Kupffer Cells Support Hepatitis B Virus–Mediated CD8+ T Cell Exhaustion via Hepatitis B Core Antigen–TLR2 Interactions in Mice

Min Li, Rui Sun, Long Xu, Wenwei Yin, Yongyan Chen, Xiaodong Zheng, Zhexiong Lian, Haiming Wei and Zhigang Tian

J Immunol published online 24 August 2015
http://www.jimmunol.org/content/early/2015/08/21/jimmunol.1500839

Supplementary Material
http://www.jimmunol.org/content/suppl/2015/08/21/jimmunol.1500839.DCSupplemental

Subscription
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Kupffer Cells Support Hepatitis B Virus–Mediated CD8+ T Cell Exhaustion via Hepatitis B Core Antigen–TLR2 Interactions in Mice

Min Li,∗ Rui Sun,∗‡ Long Xu,‡ Wenwei Yin,‡ Yongyan Chen,‡ Xiaodong Zheng,‡ Zhexiong Lian,∗‡ Haiming Wei,∗‡ and Zhigang Tian∗,†,‡

Hepatitis B virus (HBV) persistence is a fundamental process in chronic HBV infection and a key factor in all related liver diseases; however, the mechanisms have yet to be elucidated. We studied the role of TLR2 in HBV persistence using a well-established HBV-carrier mouse model generated by hydrodynamically injecting a phospho–adeno-associated virus/HBV1.2 plasmid into mice. We found that a genetic deficiency in TLR2 improves HBV elimination, whereas activating TLR2 led to more stable HBV persistence, suggesting that TLR2 activation is critical in HBV persistence. Furthermore, we noted that TLR2 activation could inhibit CD8+ T cell function, causing the exhaustion phenotype in HBV-carrier mice, because TLR2 deficiency might rescue CD8+ T cell function in a cellular adoptive experiment. TLR2 expression on Kupffer cells (KCs) was upregulated in HBV-carrier mice, which did not exist after KC depletion. In addition, similar to TLR2 deficiency, after KC depletion, CD8+ T cells were more efficiently activated in HBV-carrier mice, leading to rapid HBV elimination. KCs produced more IL-10 upon TLR2 activation in response to direct hepatitis B core Ag stimulation, and the elevated IL-10 inhibited CD8+ T cell function in HBV-carrier mice, because IL-10 deficiency or anti–IL-10R treatment resulted in CD8+ T cells with stronger antiviral function. In conclusion, KCs support liver tolerance by inducing anti-HBV CD8+ T cell exhaustion via IL-10 production after TLR2 activation by hepatitis B core Ag stimulation.

The Journal of Immunology, 2015, 195: 000–000.

The liver, receiving blood from the intestine, is a unique lymphoid organ because it usually induces immune tolerance rather than immunity (1). Because of this property of the liver, hepatotropic pathogens, such as hepatitis B virus (HBV), hepatitis C virus, and malaria, tend to establish chronic infections (2), which are initiators leading to liver fibrosis, cirrhosis, and hepatocellular carcinoma. CD8+ T cells are major players in HBV clearance (3, 4), but their function in HBV-carrier humans and mice is seriously impaired, with decreased activating receptor, increased inhibitory receptor, and lower production of the antiviral cytokines IFN-γ and TNF-α: a status known as “exhaustion” (5). However, it is unclear how the function of CD8+ T cells is impaired. Previous studies showed that regulatory T cells, γδ T cells, and myeloid-derived suppressor cells inhibit HBV-specific CD8+ T cell function and that deletion of these cells enhances the antiviral function of CD8+ T cells and HBV clearance (5, 6). Other cell types within the liver, particularly liver-resident cells, which can impair HBV-specific CD8+ T cell function, have seldom been assessed.

Kupffer cells (KCs) are liver-resident macrophages with an antigen-presenting function. Importantly, KCs secrete the inhibitory cytokine IL-10 abundantly in response to LPS (7), which is rich in the blood from the intestines, suggesting that KCs may be important players in maintaining the liver’s immunosuppressive state. Indeed, previous studies reported that KCs are critical for portal vein tolerance (8), liver allograft tolerance (9), and tolerance against soluble Ags (10). However, the role of KCs in HBV immune tolerance has seldom been reported. We proved that KCs play an important role in inhibiting Ab production in HBV-persistent mice because, following KC depletion, the hepatitis B surface Ab (anti-HBs) recall response in HBV-carrier mice after hepatitis B surface Ag (HBsAg) vaccination could be reconstituted (11). In addition, KCs might induce T cell exhaustion by upregulating galectin-9 expression in patients with chronic HBV infection and in patients with HBV-associated hepatocellular carcinoma (12, 13). However, until now, the roles of most other molecules expressed on KCs in the induction of T cell exhaustion have remained unreported.

TLR2 can recognize viral hemagglutinin and glycoprotein in addition to pathogen-associated molecular patterns derived from bacteria (14). TLR2 activation can induce both proinflammatory and regulatory effects (15). Previous studies reported that activation...
of TLR2 can induce a Th2 immune response and regulatory T cells and prevent effective antitumor immunity (16, 17). However, the role of TLR2 in HBV persistence remains unknown. An in vitro study in human THP-1 macrophages proved that TLR2 can recognize hepatitis B core Ag (HBcAg), a type of glycoprotein (18). In vivo and in vitro studies in HBV-Met cells and HBV-transgenic mice showed that TLR2 activation fails to inhibit HBV replication in contrast to the activation of other TLRs (19, 20). It was reported that TLR2 mutation might result in an increased likelihood of certain pathogenic microorganism infections in humans (21). However, whether TLR2 mutation or deficiency affects HBV persistence and HBV-induced CD8+ T cell exhaustion remains unclear and deserves further study.

A problem with studying HBV-induced immune tolerance is the lack of a suitable mouse model that is immunologically competent and maintains HBV persistence or becomes a long-term HBV carrier. In the past few years, a widely used HBV-carrier mouse model was established by hydrodynamically injecting a phospho-adeno-associated virus (pAAV)/HBV1.2 plasmid into normal mice (22). The pAAV/HBV1.2 plasmid contains the HBV fragment spanning nt 1400–3182/1–1987 and flanked by inverted terminal repeats of adeno-associated virus (AAV). These HBV-carrier mice express almost all components of HBV (e.g., HBsAg, hepatitis B core Ag [HBcAg], HBcAg, and HBV DNA), and can maintain HBV persistence for >1 y (22). Using this HBV-carrier mouse model, our previous studies demonstrated that KC-derived IL-10 induces type I regulatory T cells and subsequently maintains humoral immune tolerance. In addition, γδT cells drive myeloid-derived suppressor cell–mediated CD8+ T cell exhaustion (5, 11, 23). In the current study, we observed that TLR2 on KCs plays a key role in HBV persistence by supporting CD8+ T cell exhaustion through the secretion of IL-10 upon interaction with HBcAg. Moreover, we found that KC depletion or genetic deficiency in TLR2 or IL-10 enhances CD8+ T cell function and improves anti-HBV immunity in HBV-carrier mice. This finding provides a new target for immunotherapy of chronic HBV infection.

Materials and Methods

Animals

Male C57BL/6 (B6) mice were purchased from the Shanghai Experimental Animal Center (Shanghai, China). IL-10−/− mice (B6.129P2-Il10tm1Cgn/J) were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice were kindly provided by Professor Zhexiong Lian (University of Science and Technology of China). Tlr2−/− and Rag1−/− mice on the B6 background were bred under specific pathogen–free conditions, according to the experimental animal guidelines of the University of Science and Technology of China. All mice were housed in a specific pathogen–free facility, received human care, and were used according to the animal care regulations of the University of Science and Technology of China.

Plasmids

The plasmids used in this study were extracted using a NucleoBond Xtra Midi EF kit, according to the manufacturer’s instructions. The kit is used for endotoxin-free plasmid DNA purification. Endotoxin levels in the plasmids were <0.05 EU/μg.

HBV-carrier mouse model

To establish the HBV-carrier mouse model, 5–6-wk-old male mice were hydrodynamically injected with 6 μg pAAV/HBV1.2 plasmid, as previously reported (22).

Radioimmunoassay

The serum HBsAg, HBcAg, and anti-HBs levels were detected using commercial immunoassay kits (Beijing North Institute of Biological Technology, Beijing, China), according to the manufacturer’s instructions.

HBV DNA detection

Serum HBV DNA copies were assessed by quantitative PCR using a commercial kit for HBV DNA (AMPLify, Xiamen, China), according to the manufacturer’s instructions.

Immunohistochemistry

HBcAg+ hepatocytes in liver tissue were examined by immunohistochemistry, essentially as previously reported (11).

Cell isolation and culture

Heaptic and splenic mononuclear cells (MCNs) were prepared essentially as previously reported (24). KCs were obtained by density centrifugation (25). KCs were cultured in the presence or absence of 10 μg/ml HBcAg (ProSpec, East Brunswick, NJ) or HBsAg (HyTest, Turku, Finland) for 24 h. The cytokine levels in the supernatants were assessed using an ELISA kit (Dakewe, Beijing, China), according to the manufacturer’s instructions.

Flow cytometry

The mAbs used for flow cytometry in this study included FITC–anti-CD4, FITC–anti-CD11c, FITC–anti-CD45, FITC–anti-CD62L, FITC–anti-CD69, FITC–anti-CD86, and FITC–anti-TNF-α, PE–anti-CD28, PE–anti-CD44, PE–anti-CD80, PE–anti-CD107a, PE–anti-IFN-γ, and PE–anti-PD-1. PerCP-Cy5.5–anti-CD8a, allophycocyanin–anti-CD3, PE–Cy7–anti-NK1.1 (all from BD Biosciences, San Jose, CA), PE–anti-TLR2, PE–anti–Tim-3, and PE–anti–MHC-II, PerCP-Cy5.5–anti-F4/80 (all from eBioscience, San Diego, CA), and Alexa Fluor 647–anti-Dectin-1 (AbD, Kidlington, U.K.). Cells in a single-cell suspension were incubated with rat IgG to saturate rat FeRs before staining with the indicated fluorescently labeled mAbs for surface Ags. For HBV-specific intracellular IFN-γ and TNF-α staining, cells were stimulated with 50 μg/ml HBc93-100 peptide (synthesized by Sangon Biotech, Shanghai, China) for 7 h. Monensin (2.5 μg/ml; Sigma) was added for the last 4 h to prevent cytokine secretion. To evaluate HBV-specific CD8+ T cells, HBcAg (MGLKFRQL) peptide-specific H2-Kb pentamer (Prommune, Oxford, U.K.) was used according to the manufacturer’s instructions. The stained cells were collected using a BD LSR II flow cytometer (BD Biosciences) and analyzed using FlowJo 7.6 software.

Bone marrow–derived macrophage induction

Bone marrow cells of wild-type (WT) and Tlr2−/− mice were cultured in the presence of 20 ng/ml M-CSF (PeproTech, Rocky Hill, NJ) for 7 d to induce macrophage differentiation. Bone marrow–derived macrophages (BMDMs) were cultured in the presence or absence of 10 μg/ml HBcAg (ProSpec) and different concentrations of anti-TLR2 (eBioscience) for 24 h.

Quantitative real-time PCR

After culturing BMDMs from WT and Tlr2−/− mice, we detected Th2 and II-10 mRNA levels, as described previously (5). The primer sequences used were as follows: GAPDH: sense, 5′-GGCATGGACTGTGGTCATGA-3′, and antisense, 5′-GGCATGGACTGTGGTCATGA-3′; IL-10: sense, 5′-ATGCCTGG-TCTCAGAC-3′, and antisense, 5′-GTCCCTGATTAAAGGATCGT-3′; and TLR2: sense, 5′-TGGTGTCTGGAGTCTGCTGTG-3′, and antisense, 5′-GGATGAGGACTGTTGGCTCATGA-3′.

Cell transfer

Splenocytes (2 × 10^7) from control mice, HBV-carrier WT mice, and HBV-carrier Th2−/− mice were transferred i.v. into Rag1−/− mice, followed by HBcAg vaccination 3 d later. For CD8+ T cell transfer, 2 × 10^6 CD8+ T cells purified from the splenocytes of pAAV-null injected control mice, HBV-carrier WT mice, or HBV-carrier Th2−/− mice by MACS were transferred i.v. into C57BL/6 mice, followed by hydrodynamic injection of the pAAV/HBV1.2 plasmid 3 d later.

Cell depletion

Mice were injected i.v. with 200 μl clodronate liposomes to deplete KCs or PBS liposomes as a control 48 h before hydrodynamic injection of the pAAV/HBV1.2 plasmid. Clodronate liposomes and PBS liposomes were purchased from Dr. N. van Rooijen (Vrije Universiteit, Amsterdam, The Netherlands).

CD8+ T–KC coculture system

For the CD8+ T–KC coculture assay, 1 × 10^5 purified CD8+ T cells were cocultured or not with 3 × 10^5 F4/80+ KCs from control mice or
HBV-carrier mice, in the presence or absence of 10 μg/ml anti–IL-10R (BD Biosciences), and activated with anti-CD3/anti-CD28 for 48 h. The cytokine levels in the supernatant were evaluated using an ELISA kit (Dakewe), according to the manufacturer’s instructions.

**HBsAg vaccination**

Mice were immunized with 1 μg HBsAg vaccine (Kangtai, Shenzhen, China) per mouse, and serum anti-HBs levels were assessed at the indicated time points.

**Statistical analysis**

All data are expressed as the mean ± SEM. For statistical analyses, an unpaired two-tailed Student t test was used, and data were considered statistically significant when differences reached p < 0.05.

**Results**

**TLR2 deficiency improves HBV elimination**

To study the mechanism of HBV persistence, we used an HBV-carrier mouse model in which the pAAV/HBV1.2 plasmid was hydrodynamically injected into the mouse to mimic chronic HBV infection. These HBV-carrier mice exhibited persistent HBsAg and normal alanine transaminase levels in the serum, and 90% were HBsAg+ at 6 wk postinjection (Supplemental Fig. 1A–C). In our model, mice sustained HBsAg positivity for 3 to 6 mo (11, 22, 26). Moreover, HBV-carrier mice could not mount a humoral immune response to peripheral HBsAg vaccination (Supplemental Fig. 1D, 1E). Thus, the model is suitable for studying the mechanisms of HBV persistence–induced immune tolerance.

To determine whether TLR2 is an important player in HBV persistence, we injected the pAAV/HBV1.2 plasmid into Tlr2−/− and WT B6 mice, as previously reported (5, 11, 23, 26). HBsAg, HBeAg, and HBV DNA serum concentrations of HBV-carrier Tlr2−/− mice were clearly lower than those of HBV-carrier WT mice at the indicated time points (Fig. 1A–C), suggesting the existence of increased anti-HBV immunity in Tlr2−/− mice. Furthermore, HBV-carrier Tlr2−/− mice had less HbcAg protein expression in liver tissues than did HBV-carrier WT mice (Fig. 1D). To further explore the role of TLR2 in HBV persistence, B6 mice preinjected with the pAAV/HBV1.2 plasmid were injected i.v. with the TLR2 agonist Pam3CSK4 once a week to activate TLR2. Consistently, TLR2 activation inhibited the anti-HBV immunity of mice, because serum concentrations of HBsAg at several time points were higher in mice treated with Pam3CSK4 than in mice treated with saline (Fig. 1E). Taken together, these results suggest that TLR2 deficiency enhances the immune response to HBV in mice.

**Anti-HBV CD8+ T cells are not exhausted in Tlr2−/− mice**

To determine whether HBV-carrier Tlr2−/− mice had enhanced humoral immune responses, pAAV/null plasmid–injected control mice, HBV-carrier WT mice, and HBV-carrier Tlr2−/− mice were
immunized with HBsAg vaccine, as shown in Supplemental Fig. 2A. Compared with control mice, which produce anti-HBs, we did not detect any serum anti-HBs Ab in HBV-carrier Tlr2<sup>-/-</sup> mice or in HBV-carrier WT mice (Supplemental Fig. 2B). In contrast to splenocytes obtained from control mice, splenocytes from HBV-carrier WT mice or Tlr2<sup>-/-</sup> mice transferred into Rag1<sup>-/-</sup> mice did not produce anti-HBs in the recipient mice after HBsAg vaccination (Supplemental Fig. 2C, 2D). These results raise the possibility that TLR2 does not affect the Ab response. We also found that hepatic CD8<sup>+</sup> T cells from naive WT and Tlr2<sup>-/-</sup> mice were similar with regard to their percentages, numbers, activation, and production of CD107a, IFN-γ, and TNF-α (Supplemental Fig. 3), eliminating the possibility that TLR2 deficiency directly influences the function of CD8<sup>+</sup> T cells in normal mice.

Previous studies demonstrated that chronic infection typically leads to CD8<sup>+</sup> T cell exhaustion (27). Indeed, compared with pAAV/null plasmid-injected control mice, HBV-carrier mice had fewer CD8<sup>+</sup> T cells (Supplemental Fig. 4A). Moreover, we observed less expression of the activating markers CD28, CD44, and CD69 and more expression of the inhibiting markers PD-1 and Tim-3 on CD8<sup>+</sup> T cells in HBV-carrier mice (Supplemental Fig. 4B). After Hbc93-100 peptide stimulation, HBV-carrier mice had fewer CD8<sup>+</sup> T cells producing antivirus cytokines (IFN-γ and TNF-α) compared with control mice (Supplemental Fig. 4C, 4D). These results suggested that CD8<sup>+</sup> T cells in HBV-carrier mice are in an exhausted state. Importantly, the percentage and number of HBV-specific CTLs were elevated in HBV-carrier Tlr2<sup>-/-</sup> mice (Fig. 2A). Moreover, these HBV-specific CTLs in HBV-carrier Tlr2<sup>-/-</sup> mice expressed reduced PD-1 (Fig. 2B). Using Hbc93-100 peptide stimulation in vitro to evaluate HBV-specific responses (5, 28), we observed increased IFN-γ and TNF-α production in hepatic CD8<sup>+</sup> T cells from HBV-carrier Tlr2<sup>-/-</sup> mice compared with HBV-carrier WT mice (Fig. 2C, 2D). Furthermore, hepatic CD8<sup>+</sup> T cells exhibited increased CD44 and CD69 expression and decreased PD-1 and Tim-3 expression in HBV-carrier Tlr2<sup>-/-</sup> mice (Fig. 2E), indicating that the exhausted HBV-specific CD8<sup>+</sup> T cells in HBV-carrier mice were rescued in TLR2-deficient mice. To further determine whether the enhanced CD8<sup>+</sup> T cells were sufficient to promote HBV elimination, we transferred CD8<sup>+</sup> T cells from pAAV/null plasmid-injected control mice, HBV-carrier WT mice, and HBV-carrier Tlr2<sup>-/-</sup> mice into Cd8<sup>2/-</sup> mice, followed by the hydrodynamic injection of the pAAV/ HBV1.2 plasmid 3 d later. Indeed, Cd8<sup>2/-</sup> mice treated with CD8<sup>+</sup> T cells from HBV-carrier Tlr2<sup>-/-</sup> mice had lower HBsAg levels (Fig. 2F). Thus, TLR2 deficiency combats HBV persistence, at least in part, by enhancing CD8<sup>+</sup> T cell function.

**FIGURE 2.** Anti-HBV CD8<sup>+</sup> T cells are not exhausted in Tlr2<sup>-/-</sup> mice. (A–D) Hepatic MNCs were isolated 3 wk after the pAAV/HBV1.2 plasmid injection. (A) Percentage and number of HBc93 pentamer<sup>+</sup> CD8<sup>+</sup> T cells, gated on CD8<sup>+</sup> T cells. (B) PD-1 expression on HBc93 pentamer<sup>+</sup> CD8<sup>+</sup> T cells, gated on HBc93 pentamer<sup>+</sup> CD8<sup>+</sup> T cells. (C and D) Percentage and total number of hepatic HBc93-specific IFN-γ or TNF-α CD8<sup>+</sup> T cells, gated on CD8<sup>+</sup> T cells. (E) Percentage of CD44<sup>+</sup>, CD69<sup>+</sup>, PD-1<sup>+</sup>, and Tim-3<sup>+</sup> CD8<sup>+</sup> T cells 7 wk after the pAAV/ HBV1.2 plasmid injection, gated on CD8<sup>+</sup> T cells. (F) Reconstituted Cd8<sup>2/-</sup> mice transferred with CD8<sup>+</sup> T cells from control, HBV-carrier WT, or HBV-carrier Tlr2<sup>-/-</sup> mice were hydrodynamically injected with the pAAV/ HBV1.2 plasmid. Serum HBsAg was detected by IRMA 1 wk postinjection. All experiments were repeated at least three times. Results are expressed as the mean ± SEM (n = 3–5 mice/group). *p < 0.05, **p < 0.01, unpaired two-tailed Student t test.
KCs induce CD8+ T cell exhaustion in a TLR2-dependent manner

Because several cell types express TLR2, we wanted to determine which types of TLR2-expressing cells play a role in HBV persistence. We found that KCs from HBV-carrier mice expressed more TLR2 than did those from control mice (Fig. 3A). TLR2 expression was not detected on KCs from Tlr2−/− mice (data not shown). TLR2 expression on hepatocytes (HC), DCs, NK cells, CD4+ T, and CD8+ T cells in the liver. (C) BMDMs were cultured or not with 10 μg/ml HBcAg for 24 h, and Tlr2 mRNA expression in BMDMs was analyzed by quantitative real-time PCR. (D) Mice were treated with a single i.v. injection of 200 μl clodronate liposomes or PBS liposomes. Thirty hours later, the percentage of F4/80+ KCs in liver nonparenchymal cells was detected by flow cytometry. (E) B6 mice and Tlr2−/− mice received a single i.v. injection of 200 μl clodronate liposomes or PBS liposomes, followed by hydrodynamic injection of the pAAV/HBV1.2 plasmid 2 d later. The serum levels of HBsAg were determined. Experiments in (A) and (E) were repeated twice. Results are expressed as the mean ± SEM (n= 3–5 mice/group). *p < 0.05, **p < 0.01, unpaired two-tailed Student t test.

FIGURE 3. KC depletion or TLR2 deficiency disrupts HBV tolerance. (A and B) B6 mice were hydrodynamically injected with the pAAV/null or pAAV/HBV1.2 plasmid. Five days later, TLR2 expression on various cell types was examined by flow cytometry. (A) Cells were gated on F4/80+ cells (KCs), and the percentage of TLR2+ KCs and TLR2 mean fluorescence intensity of KCs were analyzed. (B) TLR2 expression on hepatocytes (HC), DCs, NK cells, CD4+ T, and CD8+ T cells in the liver. (C) BMDMs were cultured or not with 10 μg/ml HBcAg for 24 h, and Tlr2 mRNA expression in BMDMs was analyzed by quantitative real-time PCR. (D) Mice were treated with a single i.v. injection of 200 μl clodronate liposomes or PBS liposomes. Thirty hours later, the percentage of F4/80+ KCs in liver nonparenchymal cells was detected by flow cytometry. (E) B6 mice and Tlr2−/− mice received a single i.v. injection of 200 μl clodronate liposomes or PBS liposomes, followed by hydrodynamic injection of the pAAV/HBV1.2 plasmid 2 d later. The serum levels of HBsAg were determined. Experiments in (A) and (E) were repeated twice. Results are expressed as the mean ± SEM (n= 3–5 mice/group). *p < 0.05, **p < 0.01, unpaired two-tailed Student t test.
resulted in significantly reduced serum HBsAg levels in the HBV-carrier WT mice, and no difference in serum HBsAg levels was noted between HBV-carrier WT mice and HBV-carrier Tlr2<sup>−/−</sup> mice after KC depletion (Fig. 3E), indicating that KCs may exert their function in tolerance via TLR2. Furthermore, consistent with HBV-carrier Tlr2<sup>−/−</sup> mice, KC-depleted HBV-carrier mice had a higher proportion and number of hepatic CD8<sup>+</sup> T cells than did HBV-carrier WT mice 3 wk after HBV plasmid injection (Fig. 4A). In addition, after HBc93-100 peptide stimulation, the proportion of hepatic CD8<sup>+</sup> T cells producing the antiviral cytokines IFN-γ and TNF-α was higher in KC-depleted HBV-carrier mice than in HBV-carrier WT mice (Fig. 4B, 4C), indicating that CD8<sup>+</sup> T cells exhibited enhanced anti-HBV ability in KC-depleted HBV-carrier mice. We also observed higher expression of the activating markers CD28, CD44, and CD69 by CD8<sup>+</sup> T cells in KC-depleted HBV-carrier mice than those in HBV-carrier WT mice (Fig. 4D), suggesting that CD8<sup>+</sup> T cells were activated more efficiently after KC depletion in HBV-carrier mice. Thus, these results indicate that KC depletion resulted in stronger CD8<sup>+</sup> T cell function after injection of the pAAV/HBV1.2 plasmid, suggesting that KCs inhibit CD8<sup>+</sup> T cell function in HBV-carrier mice. Taken together, these results suggest that the inhibitory function of KCs on CD8<sup>+</sup> T cells is dependent on TLR2 in HBV-carrier mice.

IL-10 from HBcAg-responsive KCs drives CD8<sup>+</sup> T cell exhaustion

We next explored the role of TLR2 in inhibiting CD8<sup>+</sup> T cell function in KCs. We hypothesized that KCs are inhibitory to a greater extent through TLR2 upregulation after the pAAV/HBV1.2 plasmid injection. Although the pAAV/HBV1.2 plasmid injection did not alter the expression of CD80 and CD86, we observed lower MHC class II expression on KCs from HBV-carrier WT mice than on those from pAAV/null plasmid-injected control mice (Fig. 5A, 5B). Additionally, the expression of Dectin-1, a marker of M2 macrophages, was increased on KCs from HBV-carrier WT mice compared with control mice (Fig. 5A, 5B). Therefore, these data suggested that KCs from HBV-carrier WT mice exhibit an immunosuppressive capacity. Furthermore, as expected, TLR2 was required for M2 polarization of KCs, because MHC class II and Dectin-1 expression on KCs was restored in HBV-carrier Tlr2<sup>−/−</sup> mice (Fig. 5A, 5B). Moreover, KCs from WT mice produced abundant levels of the inhibitory cytokine IL-10 after HBcAg, but not HBsAg, stimulation.
process was TLR2 dependent because KCs from Tlr2−/− mice could not produce IL-10 in response to HBcAg (Fig. 5C). Consistent with the results in KCs, BMDMs expressed significantly more Il-10 mRNA after HBcAg stimulation in a TLR2-dependent manner, because blocking TLR2 signaling by mAb treatment or by a genetic deficiency in TLR2 led to a significant reduction in IL-10 expression in response to HBcAg stimulation (Fig. 5D).

Taken together, these results indicate that KCs produce IL-10 upon TLR2 activation by HBcAg stimulation.

Next, to determine whether elevated IL-10 directly inhibits CD8+ T cell function, Il-10−/− mice were used in our study. After the pAAV/HBV1.2 plasmid injection, the proportion of CD8+ T cells in Il-10−/− mice was increased (Fig. 6A). In addition, the expression of the activating markers CD44 and CD69, as well as CD127, which is important for T cell development and survival, was increased (Fig. 6B). Moreover, the percentage and number of IFN-γ–producing CD8+ T cells were significantly higher in Il-10−/− mice than in WT mice after in vitro HBc93–100 peptide stimulation (Fig. 6C), suggesting that IL-10 deficiency resulted in CD8+ T cells with stronger antiviral function. Moreover, a coculture assay proved that the secretion of IFN-γ by CD8+ T cells could be inhibited directly by HBV-carrier mice–derived KCs in an IL-10–dependent manner, because blocking IL-10 signaling by adding anti–IL-10R mAb restored the production of IFN-γ by CD8+ T cells (Fig. 6D). Consistently, similar to TLR2 deficiency or KC depletion, IL-10 deficiency led to robust HBV clearance (11). Taken together, KC-derived IL-10 could lead to CD8+ T cell dysfunction in HBV-carrier mice.

**Discussion**

In the current study, using a well-established HBV-carrier mouse model, we assessed the role of TLR2 in maintaining HBV-specific immune tolerance during HBV persistence. Deficiency in TLR2 led to HBV elimination, as shown by decreased HBV components in serum and liver, by rescuing CD8+ T cell function or relieving CD8+ T cell exhaustion (Figs. 1, 2). After HBV plasmid injection, TLR2 expression on KCs was upregulated (Fig. 3), and via TLR2 interaction with HBcAg, IL-10 production by KCs was greatly enhanced (Fig. 5). KC depletion or IL-10 deficiency led to HBV elimination by disrupting the liver’s immune tolerance, similar to what occurred in Tlr2−/− mice in terms of enhancing CD8+ T cell function (Figs. 4, 6). Therefore, TLR2-mediated CD8+ T cell exhaustion is promoted by KC-derived IL-10 after interaction with HBcAg in HBV-carrier mice.

In chronic viral infections established by HBV and other viruses, such as lymphocytic choriomeningitis virus, CD8+...
T cells become exhausted, which renders them dysfunctional and unable to eradicate the virus (27). Consistently, in our HBV-carrier mouse model, the functions of hepatic CD8+ T cells were clearly impaired, with the enhanced expression of PD-1 and Tim-3 and the reduced expression of CD28, CD44, and CD69 (Supplemental Fig. 4). Importantly, CD8+ T cell exhaustion in HBV-carrier mice could be rescued by TLR2 deficiency. Higher CD8+ T cell function, including PD-1 and Tim-3 downregulation, CD44 and CD69 upregulation, and higher production of IFN-γ and TNF-α (Fig. 2), all contributed to the recovery of anti-HBV capacity by CD8+ T cells. Thus, TLR2 plays an important role in HBV persistence by maintaining liver tolerance to HBV infection with CD8+ T cell dysfunction.

Previous reports showed that HBV infection can modulate TLR2 expression. Chen et al. (33) observed that the expression of TLR2 was lower in PBMCs from chronic hepatitis B patients compared with healthy controls. However, in contrast to that study, Wei et al. (34) showed that TLR2 expression on CD14+ PBMCs was significantly higher in chronic hepatitis B patients compared with healthy controls, suggesting that TLR2 may assist HBV infection. In the current study, we found that TLR2 expression was higher on KCs from HBV-carrier mice (Fig. 3). This result is consistent with Wei et al.’s finding, because CD14+ PBMCs are functionally similar to KCs, liver-resident macrophages. Importantly, because HBV infects liver directly, it is more valuable to study local responses rather than PBMCs. In our study, HBV Ag levels in serum increased after TLR2 activation by agonist injection, and HBV Ag levels in serum and liver were lower in Tlr2−/− mice (Fig. 1). These results are consistent with a previous observation that activation of TLR2 did not inhibit HBV replication in HBV-transgenic mice, in contrast to activation of TLR3, TLR4, TLR5, TLR7, and TLR9 (20). To our knowledge, this study is the first to demonstrate that TLR2 is crucial for HBV persistence in vivo. However, a previous study showed that TLR2 mediated antiviral responses during hepadnaviral infection using a woodchuck model (35). However, because the woodchuck hepatitis virus is genetically divergent from HBV, and immunological mechanisms in genetically outbred woodchuck are uncharacterized compared with humans and mice, the role of TLR2 in HBV persistence should be further studied in a mouse model.

Using the HBV-carrier mouse model, we found that Tlr2−/− mice have a stronger ability to resist HBV and that TLR2 activation promotes stable HBV persistence. Previous studies also revealed that KCs exhibit tolerogenic characteristics. In the current study, we demonstrated that KCs promote HBV persistence via TLR2 because TLR2 expression on KCs was enhanced in HBV-carrier mice, and the difference in anti-HBV ability between WT mice and Tlr2−/− mice did not exist after KC depletion (Fig. 3). Similarly to HBV-carrier Tlr2−/− mice, KC-depleted HBV-carrier mice also had enhanced CD8+ T cells.
T cell function (Fig. 4). Although circulating or resident macrophages and even DCs that engulf clodronate liposomes can undergo apoptosis, the pAAV/HBV1.2 plasmid is directly injected into hepatocytes, and KCs represent 80–90% of all tissue macrophages in the body. Therefore, we believe that KCs play a critical role in supporting CD8+ T cell exhaustion and promoting HBV persistence. In addition, the in vitro KC/CD8+ T cell coculture assay confirmed that KCs from HBV-carrier mice inhibited IFN-γ production by CD8+ T cells (Fig. 6D). Thus, we reveal a new mechanism of KC-mediated liver tolerance in HBV persistence.

The core Ags of hepatitis virus were considered to be pathogen-associated molecular patterns. Tu et al. (37) reported that hepatitis C virus core protein acts as a ligand for TLR2 on human KCs, inducing them to produce cytokines, including IL-10. Cooper et al. (18) showed that HBcAg promotes NF-κB activation of THP-1 macrophages, and this process requires TLR2. Consistent with previous findings, our murine results proved that KCs and BMDMs produce more IL-10 after interacting with HBcAg via their TLR2 and that the blockade of TLR2 signaling or a deficiency in TLR2 can inhibit the production of IL-10 (Fig. 5). In contrast, our results showed that HBsAg could not make KCs produce more IL-10 (Fig. 5), which was consistent with Wang et al.’s (38) finding that the production level of IL-10 was not present or not. To the best of our knowledge, this report is the first to show that TLR2 on KCs can respond directly to HBcAg for IL-10 production.

HBcAg suppresses the expression of IFN-β (39) and stimulates IL-10 production by T cells and monocytes (40), indicating that HBcAg acts as an immune inhibitor. In contrast, Lin et al. (41) demonstrated that HBV-carrier mice lacking HBcAg exhibited increased HBsAg levels, suggesting that HBcAg is required for mounting a positive immune response. Therefore, the immune response to HBcAg and its relation to the overall HBV infection are extremely complex. In our study, in the presence of HBcAg, which stimulates KCs to produce IL-10, HBV-carrier WT mice maintained a HBsAg+ status for >6 mo. Taken together, these results demonstrate that the role of HBcAg in HBV persistence remains inconclusive and controversial and requires further study.

Because HBV cannot infect mice to mimic HBV infection in humans, there are some limitations of our HBV-carrier mouse model, such as the lack of liver HBV infection (e.g., no infectious particle entry, no delivery between hepatocytes, no covalently closed circular DNA) and possible transient heart and liver trauma resulting from hydrodynamic injection (42). However, our HBV-carrier mice express almost all HBV components and can effectively mimic important aspects of HBV-induced tolerance (e.g., continuous expression of HBV Ags in the liver and the existence of hepatic HBV-specific CD8+ T cell exhaustion) (2, 43). Therefore, our HBV-carrier mouse model provides opportunities to study the mechanisms of HBV persistence and HBV-induced CD8+ T cell exhaustion.

The pAAV/HBV1.2 plasmid is not a virus vector. But, after hydrodynamic injection, any plasmids or virus vectors primarily enter hepatocytes (42). More importantly, through hydrodynamic injection of the pAAV/ HBV1.2 plasmid, HBV-associated Ags can be expressed only using a liver-specific promoter, so the pAAV/ HBV1.2 plasmid is hepatocyte specific. In addition, we did not find any evidence to support that the vector could integrate into the host cell genome. A previous study demonstrated that the AAV vector favors long-term transgene expression in hepatocytes (22). On the one hand, we observed different efficacies of elimini-
T cells prevents effective anti-tumor immunity induced by Pan2 lipopeptides in vivo. *Plas* 1: e18833.


Kupffer Cells Support HBV-Mediated CD8⁺ T Cell Exhaustion via HBCAg-TLR2 Interaction in Mice

Min Li, * Rui Sun, *, † Long Xu, † Wenwei Yin, † Yongyan Chen, † Xiaodong Zheng, † Zhexiong Lian, * , †

Haiming Wei, * , † and Zhigang Tian, * , †

*Hefei National Laboratory for Physical Sciences at Microscale, Hefei, Anhui 230027, China
†Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, Zhejiang 310003, China.
‡Institute of Immunology and The CAS Key Laboratory of Innate Immunity and Chronic Disease, School of Life Sciences and Medical Center, University of Science and Technology of China, Hefei, Anhui 230027, China.

Correspondence should be addressed to Dr. Zhigang Tian, School of Life Sciences, University of Science and Technology of China, 443 Huang-Shan Road, Hefei, Anhui 230027, China. E-mail: tzg@ustc.edu.cn; Tel.: +86-551-6360-0845; Fax: +86-551-6360-6783.

Running title: TLR2 promotes HBV-induced liver tolerance.
Supplemental FIGURE 1. HBV-tolerant mouse model. (A-C) C57BL/6 mice were hydrodynamically injected with the pAAV/HBV1.2 or pAAV/null control plasmid. (A) The HBsAg levels were assessed at the indicated time points. (B) The percent of HBsAg-positive mice was calculated. (C) The ALT level in serum 3 days post injection. (D and E) One week after the pAAV/HBV1.2 or pAAV/null control plasmid injection into WT mice, HBV-carrier and control mice were intramuscularly (IM) immunized twice with the HBsAg vaccine once a week. (E) The anti-HBs levels were assessed. Each experiment was repeated at least twice, and the results are expressed as the mean ± SEM (n=5-11 mice/ group). Unpaired two-tailed Student’s t-tests; NS, not significant; ND, not detected.
Supplemental FIGURE 2. TLR2 deficiency does not influence the humoral immune responses in HBV-carrier mice. (A and B) Two weeks after the pAAV/HBV1.2 or pAAV/null control plasmid injection into WT and Tlr2⁻/⁻ mice, the HBV-carrier and control mice were immunized IM with HBsAg vaccine at days -14 and 0. At 7 days and 12 days after the last HBsAg vaccination, the serum anti-HBs levels were detected. (C and D) Recipient Rag1⁻/⁻ mice received splenocytes isolated from HBV-carrier or control mice 21 days post-hydrodynamic injection. The serum anti-HBs levels were measured 1 week after two HBsAg immunizations. Each experiment was repeated at least twice, and the results are expressed as the mean ± SEM (n=3-5 mice/group). Unpaired two-tailed Student’s t-tests; ND, not detected.
Supplemental FIGURE 3. TLR2 deficiency does not affect CD8+ T cells in normal mice. (A) The percent and absolute number of hepatic CD8+ T cells from naïve C57BL/6 and Tlr2-/- mice. (B) The percent of hepatic CD44hi CD62Llo CD8+ T cells, gated on CD8+ T cells. (C) Flow cytometric analysis of CD107a, IFN-γ, and TNF-α expression by hepatic CD8+ T cells, gated on CD8+ T cells. Experiments were repeated three times, and the results are expressed as the mean ± SEM (n=3-4 mice/group). Unpaired two-tailed Student’s t-tests; NS, not significant.
Supplemental FIGURE 4. CD8⁺ T cells are exhausted in HBV-tolerant mice.

C57BL/6 mice were hydrodynamically injected with the pAAV/HBV1.2 or pAAV/null plasmid. Three weeks later, liver mononuclear cells (MNCs) were isolated.

(A) The absolute number of hepatic CD8⁺ T cells. (B) Flow cytometric analysis of CD28, CD44, CD69, PD-1 and Tim-3 expression on hepatic CD8⁺ T cells, gated on CD8⁺ T cells. (C) The total number of hepatic HBe93-specific IFN-γ⁺ CD8⁺ T cells. (D) The total number of hepatic HBe93-specific TNF-α⁺ CD8⁺ T cells. Experiments were repeated twice, and the results are expressed as the mean ± SEM (n=3-6 mice/group). Unpaired two-tailed Student’s t-tests; *p < 0.05, **p < 0.01.