The IL-1R/TLR signaling pathway is essential for efficient CD8+ T-cell responses against hepatitis B virus in the hydrodynamic injection mouse model

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The outcome of hepatitis B viral (HBV) infection is determined by the complex interactions between replicating HBV and the immune system. While the role of the adaptive immune system in the resolution of HBV infection has been studied extensively, the contribution of innate immune mechanisms remains to be defined. Here we examined the role of the interleukin-1 receptor/Toll-like receptor (IL-1R/TLR) signaling pathway in adaptive immune responses and viral clearance by exploring the HBV mouse model. Hydrodynamic injection with a replication-competent HBV genome was performed in wild-type mice (WT) and a panel of mouse strains lacking specific innate immunity component expression. We found higher levels of HBV protein production and replication in Tlr2−/−, Tlr23479−/−, 3d/Tlr24−/−, Myd88/Trif−/− and Irak4−/− mice, which was associated with reduced HBV-specific CD8+ T-cell responses in these mice. Importantly, HBV clearance was delayed for more than 2 weeks in 3d/Tlr24−/−, Myd88/Trif−/− and Irak4−/− mice compared to WT mice. HBV-specific CD8+ T-cell responses were functionally impaired for producing the cytokines IFN-γ, TNF-α and IL-2 in TLR signaling-deficient mice compared to WT mice. In conclusion, the IL-1R/TLR signaling pathway might contribute to controlling HBV infection by augmenting HBV-specific CD8+ T-cell responses.

Cellular & Molecular Immunology (2017) 14, 997–1008; doi:10.1038/cmi.2017.43; published online 31 July 2017

Keywords: CD8+ T-cell response; Hepatitis B virus; IL-1R/TLR signaling pathway; Toll-like receptor

INTRODUCTION

Hepatitis B virus (HBV), a hepatotropic noncytopathic DNA virus, affects millions of chronically HBV-infected patients and causes fatal liver cirrhosis and hepatocellular carcinoma.1 The outcome of HBV infection is determined by complex interactions between replicating HBV and the host immune system. Specific CD8+ T-cell responses against HBV proteins play a major role in viral clearance but are functionally impaired in chronically HBV-infected patients.2 Restoration of HBV-specific CD8+ T-cell response in chronically HBV-infected patients may be a promising strategy to terminate HBV persistence. This concept has been successfully proven in the woodchuck model for chronic woodchuck hepatitis virus (WHV) infection.3,4

Interleukin-1 receptor/Toll-like receptor (IL-1R/TLR) signaling is generally important for host immune responses against microbial infections and vaccine efficacy. For example, MyD88 was shown to be required for efficient pathogen clearance by inducing IFN-γ response or chemokines, which attract neutrophils, during both Mycobacterium avium and Escherichia coli infection.5,6 In another study, MyD88 was shown to play an important role in the induction of bacteria-specific memory CD8+ T-cell responses during Listeria infection.7 The effectiveness of DNA vaccination generally requires the function of TLR98,9 and vaccines with specific adjuvants based on monophosphoryl lipid A or flagellin TLR4 and TLR5, respectively.10,11 MyD88 was shown to be essential for the induction of efficient CD8+ T-cell responses by a lipopeptide
recombinant adenovirus prime/boost vaccine against herpes virus infection.15

Although the central role of adaptive immunity in controlling HBV infection is well established, the contribution of innate immunity in this regard remains largely unexplored. Recent studies have indicated that TLR-mediated innate immune responses contribute directly or indirectly to hepadnaviral replication regulation in both hepatocytes and animal models.13–17 Activation of TLR signaling pathways leads to the induction of type I interferons (IFNs) and inflammatory cytokines to trigger intracellular signaling pathways, which have been shown to inhibit hepadnaviral replication both in vitro or in vivo.13–17 As a bridge between innate and adaptive immunity, TLRs might play an important role in the induction of specific immune responses to HBV infection and HBV clearance.18,19 For example, TLR2 is expressed in activated and memory CD4+ and CD8+ T cells and may serve as a co-stimulatory molecule to enhance T-cell proliferation, survival and effector functions.20,21 Accordingly, the application of a TLR2 ligand into mice together with transferred tumor antigen (Ag)-specific CD8+ T cells resulted in increased efficacy in tumor models compared to that resulting from the application of CD8+ T cells alone.22 It has been shown that TLR2 engagement in CD8+ T cells increased T-bet transcription in a MyD88-Akt-mTOR- and protein kinase C-dependent manner.23 However, the importance of TLR functions for specific T-cell responses to HBV remains unclear. Accumulating evidence supports the hypothesis that HBV may interfere with TLR function and related IFN action. It has been consistently shown that TLR expression and function are reduced during HBV infection.15,24–26 Recently, it was shown that HBV induces the host factor RUN domain Beclin-1-containing (Rubicon) protein to antagonize IFN signaling and facilitate its own replication.27 However, no data have answered the question of whether reduced innate immunity impairs the induction of HBV-specific immune responses and HBV clearance. Here we examined HBV clearance and HBV-specific T-cell responses after hydrodynamic injection (HI) of an HBV infectious clone, pSM2, into wild-type mice (WT) and a panel of mouse strains with IL-1R/TLR signal pathway deficiency. Our results demonstrate that the IL-1R/TLR signaling pathway might be required for the priming of functional HBV-specific CD8+ T-cell responses in the HI model and for the suppression of HBV replication with subsequent HBV clearance.

MATERIALS AND METHODS

Mice
C57BL/6 WT mice 6–8 weeks of age were purchased from Harlan Winkelman Laboratories (Borchen, Germany). Thr2−/−, Thr23479−/−, 3d/Thr24−/−, Asc−/−, Myd88/Trif−/− and Irak4−/− mice on the C57BL/6 background were bred at the Institute of Medical Microbiology of the University Hospital of Essen. All animals were maintained according to the guidelines of the animal facility at the University Hospital of Essen. All the experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the district Government of Düsseldorf, Germany.

Plasmid and peptides
A replication-competent HBV clone harboring a head-to-tail tandem dimeric HBV genome, pSM2 (GenBank accession number: V01460), was provided by Dr Hans Will (Heinrich-Pette-Institute, Hamburg, Germany) and used previously in our lab.28 The peptide used in this study consisted of the K9-restricted HBV Env190–197 epitope (VWLSVIWM) and the K9-HBV Cov93–100 epitope (MGLKFQRL). The peptides and the synthetic lipopeptide Pam3CSK4 (TLR2/TLR1 ligand) were purchased from EMCE Microcollections (Tübingen, Germany).

HI in mice
Male C57/BL6 mice and Tlr2−/−, Tlr23479−/−, 3d/Thr24−/−, Asc−/−, Myd88/Trif−/− and Irak4−/− mice 8 weeks of age were hydrodynamically injected with 10 μg of pSM2 in a volume of phosphate-buffered saline (PBS) solution equivalent to 0.1 ml/g of their body weight through their tail veins within 8 s.29 The results of HI may be influenced by some factors, and therefore have significant inter-assay variation. Consequently, there are some differences between the WT control mice from experiment to experiment. For this reason, we used the control WT mice with each strain of deficient mice in parallel. The interpretation of a given experiment should be compared with the WT control group.

Isolation of lymphocytes from the blood, spleen and liver
Murine peripheral blood lymphocytes (PBLs) were isolated after lysis of red cells using erythrocyte lysis buffer (Qiagen, Hilden, Germany). Preparation of single cell suspensions of murine splenocytes (SPLs) was performed according to a protocol described previously.30 Mouse intrahepatic lymphocytes (IHLs) were isolated as described previously.31 In brief, the mouse liver was perfused immediately with 10 ml of PBS after killing. After perfusion, the liver was homogenized and digested with an enzyme solution containing 0.05% collagenase type IV (Sigma-Aldrich, St Louis, MO, USA), 0.002% DNAase I (Sigma-Aldrich) and 10% fetal bovine serum for 30 min. After digestion, the pellet was resuspended in 40% Percoll and centrifuged at 1000 g without the breaks. After removing the debris and hepatocytes from the top layer, the IHLs in the pellet were collected, washed and subjected to further analysis.

Cell surface and intracellular cytokine staining of murine lymphocytes
Up to 1 × 10^6 isolated PBLs, SPLs and IHLs per well were plated in 96-well plates in 200 μl of complete RPMI 1640 medium. The cells were stimulated for 5 h at 37 °C with the selected CD8+ T-cell epitope peptide at a final concentration of 2 μg/ml in the presence of 2 μg/ml anti-CD28 antibody (clone 37.51; BD Pharmingen, Heidelberg, Germany) and 5 μg/ml brefeldin A (Sigma-Aldrich). Unstimulated cells and cells stimulated with the cytomegalovirus-derived peptide (YILEETSVM) served as negative controls. The cells were then
Preparation of the peptide-loaded dimer and dimer staining
To stain the CD8+ T cells specific to the K b-restricted HBV
Env190–197 and Cox79–100 epitopes, recombinant soluble dimeric
mouse H-2K[b]: Ig fusion proteins (DimerX I, BD Bioscience)
were loaded with the respective peptides overnight and then
incubated with CD16/CD32 rat anti-mouse antibody (clone 2.4G2; BD Pharmingen) to
block FcRs. Then, the cells were stained with anti-CD8, 7AAD
and anti-PD-1 (clone J43; BD Pharmingen). After washing,
dimer staining was performed by incubating the dimer
for 1.5 h at 4 °C. The cells were then washed and
incubated with an anti-IgG1 antibody (clone 85.1; eBioscience)
for 30 min at 4 °C. The stained samples were run on a
FACSCalibur (Becton Dickinson) or NAVIOS Flow Cytometer
(Beckman Coulter GmbH). The data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Detection of serum HBV antigen and HBV DNA
The serum HBsAg and HBeAg levels were determined using the Architect System and HBsAg and HBeAg CMIA kits
(Abbott Laboratories, Wiesbaden-Delkenheim, Germany)
according to the manufacturer’s instructions. Serum HBV
DNA was extracted using the QiAamp DNA Blood Mini kit
(Qiagen) and quantified by real-time PCR using a Platinum
SYBR Green Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) as described.28

Statistical analyses
Statistical analyses were performed using GraphPad Prism
software version 5 (GraphPad Software Inc., San Diego, CA, USA).
Statistical differences were analyzed by the unpaired
Student t-test and the Mann–Whitney test. P-values <0.05
were considered significant, and asterisks mark significant
differences between the different groups (*indicates P<0.05,
**indicates P<0.01, ***indicates P<0.001).

RESULTS
Deficiency in the IL-1R/TLR signaling pathway led to higher
HBV replication and gene expression levels and partly
delayed HBV clearance in the HI mouse model
To investigate the role of innate immunity in HBV clearance,
we compared HBV gene expression and replication in WT,
Th2−/−, Th23479−/−, 3d/Th24−/− and Asc−/− mice after HI of
the HBV infectious clone pSM2. The 3d/Th24−/− mice lack
TLR2 and TLR4 expression and express a neutralizing mutant of
UNC93B1 (3D), which is involved in endoplasmic reticulum-endosome trafficking of endosomal TLRs, such as
TLRs 3, 7, 9, 11, 12 and 13.32 Apoptosis-associated speck-like
protein containing a CARD gene deficient (Asc−/−) mice lack an
adapter protein central to the numerous nucleotide-binding
domains and leucine-rich repeat receptor (NLR) encompassing
inflammasomes, such as absent in melanoma 2 (AIM2)-like
receptor inflammasomes.33 WT mice cleared HBsAg and
HBeAg from the blood on days 14 and 21 after HI, respectively.
Compared to WT mice, the levels of HBsAg, HBeAg and HBV
DNA were significantly higher in the Th2−/− mice on days 4
and 7 after HI (Figures 1a and g). However, the Th2−/− mice
cleared HBV markers from peripheral blood with a kinetic
comparable to that of the controls. Thus, TLR2 contributed to
the control of HBV replication but was not essential for viral
clearance in the mice. In contrast, HBsAg clearance was delayed
in the Th23479−/− (Figure 1b) and 3d/Th24−/− mice (Figure 1c)
compared to WT mice, demonstrating the relevance of the TLR
signaling pathway for HBV clearance in this model. The Asc−/−
mice cleared HBsAg and HBeAg from the peripheral blood
with the same kinetics as WT mice (Figure 1d), suggesting that
apoptosis-associated speck-like protein containing a CARD
gene (ASC)-dependent inflammasomes are not involved in
the control of HBV replication. In general, the serum HBeAg
levels were low in comparison with the HBsAg levels in the
mouse HI model. Thus, the serum HBeAg level is not a
sensitive marker and can only be used qualitatively for
monitoring HBV infection levels. We compared the HBeAg
levels between Th23479−/−, 3d/Th24−/− and WT mice and
found that Th23479−/− and 3d/Th24−/− mice had higher
HBeAg levels than WT mice (Figure 1). However, this
difference was not statistically significant due to the low overall
levels of HBeAg.

Next, we asked whether the TLR signaling pathways directly
influenced HBV clearance in the HI model. MyD88, TRIF and
IRAK4 are essential mediators of IL-1R/TLR signaling. Thus,
we examined HBV gene expression and replication in Myd88/
Trif−/− and Irrak4−/− mice. Although WT mice cleared HBsAg
and HBV DNA 14 days after HI, in both the Myd88/Trif−/−
and Irrak4−/− mice, the HBsAg and HBV DNA levels in the
serum remained positive for more than 28 days after HI
(Figures 1e, f and h). These results implied that the IL-1R/TLR
signaling pathway significantly contributes to HBV clearance.
Nevertheless, HBsAg and HBV DNA became undetectable in
Myd88/Trif−/− and Irrak4−/− mice on day 42 post HI (data not
shown).
Recent studies demonstrated that adaptive immune responses against HBV are required for HBV clearance in the HI mouse model of HBV infection.\textsuperscript{34–36} IL-1R/TLR signaling was shown to be important for the initiation of proper adaptive immune responses.\textsuperscript{19} Therefore, we investigated whether the TLRs affected the adaptive immune responses to HBV after HI in the mice. In naive mice, the TLR deficiency did not affect the CD8\(^+\) and CD4\(^+\) T-cell frequencies or the activation of CD8\(^+\) T cells by anti-CD3 and anti-CD28 antibodies (Supplementary Figures S1A–C). However, CD8\(^+\) T-cell responses to the TLR2 ligand were absent in the Tlr2\(−/−\) mice (Supplementary Figure S1D and data not shown). These results suggested that TLR or TLR signaling deficiency did not lead to the intrinsic defective activation and function of CD8\(^+\) T cells.

HBV-specific CD8\(^+\) T-cell responses after HI were impaired in TLR-deficient mice but not in Asc\(−/−\) mice

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The HBcAg-specific CD8\(^+\) T-cell response has been shown to play a key role in the control of HBV replication and gene expression in the HI model.\textsuperscript{34} Therefore, we analyzed and compared the HBV-specific CD8\(^+\) T-cell responses in the different mouse strains after HI. The frequencies of intrahepatic and splenic HBcAg- and HBsAg-specific CD8\(^+\) T cells were determined by staining with the HBV Core\(93–100\) or Env\(190–197\) peptide-loaded dimer. Because of high background with the HBV Env\(190–197\) dimer staining (Supplementary Figure S2), we only showed the results from HBV Core\(93–100\) dimer staining to describe HBV-specific CD8\(^+\) T cells in the HI mice (Figure 2a).

The HBcAg-specific CD8\(^+\) T-cell response was analyzed by monitoring the frequencies of IFN-\(γ\)-, TNF-\(α\)- and IL-2-producing CD8\(^+\) T cells after \textit{ex vivo} restimulation with specific HBV peptides (Figure 2b). Our previous study assessed a very low level of HBV-specific CD4\(^+\) T-cell response, with a frequency usually $<0.1\%$ of the CD4\(^+\) T-cell population in

\textbf{Figure 1} IL-1R/TLR signaling pathway deficiency led to higher HBV replication and gene expression levels and partly delayed HBV clearance in the HI mouse model. The HBV plasmid pSM2 or PBS was hydrodynamically injected into WT C57BL/6 mice and Tlr2\(−/−\) (a), Tlr2\(34/39\) (b), 3d/Tlr2\(−/−\) (c), Asc\(−/−\) (d), Myd88/Trif\(−/−\) (e) and Irak4\(−/−\) (f) mice. The control mice received PBS via HI. The serum levels of HBsAg, HBeAg and HBV DNA (g and h) were determined by ELISAs and real-time PCR at the indicated time points after HI. The bars represent the mean values and the standard errors of the means obtained for each group of mice. *\(P<0.05\); **\(P<0.01\). HBV, hepatitis B viral; HI, hydrodynamic injection; IL-1R/TLR, interleukin-1 receptor/Toll-like receptor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; WT, wild type.
the livers of HBV HI mice. Thus, we did not continue the analysis of HBV-specific CD4+ T-cell responses by flow cytometry in this study.

We first compared the HBV-specific CD8+ T-cell response in Tlr2−/− (Figure 3), Tlr23479−/− (Figure 4), 3d/Tlr24−/− (Figure 5) and Asc−/− (Figure 6) mice with that of WT mice. The frequency of HBcAg-specific CD8+ T cells was lower in the livers and spleens of Tlr2−/− mice compared to those of WT mice, but only the difference in the spleen was significant (Figure 3a). The frequency of cytokine-producing CD8+ T cells in the peripheral blood and spleen was also significantly reduced in the Tlr2−/− mice (Figures 3b–d). We further analyzed the multi-functionality of HBV-specific CD8+ T cells regarding their ability to produce single or multiple cytokines (Figures 3e and f). For both the HBsAg- and HBcAg-specific CD8+ T cells, the frequency of double- or triple-positive CD8+ T cells in the Tlr2−/− mice was decreased in the peripheral blood, the spleen and the liver compared to WT mice. Similarly, HBV-specific CD8+ T cells produced less cytokines in the Tlr23479−/− and 3d/Tlr24−/− mice compared to those in WT mice (Figures 4 and 5). Thus, TLR deficiency impaired the adaptive CD8+ T-cell response to HBV. These findings were consistent with higher serum levels of HBsAg and delayed HBsAg and HBeAg clearance in these mice. In contrast, the function of HBV-specific CD8+ T cells was intact in Asc−/− mice, and they cleared HBV with kinetics similar to that of WT mice (Figure 6), indicating that the ASC-dependent inflammasomes are not essential for HBV clearance in the HI mouse model.

We tried to detect HBV-specific CD8+ T-cell responses in deficient and WT mice at earlier time points (days 7 and 14 after HI) to generate data on T-cell priming. HBV-specific CD8+ T-cell responses were not detected in the liver, spleen and blood on day 7 by dimer staining or intracellular cytokine

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**Figure 2** HBV-specific CD8+ T-cell responses in the HBV HI mouse model. Lymphocytes were isolated from the blood, spleens and livers of the mice on day 28 after HI. (a) The specific CD8+ T cells against the HBCAgCor93–100 epitope in the liver and spleen were detected by Cor93–100 peptide-loaded dimer staining. The control mice received PBS for HI. (b) The functionality of the HBV-specific CD8+ T cells was determined by intracellular cytokine staining after ex vivo stimulation with the peptides Env190–197 or Cor93–100 for 5 h. The intrahepatic HBV-specific CD8+ T cells against Cor93–100 from WT, Tlr2−/−, Tlr23479−/−, 3d/Tlr24−/− and Asc−/− mice are shown in the graph. HBV, hepatitis B viral; HI, hydrodynamic injection; PBS, phosphate-buffered saline.
staining. However, on day 14 post HI, HBV-specific CD8+ T-cell responses were detected in the liver and blood but not in the spleen. HBV-specific CD8+ T-cell responses were impaired in Tlr2−/−, Tlr23479−/−, 3d/Tlr24−/−, Myd88/Trif−/− and Irak4−/− mice compared with those of WT mice (data not shown), suggesting that the defect in HBV-specific CD8+ T-cell responses in these IL-1R/TLR signaling-deficient mice may be due to inefficient priming of HBV-specific CD8+ T cells at earlier time points of infection.

Our results implicate that IL-1R/TLR signaling might play an important role in the induction of specific CD8+ T-cell responses against HBV and mediate HBV clearance. However, HBV-specific CD8+ T cells accumulated in the liver of Tlr2−/− mice despite reduced numbers of T cells in peripheral organs, which might explain why HBV clearance still occurred in the Tlr2−/− mice within 14 days (Figure 3).

Figure 3 Comparison of HBV-specific CD8+ T-cell responses in WT and Tlr2−/− mice after HI. The HBV plasmid pSM2 was hydrodynamically injected into WT C57BL/6 and Tlr2−/− mice. The WT mice received PBS as a control. Lymphocytes were isolated from the blood, spleens and livers of the mice on day 21 after HI. The specific CD8+ T cells against the HBCAg Cor93-100 epitope in the liver and spleen were detected by Cor93 peptide-loaded dimer staining. (a) The functionality of HBV-specific CD8+ T cells was determined by intracellular cytokine staining for IFN-γ, TNF-α and IL-2 after in vitro stimulation with peptides Env190-197 and Cor93-100 for 5 h (b-d). The multi-functionality of specific CD8+ T cells to Env190-197 (e) and Cor93-100 (f) was judged by the percentage of single-, double- and triple-positive CD8+ T cells for the cytokines IFN-γ, TNF-α and IL-2. The bars represent the mean values and the standard errors of the means. *P<0.05; **P<0.01. HBV, hepatitis B viral; HI, hydrodynamic injection; PBS, phosphate-buffered saline; WT, wild type.

Impaired HBV-specific CD8+ T-cell responses in Myd88/Trif−/− and Irak4−/− mice were associated with decreased T-cell function and higher PD-1 expression

HBV clearance in Myd88/Trif−/− and Irak4−/− mice after HI was delayed compared to that in WT, Tlr2−/−, Tlr23479−/− and 3d/Tlr24−/− mice, as shown in the previous section. Thus, HBV-specific CD8+ T cells may also be functionally impaired in Myd88/Trif−/− and Irak4−/− mice after HI. The frequency of HBCAg-specific CD8+ T cells in the liver was equal in WT, Myd88/Trif−/− and Irak4−/− mice (Figure 7a). However, HBV-specific CD8+ T cells in the peripheral blood, spleen and liver were functionally impaired and produced less cytokines, including IFN-γ, TNF-α and IL-2, in both the Myd88/Trif−/− and Irak4−/− mice compared with those of WT mice (Figures 7b–d). Multi-functional analysis of both HBsAg- and HBCAg-specific CD8+ T cells showed that
the frequency of double- or triple-positive CD8+ T cells in Myd88/Trif−/− and Irak4−/− mice was decreased in the peripheral blood, the spleen and the liver compared to WT mice (Supplementary Figure S3). These results imply that HBV-specific CD8+ T cells were functionally impaired in the absence of MyD88/TRIF and IRAK4, consistent with the delayed clearance of HBV in Myd88/Trif−/− and Irak4−/− mice.
Recent studies have suggested that PD-1 is involved in the induction and maintenance of CD8+ T-cell tolerance and energy.\(^3\)\(^8\)\(^,\)\(^3\)\(^9\) As shown in Figures 7e–g, most of the intrahepatic HBcAg-specific CD8+ T cells were PD-1 positive in WT, Myd88/Trif\(^{-/-}\) and Irak4\(^{-/-}\) mice. The frequency of PD-1+ HBcAg-specific CD8+ T cells was significantly higher in Irak4\(^{-/-}\) mice but normal in Myd88/Trif\(^{-/-}\) mice compared to WT mice. However, when we analyzed the PD-1 expression at a single cell level, we found that the mean fluorescence intensity of PD-1 was significantly higher in intrahepatic HBcAg-specific CD8+ T cells in the Myd88/Trif\(^{-/-}\) and Irak4\(^{-/-}\) mice compared with that of WT mice.

The functional impairment of HBV-specific CD8+ T cells may due to the insufficient infiltration or activation of antigen-presenting cells (APCs) in the liver in the absence of IL-1R/TLR signaling. Thus, we investigated the frequency and expression of co-stimulatory molecules on the intrahepatic APC-like macrophages, DCs and B cells on day 7 after HI (Supplementary Figure S4). The results showed that there was no significant difference in the APC frequency among the WT, Myd88/Trif\(^{-/-}\) and Irak4\(^{-/-}\) mice (Supplementary Figure S4A). Furthermore, the expression of co-stimulatory molecules, such as CD40, CD80 and MHC I, was nearly equal to that of the intrahepatic APCs from different mice (Supplementary Figures S4B–D). These results indicated that IL-1R/TLR signal pathway deficiency does not result in impaired recruitment and activation of APCs in the liver after HI.

**DISCUSSION**

In this study, we investigated the role of innate immunity in the control of HBV replication in the HBV infection mouse model based on HI. Compared with WT mice, Thr2\(^{-/-}\), Thr23479\(^{-/-}\), 3d/Thr24\(^{-/-}\), Myd88/Trif\(^{-/-}\) and Irak4\(^{-/-}\) mice showed higher serum levels of HBsAg and delayed clearance of HBsAg after HI. Consistently, HBV-specific CD8+ T-cell responses in these mice were reduced and functionally impaired, implying that the IL-1R/TLR signal pathway might play a significant role in the control of HBV replication. Our study suggested that the IL-1R/TLR signaling pathway might significantly contribute to controlling HBV replication, gene expression and HBV clearance. Impairment of HBV-specific CD8+ T-cell responses in Myd88/Trif\(^{-/-}\) and Irak4\(^{-/-}\) mice might enable HBV to replicate at higher levels and markedly delay viral clearance in the host.

Interestingly, although the Thr2\(^{-/-}\) mice had a significantly impaired specific CD8+ T-cell response to HBV, like that of the Thr23479\(^{-/-}\), 3d/Thr24\(^{-/-}\), Myd88/Trif\(^{-/-}\) and Irak4\(^{-/-}\) mice, they could still clear HBV with the same kinetics as WT mice. The major difference in the Thr2\(^{-/-}\) mice and the other IL-1R/TLR signaling-deficient mice was the accumulated frequency of IFN-\(\gamma\)-producing HBV-specific CD8+ T cells in the liver despite reduced frequencies in the peripheral organs compared to that of the intrahepatic APCs from different mice. The results show that the IL-1R/TLR signaling pathway might play a significant role in the control of HBV replication.

**Figure 6** Comparison of HBV-specific CD8+ T-cell responses in WT and Asc\(^{-/-}\) mice after HI. The HBV plasmid pSM2 was hydrodynamically injected into WT C57BL/6 and Asc\(^{-/-}\) mice. Lymphocytes were isolated from the spleens and livers of the mice on day 14 after HI. The specific CD8+ T cells against the HBcAg Cor93–100 epitope in the livers and spleens were detected by Cor93–100 peptide-loaded dimer staining. (a) The functionality of HBV-specific CD8+ T cells was determined by intracellular cytokine staining after in vitro stimulation with peptides Env190–197 and Cor93–100 for 5 h (b–d). HBV, hepatitis B virus; HI, hydrodynamic injection; WT, wild type.
with WT mice. The explanation for this difference would be that IFN-γ-producing CD8+ T cells are critical for HBV clearance, and therefore, sufficient IFN-γ-producing CD8+ T cells were recruited to the liver and produced IFN-γ in Tlr2−/− mice, resulting in HBV clearance. The importance of IFN-γ for HBV clearance in the HI mouse model was demonstrated in our previous study by applying the TLR3 ligand PolyI:C. Tlr2−/− mice could still clear HBV, though with slightly delayed kinetics. Even in the Myd88/Trif−/− and Irak4−/− mice, which completely lack IL-1R/TLR signaling, prolonged HBV replication was found only in some of the mice. Thus, single TLR deficiency, as tested and shown here, had little to no influence on HBV clearance. Therefore, single TLR deficiency does not generally impair the initiation of adaptive immunity.

Innate immune deficiency was found in patients with chronic hepatitis B viral infection. For example, their TLR expression and host cell responsiveness to TLR ligands were decreased. Though TLR-mediated innate immunity may generally not be activated during acute HBV infection, especially in the early phase, it could be supportive for the initiation of an HBV-specific immune response in the host. Reduced TLR expression and impaired TLR signaling during chronic HBV infection likely weakens specific immune responses to HBV.

Using the HBV HI mouse model, Yang et al. first demonstrated that host factors, such as CD4+ and CD8+ T cells, NK cells, Fas, IFN-γ, IFN receptors and TNF receptor 1, are required for elimination of the HBV transcriptional template from the liver. HBcAg93-specific CD8+ T cells are key for mediating HBV clearance from the liver.

Figure 7 Impaired HBV-specific CD8+ T-cell responses in Myd88/Trif−/− and Irak4−/− mice was associated with decreased T-cell function and higher PD-1 expression. (a) Lymphocytes were isolated from the blood, spleens and livers of the mice on day 28 after HI. The specific CD8+ T cells against the HBcAg Cor93−100 epitope in the livers and spleens were detected by Cor93−100 peptide-loaded dimer staining. The control mice received PBS. (b-d) The functionality of HBV-specific CD8+ T cells was determined by intracellular cytokine staining after ex vivo stimulation with peptides Env190−197 and Cor93−100 for 5 h. (e-g) Detection of PD-1 expression on intrahepatic HBcAg-specific CD8+ T cells. *P<0.05; **P<0.01. HBV, hepatitis B viral; HI, hydrodynamic injection; WT, wild type.
Consistently showed that efficient HBV-specific CD8+ T cells play significant roles in the control and clearance of HBV in the HI model. We found that the frequency and functional quality of HBV-specific CD8+ T cells is important for viral control and clearance.

Recently, Tzeng et al. demonstrated that TNF-α but not IFN receptors, RIG-1, MDA-5, MYD88, NLRP3, ASC and IL-1 receptor (IL-1R) is required for HBV clearance using an HBV HI mouse model. IL-1R and MYD88 deficiency did not influence HBV clearance in mice. Despite some of differences in the models, the basic findings are consistent with those of our present study. The previous studies showed that HI of the pAAV/HBV1.2 plasmid led to persistent viral replication in mice. However, the PD-L1 antibody performed a PD-1/PD-L1 pathway blocking experiment with CD8+ T cells from these mice. However, the PD-L1 antibody only limits the characterization of specific T-cell responses in mice, but may also influence the experimental outcomes. For example, Tzeng et al. found that IFN receptor deficiency did not influence the clearance of HBV. However, Yang et al. came to a contradictory conclusion with another HBV clone, as a strong induction of specific T-cell responses was observed.

To answer the question of whether high PD-1 expression in HBV-specific CD8+ T cells contributed to the dysfunction of CD8+ T cells in Myd88/Trif−/− and Irak4−/− mice, we performed a PD-1/PD-L1 pathway blocking experiment with T cells from these mice. However, the PD-L1 antibody treatment did not enhance the function of HBV-specific CD8+ T cells from Myd88/Trif−/− or Irak4−/− mice (data not shown). Thus, PD-1 expression is apparently not the main reason for CD8+ T-cell dysfunction in the knockout mice. Rather, the insufficient priming or helper functions of the HBV-specific CD8+ T cells in Myd88/Trif−/− and Irak4−/− mice might lead to both functional impairment and higher expression of PD-1 in these cells. One must keep in mind that our study was performed during acute infection. While it is well accepted that PD-1 inhibits the function of antigen-specific T cells during chronic viral infection, its role during acute viral infection is less well defined. We have actually shown that PD-1 is an activation marker in CD8+ T cells rather than an exhaustion marker during acute retroviral infection, but it can also contribute to cytotoxic T lymphocyte immune escape. Additionally, one study demonstrated that antigen-specific CD8+ T cells expressed higher levels of PD-1 in the absence of CD4+ T cells during acute infection. Therefore, we suggest that the higher levels of PD-1 expression in HBV-specific CD8+ T cells was attributed to insufficient priming or helper functions in the Myd88/Trif−/− and Irak4−/− mice compared to WT mice, but this is not the mechanistic reason for the CD8+ T-cell dysfunction. The intrahepatic activation and function of APC-like macrophages, DCs and B cells are intact in the Myd88/Trif−/− and Irak4−/− mice compared to WT mice. This implied that the insufficient priming of HBV-specific CD8+ T cells was not due to activation of the APCs. However, Myd88/Trif and IRAK4 deficiency might lead to the impaired induction of inflammatory cytokines, such as IL-1β and IL-18, and other genes relevant for adaptive immunity. Some of these cytokines have immunomodulatory properties that can affect T-cell proliferation and function. Deficiency in the IL-1R/TLR signaling pathway changes these innate immune effector functions, likely impacting T-cell responses.

Our novel findings might add some evidence for the importance of innate immunity in the host immune control of HBV infection. HBV has been initially described as a stealth virus, as no pronounced innate immune responses were found in the livers of HBV-infected chimpanzees during the early phase of acute HBV infection. However, recent studies have demonstrated the activation of host innate immunity by HBV or WHV to a measurable extent in humans and cultured cells. It will be important to elucidate the role of innate immune responses for HBV clearance in such experimental systems of natural infection.

According to the data available, there is no doubt that TLR signaling contributes to HBV clearance. Clinical studies have revealed that TLR2/4 and TLR3 signaling are strongly impaired in immune cells from chronically HBV-infected patients, indicating a strong interaction between HBV products and TLR2/4 and TLR3 signaling. Activation of the TLR3 pathway has been shown to induce interferon production and lead to viral inhibition. In the woodchuck model, the expression of TLR2/4 was inversely correlated with viral titers and modulated by antiviral therapy in chronically infected woodchucks. We have also shown that TLR2 activation inhibits hepadnaviral replication in hepatocytes and reverses the tolerogenic properties of liver sinusoidal endothelial cells. Thus, TLR2/4 and TLR3 signaling plays a vital role in HBV control. There is not yet evidence of whether TLR5, TLR7, TLR8 and TLR9 are involved in HBV control. However, our recent study with the TLR9 ligand in the woodchuck model clearly demonstrated that TLR9 activation may contribute to viral inhibition but is insufficient to completely control hepadnaviral infection. TLR7 activation may be a promising way to inhibit HBV replication, though TLR7 signaling is apparently not involved in HBV control and not impaired in patients with chronic HBV infection (Huang et al., manuscript in preparation, personal communication).

Given the importance of TLR-mediated innate immunity for the initiation of specific immune responses, TLR activation may be an additional option for combined immunotherapeutic approaches for the treatment of chronic HBV infection. Several studies have shown that the therapeutic activation of TLR2, TLR7, TLR8 and TLR9 could enhance HBV-specific T- or B-cell responses in the liver and lead to HBV clearance in murine models. More importantly, a TLR7 ligand, GS-9620, has been examined and showed strong antiviral effects against HBV by inducing IFN responses in woodchuck and chimpanzee models. Recently, the TLR8 ligand ssRNA40 was found to selectively activate liver-residential innate immune cells to produce high levels of the antiviral cytokine IFN-γ not only in healthy human livers, but also in chronically HBV- or HCV-infected livers. These studies implied that TLR agonists facilitate the restoration of cellular & molecular immunology.
HBV-specific immune responses and thereby achieve viral clearance in chronically HBV-infected patients.18

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
We thank Thekla Kemper for excellent technical assistance. This work was supported in part by grants from the Deutsche Forschungsgemeinschaft (DFG Transregio TRR60 and GRK1045/2).

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