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REVIEW

The Incidence and Significance of Pattern-Recognition Receptors in Chronic Viral Hepatitis Types B and C in Man

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Abstract Chronic viral hepatitis B and C are among the most common and devastating liver diseases worldwide. Immune response plays a crucial role in the course of both diseases. In spite of the importance of the adaptive arm of the immune response, there is a growing role of innate immunity, the earliest confronted with viral attack. Pattern-recognition receptors (PRRs) and, in particular, Toll-like receptors (TLRs) are molecules which are able not only to recognize foreign invaders, but also quickly mount an antiviral defense. Activation of PRRs has been demonstrated in both hepatitis types, i.e. in situ in the liver and on while blood cells. Both viruses, HCV and HBV, are able to subvert the PRR-mediated antiviral response by means of various proteins and enzymes. HCV acts via the non-structural proteins NS2 and NS3/4A, while HBV HBeAg is inversely correlated with TLR activity. Viral counterattack is particularly directed toward dendritic cells, those creating the link with the adaptive immune response. Apart from TLRs, other PRRs such as RIG-1 and MDA-5 are also able to recognize viral infection and participate in the activation of type I interferon synthesis. TLRs manifest gene polymorphism, which was shown to affect several consequences associated with chronic viral hepatitis such as liver cirrhosis and the outcome of liver allotransplantation. There have been numerous attempts to take advantage of the existence and activity of PRRs for the patients' benefit. Several authors examined the role of TLR synthetic agonists as inducers of TLR activation. In hepatitis C the most promising agonists appear to be TLR3, 7, and 9 for potential antiviral therapy. PRRs may also act as potent adjuvants in HBV vaccines. Their baseline mRNA levels may have predictive value in the course of antiviral therapy.

Keywords Innate immunity · Pattern recognition receptors · Viral hepatitis

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Introduction

Apart from its several functions, the liver, the largest internal compact structure of the body, is now considered to be an immunological organ. It is the site of production of a variety of immune molecules and mediators such as complement components, acute-phase proteins, and some cytokines and chemokines. It has vast amounts of NK and NKT cells and macrophages, the latter represented by Kupffer cells. Several other morphological elements participate in the various immunological phenomena of the liver, such as hepatic stellate cells (HSCs), liver sinusoidal endothelial cells, and dendritic cells (DCs).

The liver possesses the unique ability to prevent systemic immunization by food antigens. At the same time, hepatocytes show relatively low expression of MHC class I antigens. This is reflected in the fairly easy acceptance of liver allografts by grafted patients. Although the liver possesses substantial numbers of cells of adaptive immunity such as T and B cells, elements of innate immunity

definitely predominate in its immunological profile (Crispe 2009).

Beyond the above-mentioned immune parameters, particular attention should be given to the family of so-called pattern-recognition receptors (PRRs), widely represented in the liver. This family consists of at least seven groups of molecules, both cell surface bound and cytoplasmic, able to recognize various substances potentially dangerous to an individual. The family encompasses Toll-like receptors (TLRs), NOD-like receptors, retinoic acid-inducible gene 1-like helicases (RIG)-like receptors, C-lectin receptors, scavenger receptors, and others. Of them, the TLRs have been the most extensively studied. For the sake of brevity, only their short characteristics will be given below because the general properties of TLRs are comparable to other groups of PRRs. Information about the latter will, however, be provided in the respective paragraphs as needed.

TLRs are evolutionary conserved structures initially demonstrated in the fruit fly *Drosophila melanogaster* serving as an element governing its body's longitudinal growth and as an anti-fungal agent. Soon thereafter, structures homologous to TLRs were traced in higher animals, including man. They are able to recognize a variety of molecules present in various pathogens essential for their growth and survival, such as sugars, lipopolysaccharides, glycolipids, RNA and DNA, and others, collectively called pathogen-associated molecular patterns (PAMPs). According to the broad specificity of the recognized PAMPs, they are divided into subfamilies, ranging from TLR1 to TLR11 in man (Table 1). As far as immune cells are concerned, the majority of TLRs are expressed on the cell surface (TLRs1, 2, 4, 5, 6, and 10). Some, however, such as TLR3, 7, 8, and 9, are located intracellularly. In structural terms, a typical TLR consists of extracellular, transmembrane, and intracellular domains. The latter, termed TIR, is structurally homologous to the interleukin (IL)-1 receptor. The extracellular domain consists of multiple leucine-rich repeats. Interaction of TLR with the respective ligand results in a series of intracellular biochemical events leading to the activation and transcription of genes with subsequent secretion of various proteins, predominantly proinflammatory cytokines and cell-adhesion molecules, and up-regulated expression of MHC molecules and other consequences (Takeda et al. 2003).

Among the various liver disorders, viral hepatitis, especially induced by hepatotropic viruses B (HBV) and C (HCV), poses important problems, both clinical and social. In spite of the application of an extensive array of antiviral drugs, the viruses cannot be totally eliminated from the body of the patient and the disease becomes chronic and may lead to severe consequences such as liver cirrhosis and hepato-cellular carcinoma. The number of hepatitis B-infected people worldwide is estimated at 2 billion and those with hepatitis C at 200 million. In the case of hepatitis B, prevention of the infection became possible because a specific vaccine has been available since 1995. Unfortunately, there is no vaccine for hepatitis C due to the great variability of the viral genome (Lok and McMachon 2001; Rantala and van de Laar 2008).

The course of disease and the specific antiviral immune response are similar in both conditions. The viruses induce cell-mediated immunity and the formation of cytolytic T cells, which destroy a portion of the infected hepatocytes. Damage to the liver structure depends mainly on the intensity of the cell-mediated immune response, not on the viruses themselves, because they are not cytopathic.

By and large, the mechanisms of adaptive immunity are unable to eradicate hepatotropic viruses and were shown to contribute to substantial liver damage. This raised interest in the agents and mechanisms of innate immunity in combating these diseases, taking into account the potent armamentarium of this form of immunity in the liver. In particular, PRRs seemed to be especially attractive due to their broad distribution and great cell-signaling capacity. Besides, it has been noted that viral proteins often inhibit mechanisms of innate immunity via PRRs. For example,

Table 1 General and HBV/HCV associated TLR ligands

TLR	CD	Ligands	Ligands in HBV/HCV-infected liver
TLR1	CD281	Triacyl lipoproteins	–
TLR2	CD282	Peptidoglycan, lipoteichoic acid	HCVc (core protein), HbeAg
TLR3	CD283	Viral dsRNA	Viral HCV dsRNA
TLR4	CD284	Lipopolysaccharides	–
TLR5	CD285	Flagellin	–
TLR6	CD286	Lipoteichoic acid, triacyl lipoproteins	–
TLR7	CD287	Viral ssRNA (resiquimod)	Viral HCV ssRNA
TLR8	CD288	Viral ssRNA	Viral HCV ssRNA
TLR9	CD289	DNA—bacterial and viral	Viral HBV DNA
TLR10	CD290	Unknown	–
TLR11		Profilin	–

liver macrophages, i.e. Kupffer cells, have abundant expression of TLRs. It was demonstrated that cross-talk between Kupffer cells and NK cells takes place via TLRs. Kupffer cells' TLRs respond to TLR ligands and enhance the antiviral activity of NK cells by secretion of IL-18. It is suspected that subversion of this process by hepatotropic viruses might be a possible cause of viral persistence (Tu et al. 2008). This explains the greatly increased interest of researchers in the virus-associated innate response and liver diseases (Kanto 2008).

Incidence and Putative Function of PRRs in Chronic Viral Hepatitis B and C

Of the TLRs, TLR4 has been the most extensively studied. Its expression was found to be higher in the hepatocytes of patients with hepatitis B than in those of healthy controls. Moreover, the intensity of the immunohistochemical reaction positively correlated with grading scores (Wei et al. 2008). We were able to demonstrate TLR4, TLR2, and TLR3 staining of hepatocytes in the livers of children with hepatitis C. The reaction was both cytoplasmic and cell membrane bound, but the former predominated (Mozer-Lisewska et al. 2005).

It is of interest that peripheral blood leukocytes have also been shown to either up-regulate or down-regulate TLR expression in chronic viral hepatitis. In hepatitis C patients, mRNA levels for TLRs2, 6, 7, 8, 9, and 10 were increased in both monocytes and T cells (Dolganiuc et al. 2006). In contrast, the expressions of TLR1, 2, 4, and 6 mRNA transcripts were significantly lower in the peripheral mononuclear blood cells (PBMCs) of patients with chronic hepatitis B compared with chronic HCV infection and healthy controls. In another study comprising 41 patients with chronic HBV infection and 11 healthy controls, all HBV-positive patients had lower TLR7 and TLR9 mRNA expression and TLR7 at the protein level, but TLR9 protein was increased and correlated with serum HBV DNA. This might suggest links between TLR9 expression and viral proliferation. The authors did not comment their findings (Xu et al. 2008). Nevertheless, in in vitro studies it was shown that when HBV-positive liver cell lines were co-transfected with TLR adaptors, leading to up-regulation of TLR activity, the levels of HBV mRNA and DNA were dramatically reduced (Guo et al. 2009). On the other hand, TLR2 expression on PBMCs at the protein level was markedly decreased and it related to HBV genotype C (Chen et al. 2008). The discrepancies between hepatitis B and C such as differences in TLR mRNA transcripts in PBMCs are hard to explain. However, some novel data were obtained by searching DNA from hepatocytes and infiltrating lymphoid cells by complex cDNA microarray, which allowed discerning the hepatitis B phenotype as proapoptotic and favoring DNA repair, while the hepatitis C phenotype was found to be more inflammatory and anti-apoptotic (Honda et al. 2006). This indicates that different signaling pathways, which may be reflected in TLR signaling, might be involved in the development of hepatitis B and C.

We demonstrated up-regulation of TLR2, 3, and 4 on lymphocytes and granulocytes in children with chronic hepatitis C infection (Mozer-Lisewska et al. 2006). Up-regulation of mRNA of TLR2 and TLR4 was also found on the PBMCs of adults with chronic HCV infection, showing, in addition, significant correlation with viral load and serum tumor necrosis factor (TNF) level. In contrast, the cells of patients with liver cirrhosis demonstrated down-regulation of TLR4, but not TLR2 (Shehata et al. 2006). In another study, up-regulation of TLR2 and TLR4 expression on monocytes from HCV-infected patients correlated with TNF and ALT levels, but was not related to viral load (Riordan et al. 2006).

In HBeAg-positive patients with chronic hepatitis B, expression of TLR2 was significantly decreased on hepatocytes, Kupffer cells in blood monocytes, compared with HBeAg-negative patients and controls. Still, the HBeAg-negative patients had much higher TLR2 values than the controls. The HBeAg-positive patients had reduced cytokine (TNF-α) production and phospho-p38 kinase expression. Absence of HBeAg correlated with up-regulation of the TLR2 pathway, HBV replication, and increased TNF-α levels (Visvanathan et al. 2007) These data clearly indicate a negative role of TLR2 in HBV chronic infection associated with the presence or absence of HBeAg.

Very little is known about sensing by TLRs hepatotropic viruses. It was shown recently by in vitro assays that HCV core protein is sensed by TLR2 but not by TLR4, in spite of the fact that mRNA for both TLRs could be easily demonstrated on primary human hepatocytes. In contrast, recombinant HCV infectious virions did not significantly activate either TLR2 or TLR4 signaling pathways in those TLR-expressing cells (Hoffmann et al. 2009). The authors concluded that HCV core protein is apparently absent from enveloped virions. TLR7, present in normal and HCV-infected hepatocytes, was found following activation to induce several antiviral genes, including interferon (IFN) regulatory factor-7 (IRF7) as well as IRF3 in the hepatocyte HCV^{+} cell line Huh-7 (Lee et al. 2006). Out of ten TLRs, only TLR4 was found to be strongly activated (up to sevenfold) following HCV infection. This resulted in IFN-β production and IL-6 secretion from B cells (Machida et al. 2006). TLR4 was also shown to be responsible for inflammatory signaling of HSCs (Paik et al. 2003). Human HSCs activated via TLR3 resulted in the production of IFN-β and other antiviral cytokines (Wang et al. 2009).

These data imply that the host antiviral response is initiated by the reactivity of TLRs.

Recent data provide evidence that TLRs act through adaptor molecules such as myeloid differentiation factor 88, the Toll/IL-1 receptor domain-containing adaptor protein, and others which lead to the activation of transcription factors such as NF-κB and activator protein (AP-1). Moreover, it has become obvious that TLR signaling in the liver and in particular in viral hepatitis is apparently not limited to the cells of the immune system, but includes hepatocytes, endothelial cells, biliary epithelial cells, and HSCs (Seki and Brenner 2008).

Subverting the Effects of Viral Products on Various Cells of the Liver

Hepatitis core protein (HCVc), a TLR2 ligand, has been shown to depress the antiviral activities of Kupffer cells in chronically infected patients. HCVc down-regulated TLR3-mediated secretion of IFN-α and IFN-β and the surface expression of the cytotoxic molecule TRAIL (Tu et al. 2010).

The inhibitory effects of both HBV and HCV are especially evident in DCs. The latter are crucial for mounting specific adaptive immunity, which seems to be a definitive disadvantage for these pathogens. Several reports in the literature confirm detrimental action of both viruses on DC. For example, both plasmocytoid and myeloid intrahepatic DCs were quite abundant and activated in chronic hepatitis B liver failure. pDCs were able to produce IFN-α, which subsequently induced cytokine production in liver lymphocytes via TLR9 ligation. The low frequency of peripheral pDCs resulted, however, in markedly reduced IFN-α production, which was especially evident in non-survivors (Zhang et al. 2008). HBV surface antigen (HBsAg) was found to block IFN-α production by pDCs via inhibition TLR9-mediated activation of these cells (Xu et al. 2009). In patients with chronic HBV infection, pDCs displayed substantial reduction (up to 50%) of TLR9 expression, which correlated with the fall in IFN-α production in vitro. Moreover, the frequency of pDCs in these patients was much lower, inversely correlating with serum ALT levels and HBV viremia (Xie et al. 2009). HCV was found to be a poor inducer of IFN-α by pDCs compared with other viruses such as influenza and herpes virus-1. This was due to down-regulation of the expression of TLR9 and the transcription factor IRF7 (Gondois-Rey et al. 2009).

Hepatitis C core protein was shown to associate with the gC1q receptor present on mDCs. This resulted in skewing cytokine production by $CD4^+$ T cells toward Th2-type cytokines, probably by inhibition of TLR-mediated IL-12 production. Such a shift might favor viral persistence (Waggoner et al. 2007). pDCs, but not mDCs, isolated from the peripheral blood of hepatitis C patients exposed to TLR ligands have shown poor activation and low IFN-α expression. This resulted in impaired boosting of naïve $CD4^+$ T cells (Yonkers et al. 2007).

The HCV non-structural proteins NS2 and NS3/4A (serine protease) were found to be potent inhibitors of cytokine gene expression. In in vitro studies, these proteins inhibited the expressions of various type I IFNs and several chemokine gene promoters (Kaukinen et al. 2006). Previously, NS3/4A serine protease was shown to cause proteolysis of the Toll-IL-1 receptor domain-containing adaptor TRIF for activating IRF3 as well as NF-κB transcription factors. Cleavage of TRIF poly1:C-activated signaling took place through the TLR3 pathway. This limits the expression of several host defense genes, which leads to promoting persistent HCV infection (Li et al. 2005).

Apart from TLRs, an important member of antiviral innate immunity is cytoplasmic RIG-1, another member of the PRR family. It is generally accepted that in hepatocytes, RIG-1 plays an essential role in the recognition of the HCV viral genome. RIG-1 binds PAMP RNA and leads to signaling of IRF3. This results in the activation of further signaling to induce IFN-α and the production of various proinflammatory cytokines. IFN subsequently induces more than 300 effector molecules, termed IFN-stimulated genes (ISGs), that form an antiviral state in infected cells. Several viral strategies of HCV are able to disrupt this innate immune response and ISG function (Saito and Gale 2008).

Viral NS3/4A protease was also found to disrupt RIG-1 signaling of downstream IRF3 and NF-κB activation. It down-regulated the expression of host antiviral defense genes and interrupted an IFN amplification loop crucial for the suppression of HCV replication (Foy et al. 2005).

The mitochondrial antiviral signaling (MAVS) protein functions as a target for intracellular receptors of the RIG-1-like helicase family, resulting in a triggering cascade upon the recognition of virus. It was shown that HCV NS3/4A protease disrupts MAVS oligomer, which leads to the loss of antiviral signaling (Baril et al. 2009). Other non-structural HCV proteins such as NS4B were also shown to block dsRNA triggered RIG-1-mediated IFN expression (Tasaka et al. 2007).

Apart from TLR5 and RIG-1, another PPR member, melanoma differentiation-associated gene-5 (MDA-5), is also able to recognize viral infection and participate in the activation of IRF3. Each of the above requires adaptor proteins such as TRIF for TLR3 and Cardif (MAVS) for RIG-1. The precise signaling pathway of MDA-5 is not known. The final effect of down-stream signaling of the

above PRRs is the entry of transcription factors into the cell nucleus and induction of the synthesis of IFN type 1.

It was also found that HCV NS proteins are inhibitors of cytokine/chemokine gene expression. The mechanism of action was due to cleavage by NS3/4A serine protease of the RIG-1 adaptor protein Cardif. Co-localization of Cardif and NS3/4A at the mitochondrial membrane suggested that the latter is the site for proteolytic cleavage (Kaukinen et al. 2006).

Quantitative determination of mRNA of TLR3 and 7 and RIG-1 may be of value in the assessment of the clinical course of chronic hepatitis C. It was found that these values were significantly down-regulated in HCV patients compared with healthy controls. Moreover, TLR3 and 7, but not RIG-1, correlated with IFN-α levels (Atencia et al. 2007). Some PRR–viral interactions in infected hepatocytes are schematically depicted in Fig. 1.

Significance of TLR Gene Polymorphisms for Disease Course in Chronic Viral Hepatitis

It later became evident that PRRs, mainly TLRs, although conserved in evolution, nevertheless undergo biological alterations such as mutations and polymorphisms. The significance of TLR polymorphisms for susceptibility to infectious diseases was already noted some time ago (Schroder and Schumann 2005). These polymorphisms were also found to have significant impact on the course of several conditions of liver pathology, including viral hepatitis. TLR polymorphism turned out to influence the fate of liver transplant for chronic hepatitis C. The association

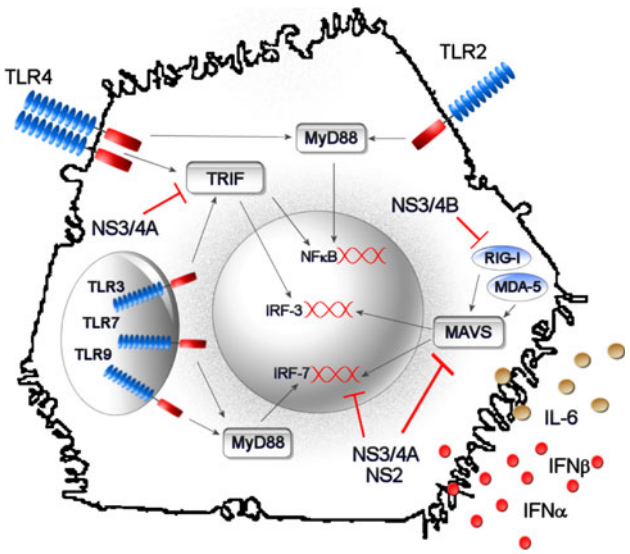

Fig. 1 PRR–viral interactions in a schematic HCV-infected hepatocyte

was assessed between single-nucleotide polymorphisms (SNPs) in genes that encode TLR2 and TLR4 in a historical cohort of 92 liver transplant patients. Homozygous TLR2 Arg7536Ln polymorphism was associated with liver allograft rejection and mortality (Eid et al. 2007). TLR3 gene polymorphisms, also studied for SNPs, were shown to be without any association with clinical parameters of chronic hepatitis C, but conferred a different susceptibility for HCV subtype infection (Askar et al. 2009). In contrast, one variant (c.1120T>G) of TLR7 in male patients with chronic hepatitis C was more common in those with no or weakly manifested inflammation and in those with no or little advanced liver fibrosis (Schott et al. 2007). In a detailed analysis of the TLR4 locus while studying 61 SNPs within a linkage disequilibrium area across a region of 76 kbp, 15 SNPs were identified to be associated with the risk of liver cirrhosis in patients with chronic hepatitis C (Li et al. 2009). It was not possible to find any data related to polymorphisms of other PRRs in chronic viral hepatitis.

PRRs and Antiviral Therapy

The primary goal of investigators involved in PRR research in hepatitis was, apart from basic knowledge, to search for the possibility of applying these agents in the treatment of patients. There are some data in the literature suggesting that PRRs may indeed inhibit viral replication. For example, it was shown that TLR2 signaling may inhibit HBV replication in vivo (Isogawa et al. 2005). In experiments on mice it was found that supernatants from TLR1-9 stimulated non-parenchymal liver cells, such as Kupffer cells, DCs, and LSECs, resulting in potent suppression of HCV replication through induction of IFN-β and expression on ISG (Broering et al. 2008). On the other hand, inhibition of PRR signaling was found by others to be to the virus' advantage (Breiman et al. 2005). Thus it seemed sensible that manipulation of PRR activity might be therapeutically advantageous for hepatitis patients. Attention was focused on TLR agonists, both natural and synthetic, because these agents are able to raise TLR activity. For example, it was found recently that synthetic class C CpG ODN agonists of TLR9 induced robust IFN-α production in vitro by pDCs from chronic HCV patients (Libri et al. 2009). This was of interest because, as shown earlier, viral products act antagonistically toward type 1 IFN synthesis. An up-regulation of innate immunity was also shown in vivo in patients with chronic HCV infection after parenteral administration of the TLR9 synthetic agonist CpG10101. There were increases in some cytokines, IFN-γ-inducible protein 10, type 1 IFN, and 2′,5′-oligoadenylate synthetase (OAS), a potent antiviral biomarker. This effect was dose

dependent and was associated with a decrease in HCV RNA (McHutchison et al. 2007).

Another group of investigators studied the effect of agonists incubated with human PBMCs in in vitro. Culture supernatant was then added to HCV replicon cells to assess its antiviral activity manifested by the induction of type 1 IFN and OAS. Several TLR agonists, including those of TLR3, 4, 7, 8, and 9, induced an antiviral effect. Some of them, namely agonists of TLR4 and 8, also stimulated proinflammatory cytokines, which might result in adverse side effects. Thus the conclusion from the above-described experiments was that agonists of TLR3, 7, and 9 are the most attractive agents for potential antiviral therapy in chronic hepatitis C (Thomas et al. 2007). On the other hand, from experiments in mice one should be aware of the fact that the activation of TLR9 expressed on HSCs enhanced liver fibrosis (Gabele et al. 2008).

An attempt was also made to apply resiquimod, a TLR7 agonist and a derivative of imiquimod, orally in patients with chronic hepatitis C. Orally administered resiquimod resulted in a marked, albeit transient, reduction of viremia, but severe side effects forced discontinuation of such treatment (Pockros et al. 2007). Both compounds were found effective in various pathological conditions when applied topically, such as human papilloma virus infection, skin cancers, and actinic keratosis, but attempts of systemic therapy of hepatitis C were not successful (Miller et al. 2008).

In patients with hepatitis C and genotype 1 treated with pegylated IFN and ribavirin, low expression of TLR correlated with good response. The authors claimed that low values of TLR3 were the best cutoffs to predict response (Yuki et al. 2010).

TLRs may also act as potent adjuvants in vaccination trials. Levamisole, a well-known compound stimulating cell-mediated immunity, was found to up-regulate response to HBV vaccine containing HBsAg. Levamisole is known to stimulate the expressions of TLR7 and 8 as well as their down-stream proinflammatory cytokines. Such combined treatment might be implemented in chronically infected patients to clear viral infection due to the induction of strong antigen-specific cell-mediated immunity (Zhang et al. 2009).

It has also been shown in in vitro tests that a synthetic TLR9 agonist, CpG oligodeoxy-nucleotide, a potent inducer of cytokines, reduced the effective concentration of lamivudine by up to 50% when cultured with cells transduced with HBV. Viral replication was inhibited as well as HBsAg and HBeAg secretion (Vincent et al. 2009).

In another study, baseline TLR2-9 mRNA levels in PBMCs were compared in HCV^+ patients treated with pegylated IFN and ribavirin. After 48 weeks of treatment the patients were divided into non-responders and sustained responders. It was shown that the sustained responders had much higher levels of the TLR4, 6, and 9 mRNA than non-responders and controls. This was accompanied by high mRNA of IFN-γ and nuclear factor of activated T cells (He et al. 2006).

The above-presented data do not offer an alternative to the current methods of the antiviral treatment of hepatitis, but show potential novel approaches, perhaps as an adjunct acting in concert with those currently used.

Concluding Remarks

It seems that mechanisms of innate immunity are of importance in HBV and HCV infection of the human liver. PRRs and, in particular, TLRs appear to exhibit a pivotal role in the pathogenesis of liver infectious diseases due to their unique ability for rapid signal transduction. The importance of TLRs in antiviral immunity is reflected by various means aimed to inhibit their function generated by the viral genomes, such as the NS proteins of HCV. TLR agonists provide novel tools with antiviral activity potential. The significance of other PRRs in the pathogenesis of viral hepatitis, with the possible exception of RIG-1, remains to be elucidated.

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