



Original Article



Gut Microbiota Dysbiosis Strengthens Kupffer Cell-mediated Hepatitis B Virus Persistence through Inducing Endotoxemia in Mice

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Abstract

Background and Aims: Change of gut microbiota composition is associated with the outcome of hepatitis B virus (HBV) infection, yet the related mechanisms are not fully characterized. The objective of this study was to investigate the immune mechanism associated with HBV persistence induced by gut microbiota dysbiosis. **Methods:** C57BL/6 mice were sterilized for gut-microbiota by using an antibiotic (ABX) mixture protocol, and were monitored for their serum endotoxin (lipopolysaccharide [LPS]) levels. An HBV-replicating mouse model was established by performing HBV-expressing plasmid pAAV/HBV1.2 hydrodynamic injection (HDI) with or without LPS, and was monitored for serum hepatitis B surface antigen, hepatitis B e antigen, HBV DNA, and cytokine levels. Kupffer cells (KCs) were purified from antibiotic-treated mice and HBV-replicating mice and analyzed for IL-10 production and T cell suppression ability. **Results:** ABX treatment resulted in increased serum LPS levels in mice. The KCs separated from both ABX-treated and LPS-treated HBV-replicating mice showed significantly increased IL-10 production and enhanced ability to suppress IFN- γ production of TCR-activated T cells than the KCs separated from their counterpart controls. HDI of pAAV/HBV1.2 in combination with LPS in mice led to a delayed HBV clearance and early elevation of serum IL-10 levels compared to pAAV/HBV1.2 HDI alone. Moreover, IL-10 function blockade or KC depletion led to accelerated HBV clearance in LPS-treated HBV-replicating mice. **Conclusions:** Our results suggest that dysbiosis of the gut microbiota in mice leads to endotoxemia, which induces KC IL-10 production and strengthens KC-mediated T cell suppression, and thus fa-

cilitates HBV persistence.

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Introduction

Hepatitis B virus (HBV) chronically infects 250 million people worldwide and continues to be a global public health burden. Chronic HBV infection may lead to hepatic cirrhosis and hepatocellular carcinoma, resulting in nearly 1 million deaths annually.¹ Studies in recent years have significantly improved our knowledge on understanding the mechanisms of HBV persistence. It has been shown that HBV uses multiple mechanisms to dampen host adaptive immunity, especially the anti-HBV T cell responses, to facilitate its persistence.^{2,3}

In recent years, increased evidence has suggested that the gut microbiota, which affects local and systemic immune responses,^{4,5} plays an important role in determining the outcome of HBV infection. On the one hand, compositional and functional changes in the gut microbiota have been observed in HBV-replicating mouse models⁶ and chronically HBV-infected patients,⁷ and is believed to be associated with disease progression.⁸ On the other hand, changes in gut microbiota composition may influence the outcome of HBV infection. In the HBV hydrodynamic injection (HDI) mouse model, depletion of gut microbiota by antibiotics prior to HBV challenge results in HBV persistence and impaired anti-HBV adaptive immunity.⁹ Moreover, using fecal microbiota transplantation to reconstitute the gut microbiota was shown to facilitate hepatitis B e antigen (HBeAg) clearance in chronic hepatitis B (CHB) patients.¹⁰ These results indicated stabilized gut microbiota play an important role in regulating the host immune response for HBV clearance. However, the detailed mechanism by which the intrahepatic anti-HBV immunity is regulated by the gut microbiota remains largely unknown.

In this study, we used an antibiotic (ABX) mixture treatment mouse model and the HBV HDI mouse model to ex-

Keywords: Chronic hepatitis B; Gut microbiota; Kupffer cells; T cell; IL-10.

Abbreviations: CD, cluster of differentiation; dpi, days post-injection; ELISA, enzyme-linked immunosorbent assay; HBeAg, hepatitis B e Antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HDI, hydrodynamic injection; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; KCs, Kupffer cells; MCP, monocyte chemoattractant protein; NS, normal saline; PBS, phosphate-buffered saline; PC, positive control; PD-L, programmed death-ligand; RBC, red blood cell; SPLs, splenocytes; TCR, T cell receptor; TNF, tumor necrosis factor; TLR, Toll-like receptor.

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amine the possible mechanism of regulating liver immunity by the gut microbiota during HBV infection.

Methods

Mice

Six to eight week-old wild-type male C57BL/6 mice were purchased from Hunan Slack King Laboratory Animal Co., Ltd. (Changsha, China). All animals were bred and kept in the Animal Care Center of Tongji Medical College (Wuhan, China) under specific pathogen-free conditions. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Tongji Medical College, Huazhong University of Science and Technology, China (IACUC number: 612) and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

HDI in mice and production of LPS mice and HBV+LPS mice

HDI was performed by using HBV plasmids pAAV/HBV1.2 to establish HBV replication in mice, as described previously.¹¹ In brief, mice were injected with 20 µg pAAV/HBV1.2 through the tail vein within an 8-s time window, in a volume of normal saline equivalent to 0.1 mL/g of the mouse body weight. Lipopolysaccharide (LPS) mice were subjected to HDI with 1 µg LPS (InvivoGen, San Diego, CA, USA). HBV+LPS mice were subjected to HDI with 20 µg pAAV/HBV1.2, in combination with 1 µg LPS.

Production of ABX mice

ABX mice were treated with antibiotics (ampicillin, neomycin, metronidazole, and vancomycin) in drinking water, according to an antibiotic mixture protocol for gut-sterilization.⁹

Cell isolation

Isolation of murine Kupffer cells (KCs) was performed, as described previously.¹² KCs were isolated from nonparenchymal liver cells by using CD11b+ microbeads (Miltenyi, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Preparation of single-cell suspensions of murine splenocytes was performed as described previously.¹³

Detection of IL-10 and IFN-γ levels in the serum and supernatants

IL-10 levels in the serum and in the cell-free supernatants were determined by the corresponding cytokine ELISA kits (eBioscience, San Diego, CA, USA), according to the corresponding manufacturer's instructions. Red blood cell (RBC)-depleted splenocytes were cultured at 1×10^6 cells per well with or without KCs in a total volume of 200 µL at a ratio of 2:1 (splenocytes: KCs). Splenocytes were stimulated with anti-CD3 (1 µg/mL) and anti-CD28 (1 µg/mL) (BD Bioscience, Franklin Lakes, NJ, USA). Interferon (IFN)-γ levels in the cell-free supernatants were determined by the corresponding cytokine enzyme-linked immunosorbent assay (ELISA) kits (eBioscience), according to the corresponding manufacturer's instructions.

Detection of serum cytokines and LPS

Serum IL-10, IL-6, IL-12, TNF-α, MCP-1, and IFN-γ levels were determined by Cytometric Bead Array Mouse Inflammation Kit (BD Biosciences), according to the manufacturer's instructions. Serum LPS levels were determined by End-point Chromogenic TAL Endotoxin Testing Assay (Bioendo Technology, Xiamen, China), according to the manufacturer's instructions.

Detection of serological HBV antigens and DNA

Serum was collected from the retro-orbital sinus of the mouse at the indicated time points. Serum hepatitis B surface antigen (HBsAg) and HBeAg levels were measured by the corresponding ELISA kits (Kehua, Shanghai, China), according to the manufacturer's instructions. Serum HBV DNA copies were measured by a diagnostic kit for HBV DNA (Sansure, Changsha, China) using quantitative real-time PCR, according to the manufacturer's instructions.

Flow cytometry

For surface staining, KCs were stained with CD80 (eBioscience), CD86 (BioLegend, San Diego, CA, USA), and PD-L1 (eBioscience) antibodies for 15 min at 4°C in darkness. Freshly isolated cells were used for all assays, and approximately 20,000–40,000 T cells were acquired for each sample using a FACS Canto II flow cytometer (BD Biosciences). Data analysis was performed using FlowJo software V10.0.7 (Tree Star, Ashland, OR, USA). Cell debris and dead cells were excluded from the analysis based on scatter signals and Fixable Viability Dye eFluor 506.

IL-10 blockade and KC depletion

Purified rat anti-mouse IL-10 receptor (IL-10R) monoclonal antibodies (Clone 1B1.3a) were purchased from Bio X Cell (Lebanon, NH, USA). For IL-10 blockade, mice were intraperitoneally injected with 250 µg anti-mouse IL-10 receptor antibody at 1, 24 and 96 h before HBV and LPS HDI. For KC depletion, mice were injected via the tail vein with 200 µL of clodronate-liposome (5 mg/mL) or a phosphate-buffered saline (PBS)-liposome (LIPOSOMA BV, Netherlands) suspension at 24 h before HBV and LPS HDI.

Statistical analysis

Nonparametric *t*-test was used when comparing two groups. One-way analysis of variance was used with a Tukey post hoc test (GraphPad Prism software; GraphPad, San Diego, CA, USA) when more than two groups were compared. Statistical significance was considered when the *p* value was less than 0.05.

Results

Gut microbiota sterilization by antibiotics results in increased serum LPS levels and enhanced IL-10 production and T cell suppression of KC

LPS is the main component of the outer membrane of Gram-

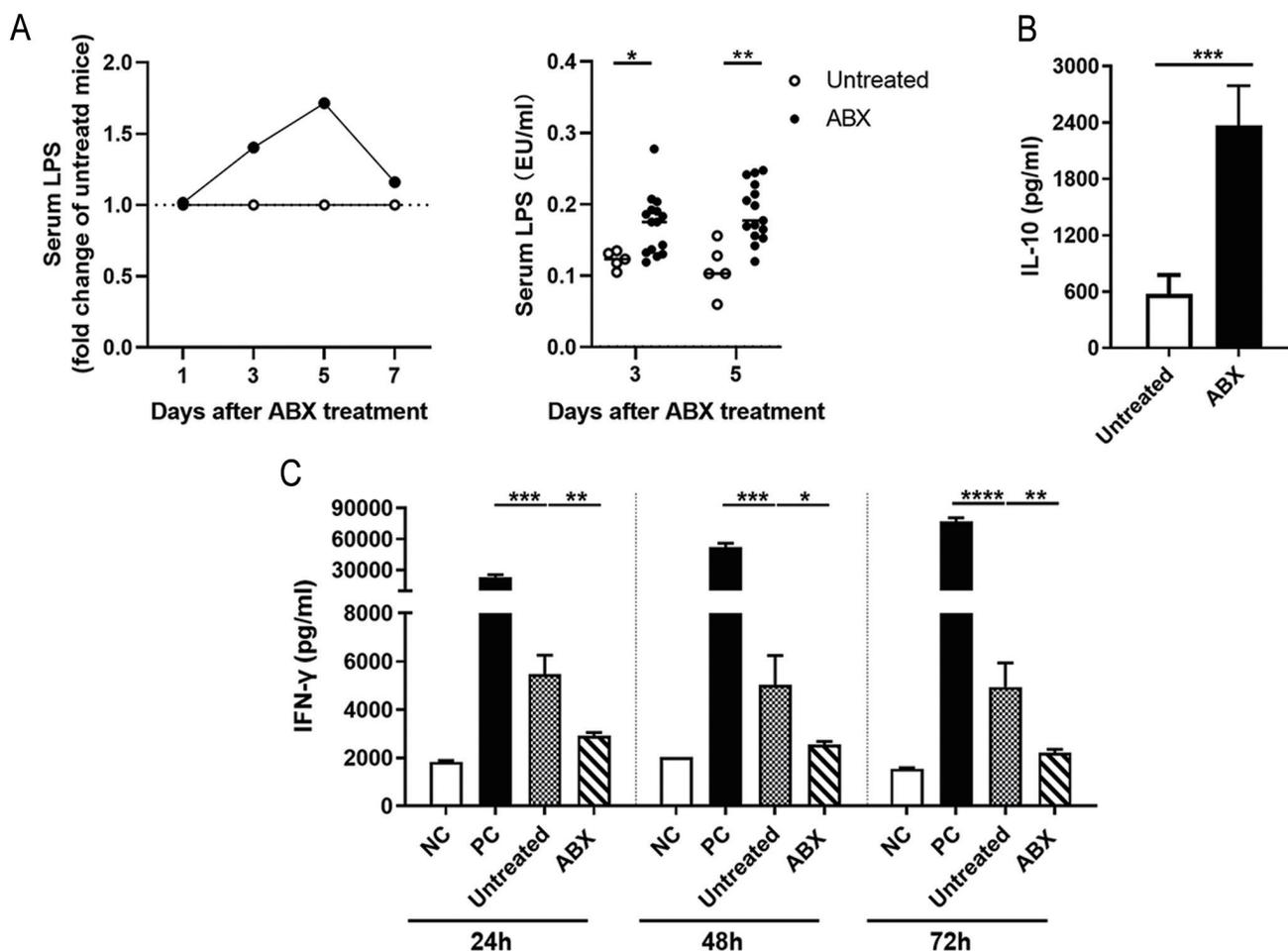


Fig. 1. Gut microbiota sterilization by antibiotics results in increased serum LPS levels and enhanced IL-10 production and T cell suppression of KCs. (A) C57BL/6 mice were treated with antibiotics (ABX; ampicillin, neomycin, metronidazole, and vancomycin) in drinking water (ABX, $n=15$; Untreated, $n=5$) and the kinetics of serum LPS were monitored by ELISA. (B) KCs were purified from the liver of mice 30 days post-ABX treatment and cultured *in vitro*. After a whole night, the amount of IL-10 in the culture supernatant was determined by ELISA. (C) KCs were co-cultured with anti-CD3/anti-CD28 (1 $\mu\text{g/mL}$)-stimulated splenocytes from naive mice at a ratio of 1:2 (KCs:splenocytes [SPLs]). After 24, 48 and 72 h, the amount of IFN- γ in the culture supernatant was determined by ELISA. Anti-CD3/anti-CD28-stimulated SPLs were used as a positive control (PC) and unstimulated SPLs were used as a negative control (NC). Unpaired *t*-test was applied. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; KCs, Kupffer cells.

negative bacteria and is an endotoxin mainly released by *Enterobacteriaceae*. Therefore, we first investigated the influence of gut microbiota sterilization on serum LPS concentration. Adult C57BL/6 mice were gut-sterilized using a well-established antibiotic (ABX) mixture protocol⁹ and serum LPS levels were monitored by ELISA every other day for a week from the ABX treatment. The ABX treatment resulted in statistically significant increases in serum LPS concentrations up to 70% on 3 days and 5 days post-treatment in mice compared to untreated controls (Fig. 1A).

KCs are the largest population of tissue-resident macrophages, and LPS stimulation induces their activation and secretion of immunosuppressive mediators, such as IL-10.¹⁴ We next examined how gut microbiota sterilization influences the IL-10 production and suppressive function of KCs for T cell activation. KCs were purified from the liver of mice 30 days post-continuous ABX treatment, and were examined for IL-10 secretion by ELISA. KCs of ABX-treated mice showed significantly increased IL-10 production compared to those of control mice (Fig. 1B). KCs were also cocultured with anti-CD3/anti-CD28-activated T cells and the IFN- γ production by T cells was measured after 24, 48 and 72 h by ELISA. Consist-

ent with our previous report,¹⁵ KCs separated from untreated control mice strongly suppressed the IFN- γ production of TCR-activated T cells. Compared to control KCs, KCs from ABX mice showed a significantly enhanced ability in suppressing the IFN- γ production of TCR-activated T cells (Fig. 1C). Taken together, these results showed that gut microbiota sterilization by antibiotics resulted in increased serum LPS levels and enhanced IL-10 production and T cell suppression of KCs.

LPS stimulation induces strengthened suppressive phenotype and T cell suppression of KC in HBV-replicating mice

Next, we investigated the impact of LPS stimulation on KC phenotype and function in the context of intrahepatic HBV replication. C57BL/6 mice were subject to HDI with normal saline (NS), LPS, HBV-replicating plasmid pAAV/HBV1.2 in combination with LPS (HBV+ LPS) or not (HBV), and KCs were purified from the liver of those mice 3 h after HDI. Flow cytometry analysis demonstrated that KCs separated from LPS mice showed significantly decreased CD80 and

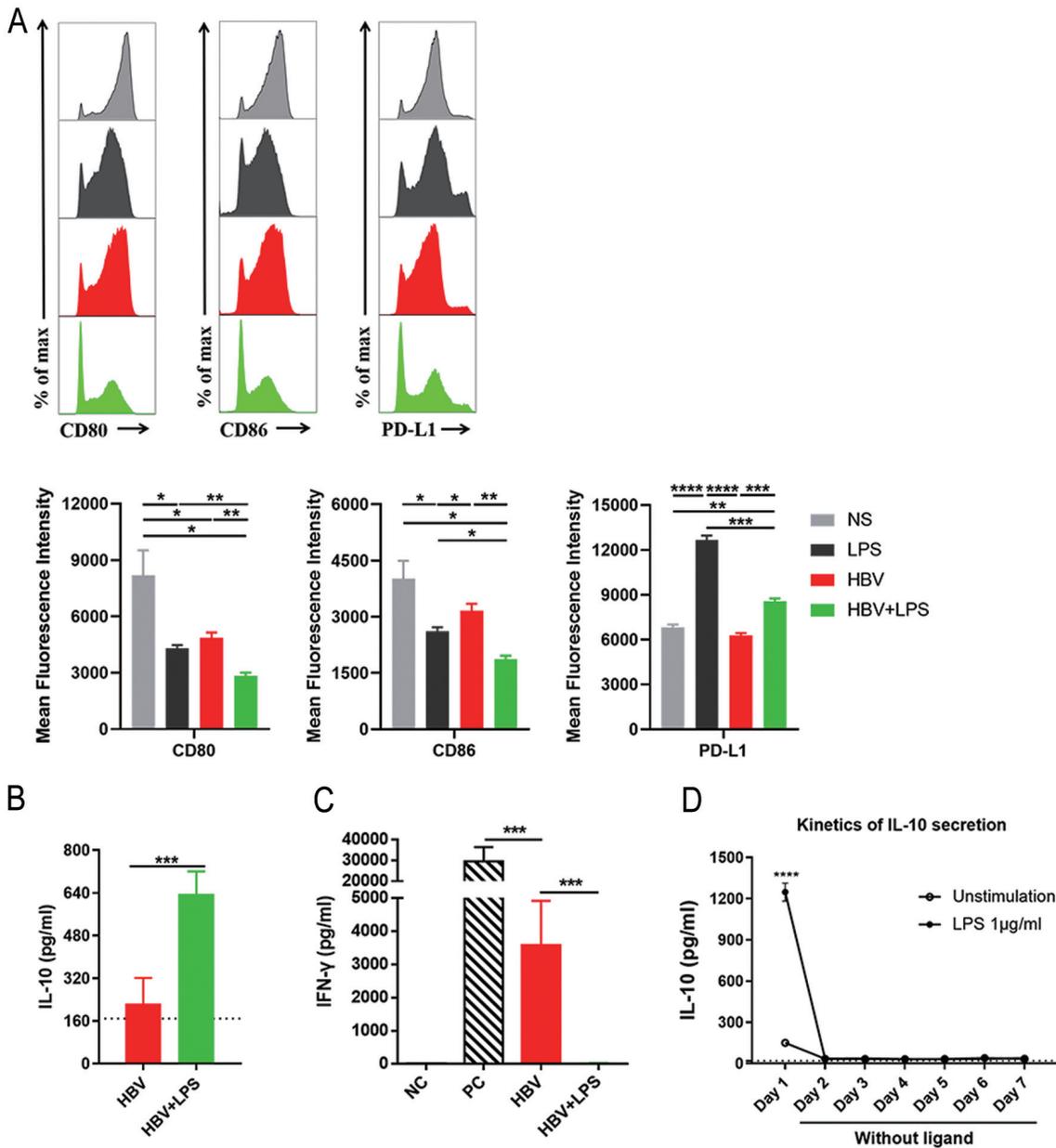


Fig. 2. LPS stimulation induces strengthened suppressive phenotype, enhanced IL-10 production and T cell suppression of KCs in HBV-replicating mice. C57BL/6 mice were subject to HDI with the NS, LPS, pAAV/HBV1.2 plasmid in combination with LPS (HBV+LPS) or not (HBV). After 3 h, KCs were purified from the liver of those mice. (A) CD80, CD86, and PD-L1 expression on KCs were analyzed by flow cytometry. (B) KCs were purified and cultured *in vitro*. After a whole night, the amount of IL-10 in the culture supernatant was determined by specific ELISA. (C) KCs were co-cultured with anti-CD3/anti-CD28 (1 μg/mL)-stimulated SPLs at a ratio of 1:2 (KCs:SPLs). After 48 h, the amount of IFN-γ in the culture supernatant was determined by specific ELISA. Anti-CD3/anti-CD28-stimulated SPLs were used as a PC and unstimulated SPLs were used as an NC. (D) KCs were stimulated by 1 μg/mL LPS for 24 h (day 1), washed, and cultured for another 6 d without stimulation. Culture medium was changed every 24 h. IL-10 secretion by KCs was determined by ELISA. Unpaired *t*-test was used. **p*<0.05, ***p*<0.01, ****p*<0.001. ELISA, enzyme-linked immunosorbent assay; HBV, hepatitis B virus; HDI, hydrodynamic injection; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; KCs, Kupffer cells; PC, positive control; SPLs, splenocytes.

CD86 expressions and increased PD-L1 expression compared to those of NS HDI mice (Fig. 2A). Consistently, the expression of CD80 and CD86 on KCs was significantly decreased and the PD-L1 expression was significantly increased in HBV+LPS mice compared with those of HBV mice (Fig. 2A). Moreover, KCs of HBV+LPS mice secreted a significantly increased amount of IL-10 than HBV mice (Fig. 2B). Consistently, KCs of HBV+LPS mice showed a significantly enhanced ability in suppressing the IFN-γ production

of TCR-activated T cells than KCs from HBV mice (Fig. 2C). The direct effect of LPS stimulation on inducing KC IL-10 production was also examined *in vitro*. KCs were purified from the liver of untreated mice and stimulated by LPS *in vitro* for 24 h and monitored for IL-10 production up to 7 days. As shown in Figure 2D, significant up-regulation of IL-10 production by LPS-stimulated KCs was observed in the first day when LPS was presented in the medium. However, no IL-10 production by LPS-stimulated KCs was detected from days 2

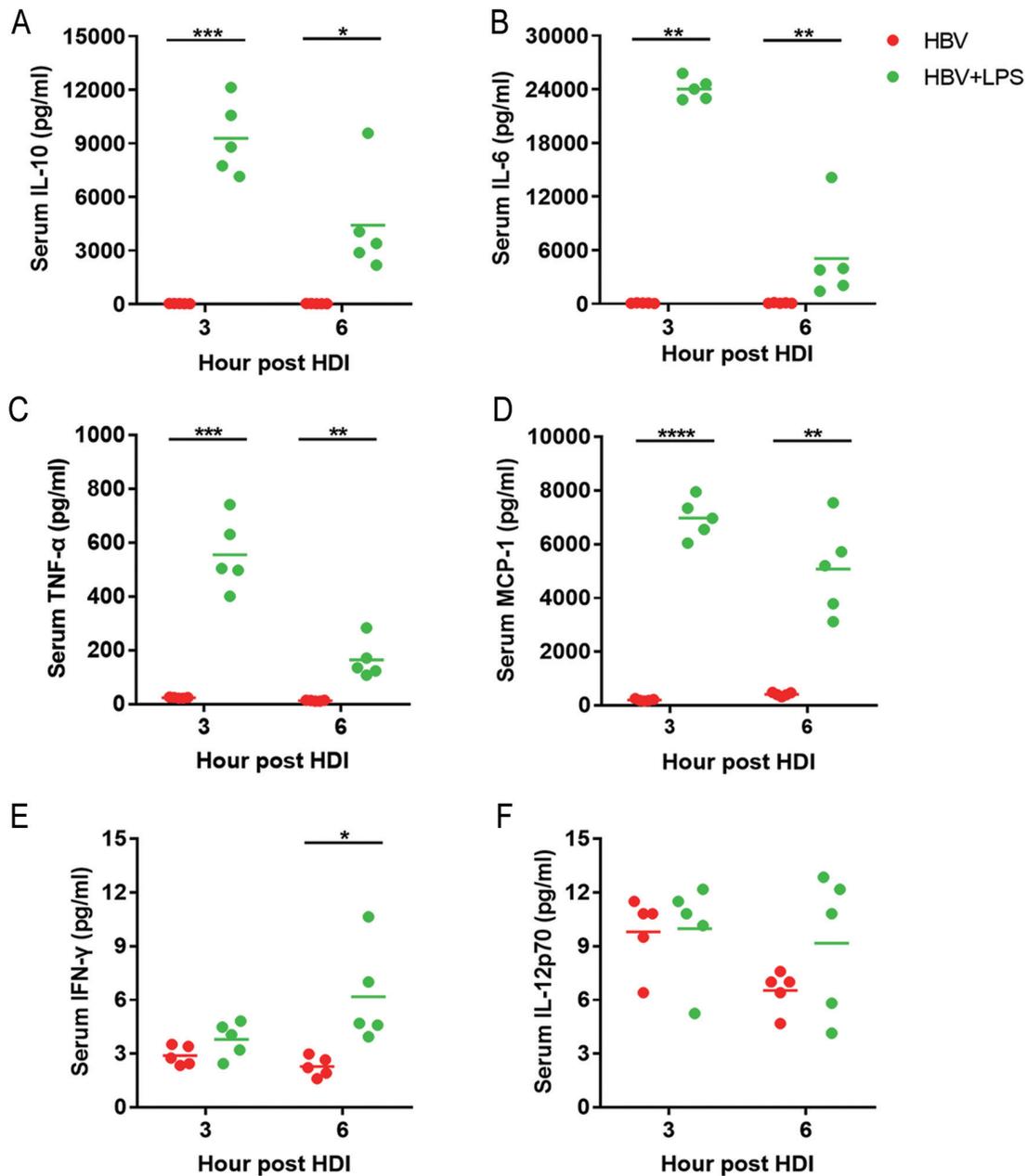


Fig. 3. LPS stimulation induces potent proinflammatory cytokine production in HBV-replicating mice. C57BL/6 mice were subject to HDI with the pAAV/ HBV1.2 plasmid in combination with LPS (HBV+LPS) or not (HBV). The kinetics of (A) serum IL-10 (B) serum IL-6 (C) serum TNF-α (D) serum MCP-1 (E) serum IFN-γ (F) serum IL-12p70 were monitored by cytometric bead array. Unpaired t-test was used. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. HBV, hepatitis B virus; HDI, hydrodynamic injection; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein; TNF, tumor necrosis factor.

to 7 in the supernatant when LPS was washed away. These results suggested that in the context of intrahepatic HBV replication, LPS stimulation could induce strengthened suppressive phenotype and T cell suppression of KCs in mice.

LPS stimulation induces potent proinflammatory cytokine production in HBV-replicating mouse and results in delayed HBV clearance

We further detected proinflammatory cytokine levels in the

sera of HBV and HBV+LPS mice at 3 and 6 h post-HDI. The concentrations of IL-10, IL-6, TNF-α, MCP-1, IFN-γ, and IL-12 were maintained at low levels in the sera of HBV mice at the detected early time points post-HDI (Fig. 3A-F). In a sharp contrast, HBV+LPS mice showed significant increases in serum IL-10, IL-6, TNF-α, and MCP-1 levels at both 3 and 6 h post-HDI (Fig. 3A-D). In general, the serum IFN-γ and IL-12 levels were comparable between the two groups, and there was only a slight increase of IFN-γ levels in HBV+LPS mice observed at 6 h post-HDI (Fig. 3E-F).

Next, the impact of LPS stimulation on HBV clearance was examined by monitoring serum HBsAg and HBV DNA.

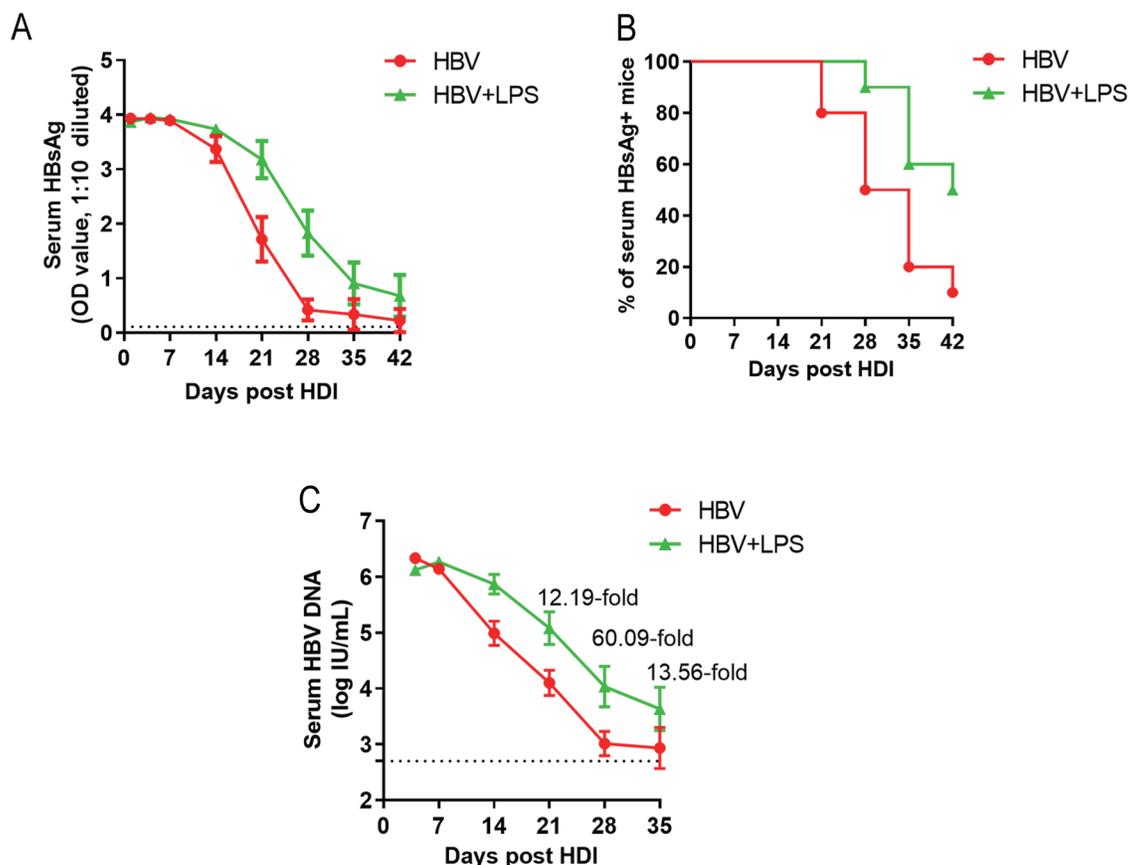


Fig. 4. LPS stimulation results in delayed HBV clearance in HBV-replicating mice. C57BL/6 mice were subject to HDI with the pAAV/HBV1.2 plasmid in combination with LPS (HBV+LPS) or not (HBV). (A) The kinetics of serum HBsAg levels were monitored by ELISA. (B) The percentages of HBsAg-positive mice were analyzed. (C) The kinetics of serum HBV DNA copies were monitored by real-time PCR. ELISA, enzyme-linked immunosorbent assay; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HDI, hydrodynamic injection; LPS, lipopolysaccharide.

As shown in Figure 4A, HBV+LPS mice showed higher serum HBsAg levels than HBV mice starting at 21 days post-HDI (dpi). At 42 days post-injection (dpi), serum HBsAg remained positive in 50% of HBV+LPS mice, but in only 10% of HBV mice (Fig. 4B). Moreover, HBV+LPS mice also showed higher serum HBV DNA levels than HBV mice. LPS treatment resulted in a 13.56-fold increase in serum HBV DNA levels at 35 dpi (Fig. 4C). Taken together, these results suggested that LPS stimulation in HBV-replicating mice induced potent proinflammatory cytokine production and resulted in a delay of HBV clearance.

IL-10 functional blockade and KC depletion results in accelerated HBV clearance in LPS treated HBV-replicating mice

We then examined whether the LPS-induced delay of HBV clearance in mice is mediated by IL-10 production and KCs. First, mice were treated with IL-10 receptor (IL-10R) blocking antibody (anti-IL-10R) prior to HBV and LPS HDI. As shown in Figure 5A, HBsAg clearance was significantly accelerated in anti-IL-10R-treated mice compared to untreated control mice. All anti-IL-10R-treated mice became serum HBsAg-negative at 4 dpi, whereas all control mice remained serum HBsAg-positive at least at 26 dpi (Fig. 5A). Next, mice were injected with clodronate-liposome to deplete KCs^{16,17} prior to HBV and LPS HDI. KC depletion resulted in signifi-

cant decreases in serum IL-10 levels at both 3 and 6 h post-HBV HDI in clodronate-liposome treated mice compared to PBS-liposome treated mice (Fig. 5B), suggesting KCs were the main source of IL-10 production in LPS-treated HBV-replicating mice. Moreover, KC depletion also resulted in accelerated HBsAg clearance in mice, as 80% of PBS-liposome treated control mice remained serum HBsAg-positive, but only 20% of clodronate-liposome treated mice were so at 26 dpi (Fig. 5C). These results suggested that LPS-induced HBV persistence in mice is mediated by IL-10 and KCs.

Discussion

In a previous study, Chou *et al.*⁹ observed that adult C3H/HeN mice rapidly cleared HBV, while their young counterparts showed HBV persistence in the pAAV/HBV1.2 HDI mouse model. Sterilization of gut microbiota using antibiotics prevented adult mice from rapid HBV clearance.

In contrast, depleting KCs or abolishing the Toll-like receptor (TLR)4 signaling pathway in young mice prior to HBV HDI resulted in rapid viral clearance. The authors thus suggested that the gut microbiota sterilization-induced HBV immune tolerance in mice was highly probably mediated by KCs via the activation of TLR4 by its ligand LPS. Based on these observations and speculations, in the current study we further demonstrated that sterilizing gut microbiota by antibiotics results in an increase of serum LPS level in

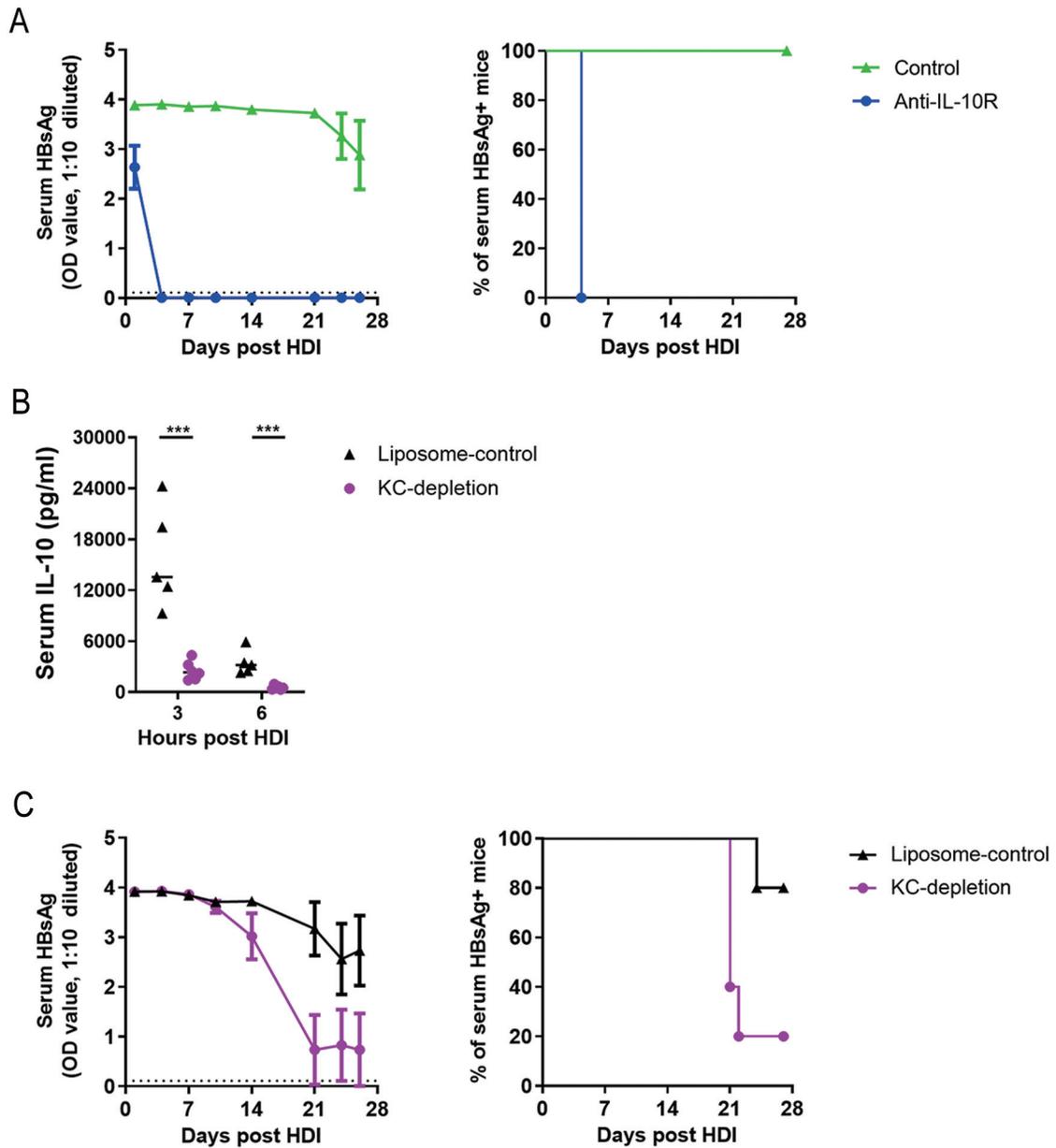


Fig. 5. IL-10 functional blockade and KC depletion results in accelerated HBV clearance in LPS-treated HBV-replicating mice. (A) C57BL/6 mice were subject to HDI with the pAAV/HBV1.2 plasmid in combination with LPS, either intraperitoneally injected with anti-mouse IL-10 receptor (Anti-IL-10R) or not (Control). (B) C57BL/6 mice were subject to HDI with the pAAV/HBV1.2 plasmid in combination with LPS, either intravenously injected with clodronate-liposome (KC-depletion) or PBS-liposome (Liposome-control). The kinetics of serum IL-10 were monitored by ELISA. (C) The kinetics of serum HBsAg levels were monitored by ELISA (left). The percentages of HBsAg-positive mice were analyzed (right). ELISA, enzyme-linked immunosorbent assay; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HDI, hydrodynamic injection; IL, interleukin; LPS, lipopolysaccharide; KC, Kupffer cell; PBS, phosphate-buffered saline.

mice. Consistently, KCs from both gut-sterilized mice and LPS-treated HBV-replicating mice produce a significantly increased amount of IL-10 and show enhanced ability in suppressing T cell activation. This LPS-induced functional change of KCs results in delayed HBV clearance in mice.

The liver is not in direct contact with gut microbiota. However, it constantly encounters bacterial components, such as LPS generated from *Enterobacteriaceae*, translocated from the gut into the portal vein. It has been shown that LPS can disrupt expression of the tight junction protein OZ-1 in the intestinal tract, which increases the permeability of the intestinal mucosa.¹⁸ This in turn allows more LPS to enter

the blood flow through the portal venous system and leads to low-grade endotoxemia.¹⁹ Therefore, in cases such as gut microbiota sterilization or dysbiosis, increased LPS release into the intestinal tract may result in increased intrahepatic LPS stimulation. Moreover, it has also been shown recently by Guo *et al.*²⁰ that ABX treatment in mice results in translocation of live commensal gut bacteria and their components, including LPS into the liver. Therefore, gut microbiota sterilized mice may have an increased intrahepatic LPS stimulation, with even the serum LPS concentrations in these mice returning to physiological levels. LPS is mainly sensed by TLR4, which is constitutively expressed on the

surface of macrophages.^{21–23}

KCs are the largest population of liver-resident macrophages, and activation of the TLR4 signaling pathway by LPS induces the production of proinflammatory cytokines, including IL-10, by KCs.^{14,24–26} IL-10 is a cytokine with pleiotropic functions, and in many cases, it has been considered as an immunosuppressive cytokine that can attenuate inflammatory responses and suppress T cell activation and proliferation via the IL-10/IL-10R pathway.²⁷ During HBV infection, elevated serum IL-10 level has been observed in both the acute phase²⁸ and chronic phase.²⁹ It is believed that KCs are one of the main sources of IL-10 production during CHB, and this KC-derived IL-10 plays an important role in maintaining liver immune tolerance and participates in HBV persistence.³⁰ We have recently reported that the HBV particle directly stimulates KCs to produce IL-10 via activation of the TLR2 signaling pathway.¹⁵ This effect strengthens liver tolerance by promoting KC-mediated CD8 T cell suppression.¹⁵ In line with these findings, blocking the function of IL10 or depleting KCs in HBV-carrier mice leads to rapid HBV elimination and improved anti-HBV CD8 T cell responses.^{31,32}

In the current study, we further demonstrate that gut microbiota dysbiosis may also trigger IL-10 production by KCs in the context of HBV infection, which plays a key role in mediating HBV persistence. Our finding has implications for the understanding of mechanisms of regulating intrahepatic immune response by the gut microbiota and their relationship with HBV infection.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study concept and design (JL), acquisition of data (WZ, JzL, XX, SY, DZ, HH), analysis and interpretation of data (WZ, JzL, JL), drafting of the manuscript (WZ, JzL, XX), critical revision of the manuscript for important intellectual content (WZ, JL), administrative, technical, and material support (JL, DY), and study supervision (JL, DY).

Data sharing statement

The raw data of the manuscript are deposited in the database of the Department of Infectious Diseases, Union Hospital, Tongji Medical College, Huazhong University of Sci-

ence and Technology, and can be provided to inquirers upon reasonable requests.

References

- [1] Yuen M, Chen D, Dusheiko G, Janssen H, Lau D, Locarnini S, *et al*. Hepatitis B virus infection. *Nat Rev Dis Primers* 2018;4:18035. doi:10.1038/nrdp.2018.35.
- [2] Maini M, Burton A. Restoring, releasing or replacing adaptive immunity in chronic hepatitis B. *Nat Rev Gastroenterol Hepatol* 2019;16(11):662–675. doi:10.1038/s41575-019-0196-9.
- [3] Bertolotti A, Ferrari C. Adaptive immunity in HBV infection. *J Hepatol* 2016;64:S71–S83. doi:10.1016/j.jhep.2016.01.026.
- [4] Belkaid Y, Naik S. Compartmentalized and systemic control of tissue immunity by commensals. *Nat Immunol* 2013;14(7):646–653. doi:10.1038/ni.2604.
- [5] Molloy MJ, Bouladoux N, Belkaid Y. Intestinal microbiota: shaping local and systemic immune responses. *Semin Immunol* 2012;24(1):58–66. doi:10.1016/j.smim.2011.11.008.
- [6] Zhu Q, Xia P, Zhou X, Li X, Guo W, Zhu B, *et al*. Hepatitis B virus infection alters gut microbiota composition in mice. *Front Cell Infect Microbiol* 2019;9:377. doi:10.3389/fcimb.2019.00377.
- [7] Wang J, Wang Y, Zhang X, Liu J, Zhang Q, Zhao Y, *et al*. Gut microbial dysbiosis is associated with altered hepatic functions and serum metabolites in chronic hepatitis B patients. *Front Microbiol* 2017;8:2222. doi:10.3389/fmicb.2017.02222.
- [8] Tripathi A, Debelius J, Brenner DA, Karin M, Loomba R, Schnabl B, *et al*. The gut-liver axis and the intersection with the microbiome. *Nat Rev Gastroenterol Hepatol* 2018;15(7):397–411. doi:10.1038/s41575-018-0011-z.
- [9] Chou HH, Chien WH, Wu LL, Cheng CH, Chung CH, Horng JH, *et al*. Age-related immune clearance of hepatitis B virus infection requires the establishment of gut microbiota. *Proc Natl Acad Sci U S A* 2015;112(7):2175–2180. doi:10.1073/pnas.1424775112.
- [10] Ren YD, Ye ZS, Yang LZ, Jin LX, Wei WJ, Deng YY, *et al*. Fecal microbiota transplantation induces hepatitis B virus e-antigen (HBeAg) clearance in patients with positive HBeAg after long-term antiviral therapy. *Hepatology* 2017;65(5):1765–1768. doi:10.1002/hep.29008.
- [11] Huang L, Wu H, Chen P, Chen D. An immunocompetent mouse model for the tolerance of human chronic hepatitis B virus infection. *Proc Natl Acad Sci U S A* 2006;103(47):17862–17867. doi:10.1073/pnas.0608578103.
- [12] Liu J, Huang X, Werner M, Broering R, Yang D, Lu M. Advanced method for isolation of mouse hepatocytes, liver sinusoidal endothelial cells, and Kupffer cells. *Methods Mol Biol* 2017;1540:249–258. doi:10.1007/978-1-4939-6700-1_21.
- [13] Wu W, Dietze K, Gibbert K, Lang K, Trilling M, Yan H, *et al*. TLR ligand induced IL-6 counter-regulates the anti-viral CD8(+) T cell response during an acute retrovirus infection. *Sci Rep* 2015;5:10501. doi:10.1038/srep10501.
- [14] Knolle P, Schlaak J, Uhrig A, Kempf P, Meyer zum Buschenfelde KH, Gerken G. Human Kupffer cells secrete IL-10 in response to lipopolysaccharide (LPS) challenge. *J Hepatol* 1995;22(2):226–229. doi:10.1016/0168-8278(95)80433-1.
- [15] Liu J, Yu Q, Wu W, Huang X, Broering R, Werner M, *et al*. TLR2 stimulation strengthens intrahepatic myeloid-derived cell-mediated T cell tolerance through inducing Kupffer cell expansion and IL-10 production. *J Immunol* 2018;200(7):2341–2351. doi:10.4049/jimmunol.1700540.
- [16] Sitia G, Iannaccone M, Aiolfi R, Isogawa M, van Rooijen N, Scozzesi C, *et al*. Kupffer cells hasten resolution of liver immunopathology in mouse models of viral hepatitis. *PLoS Pathog* 2011;7(6):e1002061. doi:10.1371/journal.ppat.1002061.
- [17] Van Rooijen N, Sanders A. Kupffer cell depletion by liposome-delivered drugs: comparative activity of intracellular clodronate, propamide, and ethylenediaminetetraacetic acid. *Hepatology* 1996;23(5):1239–1243. doi:10.1053/jhep.1996.v23.pm008621159.
- [18] Brun P, Castagliuolo I, Di Leo V, Buda A, Pinzani M, Palu G, *et al*. Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis. *Am J Physiol Gastrointest Liver Physiol* 2007;292(2):G518–525. doi:10.1152/ajpgi.00024.2006.
- [19] Frazier TH, DiBaise JK, McClain CJ. Gut microbiota, intestinal permeability, obesity-induced inflammation, and liver injury. *J Parenter Enteral Nutr* 2011;35(5 Suppl):14S–20S. doi:10.1177/0148607111413772.
- [20] Guo W, Zhou X, Li X, Zhu Q, Wang J. Depletion of gut microbiota impairs gut barrier function and antiviral immune defense in the liver. *Front Immunol* 2021;12:636803. doi:10.3389/fimmu.2021.636803.
- [21] Raetz C, Whitfield C. Lipopolysaccharide endotoxins. *Annu Rev Biochem* 2002;71:635–700. doi:10.1146/annurev.biochem.71.110601.135414.
- [22] Miyake K. Innate recognition of lipopolysaccharide by Toll-like receptor 4-MD-2. *Trends Microbiol* 2004;12(4):186–192. doi:10.1016/j.tim.2004.02.009.
- [23] Miller S, Ernst R, Bader M. LPS, TLR4 and infectious disease diversity. *Nat Rev Microbiol* 2005;3(1):36–46. doi:10.1038/nrmicro1068.
- [24] Li P, He K, Li J, Liu Z, Gong J. The role of Kupffer cells in hepatic diseases. *Mol Immunol* 2017;85:222–229. doi:10.1016/j.molimm.2017.02.018.
- [25] Tacke F. Targeting hepatic macrophages to treat liver diseases. *J Hepatol* 2017;66(6):1300–1312. doi:10.1016/j.jhep.2017.02.026.
- [26] Nakamoto N, Kanai T. Role of toll-like receptors in immune activation and tolerance in the liver. *Front Immunol* 2014;5:221. doi:10.3389/fimmu.2014.00221.

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- [27] Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001;19:683–765. doi:10.1146/annurev.immunol.19.1.683.
- [28] Fioravanti J, Di Lucia P, Magini D, Moalli F, Boni C, Benechet AP, *et al*. Effector CD8+ T cell-derived interleukin-10 enhances acute liver immunopathology. *Journal of hepatology* 2017;67(3):543–548. doi:10.1016/j.jhep.2017.04.020.
- [29] Das A, Ellis G, Pallant C, Lopes AR, Khanna P, Peppas D, *et al*. IL-10-producing regulatory B cells in the pathogenesis of chronic hepatitis B virus infection. *J Hepatol* 2012;189(8):3925–3935. doi:10.4049/jimmunol.1103139.
- [30] Thomson AW, Knolle PA. Antigen-presenting cell function in the tolerogenic liver environment. *Nat Rev Immunol* 2010;10(11):753–766. doi:10.1038/nri2858.
- [31] Xu L, Yin W, Sun R, Wei H, Tian Z. Kupffer cell-derived IL-10 plays a key role in maintaining humoral immune tolerance in hepatitis B virus-persistent mice. *Hepatology* 2014;59(2):443–452. doi:10.1002/hep.26668.
- [32] Li M, Sun R, Xu L, Yin W, Chen Y, Zheng X, *et al*. Kupffer cells support hepatitis B virus-mediated CD8+ T cell exhaustion via hepatitis B core antigen-TLR2 interactions in mice. *J Immunol* 2015;195(7):3100–3109. doi:10.4049/jimmunol.1500839.