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RESEARCH ARTICLE

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.

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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 TLR9[8,9] 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

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recombinant adenovirus prime/boost vaccine against herpes virus infection.[12]

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-interacting cysteine-rich-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 Winkelmann Laboratories (Borchen, Germany). *Tlr2*$^{-/-}$, *Tlr23479*$^{-/-}$, *3d/Tlr24*$^{-/-}$, *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 K^b-restricted HBV $Env_{190–197}$ epitope (VWLSVIWM) and the K^b-HBV $Cor_{93–100}$ epitope (MGLKFRQL). The peptides and the synthetic lipopeptide Pam3CSK4 (TLR2/TLR1 ligand) were purchased from EMC Microcollections (Tübingen, Germany).

HI in mice

Male C57/BL6 mice and *Tlr2*$^{-/-}$, *Tlr23479*$^{-/-}$, *3d/Tlr24*$^{-/-}$, *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

incubated for 30 min at 4 °C with the anti-CD8 (clone 56.6-7; BD
Pharmingen) and anti-CD4 (clone L3T4; BD Pharmingen) antibodies and 7-aminoactinomycin D (7AAD) (Becton Dickinson, Heidelberg, Germany) to exclude dead cells. After washing, intracellular cytokine staining was performed according to the manufacturer's instructions using the Cytofix/Cytoperm Plus kit (BD Pharmingen) with the following antibodies: anti-IFN-γ (clone XMG1.2; BD Pharmingen), anti-TNF-α (clone MP6-XT22; eBioscience, Thermo Fisher Scientific, Waltham, MA, USA) and anti-IL-2 (clone JES6-5H4; eBioscience). The stained cells were analyzed on the FACSCalibur (Becton, Dickinson, Heidelberg, Germany) or NAVIOS Flow Cytometer (Beckman Coulter, Brea, CA, USA). The data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Preparation of the peptide-loaded dimer and dimer staining
To stain the $CD8^+$ T cells specific to the K^b-restricted HBV $Env_{190–197}$ and $Cor_{93–100}$ epitopes, recombinant soluble dimeric mouse H-2K[b]: Ig fusion proteins (DimerX I, BD Bioscience) were loaded with the respective peptides overnight and then used to stain mouse lymphocytes according to the technical instructions. The cells were first 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 and cells 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. The percentage of specific $CD8^+$ T cells in the liver was calculated based on the percentage of $dimer^+$ $CD8^+$ T cells within the $CD8^+$ T-cell population of viable lymphocytes recovered from each liver.

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, *Tlr2*$^{-/-}$, *Tlr23479*$^{-/-}$, *3d/Tlr24*$^{-/-}$ and *Asc*$^{-/-}$ mice after HI of the HBV infectious clone pSM2. The 3d/Tlr24$^{-/-}$ 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 *Tlr2*$^{-/-}$ mice on days 4 and 7 after HI (Figures 1a and g). However, the *Tlr2*$^{-/-}$ 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 *Tlr23479*$^{-/-}$ (Figure 1b) and *3d/Tlr24*$^{-/-}$ 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 *Tlr23479*$^{-/-}$, *3d/Tlr24*$^{-/-}$ and WT mice and found that *Tlr23479*$^{-/-}$ and *3d/Tlr24*$^{-/-}$ 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 *Irak4*$^{-/-}$ mice. Although WT mice cleared HBsAg and HBV DNA 14 days after HI, in both the *Myd88/Trif*$^{-/-}$ and *Irak4*$^{-/-}$ 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 *Irak4*$^{-/-}$ mice on day 42 post HI (data not shown).

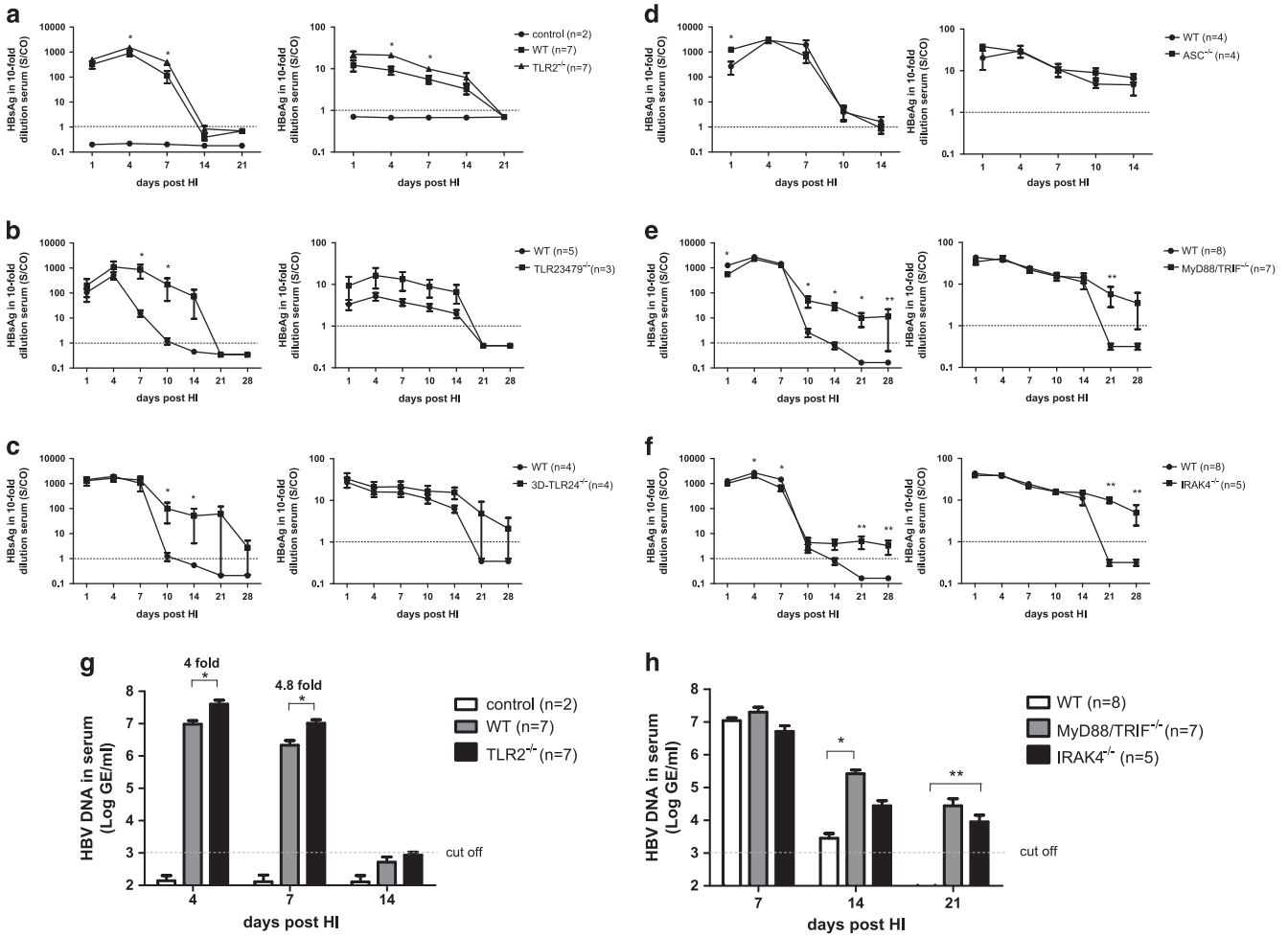

Figure 1 IL-1R/TLR signaling pathway deficiency led to higher HBV replication and gene expression levels and partly delayed HBV clearance in the HI mouse model. The HBV plasmid pSM2 or PBS was hydrodynamically injected into WT C57BL/6 mice and *Tlr2*$^{-/-}$ (**a**), *Tlr23479*$^{-/-}$ (**b**), *3d/Tlr24*$^{-/-}$ (**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.

HBV-specific CD8$^+$ T-cell responses after HI were impaired in TLR-deficient mice but not in *Asc*$^{-/-}$ mice

Recent studies demonstrated that adaptive immune responses against HBV are required for HBV clearance in the HI mouse model of HBV infection.[34–36] IL-1R/TLR signaling was shown to be important for the initiation of proper adaptive immune responses.[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.

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.[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 $Cor_{93–100}$ 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 function of HBV-specific CD8$^+$ T cells was analyzed by monitoring the frequencies of IFN-γ-, TNF-α- and IL-2-producing CD8$^+$ T cells after *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

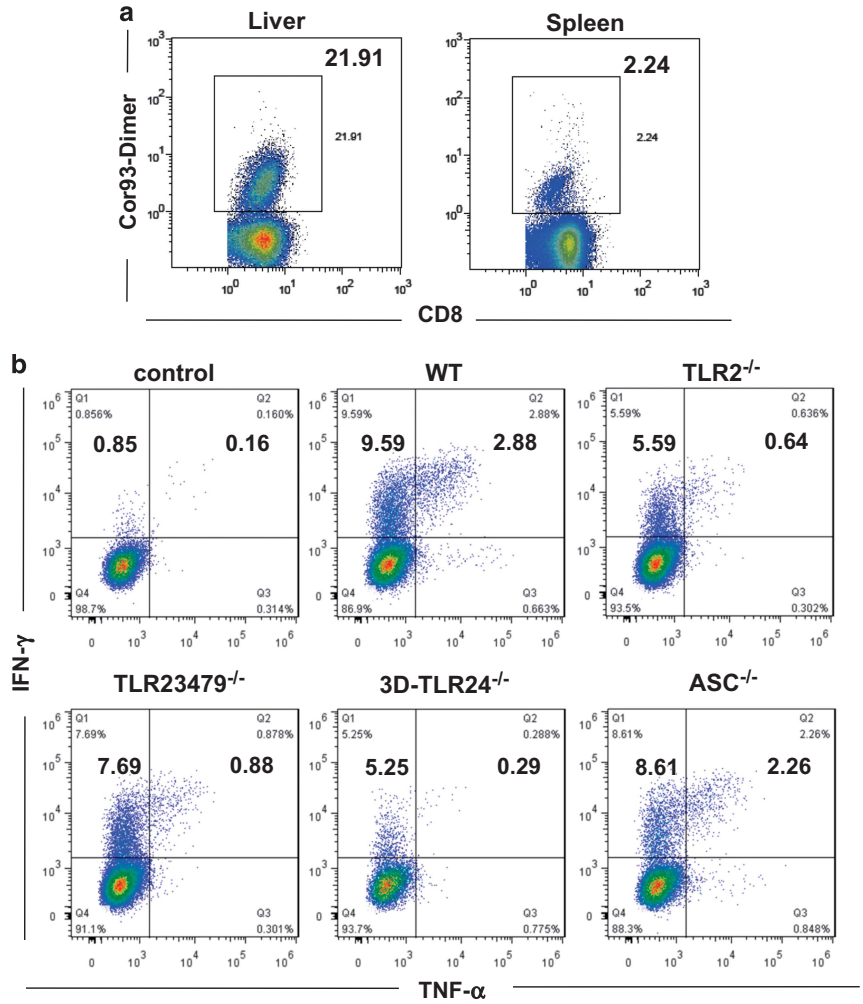

Figure 2 HBV-specific CD8$^+$ T-cell responses in the HBV HI mouse model. Lymphocytes were isolated from the blood, spleens and livers of the mice on day 28 after HI. (**a**) The specific CD8$^+$ T cells against the HBcAgCor$_{93–100}$ epitope in the liver and spleen were detected by Cor$_{93–100}$ peptide-loaded 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 Env$_{190–197}$ or Cor$_{93–100}$ for 5 h. The intrahepatic HBV-specific CD8$^+$ T cells against Cor$_{93–100}$ 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.

the livers of HBV HI mice.[37] 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

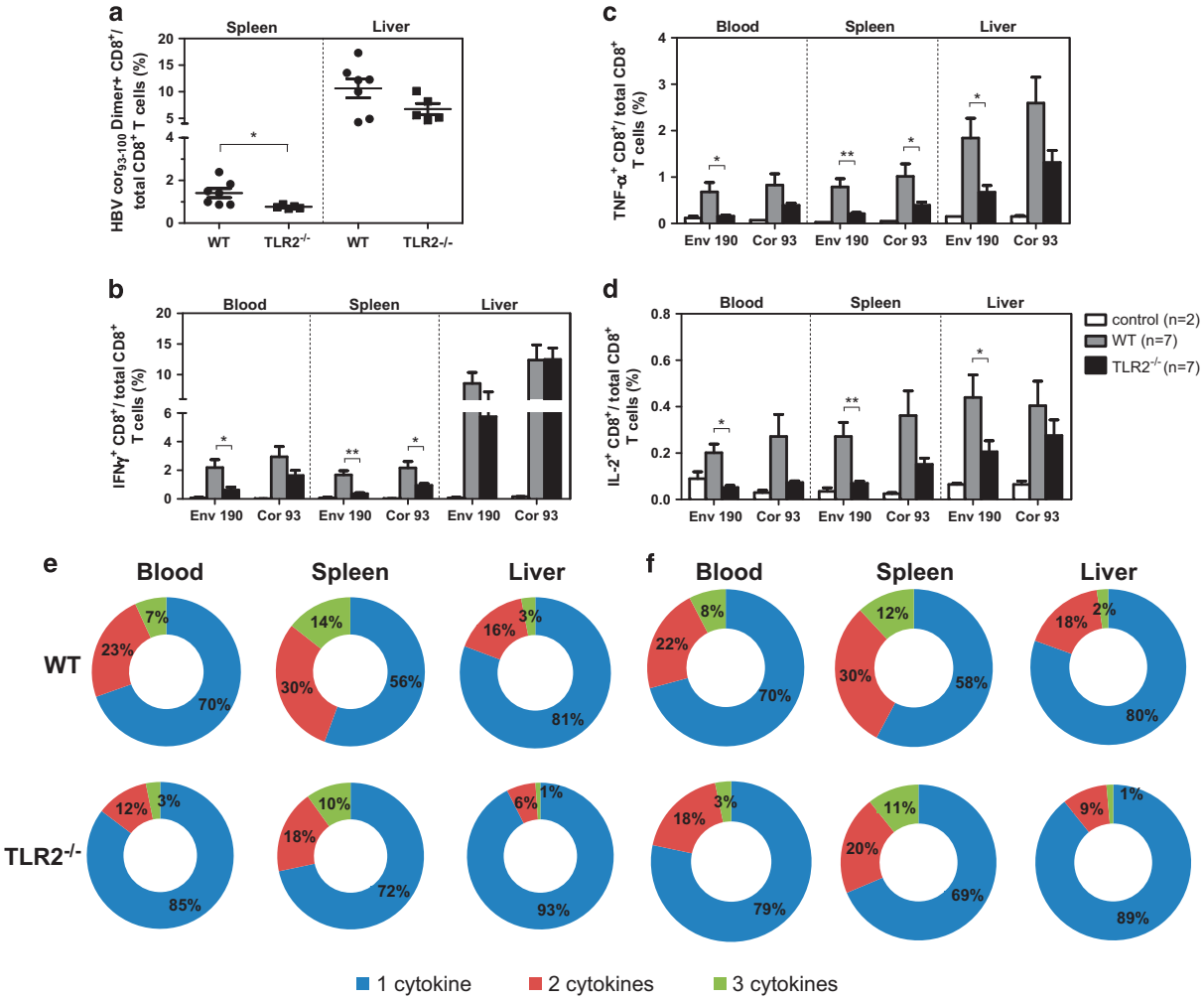

Figure 3 Comparison of HBV-specific CD8$^+$ T-cell responses in WT and *Tlr2*$^{-/-}$ mice after HI. The HBV plasmid pSM2 was hydrodynamically injected into WT C57BL/6 and *Tlr2*$^{-/-}$ mice. The WT mice received PBS as a control. Lymphocytes were isolated from the blood, spleens and livers of the mice on day 21 after HI. The specific CD8$^+$ T cells against the HBcAg Cor_{93-100} epitope in the liver and spleen were detected by Cor_{93-100} 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 $Env_{190-197}$ and Cor_{93-100} for 5 h (**b**–**d**). The multi-functionality of specific CD8$^+$ T cells to $Env_{190-197}$ (**e**) and Cor_{93-100} (**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.

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).

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

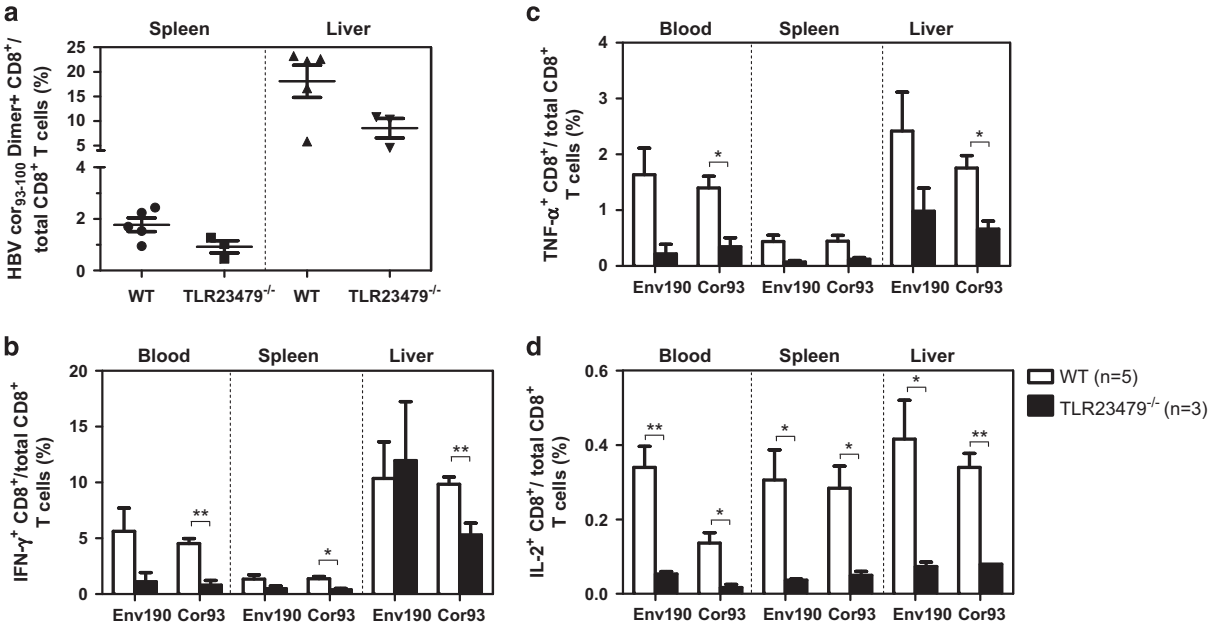

Figure 4 Comparison of HBV-specific CD8+ T-cell responses in WT and *Tlr23479*$^{-/-}$ mice after HI. The HBV plasmid pSM2 was hydrodynamically injected into WT C57BL/6 mice and *Tlr23479*$^{-/-}$ mice. Lymphocytes were isolated from the blood, spleens and livers of the mice on day 28 after HI. The specific CD8+ T cells against the HBcAg $Cor_{93–100}$ epitope in the liver and spleen were detected by $Cor_{93–100}$ 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 $Env_{190–197}$ and $Cor_{93–100}$ for 5 h (**b**–**d**). HBV, hepatitis B viral; HI, hydrodynamic injection; WT, wild type.

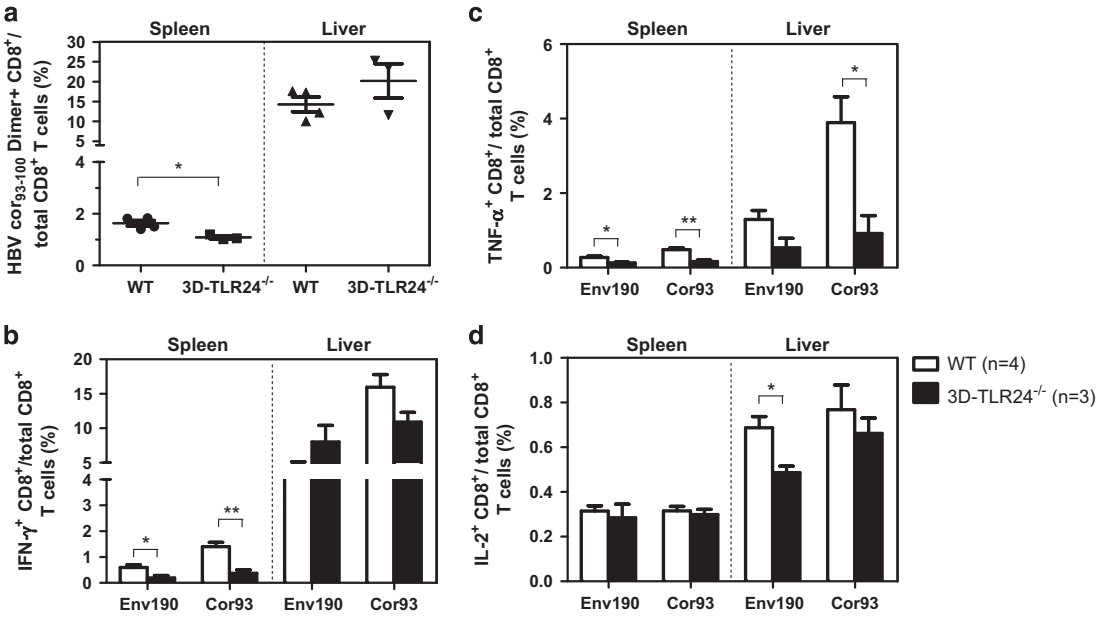

Figure 5 Comparison of HBV-specific CD8+ T-cell responses in WT and *3d/Tlr24*$^{-/-}$ mice after HI. The HBV plasmid pSM2 was hydrodynamically injected into WT C57BL/6 and *3d/Tlr24*$^{-/-}$ mice. Lymphocytes were isolated from the spleens and livers of the mice on day 28 after HI. The specific CD8+ T cells against the HBcAg $Cor_{93–100}$ epitope in the liver and spleen were detected by $Cor_{93–100}$ 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 $Env_{190–197}$ and $Cor_{93–100}$ for 5 h (**b**–**d**). HBV, hepatitis B viral; HI, hydrodynamic injection; WT, wild type.

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.

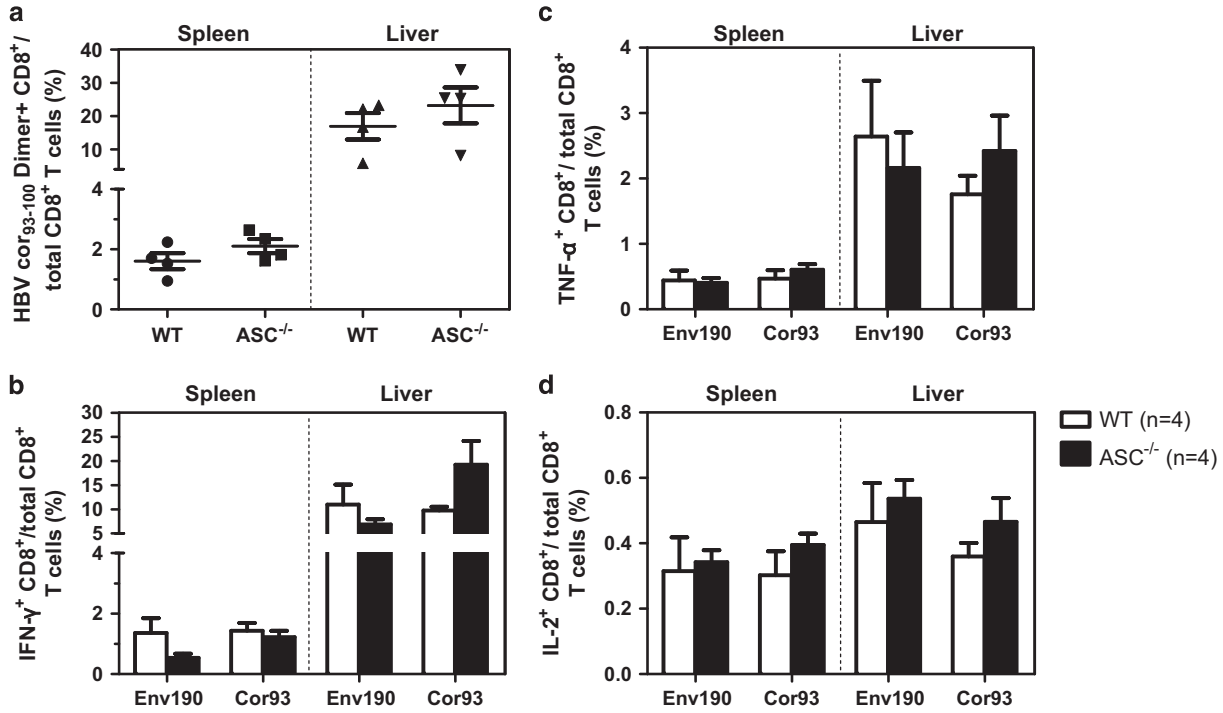

Figure 6 Comparison of HBV-specific CD8+ T-cell responses in WT and *Asc*$^{-/-}$ mice after HI. The HBV plasmid pSM2 was hydrodynamically injected into WT C57BL/6 and *Asc*$^{-/-}$ mice. Lymphocytes were isolated from the spleens and livers of the mice on day 14 after HI. The specific CD8+ T cells against the HBcAg Cor_{93-100} epitope in the livers and spleens were detected by Cor_{93-100} 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 $Env_{190-197}$ and Cor_{93-100} for 5 h (**b**–**d**). HBV, hepatitis B viral; HI, hydrodynamic injection; WT, wild type.

Recent studies have suggested that PD-1 is involved in the induction and maintenance of CD8+ T-cell tolerance and energy.[38,39] 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, *Tlr2*$^{-/-}$, *Tlr23479*$^{-/-}$, *3d/Tlr24*$^{-/-}$, *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 *Tlr2*$^{-/-}$ mice had a significantly impaired specific CD8+ T-cell response to HBV, like that of the *Tlr23479*$^{-/-}$, *3d/Tlr24*$^{-/-}$, *Myd88/Trif*$^{-/-}$ and *Irak4*$^{-/-}$ mice, they could still clear HBV with the same kinetics as WT mice. The major difference in the *Tlr2*$^{-/-}$ mice and the other IL-1R/TLR signaling-deficient mice was the accumulated frequency of IFN-γ-producing HBV-specific CD8+ T cells in the liver despite reduced frequencies in the peripheral organs compared

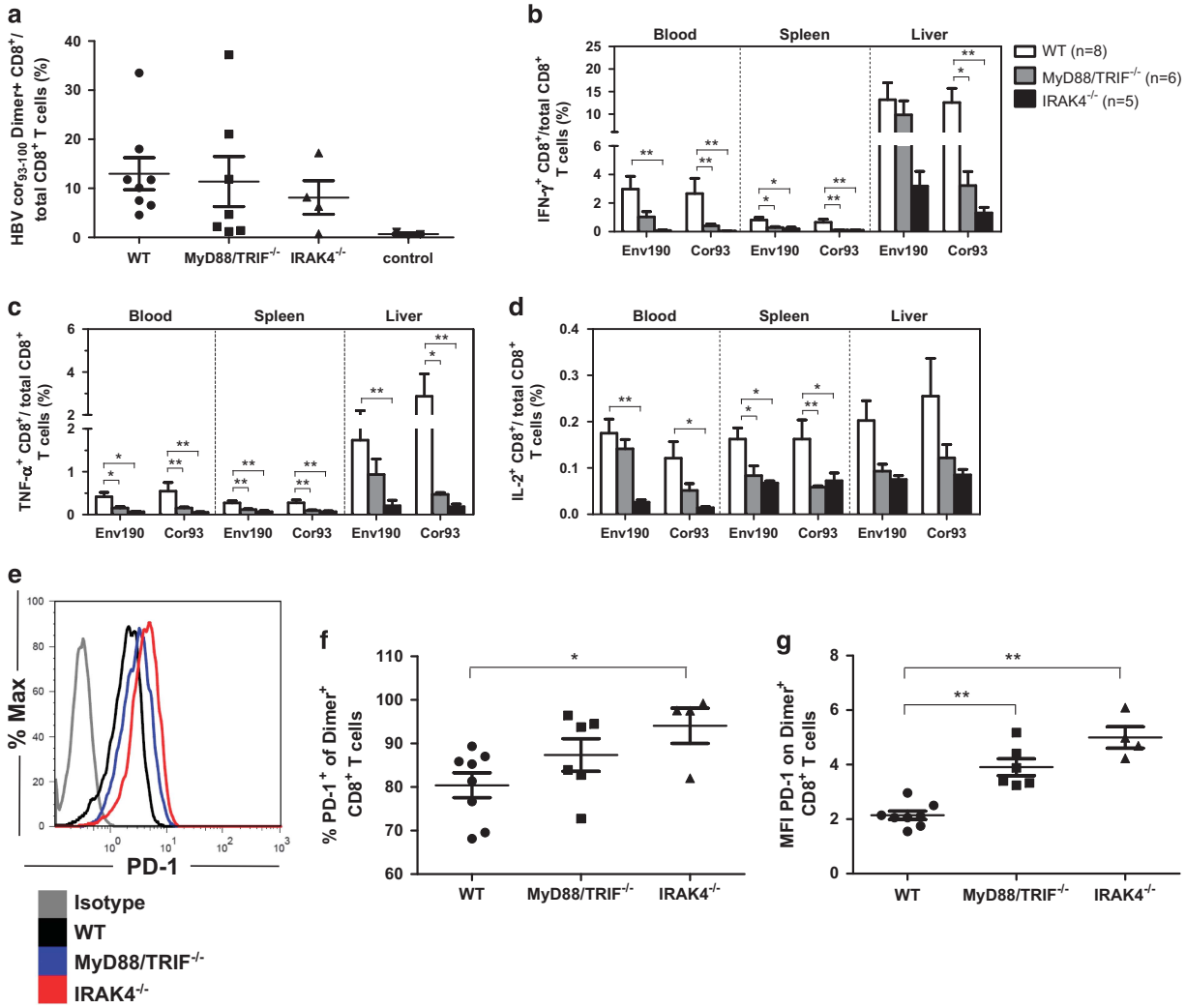

Figure 7 Impaired HBV-specific CD8+ T-cell responses in *Myd88/Trif*$^{-/-}$ and *Irak4*$^{-/-}$ mice was associated with decreased T-cell function and higher PD-1 expression. (**a**) Lymphocytes were isolated from the blood, spleens and livers of the mice on day 28 after HI. The specific CD8+ T cells against the HBcAg $Cor_{93–100}$ epitope in the livers and spleens were detected by $Cor_{93–100}$ 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 $Env_{190–197}$ and $Cor_{93–100}$ 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.

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.[40] *Tlr23479*$^{-/-}$ 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.[24–26] 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.[41,42] 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.[34,43] Our study

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.*[44] 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 of some 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 and resulted in a weak T-cell response, which was only detectable by the ELISpot assay and not by flow cytometry.[40,44] This not 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.*[34] 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,[45] but it can also contribute to cytotoxic T lymphocyte immune escape.[46] 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.[47] 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.[41] However, recent studies have demonstrated the activation of host innate immunity by HBV or WHV to a measurable extent in humans,[42,48] woodchucks[49] and cultured cells.[50,51] 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,[25,52] 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.[14] In the woodchuck model, the expression of TLR2/4 was inversely correlated with viral titers and modulated by antiviral therapy in chronically infected woodchucks.[15] We have also shown that TLR2 activation inhibits hepadnaviral replication in hepatocytes [53] and reverses the tolerogenic properties of liver sinusoidal endothelial cells.[54] 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 not sufficient to completely control hepadnaviral infection.[55] 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.[40,56–58] 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.[16,59] 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.[60] These studies implied that TLR agonists facilitate the restoration of

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.

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