (27, 28). The results presented here demonstrate that the accumulation and activation of hepatic NKT cells by CpG-ODN stimulation absolutely depended on the presence of Kupffer cells and their product, IL-12. When Kupffer cells were depleted or IL-12 was neutralized, CpG-ODN-induced NKT cell accumulation and activation were both impaired. Our previous study also showed that Kupffer cells were involved in TLR3 activation-induced NK recruitment into liver (24). Taken together, these results suggest that Kupffer cells might play a critical role in the formation of a liver microenvironment with abundant innate immune cells.

Our results showed that TLR9 is not only expressed in Kupffer cells but also in NK cells, NKT cells, T cells, and DCs. TLR9 is not a surface receptor and is expressed on endosomes or other intracellular compartments, so CpG-DNA should be internalized (phagocytosis) and then recognized by TLR9 (1). In general, macrophages (including Kupffer cells) and DCs have strong phagocytosis, whereas NK cells, T cells, and NK cells have poor phagocytosis. This might explain why NKT cells and NK cells have a poor direct response to CpG-ODN stimulation. Actually, NK cells and NKT cells need help from macrophages or DCs in response to most ligands of TLRs, although NK cells and NKT cells express some TLRs (29, 30).

One question to be further studied is the role of liver DCs in response to TLR9 activation. Our data indicated that CpG-ODN could not enhance the cytotoxicity of NKT cells in the presence of liver DCs. One possible reason is that the liver is located downstream of the gut, followed by constant exposure to endotoxin, and keeps a situation known as “endotoxin tolerance,” or “cross tolerance” to other TLR ligands, which likely causes impaired responses of liver DCs to TLR stimulation. As reported, liver DCs are less strong in activating the allogeneic T cells or polarizing the naïve T cells toward Th1 responses in response to LPS (10). Moreover, IL-12 production by liver myeloid DCs is markedly weaker than that of spleen myeloid DCs following TLR7 or TLR9 stimulation (31).

It is also important to address how IL-12 is involved in the recruitment and activation of NKT cells. One study by Dr. M. L. Dustin’s group has demonstrated that IL-12 might act on NKT cells directly; in this study the combination of IL-12 and IL-18 not only promoted NKT cells to produce IL-4 and IFN-γ but also induced NKT cell arrest in liver sinusoids (32). Meanwhile, our study cannot exclude the possibility that IL-12 acts on other cell populations, such as sinusoidal endothelial cells, and then indirectly induces NKT cell recruitment by up-regulating chemokines or other molecules.

In conclusion, we demonstrate that TLR9 stimulation promotes the accumulation and activation of hepatic CD4+ NKT cells by Kupffer cell- and IL-12-dependent mechanisms. We also show that the pretreatment with CpG-ODN aggravates Con A-induced hepatitis, suggesting a possibility that TLR9 and bacterial DNA might be involved in the pathogenesis of human hepatitis.

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Disclosures

The authors have no financial conflict of interest.

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