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The Role of the Innate Immune System of the Liver in the Control of HBV and HCV*

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Abstract: Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infection are among the most frequent causes of chronic liver disease worldwide. As recent studies suggested that Toll like receptor (TLR)-based therapies may represent a promising approach in the treatment of HBV infection, we have studied the role of the local innate immune system of the liver as possible mediator of this effect. Murine non-parenchymal liver cells (NPC; Kupffer cells, KC; sinusoidal endothelial cells, LSEC) were isolated from C57/BL6 and stimulated by TLR 1-9 agonists. Supernatants were harvested and assayed for their antiviral activity against HBV in HBV-Met cells and HCV in the murine HCV replicon cell line MH1. Only supernatants from TLR 3 and -4 stimulated KC and TLR 3 stimulated LSEC were able to potently suppress HBV and HCV replication. By using neutralizing antibodies we could demonstrate that the TLR 3- but not the TLR 4 mediated effect is exclusively mediated through IFN-β. Our data indicate that TLR 3 and -4 mediated stimulation of NPC leads to production of IFN-β which can potently suppress HBV and HCV replication. This is of relevance for the local control of viral hepatitis infection by the innate immune system of the liver, the development of novel TLR-based therapeutic approaches and sheds new light on the viral crosstalk between HCV (TLR 3 stimulator) and HBV.

Key words: Hepatitis B; Kupffer cells; Sinusoidal endothelial cells; Interferon

Abbreviations: HBV, Hepatitis B virus; HCV, Hepatitis C virus; TLR, Toll like receptor; KC, Kupffer cells; LSEC, Liver sinusoidal endothelial cells; IFN, Interferon; NPC, Non-parenchymal liver cells.

IMMUNE SYSTEM OF THE LIVER – NON-PARENCHYMAL LIVER CELLS

The liver is a unique immunological organ in which protein- and antigen-rich blood from the gastrointestinal tract is passed through the sinusoids in close contact to antigen-presenting cells (APC) and lymphocytes (21). The liver lobule is formed by parenchymal cells, i.e., hepatocytes and NPC. Heptocytes constitute only about two thirds of the total cell population in the liver. The remaining population of NPC includes Kupffer cells (KC), liver sinusoidal endothelial cells (LSEC), stellate cells, biliary cells and intrahepatic lymphocytes. Resident APC include KC, LSEC and

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dendritic cells (DC). The liver is known as a site of tolerance induction rather than induction of immunity, and the three types of APC may contribute in different ways to maintain the local microenvironment homeostasis (16).

Fig.1 schematically shows that LSEC, which comprise about 50% of the NPC, form a fenestrated monolayer that separates hepatocytes from the passing blood. LSEC take up antigens by receptor-mediated endocytosis and/or phagocytosis with similar efficacy as dendritic cells load the processed peptides onto major histocompatibility complex (MHC) class I and II molecules and present them to passenger lymphocytes. Kupffer cells account for approximately 20% of the NPC in the liver. They are located in the hepatic sinusoids where they come in close contact to blood and passing lymphocytes (Fig. 1). In this exposed location, Kupffer cells are not only responsible for the phagocytosis of passing organisms and debris but also for induction and maintenance of tolerance (27).

TLR (toll-like-receptor) SIGNALING PATHWAYS

In 1908 Elie Mechnikov, who won the Nobel Prize for his discovery of cellular immunity mentioned endotoxins and nucleic acids as activators of phagocytes, speculated that their use might prevent infection during surgery in his Nobel lecture. It took 90 years to discover the receptors for these microbial products. The path scientists traveled to get to TLRs was a circuitous one, winding through studies of dorsoventral polarity in the developing Drosophila embryo, the search for drugs to treat arthritis, and the dawn of the genomic era (20). To date, thirteen mammalian TLRs (10 in humans and 13 in mice) with different pathogen recognition profiles have been identified (1).

TLRs possess two domains, a unique extracellular domain and a conserved cytoplasmic TIR domain. The extracellular domains of these receptors contain blocks of repeats of a 24-amino-acid motif, called a leucine-rich repeat (LRR). Within the TIR domain, the regions of homology comprise three conserved boxes, which are adjacent and display most of their side chains for interaction with adaptor molecules. MyD88 (myeloid differentiation primary-response protein 88), identified as first adaptor in IL-1R signaling, used by all TLRs with the exception of TLR 3 and certain TLR 4 signaling pathways (28). Mal (MyD88-adaptor-like protein) or namely TIRAP (TIR-domain-containing adaptor protein) was identified as second adapter indicating specificity in MyD88 recruitment in the case of TLR 2 and TLR 4 signaling. Two further adapters were then described: TRIF (TIR-domain-containing adaptor protein inducing IFN-β), which is used by TLR3 and TLR4 and couples to another transcription factor, IRF3, thus leading to IFN-β production, and TRAM (TRIF-related adaptor molecule), that has a role in LPS signaling. In contrast to the other four adaptors, SARM (sterile α- and armadillo-motif containing protein) is a negative regulator of NF-κB and IRF activation.

NON-PARENCHYMAL LIVER CELLS AND THE TLR SYSTEM

Nonparenchymal liver cells (NPCs: Kupffer cells, KCs; sinusoidal endothelial cells, LSECs) play an indispensable role in pathogen clearance and innate

and early adaptive immune responses (15). TLR recognize molecular patterns specific to microbial pathogens and serve as an important link between innate and adaptive immunity. Although a lot of progress has been made in clarifying TLR4 signaling pathways in murine NPCs, relatively little is known about how NPCs respond to other TLR ligands, especially five viruses associated TLRs (TLR3, -4, -7, -8 and -9).

To investigate the diversification of TLR signaling pathways in murine NPCs, we isolated KCs and LSECs from wild-type C57BL/6 mice and examined their responses to TLR1-TLR9 agonists (Wu *et al*., in preparation; Fig. 2, Table 1 and 2). We demonstrate that KCs strongly express TLR1-4 & 6-9 (low levels of TLR 5), and LSECs preferentially express TLR1-4 & 6-8 (low levels of TLR5 & 9). In accordance with these TLR expression profiles, KCs respond to TLR3 and TLR4 ligands by producing IFN-β, to all TLR1-TLR9 ligands by producing TNF-α and IL-6, and to TLR1 and -8 ligands by significantly upregulating MHC-II and costimulatory molecules. Similarly, LSECs respond to TLR3 ligands by producing IFN-β, to TLR1-4 and -6 ligands by producing TNF-α and IL-6, and to TLR8 ligands by significantly upregulating MHC-II and costimulatory molecules. Myeloid dendritic cells (mDC), as a control for classical APCs, constitutively produced high levels of TNF-α and IL-6, very low levels of IFN-α but no IFN-β and expressed high levels of MHC and costimulatory molecules. Our results indicate that the expression of distinct sets of TLRs and the consequent activation profiles of KCs, LSECs and mDCs support the concept that KCs and LSECs have developed through cell-type specific TLR signaling pathways to control the local immune response in health and disesae.

HEPATITIS B

The hepatitis B virus (HBV), a noncytopathic, parenterally transmitted DNA virus, belongs to the Orthohepadnavirus genus of the Hepdnavirus family. Whereas infection during adulthood is frequently cleared, vertical transmission from mother to child leads to persistent infection leading to liver cirrhosis, hepatic decompensation and hepatocellular carcinoma (4). It is estimated that more than estimated 350 million patients worldwide are chronically infected with HBV.

Despite the fact that microarray analyses of serial liver biopsies of experimentally infected chimpanzees

Table 1. TLR-mediated induction of costimulatory molecules, cytokines and antiviral activity in sinusoidal endothelial cells

TLR	1	2	3	4	5	6	7	8	9
CD40 expression	-	-	-	-	-	-	-	++	-
CD80 expression	-	-	-	-	-	-	-	++	-
CD86 expression	-	-	-	-	-	-	-	++	-
MHC II expression	-	-	-	-	-	-	-	+++	-
IFN-α	-	-	-	-	-	-	-	-	-
IFN-β	-	-	++	-	-	-	-	-	-
TNF-α	+	++	++	++	-	+	+	+	-
IL-10	-	-	++	++	-	-	-	-	-
EMCV	-	-	↓↓↓	-	-	-	-	-	-
HCV	-	-	↓↓↓	-	-	-	-	-	-
HBV	-	-	↓↓↓	-	-	-	-	-	-

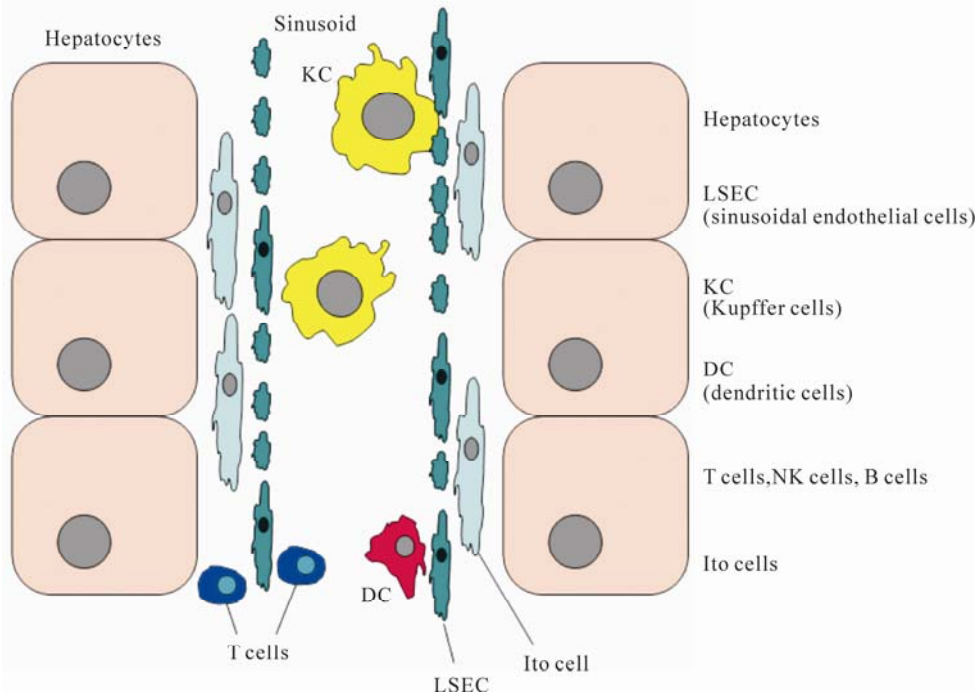

Fig. 1. The local immune system of the liver

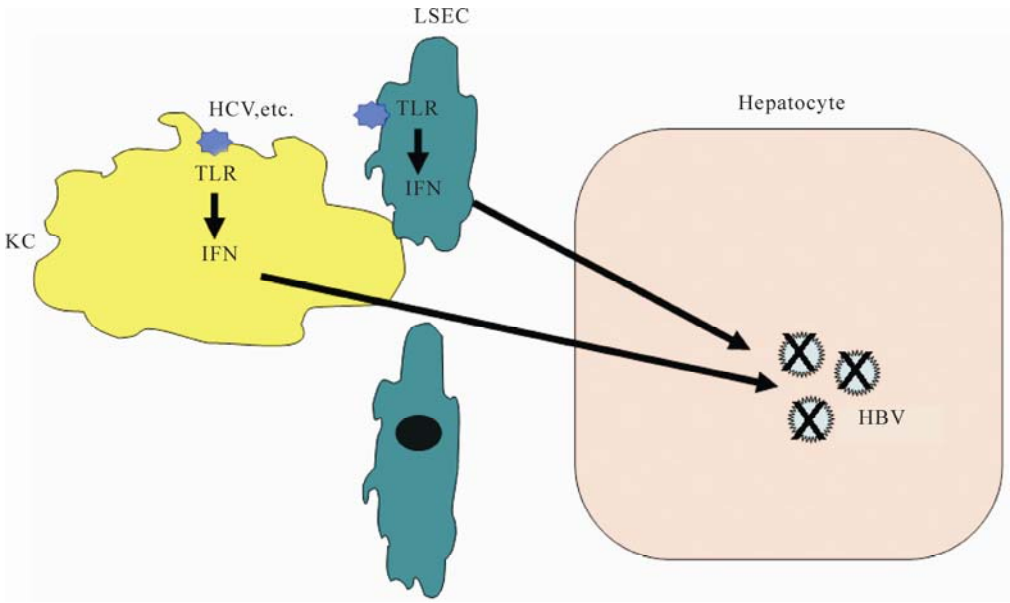

Fig. 2. Working hypothesis: Can TLR-activated NPC control HBV replication? KC: Kupffer cell, LSEC: sinusoidal endothelial cells, TLR: Toll-like receptor

indicate that HBV does not induce any detectable changes in the expression of intrahepatic genes in the early phase of infection, a role for the innate immune response in the control of early HBV replication should not be dismissed (22). In fact, most of the viral DNA can be cleared from liver and serum of

Table 2. TLR-mediated induction of costimulatory molecules, cytokines and antiviral activity in Kupffer cells

TLR	1	2	3	4	5	6	7	8	9
CD40 expression	+++	-	-	-	-	-	-	++	-
CD80 expression	+++	-	-	-	-	-	-	++	-
CD86 expression	++	-	-	-	-	-	-	++	-
MHC II expression	+++	-	-	-	-	-	-	++	-
IFN-α	-	-	-	-	-	-	-	-	-
IFN-β	-	-	+++	++	-	-	-	-	-
TNF-α	++	+++	+++	+++	++	+++	-	+++	++
IL-10	++	+++	++	++	++	+++	-	++	++
EMCV	-	-	↓↓↓	↓↓	-	-	-	-	-
HCV	-	-	↓↓↓	↓↓	-	-	-	-	-
HBV	-	-	↓↓↓	↓↓	-	-	-	-	-

experimentally infected chimpanzees several weeks prior to any manifestation of liver disease. Indeed, antiviral effects of IFN-α, IFN-β, IFN-γ as well as other mediators induced by TLR agonists, activation of natural killer T (NKT) and T cells or infection with unrelated hepatotropic viruses, such as lymphocytic choriomeningitis virus and adenovirus, have been shown in transgenic HBV-producing mice. In this model, the mechanism whereby IFN-α/-β inhibits HBV replication relies on the inhibition of formation and/or promoting the destabilization of immature HBV RNA-containing capsids in a proteasome dependent manner (23).

Resolution of infection in acute hepatitis B is typically associated with a vigorous, polyclonal, multispecific CD4+ T helper and CD8+ cytotoxic lymphocyte (CTL) response. The CD4+ T cells recognize viral peptides generally derived from phagocytosed and proteolytically cleaved HBV proteins in the context of MHC class II molecules on APCs. In contrast, CD8+ T cells recognize peptides exclusively derived from endogenously synthesized proteins (with the exception of peptides derived from the hydrophobic HBsAg) in the context of MHC class I molecules. CD4+ T cells provide help for activation and differentiation of B cells, contribute to induction and maintenance of HBV-apecific CD8+ T cells, and recruit dendritic cells to activate CD8+ T cells. Infected hepatocytes are recognized and destroyed by HBV specific CTLs directed against the HBV. It is widely believed that the most important pathway for virus elimination is killing of infected cells. However, experiments in transgenic HBV-producing mice led to the concept that HBV clearance may be primarily due to a noncytopathic mechanism in which HBV-specific CTLs, mediated mainly by inflammatory cytokines such as IFN-γ and TNF-α, abolish HBV gene expression and replication (10). Important supporting evidence favoring an important role for this non-cytopathic clearance mechanism is therefore provided by the studies in chimpanzees and human patients (11, 24, 29).

HBV AND THE INNATE IMMUNE SYSTEM OF THE LIVER

As recent studies suggested that Toll like receptor (TLR)-based therapies may represent a promising approach in the treatment of HBV infection, we have studied the role of the local innate immune system of the liver as possible mediator of this effect (Fig. 2).

Murine NPCs were isolated from C57/BL6 wild-type or MyD88–/– mice and stimulated by agonists of TLR1-TLR9. Supernatants were harvested and assayed for their antiviral activity against HBV in HBV-Met cells. No direct antiviral effect of TLR agonists could be observed. In controls (mDC), TLR1, -3, -4, -7 and -9 activation lead to production of antiviral cytokines. By contrast, only supernatants from TLR3-stimulated and TLR4-stimulated KCs and TLR3-stimulated LSECs from wild-type mice were able to potently suppress HBV replication as assessed via Southern blotting. Similar results were found with cells from MyD88–/– mice indicating that the effect was independent of this signaling pathway. Cellular HBV-RNA and HBsAg or HBeAg levels in supernatants remained unchanged. By using neutralizing antibodies we could demonstrate that the TLR3 effect was mediated through IFN-β while TLR4 stimulation led to the production of as yet undefined (non-IFN, non-TNF-α) antiviral factor(s). Our data indicate that the innate immune system of the liver can control HBV replication after activation by TLR agonists. This has implications for the development of novel TLR-based therapeutic approaches against HBV and sheds new light on the viral crosstalk between HCV and HBV (30).

HEPATITIS C

Hepatitis C virus (HCV) infection is a major cause for chronic hepatitis with more than 170 Million infected people worldwide. HCV can progress to liver cirrhosis and hepatocellular carcinoma (2). The current standard therapy consists of the combination of pegylated interferon alpha (IFN-α) with ribavirin which leads to viral clearance in 70-90% of patients infected with HCV genotype 2 or 3 but only in 50% of patients infected with genotype 1 (19).

Hepatitis C virus (HCV) primarily infects hepatocytes, but various lymphoid populations like B-cells or dendritic cells have also been shown to be HCV RNA-positive (3, 13, 14). LSEC and KC have been described to be involved in tolerance induction and the innate immune response against various pathogens. (8, 9, 25). To date, however, only little is known about their role in the antiviral defense against HCV.

The adaptive immune response directed towards HCV is a well studied field, but less is known about innate responses against this virus. It has been shown that HCV can activate the innate immune system through TLR3 and the RNA helicase RIG-I (retinoic acid-inducible gene-I) (18, 26). This is in accordance with the observation that gene expression profiles can be found in livers of HCV infected patients or chimpanzees that are indicative of a strong type I IFN-mediated local response to HCV (5, 12). On the contrary, this activation can be blocked in HCV infected hepatocytes by the viral NS3/4A protease that inhibits the virus-induced RIG-I and TLR3 signaling (7, 17) which led to the suggestion that this type I IFN response is either mediated by liver cells that are not primarily infected by HCV or in which this suppressive effect of HCV on IFN production is not effective.

The strong type I IFN response observed in HCV-positive livers may be mediated by NPC and in particular KC and LSEC that, as already mentioned, represent the largest proportion in this subset of liver cells. As described previously, murine NPC can be

activated by TLR3 and TLR4 to produce IFN-β, an approved cytokine which potently suppress HCV replication. We used the murine HCV replicon harbouring cell line MH1 to show the antiviral capacity of KC and LSEC treated with TLR ligands. The MH1 cells are highly sensitive to IFNs and supernatants from TLR 3- and 4-stimulated NPC led to potent suppression of HCV replication through IFN-β (TLR 3 treated KC and LSEC: -80%, TLR 4 treated KC: -60%). By contrast, DCs could be stimulated by TLR 2, -3, -4, -7 and -9 to induce antiviral activity (6).

The responsiveness of KC and LSEC to poly I: C, led to the assumption that this NPC exposed to HCV may be responsible for the local interferon response (5, 12) in infected patients and could be mediated by TLR signaling. This question has to be addressed in further experiments that have to focus the control of HCV replication by human NPCs. It would also be of interest if there are any differences in the capability of NPC to produce antiviral cytokines depending on the stage of liver disease.

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