Role of Toll-like receptors in liver health and disease

Ruth BROERING*, Mengji LU† and Joerg F. SCHLAAK*

*Department of Gastroenterology and Hepatology, University Hospital of Essen, University Duisburg-Essen, 45122 Essen, Germany, and †Institute of Virology, University Hospital of Essen, University Duisburg-Essen, 45122 Essen, Germany

ABSTRACT

TLRs (Toll-like receptors), as evolutionarily conserved germline-encoded pattern recognition receptors, have a crucial role in early host defence by recognizing so-called PAMPs (pathogen-associated molecular patterns) and may serve as an important link between innate and adaptive immunity. In the liver, TLRs play an important role in the wound healing and regeneration processes, but they are also involved in the pathogenesis and progression of various inflammatory liver diseases, including autoimmune liver disease, alcoholic liver disease, non-alcoholic steatohepatitis, fibrogenesis, and chronic HBV (hepatitis B virus) and HCV (hepatitis C virus) infection. Hepatitis viruses have developed different evading strategies to subvert the innate immune system. Thus recent studies have suggested that TLR-based therapies may represent a promising approach in the treatment in viral hepatitis. The present review focuses on the role of the local innate immune system, and TLRs in particular, in the liver.

INTRODUCTION

Innate immunity

Although the innate immune system is involved in many inflammatory processes, it is particularly relevant in the early and late phases of viral and bacterial infections [1]. Effectors of the innate immune system, the PRRs (pathogen recognition receptors), are activated immediately after exposure to infectious agents and may subsequently limit their replication [2]. PRRs detect evolutionarily highly conserved structures, the so-called PAMPs (pathogen-associated molecular patterns). Here, the so-called TLRs (Toll-like receptors) play a pivotal role. Activation of the TLR system leads to the expression of pro-inflammatory as well as anti-inflammatory cytokines. TLR7, TLR8 and TLR9

Key words: cytokine, hepatitis, inflammation, innate immune system, liver, Toll-like receptor.

Abbreviations: ALD, alcoholic liver disease; AP1, activator protein 1; APC, antigen-presenting cell; BEC, bile duct epithelial cell; CHB, chronic hepatitis B; DC, dendritic cell; dsRNA, double-stranded RNA; eIF, eukaryotic initiation factor; ERK1/2, extracellular-signal-regulated kinase 1/2; HBsAg, hepatitis B e antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HNF, hepatocyte nuclear factor; HSC, hepatic stellate cell; IFN, interferon; IL, interleukin; IL-1R, IL-1 receptor; IRAK, IL-1R-associated kinase; IRF, IFN regulatory factor; ISG, IFN-stimulated gene; ISS, immunostimulatory sequence(s); JNK, c-Jun N-terminal kinase; KC, Kupffer cell; LPS, lipopolysaccharide; LSEC, liver sinusoidal endothelial cell; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation factor 88; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NF-κB, nuclear factor κB; Iκκ, Iκκ kinase; NEMO, NF-κB essential modulator; NPC, non-parenchymal liver cell; NTR, non-translated region; ODN, oligodeoxynucleotide; PAMP, pathogen-associated molecular pattern; PBC, primary biliary cholangitis; PBMC, peripheral blood mononuclear cell; PRR, pathogen recognition receptor; PSC, primary sclerosing cholangitis; RIG-I, retinoic acid-inducible gene I; RNAi, RNA interference; ROS, reactive oxygen species; siRNA, small interfering RNA; TGF-β, transforming growth factor-β; TAK1, TGF-β-activated kinase 1; TLR, Toll-like receptor; TIR, Toll/IL-1R; TRIF, TIR domain-containing adaptor protein; TNF, tumour necrosis factor; TRAF6, TNF-receptor-associated factor 6; TIRAP, TIR domain-containing adaptor molecule.

Correspondence: Professor Joerg F. Schlaak (email joerg.schlaak@uni-due.de).
Figure 1  TLR signalling
Activation of the TLRs leads to recruitment of the adaptor molecules MyD88, TIRAP, TRIF and TRAM. Downstream signals involve TAK1, MAPKs, TRAF3, TBK1 and IKKs, resulting in nuclear translocation of transcription factors (AP-1, NF-κB, IRF-3 or IRF-7) into the nucleus and transcription of inflammatory genes.

additionally initiate IFN (interferon)-α production after exposure to their specific ligands, whereas stimulation of TLR3 and TLR4 leads to expression of IFN-β as well as immunoregulatory cytokines [3,4].

The TLR system
All TLRs are located in the cell membrane and have a common highly conserved cytosolic domain with similarity to the IL-1R [IL (interleukin)-1 receptor] and are therefore called TIRs (Toll/IL-1Rs). Figure 1 shows that binding of specific TLR ligands leads to recruitment and activation of adapter molecules, such as MyD88 (myeloid differentiation factor 88), which is activated by all TLRs except TLR3. MyD88-dependent signalling involves IRAK (IL-1R-associated kinase) 1 and IRAK4, as well as TRAF6 [TNF (tumour necrosis factor)-receptor-associated factor 6] to dissociate the NF-κB (nuclear factor κB) inhibitor IκB. This results in translocation of NF-κB into the nucleus, leading to transcription of immunoregulatory genes [4]. MyD88 also activates MAPKs (mitogen-activated protein kinases) that stimulate the AP-1 (activator protein-1) pathway. MyD88 signalling of endosomally located TLR3, TLR7, TLR8 and TLR9 additionally activates IRF (IFN regulatory factor)-7, thereby initiating transcription of IFN genes [5]. TLR3 signalling is MyD88-independent, involving TRIF (TIR domain-containing adaptor inducing IFN-β), which results in phosphorylation of IRF-3 and subsequent induction of IFN-β expression. TRIF additionally activates TRAF6, leading to translocation of NF-κB as described above. TLR4 involves TIRAP (TIR domain-containing adaptor protein) to activate MyD88 signalling, as well as TRAM (TRIF-related adaptor molecule) and induces the TRIF-dependent induction of IFN-β [4–6].

Immune system of the liver
The liver is an immunological organ in which blood from the gastrointestinal tract, enriched with nutrients and antigens, passes through the sinusoids in close contact to APCs (antigen-presenting cells) and lymphocytes. The physiological function of the liver is protein synthesis and metabolism, as well as removal of pathogens and antigens from the blood. This requires a locally regulated immune response.

Pathogenic micro-organisms must be efficiently eliminated, while tolerance against the large number of antigens derived from the gastrointestinal tract has to be established to avoid unnecessary damage of the hepatocytes [7]. Hepatocytes represent about two-thirds of the total cell population in the liver. The remaining population consists of NPCs (non-parenchymal liver cells), including KCs (Kupffer cells), LSECs (liver sinusoidal endothelial cells), HSCs (hepatic stellate cells) and intrahepatic lymphocytes. Resident APCs include KCs, LSECs and DCs (dendritic cells).

The liver has been proposed to be a site of tolerance induction rather than induction of immunity,
and the three types of APCs may contribute in different ways to maintain the homoeostasis of the local microenvironment [8]. LSECs comprise approximately 50% of the NPCs and form a fenestrated monolayer that separates hepatocytes from the passing blood. LSECs take up antigens by receptor-mediated endocytosis and/or phagocytosis with similar efficacy as DCs, load the processed peptides on to MHC class I and II molecules and present them to passenger lymphocytes. Kupffer cells represent approximately 25% of the NPC population in the liver. They are located in the hepatic sinusoids where they are in close contact with blood and passing lymphocytes. In this exposed location, KCs are not only responsible for phagocytosis of passing organisms and debris, but also for the induction and maintenance of tolerance [9].

Role of liver cells as part of the local innate immunity

Hepatocytes express TLRs and are able to respond to stimulation with TLR ligands. The expression of TLRs has been demonstrated on primary human and murine hepatocytes, as well as different hepatoma cells including HepG2 and Huh7 cells. The functionality of the TLR pathways in such cell systems was shown by responses to the stimulation with TLR ligands [10–14]. In addition to this, investigation of murine NPCs revealed that KCs, LSECs and HSCs respond to all TLR ligands by producing TNF-α or IL-6, whereas production of IFN-β is restricted to TLR3 and TLR4 in these cells. TLR8 activation in KCs and HSCs may enhance T-cell proliferation and IFN-γ production when these NPCs are present in a mixed lymphocyte reaction. Interestingly, LSECs failed to stimulate allogenic T-cells in mixed lymphocyte reaction, despite significant up-regulation of MHC class II and co-stimulatory molecules in response to TLR8 ligands [15]. Moreover, recent work on human primary isolated liver cells has revealed that hepatocytes, LSECs, HSCs and a mixture of the remaining NPCs respond to TLR1–TLR9 ligands, except TLR9, resulting in up-regulation of TNF-α, IL-6 and IL-10. Activation of TLR3 in human hepatocytes and NPCs leads to the induction of type I and II IFNs [15a]. Thereby murine and human NPCs display a restricted TLR-mediated activation profile which may, at least in part, explain their tolerogenic function in the liver.

As mentioned above, it has been suggested that the liver favours the induction of tolerance rather than the induction of immunity. Repetitive stimulation with low-dose LPS (lipopolysaccharide) leads to a state of refractoriness which is characterized by a lack of nuclear transactivation of NF-κB and the inability to increase CD54 and CD106 expression in response to pro-inflammatory stimuli [16]. In this regard, NPCs are cross-linking the adaptive and innate immunity, orchestrating tolerance induction as well as pathogen elimination [17].

ROLE OF THE TLR SYSTEM IN LIVER DISEASES

TLRs are involved in inflammatory liver diseases

There is increasing evidence that TLRs play an important role in the pathogenesis and progression of many liver diseases. As a key component of the innate immune system, these receptors may also recognize endogenous DAMPs (damage-associated molecular patterns) during inflammatory reactions. It has been suggested that an inappropriate activation of TLRs is involved in the pathogenesis of autoimmune disorders. Although autoreactive B-cells can be found in the lymphatic system of healthy individuals, these cells normally remain quiescent. Perturbation of this homoeostasis leads to the formation of self-recognition antibodies, resulting in pathological reactions. It has been shown that activation of these B-cells requires the synergistical activation of the antigen receptor and a member of the MyD88-dependent TLRs. Inhibition studies implicated the involvement of TLR9 in these autoimmune processes [18,19]. Application of CpG-ODNs (oligodeoxynucleotides) in a transgenic mouse model, expressing a specific MHC class I molecule exclusively on hepatocytes, sufficiently inhibited tolerance in vivo. This led to activation of CD8+ T-cells and autoaggression against hepatocytes. The CpG-ODN-induced inflammation appears to cause infiltration of T-cells into the liver, as well as up-regulation of adhesion and co-stimulatory molecules on hepatocytes, resulting in infiltration of CD8+ T-cells targeting the specific antigen on hepatocytes, which represent an APC-like phenotype, inducing tissue damage [20].

Autoimmune-dependent inflammation processes in the bile duct are described in patients with PBC (primary biliary cholangitis) or PSC (primary sclerosing cholangitis). Both diseases show inappropriate activation of the TLR system. It appears that patients with PBC showed higher responsiveness of the innate immune system to pathogen-associated stimuli, resulting in the loss of tolerance. Monocytes from patients with PBC are more susceptible to activation of selective TLRs (TLR2, TLR3, TLR5 and TLR9), resulting in the secretion of pro-inflammatory cytokines. This inflammatory response may be critical for the self-tolerance and autoimmune progression [21].

TLR4 is additionally involved in the pathogenesis of PBC. BECs (bile duct epithelial cells) of liver biopsies from PBC patients showed highly elevated TLR4 expression compared with control. TLR4 expression is additionally increased in periportal hepatocytes of
PBC liver tissue, as well as in blood monocytes. The elevated TLR4 expression in PBC patients possibly cross-link bacterial pathogens and TLR4 in the inflammatory processes of PBC [22,23].

In the case of PSC, the production of autoreactive BEC-specific antibodies link adaptive and innate immunity. Binding of PSC BEC-specific antibodies activates ERK1/2 (extracellular-signal-regulated kinase 1/2) signalling and therefore the up-regulation of TLR4 but not TLR9. TLR4 activation itself leads to the production of pro-inflammatory mediators, possibly recruiting inflammatory cells. In PSC, BECs are targets of the autoimmune attack, but may additionally be active participants and mediators of their own destruction [24].

However, it has been demonstrated that TLR signalling affects inflammation in the absence of infection. There is increasing evidence that TLR activation is involved in the innate immune recognition of allografts after solid organ transplantation. Here, the TLR-driven MyD88-dependent signalling pathway is important for DC maturation, CD8+ alloimmune priming and subsequent Th1-dependent alloimmunity. In the absence of TLR2, organ recipients had a delayed rejection. In addition, a non-TLR MyD88-dependent pathway, involving IL-1 and IL-18, plays a role in allograft rejection, as caspase 1-deficient recipients also showed a delayed allograft survival. This is not surprising in view of the pro-inflammatory functions of IL-1 and its role in the induction of acute-phase proteins as well as adhesion molecules [25,26].

Role of TLR in alcoholic liver disease
Alcohol abuse is a leading cause of morbidity and mortality worldwide. The manifestation of ALD (alcoholic liver disease) ranges from steatosis to steatohepatitis to fibrosis, cirrhosis and can result in development of HCC (hepatocellular carcinoma). Alcohol-induced liver injury involves parenchymal and non-parenchymal liver cells, as well as recruited immune cells that contribute to inflammation and liver damage [27].

Alcohol ingestion increases permeability of the gut mucosa to LPS. This leads to increased endotoxin levels in the liver and therefore activates TLR4 on parenchymal and non-parenchymal liver cells. KCs, HSC, LSECs and hepatocytes respond to LPS with an increase in the production of pro-inflammatory cytokines [28]. Exposure to LPS during chronic alcohol consumption results in increased production of inflammatory mediators, as well as the induction of ROS (reactive oxygen species), leading to progression of liver injury [29,30]. This signalling was abrogated in TLR4-deficient mice, but not in MyD88-deficient mice. Activation of IRF-3 was not affected by chronic alcohol treatment. However, the expression of IRF-7- and IRF-3-inducible genes was found in KCs of alcohol-fed wild-type mice. Alcohol feeding additionally activates NF-κB in a TLR4-dependent but MyD88-independent manner [31].

Chronic alcohol consumption is additionally associated with the increased expression of TLR1, TLR2, TLR4 and TLR6–TLR9. The enhanced TLR expression further potentiates the induction of the pro-inflammatory TNF-α in response to LPS [32].

TLR in progression of NASH (non-alcoholic steatohepatitis)
Overweight and obesity are increasing diseases in Western countries. They are associated with the progression of Type 2 diabetes, hypertension and NAFLD (non-alcoholic fatty liver disease). NAFLD is one of the most common forms of chronic liver diseases. Liver biopsies of NAFLD patients showed pathological changes ranging from steatosis to steatohepatitis (NASH), leading in cirrhosis and HCC [33]. It has been reported that the hepatic expression levels of inflammatory mediators are modified in morbidly obese patients even without pathohistological manifestations. Furthermore, the liver of NAFLD patients could be more responsive to activators of the TLR pathway. The progression of NASH is associated with recruitment of T-cells and a Th1 response leading to inflammation [34].

The up-regulation of key molecules required for the TLR signalling pathways (CD14 and TLR4) led to a higher responsiveness to LPS or saturated fatty acids. It is known that saturated fatty acids can activate macrophages via TLR4. Hepatic inflammation in NAFLD patients and the development of liver complications are associated with this activation of KCs [35,36].

Impact of TLRs on wound healing, fibrogenesis and regeneration processes
The pathological mechanisms that are involved in wound healing include tight interactions between the innate and adaptive immune system and NPCs. Previous studies that investigated the role of the TLR system in liver fibrosis largely focused on TLR4 and TLR9. LPS has been shown to play a role by enhancing TGF-β (transforming growth factor-β) signalling. Although stimulation of HSCs with LPS alone does not effect their transformation into myofibroblasts, repetitive treatment with LPS strongly enhances responsiveness of HSCs to TGF-β, a pro-fibrogenic cytokine predominantly secreted from activated KCs. This increased response of LPS-pre-treated HSCs to TGF-β has been associated with a TLR4-dependent down-regulation of the TGF-β receptor in HSCs, thereby negatively regulating the TGF-β signalling [32,37]. This lead to the suggestion that LPS affects hepatic fibrosis via the TLR4-dependent modification of TGF-β signalling in HSCs and thereby connecting inflammatory and fibrogenic pathways [38].
The innate immune system additionally plays a role in liver regeneration. The liver can restore major tissue loss by regeneration. Rapid hepatocyte proliferation with complete restoration could be shown in mouse and human livers [39]. TLR activation of NPCs leads to the activation of the transcription factor NF-κB, subsequent production of cytokines, including TNF-α, IL-6 and IL-10, and recruitment of immune cells leading to local inflammation. Regeneration processes after liver tissue damage are partially regulated by the same signalling cascades and secretion of TNF-α and IL-6. These cytokines prime hepatocytes into a state in which they are susceptible for growth factors [40,41].

**TLR in hepatocellular tumour progression**

HCC is among the most frequent causes for cancer deaths worldwide. Chronic liver damage induced by inflammation, alcohol, obesity or viral infection represents a high risk factor for the development of fibrosis-associated HCC [42]. As all of these diseases are associated with an uncontrolled innate immune system, it appears likely that TLRs are also involved in the progression of HCC. Analysis of the cell types involved in the process of tumorigenesis indicated that KCs may play a role. Although KCs mostly express all of the TLRs, hepatocytes have low TLR2 and TLR4 expression levels and weak responses upon activation [43,44]. However, TLR2 expression in hepatocytes is induced after exposure to LPS or TNF-α, suggesting that hepatocytes become more sensitive during inflammatory conditions [45].

Stimulation of the TLR system and subsequent activation of NF-κB and JNK (c-Jun N-terminal kinase) pathways are critical modulators for the production of the cytokines associated with tumour progression. It is proposed that NF-κB activation is directly associated with tumour cell proliferation. There is strong evidence that NF-κB participates in the transcriptional regulation of the expression of growth factors. The NF-κB-dependent cytokine TNF-α plays a central role in inflammatory process as well as in cell proliferation [46]. Higher expression levels of IKK (IkB kinase) α and IKKβ, regulators of NF-κB activation, are necessary to produce the malignant properties of liver cancer. Acute increases in hepatocyte death accompanied by an increase in ROS was observed in hepatocyte-specific IKKβ-knockout mice after carcinogen administration. Hepatocyte death combined with inflammation enhanced proliferation due to the strong regenerative capacity of the liver [47]. In addition, the IKK subunit NEMO (NF-κB essential modulator) is essential for activating NF-κB. Hepatocyte-specific knockdown of NEMO causes spontaneous development of HCC in mice. Therefore NEMO-mediated NF-κB activation in hepatocytes plays an essential physiological role in TLR-induced liver damage and HCC progression [48].

The JNK pathway has been additionally associated with tumour progression. JNK was identified as a protein kinase that phosphorylates c-Jun, the product of a well-characterized oncogene. Its phosphorylation may be relevant in HCC development [49]. The tumorigenic effect of JNK in the liver is mediated through positive gene regulation or by molecules involved in cell proliferation such as cyclins and CDKs (cyclin-dependent kinases), and metastatic factors such as MMP9 (matrix metalloproteinase 9), VEGF (vascular endothelial growth factor) and others [50]. Studies of hepatocarcinogenesis in animal models revealed that regulation of ROS-mediated JNK activation is critical for developing cancer [51,52]. TLR signalling involves TAK1 (TGF-β-activated kinase 1) to activate both the NF-κB and JNK pathways. Hepatocyte-specific TAK1-deficient mice show spontaneous hepatocyte death, compensatory proliferation, inflammatory cell infiltration and fibrosis, with older mice developing multiple tumour nodules. These results indicate that TAK1 is an essential component of cellular homoeostasis in the liver [53].

**TLR in chronic viral hepatitis: interplay of forces**

Chronic infection with HBV (hepatitis B virus) and HCV (hepatitis C virus) are associated with the progression of fibrosis, cirrhosis and HCC [54]. As the interaction between hepatitis viruses and the innate as well as the adaptive immune system determines the outcome of infection, studies regarding specific interactions between viral proteins and components of the immune system have generated important information about the establishment of chronicity of these infections.

**Role of innate immunity in the pathogenesis of HBV infection**

HBV is a hepatotropic non-cytopathic DNA virus and belongs to the *Hepadnaviridae* family. An estimated 300 million individuals worldwide have been chronically infected with HBV with a high local prevalence in Asia and Africa. It has been suggested that cell-mediated immune responses play an essential role for viral clearance [55]. In contrast with individuals with self-limiting resolving HBV infection, patients that progress to chronic infection generally fail to develop adequate HBV-specific immune responses. Although HBV infection can be prevented by vaccination, either PEGylated IFN-α or nucleoside/nucleotide analogues are used for therapy of chronic hepatitis B.

The role of innate immune responses during the early phase of HBV infection has been investigated in different experimental systems. Wieland et al. [56] examined the liver transcriptome in three chimpanzees during acute HBV infection. Their results indicated that HBV does not induce any genes during entry and
expansion, suggesting it is a ‘stealth virus’ in the early stage of the infection. During viral clearance, a large number of IFN-γ-regulated genes are induced as specific T-cells infiltrate into liver [56]. Thus HBV and HCV infections differ strongly in the early phase, as HCV induces an early strong IFN-α response in chimpanzees [57]. HBV may actively inhibit the induction of an early IFN response in infected hosts [58]. Wu et al. [59] described a modulatory effect of TLR-treated KCs and LSECs on HBV replication using an in vitro co-culture model (HBV-Met cells). TLR3- and TLR4-stimulated KCs as well as TLR3-activated LSECs induced a MyD88-independent response affecting HBV replication. The HBV-suppressing effect induced by TLR3 ligands was mediated by IFN-β, whereas TLR4-activated KCs additionally induced cytokines of an undefined nature [12]. Further experiments revealed that hepatocytes and NPCs pre-treated with HBV-Met cell supernatants, HBsAg (hepatitis B surface antigen) and HBeAg (hepatitis B e antigen) as well as HBV virions abrogated TLR-induced antiviral activity, correlating with suppressed IFN-β expression and decreased activation of IRF-3, NF-κB and ERK1/2. In HBV-infected HBV-Met cells, TLR stimulation did not induce antiviral cytokines in comparison with that observed in primary hepatocytes. Accordingly, suppression of HBV replication by siRNA (small interfering RNA) led to the activation or expression of pro-inflammatory transcription factors and cytokines [59]. These findings might explain why HBV, in contrast with HCV, does not induce a strong initial type I IFN response and thus appears to be a ‘stealth virus’ [60].

Although HBV does not induce an IFN response during the early phase of infection, it may be recognized by liver resident cells and thereby activate innate immune responses without IFN induction. Recently, Hösel et al. [61] showed in vitro that HBV is recognized by hepatic NPCs, mainly by KCs, upon infection of primary human liver cells. This recognition induces the release of the inflammatory cytokines IL-6, IL-8, TNF-α and IL-1β through an NF-κB-dependent pathway. Interestingly, IL-6 released by KCs controls HBV gene expression and replication in hepatocytes at the level of transcription. IL-6 is able to activate the MAPKs ERK1/2 and JNK. As a result, the expression of two transcription factors essential for HBV gene expression and replication, HNF (hepatocyte nuclear factor) 1α and HNF4α, was reduced [61].

Since TLR-mediated immune responses are able to down-regulate HBV replication, the virus has developed mechanisms to counteract these TLR functions. Investigation of hepatocytes and KCs from fresh liver biopsies from patients with CHB (chronic hepatitis B) showed that the expression of TLR2 on hepatocytes and KCs as well as peripheral monocytes was significantly reduced in patients with HBeAg-positive CHB in comparison with HBeAg-negative CHB and controls, whereas the level of TLR4 expression did not differ significantly between these groups. Transduction of hepatic cell lines with baculovirus expressing HBV led to a significant reduction in TNF-α production and phospho-p38 MAPK expression in the presence of HBeAg. In the absence of HBeAg, HBV replication was associated with an up-regulation of the TLR2 pathway, leading to increased TNF-α production [62]. Consistently, the TLR expression was significant lower in liver tissues and PBMCs (peripheral blood mononuclear cells) in woodchucks with chronic WHV (woodchuck hepatitis virus) infection, suggesting that TLR2 plays a role in hepadnaviral infection and pathogenesis (X. Zhang, Z. Ma, H. Liu, R. Broering, Z. Meng, J. Liu, D. Yang, J. F. Schlaak, M. Roggendorf and M. Lu, unpublished work).

In addition, HBV may block the expression of the MyD88 gene, which is essential in TLR-mediated activation of innate immune responses. The TP (terminal protein) domain of HBV polymerase was found to be responsible for this antagonistic activity. HBV polymerase can inhibit IFN-inducible MyD88 expression by inhibiting the activity of the MyD88 promoter through blocking the nuclear translocation of STAT1 (signal transducer and activator of transcription 1) and therefore represents a general inhibitor of IFN signalling [63].

Role of innate immunity in the pathogenesis of HCV infection

Chronic HCV infection is a global healthcare problem that affects approximately 2% of the world’s population. HCV, a member of the Flaviviridae family, is a hepatotropic virus that leads to chronic hepatitis and subsequent complications such as liver cirrhosis, liver failure or HCC [64,65]. Thus HCV infection is a main cause for liver transplantation in many countries. HCV infection is commonly treated with type I IFN in combination with ribavirin [66]. IFNs are antiviral cytokines produced by activated cells of the innate immune system during virus infection. Secretion of IFNs leads surrounding cells to turn on an ‘antiviral state’ to resist infection. HCV, however, is able to potentely activate the innate immune system.

Motifs of the HCV genome within the 5’- as well as the 3’-NTRs (non-translated regions) of the viral RNA have been identified as RIG-I (retinoic acid-inducible gene I)-activating PAMPs. It has been suggested that HCV RNA triggers IFN-β signalling in a RIG-I-dependent manner through its 5’- and 3’-NTR secondary structures [67,68]. In addition, it has been demonstrated that TLR3 is able to sense HCV infection in cultured hepatoma cells, which leads to the activation of IRF-3 and the expression of ISGs (IFN-stimulated genes). The HCV PAMPs, however, that trigger TLR3 signalling remain to be characterized [69]. These two independent signalling pathways are suggested to be involved in IRF-3 and NF-κB activation during viral infection, inducing an antiviral state [70].

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Despite this immune activation, HCV is able to subvert this antiviral pathway in infected cells. Studies have suggested that HCV utilizes its NS3/4A serine protease to facilitate chronic infection as it induces specific proteolysis of the TRIF adaptor molecule downstream of TLR3 signalling [71,72]. Viral NS3/4A protease additionally leads to disruption of RIG-I signalling, a second PRR sensing viral RNA structures. Binding of viral dsRNAs (double-stranded RNAs) to the cytosolic RNA helicase RIG-I recruits the mitochondria-associated CARDIF protein, which is involved in IKKε/TBK1 (TRAF-associated NF-κB activator-binding kinase 1)-mediated IRF-3 phosphorylation, and induces IFN-β. The NS3/4A protease has been described to cleave CARDIF and thereby abrogate IKKε/TBK1-induced IFN-β expression. The NS3/4A protein induces disruption of two signalling pathways, leading to inhibition of IRF-3 and NF-κB activation, thus controlling the cellular antiviral defence [71,72].

Broering et al. [14] and Wang et al. [73] have described the antiviral capacity of TLR-activated KCs, LSECs and HSCs against HCV. Murine KCs and LSECs pre-treated with TLR3 and TLR4 ligands potently suppressed HCV replication in a co-culture model. This antiviral effect was mostly mediated by IFN-β secretion. Although all TLRs were detectable in murine HSCs, only TLR3 and TLR4 agonists could stimulate cytokines inducing antiviral effects against HCV. IFN-β was the main cytokine mediating the antiviral activity of TLR3-stimulated HSC, whereas other cytokines of undefined nature were involved in TLR4-mediated antiviral effects. In human HSCs, only TLR3 stimulation led to production of antiviral cytokines. The antiviral effect was related to the up-regulation of ISGs and RIG-I in target cells [14,73].

HCV cell culture models [74–76] have allowed the generation of infectious HCV particles. Although complete HCV particles were not able to induce cytokine production, isolated HCV RNA showed immune stimulation, induced by enhanced TLR7 activation. It was shown that the HCV RNA genome includes G/U-rich motifs with immune-stimulatory capacity. It is proposed that HCV particles are degraded by endosomal proteases, which uncoat the viral genome and immunostimulatory motifs and leads to activation of the TLR7 pathway [77]. Lee et al. [78] revealed that TLR7 is able to mediate HCV immunity by IFN induction as well as through an IFN-independent manner.

Another evading strategy of HCV targets TLR7 expression, mRNA stability and signalling. Recent studies have identified a significantly decreased TLR7 expression in the presence of HCV, both in vitro and in vivo [79]. It was suggested that HCV may directly interfere with the transcriptional regulation of TLR7 mRNA. In addition, it was speculated that the level of HCV replication directly affects TLR7 expression, as indicated by reconstitution of TLR7 expression upon viral suppression. Despite decreased TLR7 expression in HCV-replicating cells, enhanced activation of IRF-7 was observed. This indicates that activation of the IRF-7 pathway during HCV infection occurs from other PRRs, as TLR7-induced IRF-7 nuclear translocation was significantly decreased in HCV-replicating cells [79].

HCV core protein additionally triggers innate immunity leading to inflammatory responses, but fails to induce antiviral cytokines [80]. Other studies have indicated that synthetic lipopeptide complexes of the HCV core protein activate the innate immune response through TLR2 and TLR4 [81]. Sato et al. [82] described an increased expression of some TLRs and inflammatory cytokines in PBMCs of chronically infected HCV patients. Dolganivc et al. [83] identified pre-activated monocytes in patients with chronic HCV infection. Elevated concentrations of IFN-γ, endotoxin and HCV core protein appeared to modulate monocyte functions, leading to the formation of MyD88–IRAK complexes and elevated NF-κB activation as well as enhanced production of TNF-α. This led to the suggestion that LPS, the HCV core protein and IFN-γ amplify inflammatory monocyte/macrophage activation, which involves the loss of TLR tolerance. These findings also led to the assumption that both host- and virus-derived factors modulate macrophages for persistent inflammation during chronic HCV infection [83].

**IFN response: pro- or anti-viral?**

In accordance with the findings described above, elevated hepatic gene expression in HCV-infected chimpanzees and patients has been characterized as a virus-induced type I IFN response. As already mentioned, HCV has evolved evading strategies, subverting the innate immunity. The increased ISG expression during HCV infection is thought to be induced by an activated local innate immune system. Activation of the innate immunity results in the expression and secretion of IFN-β and subsequent induction of ISGs, which may result in HCV eradication in acute hepatitis C infection [84–86].

Some ISGs have been demonstrated to directly suppress HCV replication. PKR (protein kinase R) phosphorylates the α subunit of eIF (eukaryotic initiation factor)-2, resulting in the suppression of HCV translation [87]. The RNA-specific adenosine deaminase 1 (ADAR1) binds to dsRNA thus destabilizing RNA secondary structures [88]. 2′-5′ OAS (2′-5′ oligoadenylate synthetase) activates the latently expressed endoribonuclease RNaseL, leading to degradation of viral and cellular RNAs [89]. ISG56 binds to an eIF3 subunit resulting in inhibition of translation [90–92]. In addition ISGs, such as MxA, 6–16 and viperin, have been reported as anti-HCV effectors during IFN therapy; the detailed mechanisms of action, however, are still unclear [86,93].

The virus-induced local type I IFN response appears to be paradoxical, as it is well known that IFNs may
Figure 2  Role of the innate immunity during pathogenesis of viral hepatitis

HCV infection directly induces type I IFNs, whereas viral proteins such as NS3/NS4 may inhibit this process. Activated cells of the innate immune system are still able to produce type I IFNs, resulting in increased ISG15 expression in infected cells which leads to increased HCV replication. On the contrary, HBV can inhibit endogenous IFN expression and TLR activation. This may explain why HCV but not HBV induces an initial type I IFN response during acute infection. In the case of HBV/HCV co-infection, HCV-activated NPCs may inhibit HBV replication by the production of type I IFNs.

inhibit HCV replication. Furthermore, patients who do not respond to IFN-based therapies have a highly elevated expression of a subset of ISGs in comparison with patients who cleared the virus [94,95]. One of these genes is ISG15, the product of which is a ubiquitin-like protein that is conjugated to a subset of target proteins. Although it is known that ISGylated proteins are not degraded by the proteasome, the main function of this ISGylation process has still to be determined. Studies have shown that ISGylation modulates IFN signalling [96,97], and ISGylation as well as ISG15 itself promote HCV replication [98,99]. These findings may explain why it is beneficial for HCV to induce a type I IFN response, as the IFN-induced ISG15 is important for efficient replication (Figure 2).

THERAPEUTIC PROPERTIES TARGETTING TLR SIGNALING

RNAi (RNA interference) activates TLR signalling

RNAi is an attractive option for the development of liver-specific drugs. However, effective cell- and tissue-specific delivery of RNAi therapeutics remains a key hurdle for the advancement of this technology. To date, the delivery of siRNA has been mediated by direct conjugation of lipid- [100], polymer- [101] or peptide- [102] based delivery systems, as well as antibody fusion proteins [103]. A major concern is, however, that unmodified siRNAs with or without cholesterol conjugation lead to strong and cell-type specific activation of the innate immune system in the liver and peripheral blood. The activation of the innate immune system by unmodified siRNAs is mediated by endosomally located TLR (Figure 3). In contrast, 2’-O-methyl-modified siRNAs do not trigger immune responses (R. Broering, C. I. Real, K. Jahn-Hofmann, L. Ickenstein, M. Jiang, K. Kleinher, G. Gerken and J. F. Schlaak, unpublished work).

Targeting host factors such as ISG15, which acts as a pro-viral factor in HCV replication and as a negative regulator of the IFN response, with this powerful RNAi-based strategy may represent an attractive therapeutic approach to overcome non-response to antiviral therapies for HCV in the future.

TLR ligands as therapeutic agents in liver diseases

In case of HCV, selective agonists of TLR7 have been proofed for therapeutic treatment. In a proof-of-concept study, Horsmans et al. [104] found that a once-daily treatment with intravenous isatoribine (800 mg) for 7 days caused a significant reduction in viral load compared with untreated patients. The fall in viral load occurred independently of HCV genotype and correlated with the induction of markers indicating an antiviral immune state. The treatment was well tolerated and showed a low frequency of mild-to-moderate side effects. Systemic administration of the TLR7 agonist isatoribine resulted in changes in immunological biomarkers and a statistically significant antiviral response [104].

ISS (immunostimulatory sequences) containing repeating sequences of CpG motifs have emerged as useful
tools for modulating immune responses involving the TLR9 pathway. Dynavax Technologies have produced a synthetic ODN containing these motifs, resulting in an unmethylated cytosine and phosphoguanosine ODN called 1018 ISS. The HBV vaccine HEPLISAV™ is comprised of 1018 ISS mixed with recombinant hepatitis B surface antigen and increases the frequency and magnitude of seroprotective responses after vaccination [105]. Furthermore, TLR7 agonists are currently being developed for the treatment of CHB.

**Direct inhibition of TLR as a therapeutic strategy**

Small molecules targeting TLR4 have been developed and tested in human studies. Lipid A mimetics bind to TLR4–MD2 without receptor activation, therefore inhibiting LPS-induced activation. Further studies identified MD-2 as a binding site. Lipid A mimetics inhibit LPS-mediated activation in vitro and in vivo [106,107]. A second TLR4 antagonists is TAK-242 [108]. TAK-242 targets the intracellular domain of TLR4 [109] and thus avoids the induction of a wide range of cytokines in mice treated with LPS. Both Lipid A mimics and TAK-242 are currently being tested in Phase III clinical trials in patients with septic shock [110]. As TLR4 signalling is involved in several inflammatory liver diseases, TLR4 inhibition might be a promising strategy for the treatment or prevention of these diseases.

**Therapeutics targeting TLR downstream signals**

As MyD88 is clearly involved in infectious diseases, cancer and autoimmune diseases, it is obviously an attractive target for treatment strategies. The MyD88 inhibitor ST2825 was specifically designed to block recruitment of IRAK1 and IRAK4, resulting in the inhibition of NF-κB activation [111]. ST2825 additionally suppresses CpG-induced B-cell proliferation and differentiation. Other MyD88 signalling inhibitors include Compound 4a, a MyD88 mimic, which inhibits the interaction of MyD88 and TIR domains [112], and RO0884, a dual inhibitor of IRAK1 and IRAK4, thus blocking IL-1β and TNF-α signalling, as well as IL-6 production in human cells [113].

**CONCLUSIONS**

TLRs have been identified as key regulators of innate and adaptive immune responses in the liver as they play a critical role in the pathogenesis and progression of many liver diseases, as well as in the regulation of tissue injury and wound healing processes. The local innate immune system represented by hepatocytes, LSECs, KCs and HSCs is involved in tolerance induction or inflammation and additionally cross-talks with the adaptive immune system. In contrast, pathogens have developed strategies to suppress or evade (e.g. HBV and HCV) the TLR system in the liver to facilitate chronicity of infection. Therefore therapeutic manipulation of the hepatic TLR system is of high interest for the development of novel treatments for inflammatory liver diseases.

**REFERENCES**


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