Lipopolysaccharide-induced innate immune responses in primary hepatocytes downregulates woodchuck hepatitis virus replication via interferon-independent pathways

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Summary

Our previous studies have shown that Toll-like receptor (TLR) ligands, Poly I:C and lipopolysaccharide (LPS), are able to activate non-parenchymal liver cells and trigger the production of interferon (IFN) to inhibit hepatitis B virus replication in vivo and in vitro. However, little is known about TLR-mediated cellular responses in primary hepatocytes. By the model of woodchuck hepatitis virus (WHV) infected primary woodchuck hepatocytes (PWHs), Poly I:C and LPS stimulation resulted in upregulation of cellular antiviral genes and relevant TLRs mRNA expression respectively. LPS stimulation led to a pronounced reduction of WHV replicative intermediates without a significant IFN induction. Poly I:C transfection resulted in the production of IFN and a highly increased expression of antiviral genes in PWHs and slight inhibitory effect on WHV replication. These results indicate that IFN-independent pathways which activated by LPS are able to downregulate hepadnaviral replication in hepatocytes.

Introduction

Hepatitis B virus (HBV) is a major cause of acute and chronic hepatitis. More than 350 million people worldwide are chronically infected with HBV and are at high risk of developing liver cirrhosis and hepatocellular carcinoma (Mailliard and Gollan, 2006). The immunological control of HBV infection was studied extensively in the last two decades (Rehermann and Nascimbeni, 2005, Guidotti and Chisari, 2006). It has been shown that HBV-specific T-cells are essential for the control of primary HBV infection while the chronic course of HBV infection is associated with weak or absent specific T-cell responses to HBV antigens (Guidotti et al., 1999; Thimme et al., 2003). However, the role of innate immune responses in HBV infection is not well understood. Further, it is not yet clear to what extent an activation of the innate immune system responses may contribute to the control of HBV infection.

Toll-like receptors (TLRs) are a group of highly conserved molecules that play a critical role in the recognition of pathogen-associated molecular patterns and in the activation of innate immune responses to infectious agents (Aderem and Ulevitch, 2000). Binding of TLR ligands to their receptors initiates the activation of complex networks of intracellular signal transduction pathways to co-ordinate the inflammatory response (Kawai and Akira, 2006). The important components of these signalling networks are the adaptor proteins MyD88 and TRIF, several protein kinases including ERK, JNK, p38 MAP kinase and PI-3k kinase, and the transcription factors interferon regulatory factor 3/5/7 (IRF3/5/7), nuclear factor kappa B (NF-κB), and activating protein 1. The activation of these transcription factors leads to the induction of type I interferons (IFNs), pro-inflammatory cytokines, or co-stimulatory molecules, which are involved in antiviral responses (Schwabe et al., 2006; Seki and Brenner, 2008).

The interaction of HBV with TLRs is yet to be fully investigated. However, increasing evidence suggests that...
immune mediators such as TLR ligands could successfully induce innate immune responses against HBV (Cooper et al., 2008) or other viral infections (Chua et al., 2008; Zucchini et al., 2008). In HBV-transgenic mice, Isogawa et al. showed that HBV replication could be inhibited by a single intravenous injection of ligands specific for either TLR 3, -4, -5, -7 or -9 within 24 h in a type I IFN-dependent manner (Isogawa et al., 2005). More recently, our group has investigated the activation of non-parenchymal liver cells like sinusoidal endothelial cells (LSECs) and Kupffer cells by TLR ligands. Our results suggest that non-parenchymal liver cells can be activated by TLR 3 and -4 ligands to produce IFN-β and other mediators which can potently suppress HBV replication in the HBV-Met cell system (Wu et al., 2007).

The study of the pathogenesis of HBV has been limited by the lack of available animal models and in vitro cell lines that support HBV infection. The woodchuck hepatitis virus (WHV) is a member of the family Hepadnaviridae. WHV and HBV have high similarities in morphology, genome structure, replication cycle, natural history of infection, etc. (Summers et al., 1978). The woodchuck model has been proven to be an informative model for studies on hepadnaviral infection and pathogenesis and for evaluation of antiviral drugs (Kulkarni et al., 2007; Lu et al., 2007). The primary hepatocytes isolated from WHV chronic infected woodchucks are naturally, persistently infected with WHV, which providing a useful cell model for study of hepadnavirus infection. In the present study, the

TLR expression profile and its mediated innate immune responses in primary woodchuck hepatocytes (PWHs) and their effect on WHV were investigated in detail. It is interesting that TLR4 ligand LPS stimulation can activate IFN-independent signalling pathways, leading to down-regulation of WHV replication in WHV-infected primary hepatocytes.

Results

TLRs expression in PWHs and its modulation by TLR ligands and IFNs

To generate tools for detection and quantification of TLR expression, partial cDNAs of woodchuck TLR3, -4, -7, -8 and -9 were cloned and sequenced. Then, based on the obtained sequences, real-time RT-PCR primers were designed to quantify the mRNA expression of TLRs in WHV non-infected PWHs and in the permanent woodchuck cell line w12/6 (Table 1). As shown in Table 2, TLR4 was highly expressed in PWHs while TLR3 expression was lower but clearly present. However, the expression of TLR7, -8 and -9 was very low or not detectable. The expression levels of TLRs in PWHs was not changed by WHV infection (Fig. S1).

The expression of TLR3 and TLR4 was enhanced upon stimulation with their ligands Poly I:C and LPS in a dose-dependent manner respectively (Fig. 1). An incubation with 2.5 µg/ml and 12.5 µg/ml of Poly I:C led to a 5.3-
and a 10.6-fold increase of TLR3 mRNA and a 2.4- and 3.7-fold increase of TLR4 mRNA respectively (Fig. 1A). A stronger increase of TLR3 and -4 expression up to 100- and 12-fold was measured after Poly I:C transfection respectively (Fig. 1B). Similarly, LPS at concentrations of 2.5 and 10 µg ml⁻¹ enhanced the expression of TLR3 and -4 (Fig. 1C). TLR3 expression was stimulated by IFN-α and to a less extent by IFN-γ while both IFNs stimulated TLR4 expression only marginally (Fig. 1D). The regulation of TLR3 and -4 by TLR ligands and IFNs observed in this study is consistent with findings in other systems like monocytes (Huang et al., 2006). Compared with PWHs, significantly lower levels of TLR3 and -4 were detected in the woodchuck cell line W12/6 (Table 2). Upregulation of TLR3 and -4 by Poly I:C, LPS, and IFNs treatment was similar as in PWHs (Fig. S2).

Table 2. Quantitative real-time RT-PCR analysis of expression of TLRs, ISGs and proinflammatory cytokines in PWH and W12/6 cells.

| Gene name | PWH     | W12/6    |
|-----------|---------|----------|
| TLR3      | 709.59 ± 74.48 | 287.18 ± 35.95 |
| TLR4      | 3730.52 ± 138.29 | 731.82 ± 1.40 |
| TLR7      | 43.46 ± 0.01  | 9.83 ± 0.01  |
| TLR8      | 132.26 ± 9.28 | 33.07 ± 10.22 |
| TLR9      | 50.17 ± 0.13  | ND        |
| MxA       | 3160.59 ± 138.67 | 1815.15 ± 202.56 |
| IP-10     | 1712.50 ± 175.98 | 1693.99 ± 450.67 |
| IFN-β     | 337.90 ± 20.36 | 127.28 ± 1.65 |
| TNF-α     | 110.84 ± 1.93  | 435.21 ± 63.22 |
| IL-1β     | 760.15 ± 44.46 | 1430.33 ± 130.75 |
| IL-6      | 199.25 ± 1.46  | 619.88 ± 37.74 |

The basal expression level of each gene is presented as copies per 10⁶ β-actin transcripts. The detection limit of real-time RT-PCR was at 100 mRNA copies per 100 ng of total RNA. ND, not detectable.

The presence of TLR3 and -4 on PWHs suggested the possibility that TLR ligands may trigger innate immune responses in these cells. Hepatocytes are a major source of chemokines and cytokines in various viral or bacterial infections (Szabo et al., 2007). Therefore, we investigated whether ligands for TLR3 and -4 were able to induce

Poly I:C and LPS triggered expression of interferon-stimulated genes and proinflammatory cytokines in PWHs

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genes related to innate immune responses and proinflammatory cytokines in these cells.

Using real-time RT-PCR, it could be demonstrated that direct stimulation with Poly I:C and LPS induced interferon-stimulated genes (ISGs, MxA and IP10, \( P < 0.05 \)) and cytokines (tumour necrosis factor-\( \alpha \), IL-1\( \beta \), \( P < 0.05 \)) expression, while IFN-\( \beta \) and IL-6 expression was not changed (Fig. 2A and B). The basal levels of these genes expression were listed in Table 2. A specific inhibitor of LPS binding to TLR4 polymyxin B significantly reduced the LPS-induced cytokine expression, indicating the requirement of TLR4 (Fig. S3). In contrast, transfection of PWHs with Poly I:C resulted in significant upregulation of the ISGs (MxA and IP10, \( P < 0.05 \)) as well as cytokines such as IFN-\( \beta \), TNF-\( \alpha \), IL-1\( \beta \) and IL-6 (Fig. 2C, \( P < 0.05 \)). The IFN production in response to Poly I:C was further verified by virus protection assay. The Poly I:C-induced IFN-\( \beta \) production was readily detectable on day 2 but decreased on day 6 (Fig. 2D). Transcripts of IFN-\( \alpha \) were not detected by a RT-PCR protocol using specific primers described previously (Lu et al., 2008a), indicating that the induction of IFN-\( \alpha \) expression did not occur at a significant level (data not shown). The results supported the initial findings that the activation of TLR3 led to significant upregulation of IFNs and ISGs (Fig. 2A–D). As TLR3 is mainly localized in intracellular compartments (Matsumoto et al., 2003), an incubation of PWHs with Poly I:C did not trigger a measurable IFN production (Fig. 2D). Similarly, Poly I:C and LPS were able to induce comparable cellular responses, such as ISGs and cytokines induction in primary mouse hepatocytes (Fig. S4).

Consistently, 3pRNA activates RIG-I leading to vigorous IFN production and significant upregulation of IFNs and ISGs (Fig. 2D and E). However, R848 and CpG failed to induce a noticeable response in PWHs, probably due to the low level expression of the corresponding receptors TLR 7/8 and -9 (Fig. 2D and E, Table 2).

### Lipopolysaccharide treatment reduced WHV replication in PWHs

As Poly I:C and LPS treatment activates innate immune responses in hepatocytes and leads to the induction of various antiviral genes (Liu et al., 2002; Li et al., 2005; Preiss et al., 2008), the antiviral activity of Poly I:C and LPS in PWHs isolated from chronic WHV-infected woodchucks was examined. Incubation with LPS led to suppression of WHV replication in PWHs (Fig. 3A). The amount of WHV replicative intermediates decreased and was reduced to 36% of the untreated controls on day 6 (Fig. 3A, upper panel), while LPS did not reduce the amounts of WHV transcripts in PWHs (Fig. 3A, middle panel), suggesting that it acts against WHV through a post-transcriptional mechanism. The WHV progeny DNA in PWHs cultures was consistently decreased by LPS for at least 6 days (Fig. 3A, lower panel, \( P < 0.05 \)).

Pol I:C and 3pRNA were able to activate the production of IFN, various proinflammatory cytokines when transfected into PWHs (Fig. 2C and D). The amounts of WHV replicative intermediates were slightly reduced in PWHs after Poly I:C and 3pRNA treatment (Fig. 3B, upper panel, and right lower panel of Fig. 3C). However, TLR7/8 ligand R848 and TLR9 ligand CpG were not able to inhibit WHV replication in PWHs (Fig. 3C).

### Upregulation of ISGs is not sufficient to reduce WHV replication

The question is raised whether Poly I:C- or LPS-induced IFN responses and/or ISG upregulation was responsible for reduction of WHV replication in PWHs. To clarify the role of IFNs on WHV in PWHs, PWHs were treated with recombinant woodchuck IFN-\( \alpha \) and -\( \gamma \) at concentrations of 500 U ml\(^{-1}\). MxA and IP10 expression increased strongly while TNF-\( \alpha \) and IL-1\( \beta \) were upregulated only marginally at 6 h (Fig. 4A). Thus, the induction of these antiviral ISGs was not inhibited by the presence of WHV. However, the amount of WHV replicative intermediates in PWHs was only slightly reduced (about 15% compared with controls) and WHV transcripts were not influenced within 6 days by treatment with IFN-\( \alpha \) and -\( \gamma \) (Fig. 4B) which is consistent with results from previous studies (Lu et al., 2002).

### MAPK/ERK and PI-3k/Akt pathways are involved in downregulation of WHV replication by LPS

Next, we addressed the question which signalling pathways are activated by LPS stimulation and are involved in suppression of WHV replication. The NF-\( \kappa B \), MAPK and PI-3k/Akt pathways are known to be activated in response to LPS in both macrophage and non-myeloid cell types, while IRF3 pathway activation occurs at a later phase (Akira and Takeda, 2004). Accordingly, the LPS-induced reduction of WHV replication may be dependent on these signalling pathways. Total cell lysates of PWHs from WHV-infected woodchucks were prepared at various time points following LPS treatment and subjected to Western blotting analysis for phosphorylated ERK1/2, Akt, JNK and p38. As indicated in Fig. 5A, LPS-stimulated PWHs exhibited an increased phosphorylation of ERK1/2 from 5 to 60 min, and phosphorylation of Akt from 15 to 120 min. Phosphorylation of JNK and p38 was relatively lower in hepatocytes compared with ERK1/2 and Akt (Fig. 5A). The ability of LPS to activate IRF3 and NF-\( \kappa B \) in hepatocytes was examined by nuclear translocation of IRF3 and the NF-\( \kappa B \) p65 subunit. The nuclear translocation of the NF-\( \kappa B \) p65 subunit occurred at 30 min after LPS
Fig. 2. Induction of ISGs and proinflammatory cytokines genes in primary hepatocytes treated with TLR ligands and RIG-I ligand 3pRNA. Total RNA were extracted from PWHs treated with different TLR ligands and 3pRNA at indicated concentrations for 6 h. The detection of relevant mRNAs was performed by real-time RT-PCR using the primers listed in Table 1. The basal expression levels of the relevant genes were listed in Table 2.

A. LPS stimulation.
B. Poly I:C stimulation.
C. Poly I:C transfection.
D. IFN production in culture medium was validated by virus protection assay. Supernatants were collected after TLR ligands or 3pRNA treatment in PWHs at indicated time points.
E. MxA and TNF-α induction after R848, CpG and 3pRNA transfection in PWHs.
Fig. 3. Antiviral effect of TLR ligands and 3pRNA in PWHs. PWHs were treated with TLR ligands or 3pRNA at indicated concentrations extent to 6 days. Encapsidated viral DNA and total RNA were prepared. WHV replicative intermediates and transcripts were detected by Southern (upper panel) and Northern (middle panel) blot hybridization analyses respectively. β-Actin mRNA was presented as loading control in the Northern blot (lower panel). WHV progeny DNA in the culture supernatants was quantified by real-time PCR. Relative level of WHV DNA was shown as percentage of the control.
A. LPS stimulation.
B. Poly I:C transfection.
C. Poly I:C stimulation and R848, CpG and 3pRNA transfection. The position of relaxed circular (RC), double stranded linear (DL) and single stranded (SS) DNAs and 3.7 kb and 2.1 kb RNAs were indicated.
stimulation while no IRF3 translocation could be observed (Fig. 5B). Further, ERK1/2 phosphorylation induced by LPS stimulation can be blocked specifically by pre-treatment with the MAPK-ERK pathway inhibitors U0126 and PD98059, not by the p38 and JNK pathway inhibitors SB203580 and SP600125 (Fig. 5C). The specific inhibitory effect of different signalling inhibitors was validated by Western blotting assay (data not shown). Due to the lack of specific antibodies, woodchuck JNK, p38 and Akt basal expression was not detected.

Further, we addressed the question whether the activation of these signalling pathways was essential for the reduction of WHV replication in PWHs by using inhibitors of the JAK-STAT, NF-κB, MAPK and PI-3K pathways. The inhibitors of JAK-STAT and NF-κB pathways showed no effect on LPS-induced ISGs and cytokines expression in hepatocytes. As shown in Fig. 6 (lower panel), AG490 and Bay11-7082 did not affect LPS-induced upregulation of MxA, IP10, TNF-α and IL-1β, and did not block the antiviral effect of LPS. However, the LPS-mediated reduction of WHV replication was abolished by U0126 and Rapamycin treatment (Fig. 6, upper panel) which was accompanied by significant reduction of LPS-induced gene expression in PWHs. These findings suggest that the MAPK/ERK and PI-3K/Akt signalling pathways play an important role in the LPS-mediated suppression of WHV replication in PWHs.

Treatment of unpurified hepatocytes with TLR ligands

Previously, we had shown that Poly I:C and LPS could activate LSECs and Kupffer cell to produce IFN-β and other undefined cytokines and led to suppression of HBV replication in HBV-met cells (Wu et al., 2007). Therefore, we tested whether a co-cultivation of hepatocytes with other cells from liver tissue (no additional washing, a mixture of hepatocytes with other non-parenchymal liver cells) in the presence of Poly I:C and LPS resulted in inhibition of WHV replication. An incubation with Poly I:C and LPS led to a significant reduction of WHV replicative intermediates and WHV progeny DNA on day 4 (Fig. 7A). Upon Poly I:C and LPS stimulation, MxA mRNA expression was increased by 25.1- and 13.1-fold respectively. and an increased expression of IFN-β and TNF-α mRNA was also detected (Fig. 7B).

These results are consistent with the previous findings in the murine model from our group (Wu et al., 2007). Apparently, Poly I:C and LPS were able to stimulate non-parenchymal liver cells, such as LSECs and Kupffer cells, to produce some undefined cytokines and lead to suppression on WHV replication. However, the techniques for isolation of non-parenchymal liver cells from woodchuck livers still need to be established and validated for further detailed analysis in the future.

Knock down of WHV replication by siRNA enhances the expression of TLR3 but not that of TLR4 in PWHs

It has been suggested that HBV infection may influence TLR expression in hepatocytes or other cell types (Visvanathan et al., 2007; Chen et al., 2008). Thus, suppression of WHV gene expression and replication may lead to changes in TLR expression in WHV-infected PWHs. Therefore, we examined whether a knock down of WHV gene expression and replication enhances the expression of TLR3 and -4 in PWHs. Previously, we have shown that the application of siRNAs directed against WHV S and X genes (siWHs and siWHx) led to a reduction of WHV transcripts in PWHs (Meng et al., 2009). An siRNA to the WHV core gene (siWHc) was ineffective to knock down
WHV transcript therefore served as a negative control for further gene silencing experiments. After transfected for 4 days, WHV replication and transcription were reduced to 30% by siWHs and siWHx, at a concentration of 100 nM (Fig. 8A, upper panel), while siWHc was not effective on WHV replication respectively. Similarly, a scrambled siRNA used as a negative control was also unable to reduce WHV replication, consistently with published data (data not shown). The results were consistent with our previous findings (Meng et al., 2009). A significant increase of TLR3 expression up to 3.8- and 12.1-fold occurred in siWHs and siWHx-treated WHV-infected hepatocytes respectively, while TLR4 expression was not changed (Fig. 8A, lower panel).

The control siWHc and scrambled siRNA, however, had no effect on TLR3 expression in WHV-infected PWHs. siRNAs had no effect on TLR3 expression in primary hepatocytes from naïve woodchucks or woodchuck cell line w12/6 (Fig. 8B). These results demonstrated that WHV may suppress TLR expression in PWHs to countermine its antiviral effect.

**Discussion**

In the present study, we demonstrated that ligands of TLR3 and TLR4 are able to activate innate immune responses in PWHs. Treatment of PWHs with LPS resulted in reduced WHV replication while Poly I:C was not effective despite strong induction of IFN production and subsequent stimulation of ISGs. These findings along with the data published by Locarnini’s group and our own group further substantiate the relevance of innate responses in the control of hepadnaviral infection and the potential use of TLR ligands for immunotherapeutic approaches against chronic HBV infection (Isogawa et al., 2005; Wu et al., 2007; Visvanathan et al., 2007).
Previous studies showed that IFNs are involved in the inhibition of HBV replication in HBV-transgenic mice as well as in HBV-transduced hepatoma cell lines (Peltekian et al., 2005; Park et al., 2005; Parvez et al., 2006). In HBV-transgenic mice, IFNs were found to be effective to inhibit HBV replication in vivo (Isogawa et al., 2005) and in vitro (Wu et al., 2007). However, exogenous IFNs added to cultured PWHs were not highly effective to suppress WHV replication despite the upregulation of ISGs (Lu et al., 2002). Likely, primary hepatocytes from chronically infected woodchucks do not respond properly to IFNs because they are exposed continuously to IFNs during the chronic course of WHV infection (Schildgen et al., 2006).

Lipopolysaccharide was able to activate the NF-κB and MAPK pathways and to stimulate the expression of pro-inflammatory cytokines in PWHs. The activation of the MAPK and PI-3K pathways was essential for the antiviral activity as has been shown previously (Chin et al., 2007; Guo et al., 2007). Consistently, the inhibitors of these two pathways abolished the ability of LPS in PWHs to reduce WHV replication. It is likely that IFNs do not play a major role for the antiviral action of LPS, as AG490, an inhibitor of the JAK-STAT pathway, did not reverse the LPS-mediated reduction of WHV replication in PWHs. Recently, Guo et al. showed that an activation of innate responses by introducing adaptor molecules in hepatoma cells could suppress HBV replication (Guo et al., 2009). However, the NF-κB pathway appeared to play a major role in HBV-transduced hepatoma cells (Lin et al., 2009; Guo et al., 2009), in contrast to primary hepatocytes. The reason for this difference needs to be investigated in the future. Taken together, these results suggest that an activation of the IFN signalling pathways does not play a major role in LPS-triggered downregulation of WHV replication.
The reduction of WHV replication by LPS is apparently mediated through a mechanism at the post-transcriptional level because the amount of WHV transcripts remained unchanged by treatment with LPS. In addition, the single stranded form of WHV replicative intermediates was less affected by LPS while the RC form was reduced significantly. Several previous publications suggested that the encapsidation of pregenomic RNAs and subsequent steps of HBV replication may represent the target of antiviral cytokines (McClary et al., 2000). These mechanisms are partly dependent on the action of IFN-γ and TNF-α (Parvez et al., 2006; Puro and Schneider, 2007). It is possible that a similar mechanism is activated in PWHs by LPS.

Previously, we tested the adenoviral vector-mediated expression of IFN-α and -γ in PWHs and in chronically WHV-infected woodchucks (Fiedler et al., 2004). It could be shown that the application of wIFN-α and -γ induced the expression of the marker genes MxA and IP10 respectively. Treatment of PWHs with the adenoviral vector expressing IFN-α led to a significant reduction of WHV replication, while a control adenoviral vector expressing β-galactosidase had no such effect. Recently, several publications indicated that an adenoviral infection of cells itself is able to trigger the activation of innate immune responses (Hartman et al., 2007; Nociari et al., 2007; Zhu et al., 2007; Muruve et al., 2008). Thus, a synergistic effect of activation of innate responses and the IFN signalling pathway might be responsible for the suppression of WHV infection in PWHs. This aspect is now under examination. Data available from our study showed that knock down of WHV transcripts by siRNA led to the upregulation of the TLR 3 expression. It is likely that the cellular innate responses are inhibited by WHV/HBV and therefore a synergistic action is needed to regain their effectiveness.

At the moment, it could not be predicted how TLR ligands may act on the hepadnaviral replication in vivo. Early studies by Korba et al. showed that LPS treatment increases WHV replication in cultured woodchuck peripheral blood cells (Korba et al., 1988; 1989). Thus, careful studies in vivo are needed to verify the findings in vitro.

In conclusion, our data indicate that the LPS-mediated activation of TLR signalling pathways in PWHs is able to reduce hepadnavirus replication which is associated with the induction of proinflammatory cytokines and antiviral genes. The present work demonstrated the usefulness of the woodchuck model for studies on liver-specific innate responses and its potential in future research on pathogenesis of hepadnaviral infections. The findings may lead to identification of new factors contributing to the control of hepadnaviral replication.

**Experimental procedures**

**Reagents**

The ligands for TLR3 (Poly I:C), TLR4 (LPS, from O26:B6 E. coli), JNK-stat pathway inhibitor AG490 and NF-κB pathway inhibitor Bay11-7082 were purchased from Sigma-Aldrich (Steinheim, Germany). The ligands for TLR7/8 (R848) and TLR9 (CpG) were kindly provided by Coley Pharma GmbH (Duesseldorf, Germany). The ERK/MAPK pathway inhibitor U0126 and TLR4

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inhibitor Polymyxin B were purchased from Invivogen (Toulouse, France). The PI-3k/Akt pathway inhibitor Rapamycin was purchased from LC Laboratories (Woburn, MA). RIG-I ligand 3pRNA was generated by in vitro transcription as described before (Hornung et al., 2006). Recombinant wIFN-α and wIFN-γ were generated by eukaryotic expression in baby hamster kidney (BHK) cells as described before (Lu et al., 2002; Lu et al., 2008a).

Woodchucks

All animals were purchased from North-eastern Wildlife (Ithaca, NY) and raised in the local animal facilities. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were reviewed and approved by the local Animal Care and Use Committee (Animal Care Center, University of Duisburg-Essen, Essen, Germany, and the district government of Düsseldorf, Germany). Eight chronic and four naive WHV-infected woodchucks were used for the present study. The serum WHV titres ranged in WHV carrier woodchucks between 10⁷ and 10⁹ genome equivalents/ml, as determined by real-time PCR (detailed information in Table S1).

Isolation of hepatocytes

Primary hepatocytes from eight chronic WHV-infected and four naive woodchucks were prepared by perfusion with collagenase IV (Sigma-Aldrich) according to the published protocols (Baccarani et al., 2003; Wu et al., 2007; Meng et al., 2009). Isolated hepatocytes were washed twice with 50 ml HBSS (PAA, Cölbe, Germany) and collected by centrifugation at 300 r.p.m. (18 g) for 10 min. The viability of hepatocytes exceeded typically 85% as assessed by trypan blue dye exclusion. The purity of hepatocyte cultures exceeded 95% by light microscopy. Each experiment was performed with at least with three different PWH preparations.

Cell culture and treatment

A permanent woodchuck fibroblastoma cell line w12/6 (kindly provided by P. Banasch in German Cancer Research Center DKFZ Heidelberg, Germany) and BHK cells were maintained in Ham’s F12 and Dulbecco modified Eagle medium supplemented with 10% fetal calf serum respectively. Primary hepatocytes (1.5 × 10⁶ per well) were cultured in 6 well plates according to an established protocol (Lu et al., 2002). Hepatocytes were cultured overnight prior to treatment. PWHs were treated with Poly I:C, LPS or other TLR ligands and wIFNs to assess their antiviral effect. Alternatively, Poly I:C was transfected into hepatocytes by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Culture supernatants and cells were collected at various time points. The wIFN levels in culture supernatants of hepatocytes were determined using specific primer sets (Table 1; Biomers, Ulm, Germany), which were designed by online software Primer3 (http://frodo.wi.mit.edu/primer3/input.htm, version 0.3.0). The real-time RT-PCR was performed in a one-step method with 100 ng of total RNA using Quantifast SYBR Green RT-PCR Kit (Qiagen) on a Light CyclerTM (Roche Diagnostics, Mannheim, Germany). Same cycling parameters were used for all genes examined in the study. The cycling parameters were set as followed: reverse transcription at 50°C for 10 min for one cycle, then 45 cycles consisting of denaturation at 95°C for 10 s and combined annealing/extension at 60°C for 30 s. Standard curves for the genes examined were determined using serial dilutions of corresponding DNA fragments amplified by RT-PCR. The relative mRNA copies of each gene were determined with help of the corresponding standard curve. For each sample, RT-PCR was performed in duplicate. The expression levels of each gene are presented as values normalized against woodchuck 10⁶ copies of β-actin transcripts.

Preparation of cell lysates and Western blotting analysis

Cultured hepatocytes were washed once with ice-cold phosphate-buffered saline and lysed in 1× SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue) supplemented with a protease inhibitor cocktail (Roche) and a phosphatase inhibitor cocktail (Sigma-Aldrich). Protein samples were subjected to SDS-PAGE and Western blotting with primary antibodies selectively recognizing phosphorylated forms of JNK (Thr183/Tyr185), Akt (Ser473), p38 MAP kinase (Thr180/Tyr182), and ERK (Thr202/Tyr204) (Cell Signalling Technology, Danvers, MA). To determine the amounts of loaded proteins, blots were stripped and reprobed with anti-ERK (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-β-actin (Sigma-Aldrich) antibodies. For the detection of the nuclear translocation of NF-κB p65 subunit and IRF3, cytoplasmic and nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, regularly controlled by trypan blue staining. TLR ligands tested in the study, particularly LPS and Poly I:C, did not cause cell death in concentrations used in the experiments.

Analysis of intracellular WHV replicative intermediates and transcripts, and quantification of WHV progeny DNA in culture supernatants

WHV replicative intermediates from intracellular core particles and total RNA were extracted from hepatocytes and detected by Southern blot and Northern blot according to the protocols published previously (Lu et al., 2002). WHV progeny DNA in the culture supernatants was extracted by using QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) and quantified by real-time PCR as described previously (Lu et al., 2008b).

RNA preparation and real-time RT-PCR assay of cellular mRNAs

Total RNA was extracted with TRIzol (Invitrogen) including a digestion with DNase Set (Qiagen). The mRNA expression of TLRs and other relevant genes in the PWHs was quantified using specific primer sets (Table 1; Biomers, Ulm, Germany), which were designed by online software Primer3 (http://frodo.wi.mit.edu/primer3/input.htm, version 0.3.0). The real-time RT-PCR was performed in a one-step method with 100 ng of total RNA using Quantifast SYBR Green RT-PCR Kit (Qiagen) on a Light CyclerTM (Roche Diagnostics, Mannheim, Germany). Same cycling parameters were used for all genes examined in the study. The cycling parameters were set as followed: reverse transcription at 50°C for 10 min for one cycle, then 45 cycles consisting of denaturation at 95°C for 10 s and combined annealing/extension at 60°C for 30 s. Standard curves for the genes examined were determined using serial dilutions of corresponding DNA fragments amplified by RT-PCR. The relative mRNA copies of each gene were determined with help of the corresponding standard curve. For each sample, RT-PCR was performed in duplicate. The expression levels of each gene are presented as values normalized against woodchuck 10⁶ copies of β-actin transcripts.

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siRNA transfection of primary hepatocytes

siRNAs targeting different region of WHV genome were purchased from Qiagen (Table S2) and their antiviral effect has been validated in PWHs previously (Meng et al., 2009). Transfection of primary hepatocytes with siRNAs was carried out on day 2 of in vitro culture. 150 pmol of siRNA and 5 μl of Lipofectamine 2000 per well were applied in a final volume of 1.5 ml Opti-MEM. After 6 h, the medium was replaced by fresh culture medium. DNA and RNA were prepared at various time points for hybridization and real-time RT-PCR assay.

Statistical analysis

The statistical analysis was carried out using GraphPad (GraphPad Software, San Diego, USA). Analysis of variance with Student’s t-test was used to determine significant differences in multiple comparisons. P < 0.05 was considered as statistically significant. Representative data from a series of at least three experiments are shown. Data are presented as means ± standard deviation.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. TLR3 and TLR4 mRNA expression in PWHs from naïve animals and WHV chronic infected animals. RNAs were
extracted from PWHs which isolated from four naïve animals and eight WHV chronic infected animals. The TLR3 and -4 mRNAs were quantified using real-time RT-PCR with primers listed in Table 1. The copy numbers of TLR3 and -4 mRNAs were normalized using β-actin as an internal control. The results are shown as fold changes compared with untreated control.

**Fig. S2.** TLR3 and TLR4 mRNA expression was upregulated by its ligands and IFN stimulation for 6 h in a woodchuck cell line w12/6. RNAs were extracted from a woodchuck cell line w12/6 treated with Poly I:C, LPS, woodchuck IFN-α, and -γ for 6 h. The TLR3 and -4 mRNAs were quantified by real-time RT-PCR with primers listed in Table 1. The copy numbers of TLR3 and -4 mRNAs were normalized using β-actin as an internal control. The results are shown as fold changes compared with untreated control.

**Fig. S3.** Polymyxin B blocked LPS-induced gene expression in PWHs. PWHs were pretreated with Polymyxin B (100 μg ml⁻¹) for 15 min, and then stimulated with 10 μg ml⁻¹ of LPS for 6 h. Total RNA was collected and the induction of MxA, IP10 and TNF-α mRNAs were detected by real-time RT-PCR.

**Fig. S4.** Induction of ISGs and proinflammatory cytokines genes in primary murine hepatocytes treated with LPS and Poly I:C. Total RNA were extracted from primary murine hepatocytes treated with LPS and Poly I:C or transfected with Poly I:C at indicated concentrations for 6 h. The detection of relevant mRNAs was performed by real-time RT-PCR using the primers purchased from Qiagen (Hilden, Germany).

**Table S1.** Woodchucks used in this study.

**Table S2.** Target sequences of siRNAs.

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