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

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*J Immunol* 2009; 182:3768-3774; doi: 10.4049/jimmunol.0800973
http://www.jimmunol.org/content/182/6/3768
TLR-9 Activation Aggravates Concanavalin A-Induced Hepatitis via Promoting Accumulation and Activation of Liver CD4\(^+\) NKT Cells\(^1\)

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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 in a large number of hepatic diseases (11). For example, in alcoholic liver disease, the activation of Kupffer cells depends on TLR4, because TLR4-mutated 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-\(\alpha\), IL-1\(\beta\), IL-18 etc.), which are partly responsible for the activation of liver mononuclear cells (MNCs) (IFN-\(\gamma\) 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’-TCCATGAGCTTCCTGAGCTT-3’, were obtained from Shanghai Institute of Immunology, Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science and Technology of China, Hefei, China

Received for publication April 1, 2008. Accepted for publication January 14, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(^1\) This work was supported by Natural Science Foundation of China Grants 30630059, 30721002, and 30730084 and Ministry of Science and Technology of China 973 Basic Science Project Grants 2007CB512405, 2007CB512807, 2007CB815800, and 2009CB522405.

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

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0800973
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-CD3e and anti-CD4; and allophycocyanin-conjugated anti-CD3 (eBioscience).

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 intercellular 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 NK 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 NK 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 NK 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 NK cells mainly consist of CD4+ and CD4− (DN and CD8−) NKT cells, we then evaluated the change of these subgroups of NK 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 enhance the cytotoxicity of hepatic CD4+ NKT cells against primary hepatocytes. C57BL/6 mice were given ODN control or CpG-ODN (10 μg/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

**FIGURE 4.** CpG-ODN activates liver CD4^+ NKT cells. C57BL/6 mice were treated with ODN control or CpG-ODN (10 μg/mouse), and FasL and intracellular cytokine (IFN-γ, IL-4) production of hepatic CD4^+ and CD4^- NKT cells was compared by flow cytometry 12 h post-CpG-ODN treatment. NKT cells were gated on NK1.1^-CD3^+ population. Bar graphs show the mean number ± SD of FasL, IFN-γ, or IL-4 positive CD4^+ or CD4^- NKT cells.

**FIGURE 5.** The effect of CpG-ODN on hepatic NKT cells depends on Kupffer cells. A, Primary Kupffer cells (KCs) or DCs sorted from C57BL/6 mice were stimulated with CpG-ODN in vitro for 24 h, purified hepatic NKT cells were added, 24 h later NKT cells were isolated, and then tested against primary hepatocytes at 10:1 cell ratio (E:T). B, IL-12 production in the supernatant of Kupffer cells was detected by ELISA. The results shown are representative of three experiments. Kupffer cells, DCs and NKT cells were gated on F4/80^-CD11c^-CD3^- and NK1.1^-CD3^- populations, respectively. C–E, C57BL/6 mice were treated with GdCl3 or anti-mouse-IL-12 Abs and then treated with CpG-ODN (10 μg/mouse) (C and D), and the proportion and expression of FasL and CD69 on hepatic NKT cells were calculated by FACS analysis (E). Bar graphs (D) show the mean percentage ± SD of NKT cells (n = 3).
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.

**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 hepatitis (19–21). 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.

**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 pretreatment aggravates Con A-induced liver injury via TLR9 signaling. A–C, C57BL/6 mice were pretreated with ODN control or CpG-ODN (10 μg/mouse), and 12 h later Con A (10 μg/g body weight) was administrated. A, Serum was collected for measuring ALT values at various time points post-Con A injection. B, Photomicrographs with H&E staining of representative C57BL/6 mouse livers obtained 24 h post-Con A injection were shown (original magnification, ×200). WT, Wild type. C, After Con A injection, kinetics of serum productions of IFN-γ, IL-4, and TNF-α were determined by ELISA. D, C57BL/6 mice and TLR9−/− mice were pretreated with ODN control (cont) or CpG-ODN (10 μg/mouse) for 12 h and then injected with Con A (10 μg/g body weight). Serum was collected for measuring ALT value at 24 h post-Con A injection. Values are shown as means ± SD (n = 4).
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 NK 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 NK 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 DCs have poor phagocytosis. This might explain why NK 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 NK cells in the presence of liver DCs. One possible reason is that the liver is located downstream of the gut, followed by constant exposure of 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 TLR9 stimulation. As reported, liver DCs are less strong in activating the allogeneic T cells or polarizing the naive T cells toward Th1 responses in response to LPS (10). More over, IL-12 production by liver myeloid DCs is markedly weaker in activating the allogeneic T cells or polarizing the naive T cells toward Th1 responses in response to LPS (10). Moreover, our data indicated that CpG-ODN stimulation auto induces immunotolerance or tolerance. Trends Immunol. 28: 74–79.

Acknowledgments
We thank Dr. Shizuo Akira (Osaka University, Osaka, Japan) for providing TLR9 gene-deficient mice. We also thank Xiaodong Zheng, Chen Ding, Xin Hou, and Hairong Wei for technical assistance.

Disclosures
The authors have no financial conflict of interest.

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