

Toll-like receptor 4 plays an anti-HBV role in a murine model of acute hepatitis B virus expression

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Abstract

AIM: Toll-like receptor 4 (TLR4) has been shown to be important for bacterial infection, especially to lipopolysaccharide signaling. Its possible role in HBV infection is studied in the present study.

MATERIALS AND METHODS: pHBV3.6 plasmid, containing full-length HBV genome was used in the murine model of acute HBV expression by hydrodynamics *in vivo* transfection. TLR4 normal or mutant mouse strain was compared to investigate the possible role of TLR4 in acute HBV expression.

RESULTS: After pHBV3.6 injection, the infiltrating leukocytes expressed TLR4 were observed nearby the HBsAg-expressing hepatocytes. The HBV antigenemia as well as the replication and transcription were higher in TLR4-mutant C3H/HeJ mice than in normal C3H/HeN mice. The HBV-specific immune responses were impaired in the liver or spleen of the C3H/HeJ mice. Their inducible nitric oxide synthase (iNOS) expression on the hepatic infiltrating cells was also impaired. When adoptively transferring splenocytes from C3H/HeN mice to C3H/HeJ mice, the HBV replication was inhibited to the level as that of C3H/HeN.

CONCLUSION: These results suggest that TLR4 plays an anti-HBV role *in vivo* through the induction of iNOS

expression and HBV-specific immune responses after HBV expression.

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Key words: TLR4; Rodent; HBV; iNOS; Liver

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INTRODUCTION

Hepatitis B virus (HBV) is an enveloped, double-strand DNA virus, and its replication is through an RNA intermediate that requires reverse transcriptase activity^[1]. HBV infection in human beings can cause chronic hepatitis and is associated with liver cirrhosis and hepatocellular carcinoma^[2,3]. One-third of the global population has been infected with HBV and about 350 million people are chronic carriers of HBV^[4]. HBV is non-cytopathic to hepatocytes and the hepatitis it causes is thought to be mediated by immune mechanism. However, the innate immune response to HBV infection is not fully understood.

Toll receptors are type I membrane proteins that was first identified in *Drosophila* and play a key role in antifungal immunity of *Drosophila*^[5]. The mammalian homologs of *Drosophila* Toll protein are called Toll-like receptors (TLRs), and there are 10 human (TLR1 to TLR10) and murine TLRs (TLR 1 to TLR9 and TLR11)^[5,6]. TLRs play a key role in host defense against microbial infection by regulating both innate and acquired immunity^[7,8]. For example, TLR4 is the receptor of Gram-negative bacterial lipopolysaccharide (LPS). After binding, the MyD88, interleukin-1 receptor-associated kinase, and tumor necrosis factor receptor associated factor 6 are activated, and then through MAP kinases and NF- κ B transcription factors^[6,9] to turn on the genes expression, which were involved in the inflammatory responses^[10]. TLR4 also plays a role in viral infections. Respiratory syncytial virus (RSV) persists longer in the lung of TLR4-deficient mice than normal mice, and RSV fusion protein can activate the human monocytes through TLR4^[11].

Hydrodynamics-based *in vivo* transfection has been

recently described. With this procedure, naked DNA can be introduced and expressed significantly in liver^[12,13]. This property allows investigators to develop hepatitis virus infection model in mouse^[14]. Recently, a murine acute HBV expression model was generated by hydrodynamics-based injection of plasmid containing full-length HBV genome by our group^[15] or others^[16]. After hydrodynamic injection of pHBV3.6, including full-length HBV genome, the HBV transcript and replicative intermediate were induced in the liver whereas the HBV-antigens, HBV-DNA, and HBV-specific antibody were detected in the sera^[15].

We are interested in the role of TLR4 during HBV infection. Using the murine model of acute HBV expression in this study, we reported that HBV expression-induced TLR4 expression has anti-HBV activity by upregulating the iNOS expression and HBV-specific immune response to help clearing the virus.

MATERIALS AND METHODS

Mice

Breeder mice of C3H/HeN and C3H/HeJ strain were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) or Charles River Japan, Inc. (Atsugi, Japan). They were fed standard laboratory chow and water *ad libitum* in the animal facility. The animals were raised and cared for according to the guidelines set up by the National Science Council of the Republic of China. Eight- to twelve-week-old male mice were used in all experiments.

Plasmids

pHBV3.6 containing all HBV open-reading frames was provided by Dr LP Ting (Department of Microbiology and Immunology, National Yang-Ming University), p(3A)SAg that encodes HBsAg was provided by Dr CC Lu (Department of Pathology, National Cheng Kung University) and pHBV^ΔPSX that encodes HBcAg was provided by Dr SJ Lo (Department of Microbiology and Immunology, National Yang-Ming University). pEGFP-N1 was obtained from Clontech (Palo Alto, CA, USA). All plasmids were prepared with Hi-speed Plasmid Midi Kit (Qiagen, Hilden, Germany).

Cells and transfection

The C3H/He bladder cancer cell line, MBT-2, was kindly provided by Dr MD Lai (Department of Biochemistry, National Cheng Kung University). Cells were maintained in Dulbecco's modified Eagle medium (Gibco BRL, Grand Island, NY, USA) and 10% fetal bovine serum (HyClone, Logan, UT, USA) at 37 °C under 50 mL/L CO₂. The cells were plated at a density of 3 × 10⁵ cells/well in six well-culture plate. One day later, cells were transfected with 1 μg of p(3A)SAg and pHBV^ΔPSX using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The medium was replaced with a fresh medium 8 h after transfection, and cells were used for T cell stimulation at 36 h after transfection.

Hydrodynamics-based *in vivo* transfection

Ten micrograms of plasmid, dissolved in Ringer's solution (NaCl 0.154 mol/L, KCl 5.63 mmol/L, CaCl₂ 2.25 mmol/L), were injected in the mouse tail vein, within 5 to 7 s, at a 12% of mouse bodyweight (around 3.0 mL) following the hydrodynamics-based transfection protocol described previously^[15].

Immunohistochemical analysis of HBsAg, TLR4, and iNOS expression

Mouse liver tissues were embedded in OCT compound (Miles Inc., Elkhart, IN, USA) and frozen in liquid nitrogen. Four micrometer cryosections were made using cryostats (Leica CM 1800, Nussloch, Germany). For TLR4 and HBsAg double staining, sections were fixed by 3.7% formaldehyde/PBS and then firstly stained with sheep-anti-HBsAg antibody (Serotec, Oxford, UK) and FITC-conjugated donkey-anti-sheep antibody (Jackson Laboratories, West Grove, PA, USA). After washing with PBS, the sections were further stained with rat-anti-mouse TLR4 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and Rhodamine-conjugated donkey-anti-rat antibody (Jackson Laboratories, West Grove, PA, USA). The nucleuses were stained by Hoechst 33258. For HBsAg and iNOS staining, the sections were fixed by cold acetone and endogenous peroxidase was inhibited by 3% H₂O₂/PBS. HBsAg and iNOS were detected with sheep polyclonal anti-HBs (Serotec, Oxford, UK) and rabbit polyclonal anti-iNOS antibodies (Chemicon, Temecula, CA, USA), respectively. Secondary antibody used was either biotinylated anti-sheep or anti-rabbit, and then incubated with avidin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA). Peroxidase stain of red color was developed by aminoethyl carbazole substrate (Zymed Laboratories, San Francisco, CA, USA) and counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany).

Northern and Southern blot hybridization of HBV transcription and replication

Total RNA was purified from mouse liver by TRI Reagent (Molecular Research Center, Inc, Cincinnati, OH, USA). The cytoplasmic DNA of mouse liver was purified as described previously by Guidotti *et al*^[17]. Thirty micrograms of total RNA or cytoplasmic DNA isolated from 30 mg of liver tissue were run on agarose gel electrophoresis, transferred to a nylon membrane and hybridized with biotin labeled HBV specific DNA probe, which was prepared by PCR amplification with the primers as: HBV1806: 5'-CCGGAAAGCTTGAGCTCTTCAAAAAGTATGGTGCTGG-3'; HBV1821: 5'-CCGGAAAGCTTCTTTTTCACCTCTGCCTAATCA-3', at 45 °C overnight. To verify the transfection efficiency, we injected pEGFP-N1 with pHBV3.6 simultaneously and detected by a specific biotin labeled DNA probe. The hybridized bands were detected by Detector AP Chemiluminescent Blotting Kit (KPL, Inc., Gaithersburg, MD, USA) and visualized by X-ray films.

HBV DNA detection in mouse sera

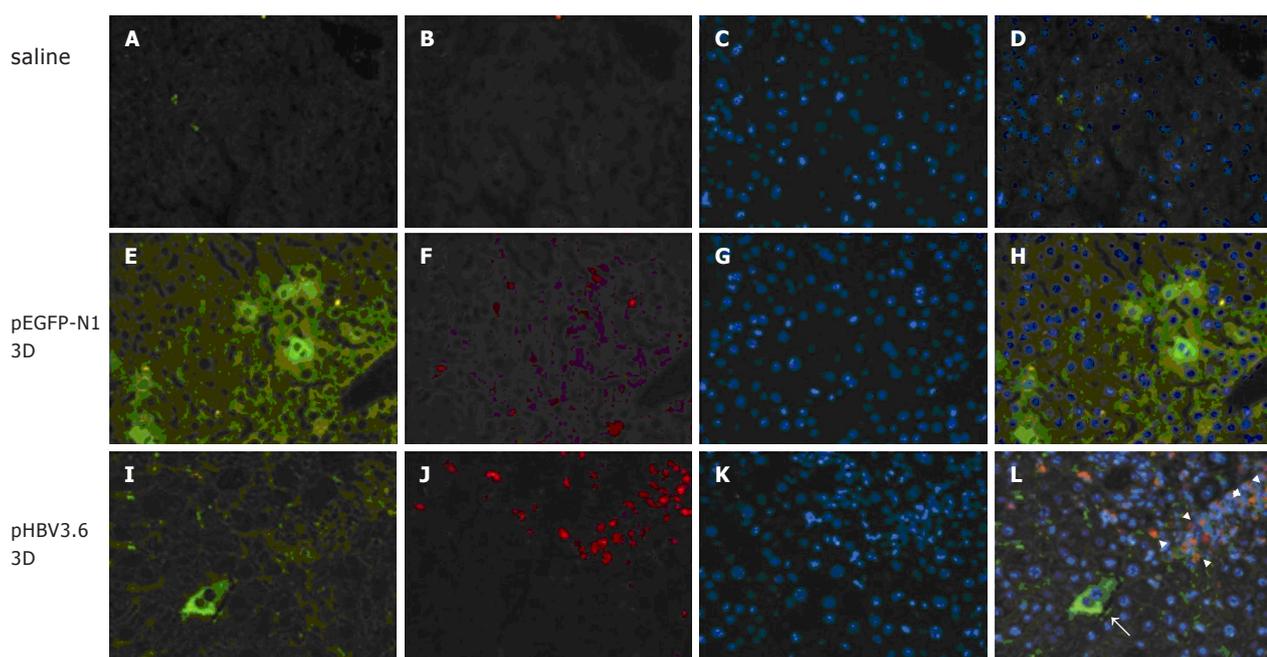


Figure 1 TLR4 expression in the liver of C3H/HeN mice after hydro-dynamic injection of pHBV3.6. Groups of four C3H/HeN mice were injected intravenously with 10 μ g of plasmid by hydrodynamics-based transfection. The liver tissues were collected at day 3 post injection and 4- μ m cryosections were made, stained with anti-HBsAg-FITC and anti-TLR4-PE antibody. The green represents EGFP-positive (E) or HBsAg-positive cells (I) and the red represents TLR4-positive cells (B, F, J). The blue represents counter staining by Hoechst 33258 dye (C, G, K). A-D: naïve; E-H: pEGFP-N1; I-L: pHBV3.6. The arrows indicate the HBsAg positive hepatocytes and the arrowheads indicate the TLR4 positive immune cells (original magnification $\times 200$)

Two hundred microliters of mouse serum was treated with 20 U DNase I for at least 12 h to remove retaining plasmid. After DNase I treatment, the serum DNA was purified by Viral DNA/RNA Isolation Kit (Maxim Biotech, INC., San Francisco, CA, USA). Five microliters of isolated DNA solution was used to detect the HBV DNA by PCR analysis. The preS2 region of surface antigen gene was amplified and visualized on an agarose gel as described previously^[18].

Detection of HBsAg and HBeAg in mouse sera

The level of HBsAg and HBeAg were determined using enzyme-linked immunosorbent assay (ELISA) kits (General Biological Corp., Taiwan, ROC) following the manufacturer's protocol.

ELISA for detecting cytokines

The intrahepatic lymphocytes (IHLs) were isolated as described previously^[15] with further removing the adhering cells. The IHLs or splenocytes were co-cultured with mitomycin C-treated (100 μ g/mL at 37 $^{\circ}$ C for 90 min) MBT-2^[19] or p(3A)SAg and pHBV[^]PSX transfected, MBT-2 (MBT-2-SC) at a ratio of 10:1 for 48 h. The co-cultured supernatants were harvested and assayed by sandwich ELISA for mouse IFN- γ , TNF- α or IL-12 (R&D Systems, Minneapolis, MN, USA).

RESULTS

Infiltrating cells in the liver expressed TLR4 after tran-

sfection of HBV gene

To investigate the association of TLR4 and HBV expression, the plasmid pHBV3.6, containing the full-length HBV genome was administrated to C3H/HeN mice by hydrodynamics *in vivo* transfection. As shown in Figure 1, the TLR4 expression could be detected on infiltrating leukocytes at day 3 post-injection (Figures 1I-L). But it was not detected in the liver of naïve C3H/HeN mice (Figures 1A-D) or pEGFP-N1 injected mice (Figures 1E-H). These results indicate that TLR4 expressed on leukocytes might involve the acute HBV expression.

HBV antigenemia and replication was higher in C3H/HeJ than in C3H/HeN

In C3H/HeJ mice, a missense mutation in the cytoplasmic domain of TLR4 impaired its ability to respond to lipopolysaccharide (LPS)^[20]. To further investigate the role of TLR4 in HBV expression, we compared the response in C3H/HeN (TLR4 normal) and C3H/HeJ (TLR4 mutation) after the pHBV3.6 *in vivo* transfection. As shown in Figure 2, the serum HBsAg (Figure 2A) or HBeAg (Figure 2B) level was higher in C3H/HeJ than in C3H/HeN mice. By Northern and Southern blot analyses, the specific HBV transcripts or HBV replication fragments were also higher in the liver of C3H/HeJ than C3H/HeN mice under similar transfection efficiency that was verified by co-injection of pEGFP-N1 (Figure 2C). Using viral DNA isolation and PCR analysis, the serum HBV-DNA showed higher level in C3H/HeJ than in C3H/HeN mice (Figure 2D). These results suggest that TLR4 is involved

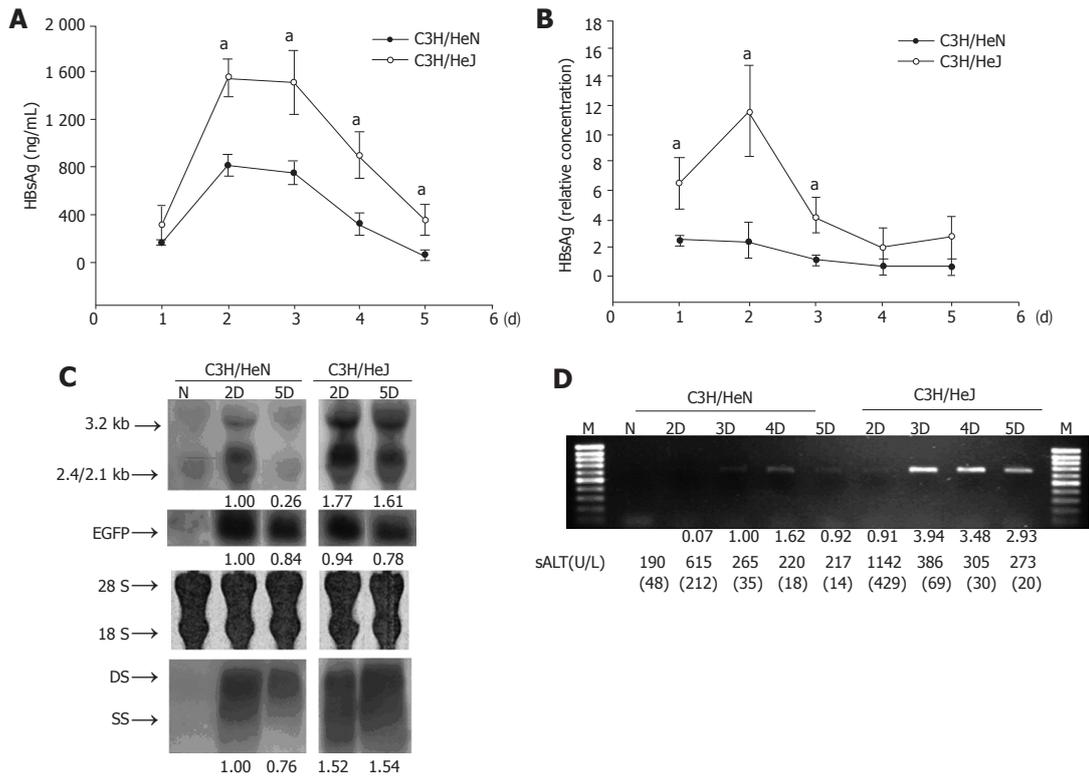


Figure 2 HBV antigenemia and replication in TLR4-mutant C3H/HeJ mice or normal C3H/HeN mice. Groups of four C3H/HeN or C3H/HeJ mice were injected intravenously with 10 μ g of pHBV3.6 and 10 μ g of pEGFP-N1 that was used as control of transfection efficiency by hydrodynamics-based transfection. The HBsAg (A) or HBeAg (B) in the serum was detected by ELISA kits as described in Materials and methods. * $P < 0.05$. (C) The total RNA or cytoplasmic DNA was purified from liver and hybridized with HBV specific probes to analyze the HBV transcription or replication. The number below each land of Northern or Southern blot was represented as the relative fold of expression comparing to d2 result of C3H/HeN mice. N, naive C3H/HeN. (D) The serum DNA was purified after treatment with 20 U DNase I and the HBV-DNA was detected by PCR method. The number below each land was represented as the relative fold of expression comparing to d3 result of C3H/HeN mice. M, 100-bp DNA ladder; N, naive C3H/HeN. The sALT level was represented as mean (SD).

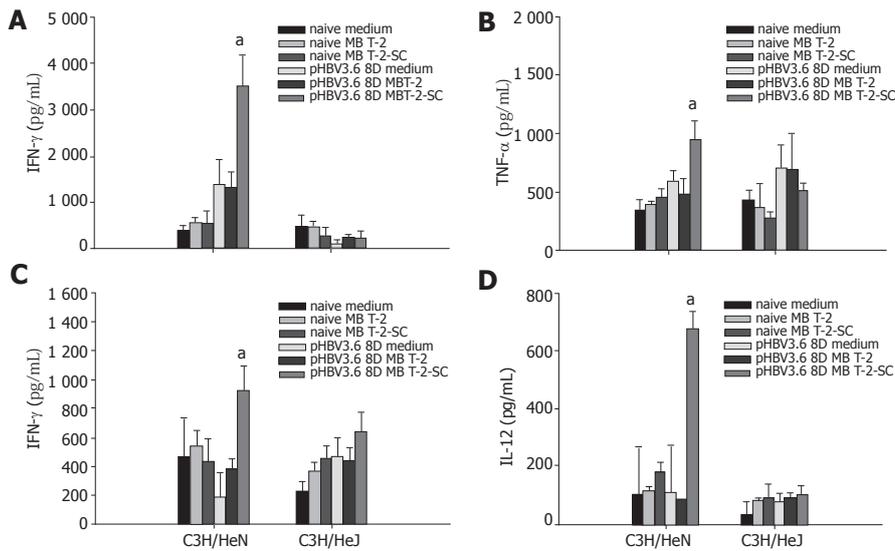


Figure 3 HBV-specific immune responses in the liver or spleen of C3H/HeN or C3H/HeJ mice after hydrodynamic-injection of pHBV3.6. Groups of four C3H/HeN or C3H/HeJ mice were injected intravenously with 10 μ g of pHBV3.6 by hydrodynamics-based transfection. The intrahepatic lymphocytes (A and B) or spleenocytes (C and D) were isolated at d 8 post injection. After being co-cultured with HBsAg and HBcAg expressing syngenic MBT-2 cells (MBT-2-SC) for 48 h, the IFN- γ (A and C), TNF- α (B) or IL-12 (D) in the culture supernatant was analyzed by ELISA method. * $P < 0.05$ vs naive MBT-2-SC group.

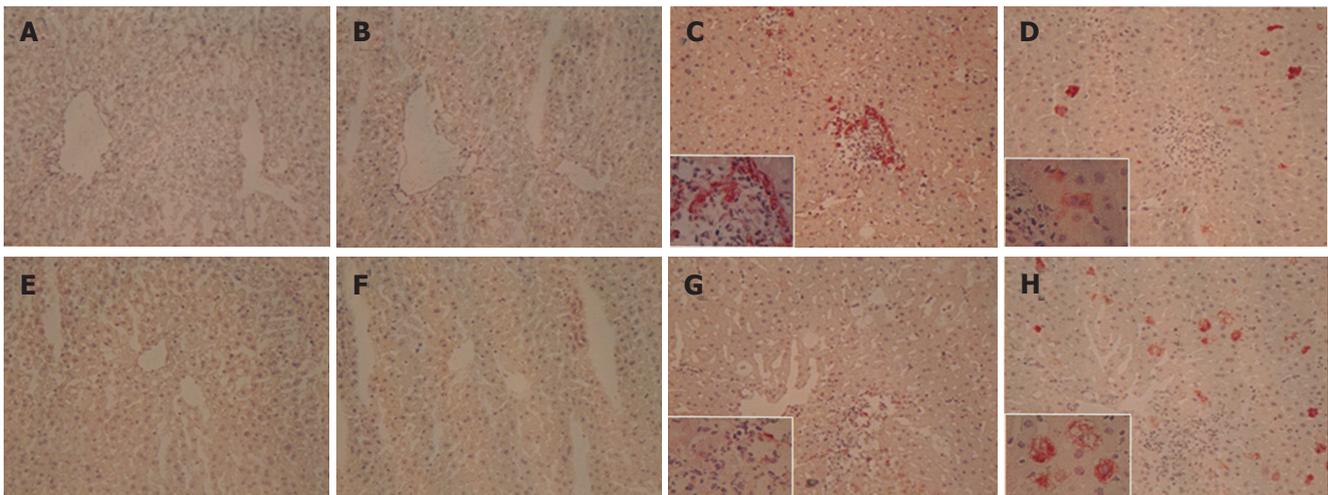


Figure 4 iNOS expression in the liver of C3H/HeN or C3H/HeJ mice after hydrodynamic-injection of pHBV3.6. Groups of four C3H/HeN (C, D) or C3H/HeJ (G, H) mice were injected intravenously with 10 μ g of pHBV3.6 by hydrodynamics-based transfection. Serial sections of frozen liver tissue were made and stained with anti-HBs antibody (B, D, F, H) or anti-iNOS antibody (A, C, E, G). Red color was developed and indicated the positive staining. The insets in (C), (D), (G), and (H) represent twofold magnification of positive staining. A, B, E, F, saline injected control mice; C, D, G, H, day 2 post injection (original magnification \times 200).

in host responses to HBV replication and a mutation in C3H/HeJ impaired its ability to clear the HBV virus.

HBV-specific immune responses were defective in TLR4 mutant C3H/HeJ mice after hydrodynamic injection of pHBV3.6

To investigate the effect of TLR4 on immune responses to HBV, intrahepatic lymphocytes (IHLs) or splenocytes from pHBV3.6-injected C3H/HeN or C3H/HeJ mice were isolated and stimulated with HBsAg- and HBcAg-expressing syngenic MBT-2 cells. The IFN- γ and TNF- α production of IHLs from C3H/HeN mice was significantly increased at 48 h after HBsAg and HBcAg stimulation. But this was not observed in IHLs from C3H/HeJ mice (Figures 3A and B). The IFN- γ and IL-12 production of splenocytes of C3H/HeN mice was also significantly increased in C3H/HeN mice, but not in C3H/HeJ mice (Figures 3C and D). These data suggest that TLR4 mutation in C3H/HeJ affects the HBV specific immune responses (IFN- γ and TNF- α production in liver or IFN- γ and IL-12 production in spleen).

Induction of iNOS was also defective in TLR4 mutant C3H/HeJ mice after hydrodynamic injection of pHBV3.6

It was reported that the activation of TLR4 signaling can induce the iNOS expression^[21], and we also reported that iNOS plays an anti-HBV role in acute HBV expression^[15]. Therefore, the iNOS expressed was compared in wild type and TLR4 mutant mice after pHBV3.6 transfection. The HBsAg-expressing hepatocytes were equivalent among C3H/HeN and C3H/HeJ mice after pHBV3.6 injection, indicating the transfection efficiency was similar in both strains of mice. But iNOS stainings were detected on the infiltrating leukocytes nearby the HBsAg-expressing hepatocytes in the liver of pHBV3.6-injected C3H/HeN (Figures 4C and D) whereas the expression of iNOS on

infiltrating leukocytes was impaired in the liver of C3H/HeJ mice (Figures 4G and H). These data suggested that the mutation of TLR4 influences the induction of iNOS expression during acute HBV expression and may further affect the clearance of HBV.

HBV replication was reduced in C3H/HeJ mice after adoptive transfer of splenocytes from C3H/HeN mice

To further confirm, if TLR4-expressing immune cells provide the protection role to HBV expression, the splenocytes from C3H/HeN mice were injected into C3H/HeJ mice intravenously at the day before pHBV3.6 injection. The HBsAg antigenemia was higher in C3H/HeJ than in C3H/HeN mice, but the adoptive transfer of C3H/HeN splenocytes into the C3H/HeJ mice reduced the serum HBsAg to the level similar to that in C3H/HeN mice (Figure 5A). The HBV transcription or replication in liver (Figure 5B) or HBV-DNA in sera (Figure 5C) was also reduced as well under similar transfection efficiency as verified by co-injection of pEGFP-N1. But when adoptive transfer of splenocytes from C3H/HeJ mice to C3H/HeN mice was carried out, there was no effect to HBsAg in sera (Figure 5A), HBV replication in liver (Figure 5B), or HBV-DNA in sera (Figure 5C). These results indicate that the TLR4 enhances the immune functions to help clear the HBV replication.

DISCUSSION

In this study, we investigated the role of TLR4 in a murine acute HBV expression model. The HBV antigenemia, serum HBV-DNA, as well as HBV transcription and replication in liver were higher in TLR4 mutant C3H/HeJ than TLR4 normal C3H/HeN. This is probably caused by the impaired induction of iNOS and HBV specific immune response because of TLR4 mutation in C3H/HeJ

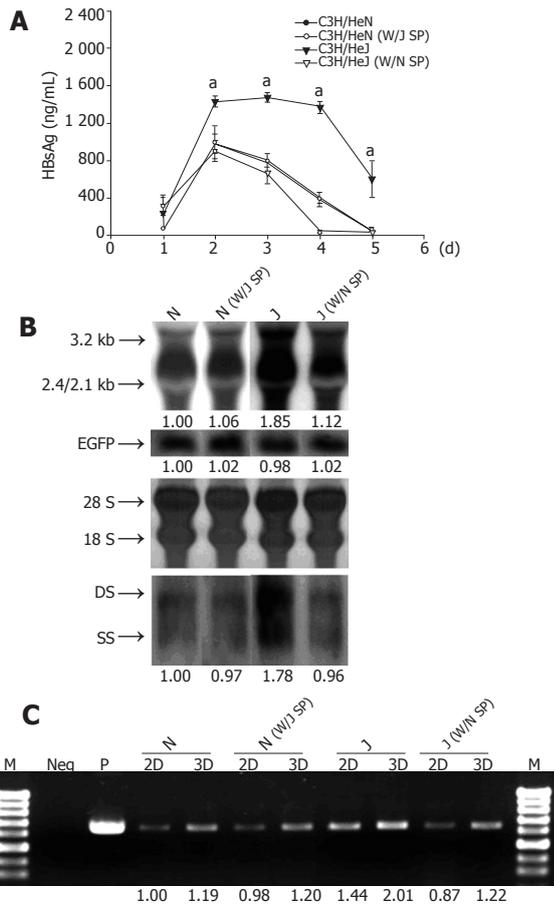


Figure 5 HBV replication was reduced in C3H/HeJ mice after adoptive transfer of splenocytes from C3H/HeN mice. 5×10^6 splenocytes from C3H/HeN or C3H/HeJ mice were injected into C3H/HeJ or C3H/HeN mice intravenously at the day before pHBV3.6 injection, respectively. (A) The HBsAg in sera was detected as indicated day by ELISA. $^*P < 0.05$. (B) The total RNA or cytoplasmic DNA of liver was isolated at day 3 and was further analyzed for HBV transcription or replication by Northern or Southern blot. The transfection efficiency was verified by co-injection of pEGFP-N1 and EGFP RNA confirmation. The number below each land of Northern or Southern blot was represented as the relative fold of expression comparing to C3H/HeN mice. N, C3H/HeN; J, C3H/HeJ; SP, splenocytes. (C) The HBV-DNA in sera was isolated as on the indicated day and detected by PCR method. The number below each land was represented as the relative fold of expression comparing to day 2 result of C3H/HeN mice. M, 100-bp DNA ladder; Neg, no template control; P, pHBV3.6 5 ng; N, C3H/HeN; J, C3H/HeJ; SP, splenocytes.

mice, and suggests that TLR4 plays an antiviral role in HBV replication. Recently, Isogawa *et al*^[22] reported that TLR signaling which includes TLR4 could inhibit HBV replication in transgenic mice model and the mechanism might be through the induction of type I interferon. Our finding of iNOS and HBV specific immune response has increased further understanding on anti-HBV response in addition to the induction of type I interferon.

The TLRs are pattern-recognition receptors that have an important role in mammalian immunity^[7,8]. At least 10 TLRs are expressed on a variety of cell types including immune cells, endothelial cells^[23], cardiac myocytes^[24], and intestinal epithelial cells^[25]. Most TLR ligands are conserved pathogen-associated molecular patterns of microbes and the TLR signals serve as a sensor to the presence of infection^[13]. TLR4 is the first identified mammalian

TLR that expresses predominantly on macrophages and dendritic cells (DCs)^[5]. A point mutation (His⁷¹²→Pro⁷¹²) in the Toll/interleukine-1 receptor domain of TLR4 gene causes the C3H/HeJ mice to become defective after LPS challenge^[20,26]. TLR4 has been shown to initiate a response to the fusion protein of RSV^[11]. Prolonged RSV infection was found in TLR4-deficient mice because of the impaired recruitment of natural killer cells and CD14⁺ cells in the lung tissue as well as the impaired IL-12 production^[27]. Two vaccinia virus ORFs, A46R, and A52R, have been shown to share amino acid sequence similarity to TIR domain, and these two proteins can partially or potentially inhibit the IL-1 and TLR4 mediated NF- κ B activation^[28]. These reports indicate that TLR4 also has anti-virus activity in addition to its anti-bacterial function.

In our study, the IHLs nearby the HBsAg-positive hepatocytes (which represented HBV-replicating hepatocytes) expressed TLR4 post pHBV3.6 hydrodynamic transfection (Figure 1). The HBV replication was higher in TLR4 mutant C3H/HeJ than C3H/HeN mice (Figure 2). This is probably caused by the impaired specific anti-HBV immunity in liver or spleen (Figure 3). When adoptively transferring splenocytes from TLR4 normal C3H/HeN mice to TLR4 mutant C3H/HeJ mice, the anti-HBV responses could be upregulated (Figure 5). These results indicate that anti-HBV specific immune responses may be initiated by TLR4 activation. Activation of DCs by LPS through TLR4 has been found to induce IL-12 production and elicit the Th1 responses against intracellular pathogens^[29]. In our study, the IL-12 production in splenocytes after HBsAg or HBcAg stimulation (Figure 3D) and IFN- γ production in IHLs (Figure 3A) was also impaired in the C3H/HeJ mice. These results correlated with the impaired function of TLR4 in the C3H/HeJ mice.

TLR4 can signal to induce iNOS expression. After TLR4 agonist stimulation, iNOS gene could be induced through MyD88-dependent (NF- κ B) or -independent (IFN- β and STAT-1) pathway in murine macrophage cell line^[21]. We have also reported the role of iNOS in anti-HBV response^[15]. The iNOS induction in the infiltrating immune cells was impaired in C3H/HeJ mice (Figure 4). Therefore, the iNOS may also involve in the TLR4-mediated anti-HBV responses. In conclusion, the present study reports that TLR4 plays an anti-HBV role in acute HBV expression through induction of iNOS expression and specific anti-HBV immune responses.

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