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Toll-like receptors as targets in chronic liver diseases

Ali Mencin, **Johannes Kluwe**, and **Robert F. Schwabe**
Department of Medicine, Columbia University, College of Physicians and Surgeons, New York, NY 10032

Abstract

Toll-like receptors (TLRs) recognize pathogen associated molecular patterns (PAMPs) to detect the presence of pathogens. In addition to their role in innate immunity, TLRs also play a major role in the regulation of inflammation, even under sterile conditions such as injury and wound healing. This involvement has been suggested to be depend, at least in part, on the ability of TLRs to recognize several endogenous TLR ligands termed damage associated molecular patterns (DAMPs). The liver not only represents a major target of bacterial PAMPs in many disease states but also upregulates several DAMPs following injury. Accordingly, TLR-mediated signals have been implicated in a number of chronic liver diseases. Here, we will summarize recent findings on the role TLRs and TLR ligands in the pathophysiology of liver fibrosis and cirrhosis, viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease and hepatocellular carcinoma, and highlight the potential role of TLR agonists, antagonists and probiotics for the treatment of chronic liver disease.

Keywords

Bambi; hepatocyte; hepatic stellate cell; E5564; TAK-242

Introduction

Toll-like receptors (TLRs) are a highly conserved group of pattern recognition receptors that function as pathogen sensors in vertebrate and invertebrate species. The recognition of specific signature molecules termed pathogen associated molecular patterns (PAMPs) by TLRs is a cornerstone of the innate immune system, and enables it to rapidly mount protective responses against invading pathogens [1,2,3,4]. In addition to their role in the innate immune system, TLRs contribute significantly to a number of other processes including adaptive immune responses, regulation of sterile inflammation, wound healing and promotion of epithelial regeneration and carcinogenesis [5,6,7]. Toll-like receptors play a major role in liver physiology and pathophysiology due to the liver's anatomic association with the intestine and its exposure to relatively large amounts of intestinally derived PAMPs in healthy and disease states [8,9,10].

I. TLR signaling

Toll-like receptors and co-receptors

The human TLR family consists of currently 10 members, which are structurally characterized by the presence of a leucine-rich repeat (LRR) domain in their extracellular domain and a Toll/interleukin (IL)-1 receptor (TIR) domain in their intracellular domain [11]. The existence of a

Correspondence: Robert F. Schwabe; Department of Medicine, Columbia University, College of Physicians&Surgeons, Russ Berrie Pavilion, Room 415, New York, NY 10032; rfs2102@columbia.edu; Tel. (212) 851-5462, Fax. (212) 851-5461.

large number of TLRs enables the innate immune system to discriminate between PAMPs that are characteristic of different microbial classes and launch specific defense mechanisms [11, 12]. TLR4 is best known for sensing gram-negative bacteria by detecting lipopolysaccharide (LPS), a membrane component of gram-negative bacteria. TLR2 senses gram-positive infection by recognizing cell membrane components such as leipoteichoic acid, peptidoglycan, and various lipopeptides and lipoproteins. TLR3 and TLR7 sense viral infections by recognizing double-stranded and single-stranded RNA, respectively. TLR9 recognizes non-methylated CpG-containing DNA from bacteria and viruses. The molecular basis for ligand recognition is largely unknown but it has been suggested that leucine rich repeated domains in the extracellular domain of TLRs may be involved in ligand binding [13]. Activation of some TLRs such as TLR2 and TLR4 additionally requires the presence of co-receptors. Two of these co-receptors, CD14 and MD-2, also contain leucine rich repeat domains suggesting their involvement in ligand recognition and binding. Activation of TLR4 by LPS absolutely requires the presence of the co-receptor MD-2 for signaling, whereas some TLR4-mediated signals may still be generated in the absence of CD14 [14]. MD-2 is not only required for cell-surface expression of TLR4 [15], but also appears to be essential for the activation of the TLR4 signaling cascade [16]. The most recent model of LPS-mediated TLR4 activation suggests that CD14 catalyzes the binding of LPS to the MD-2-TLR4 complex. Binding of LPS to MD-2 induces a conformational change in MD-2 which then allows this complex to bind to a second TLR4 receptor thus achieving TLR4 homo-dimerization and signaling [17]. TLR2 ligands require the presence of CD36 for efficient signal induction by some ligands but the exact role of CD36 in TLR2 signaling remains to be elucidated [18]. Although each TLR detects specific ligands, many of the signaling molecules that mediate intracellular response are shared by the TLRs (see Figure 1). All TLRs signal through one or two adapter molecules termed MyD88 and Trif which explains why responses to different TLR ligands often generate similar downstream signals [11,12]. Thus, it appears that the existence of a large number of TLRs mainly serves to enable an efficient and specific detection of a wide range of PAMPs rather than generating highly tailored immune responses.

TLR-mediated signals are transduced through two major pathways, the “MyD88-dependent pathway” and the “MyD88-independent pathway”. MyD88 is an essential part of the signaling cascade of all TLRs except for TLR3. In contrast, Trif only interacts with TLR3 and TLR4, and is responsible for mediating MyD88-independent signals (see Figure 1). Both MyD88-dependent and MyD88-independent pathways initiate the transcription of a specific set of genes involved in proinflammatory, antiviral, and antibacterial responses [11]. In TLR4-signaling, MyD88 mediates an upregulation of inflammatory cytokines through activation of NF-κB whereas the MyD88-independent pathway contributes to both interferon upregulation through IRF-3, and inflammatory gene induction through NF-κB (see Figure 1). The MyD88-dependent and the MyD88-independent pathway mediate NF-κB and subsequent inflammatory cytokine production through different mechanisms and kinetics: NF-κB induction in the MyD88-dependent pathway occurs with fast kinetics whereas NF-κB activation in the MyD88-independent pathway occurs with slower kinetics. TLR3 interacts with Trif resulting in an upregulation of interferon and inflammatory cytokines through the MyD88-independent pathway in a similar fashion as the MyD88-independent pathway in TLR4 signaling (see above). In TLR7 and TLR9 signaling, MyD88 induces an IRF-7-dependent upregulation of interferon production and NF-κB-dependent upregulation of inflammatory cytokines. In TLR2 signaling, MyD88 upregulates inflammatory cytokines through an NF-κB dependent pathway.

Negative regulation of TLR signaling

Although strong proinflammatory and anti-viral responses after TLR stimulation may be beneficial in the short term to eradicate pathogens, a prolonged or exaggerated activation of TLR signaling may have deleterious effects. Therefore, several mechanisms have evolved that

negatively regulate TLR-induced cellular responses [19]. These mechanisms act at the receptor level (RP105, ST2 and SIGGIR expression, TLR downregulation or degradation), at the level of adapter molecules such as MyD88 and TIRAP, or receptor proximal kinases such as IRAK. Mechanisms to achieve a reduction in TLR signaling include decreased TLR4 transcription [20], proteolytic degradation of TLR4, TLR 9 and TIRAP after being marked for degradation by the E3 ubiquitin ligase Triad3A [21] or SOCS-1 [22], expression of inhibitory TIR-domain containing receptors such as ST2 and SIGIRR [23,24] or LRR-domain containing receptors such as RP105 [25], and expression of non-functional signaling molecules such as MyD88s [26,27], IRAK-M [28], IRAK2c and IRAK2d [29].

II. TLR ligands

Pathogen associated molecular patterns (PAMPs)

Early detection of infection is advantageous in mounting an efficient defense against pathogens. Accordingly, even minuscule amounts of PAMPs such as lipopolysaccharide (LPS), lipopeptides, unmethylated DNA, and double-stranded RNA evoke intense inflammatory reactions. Many proinflammatory effects of PAMPs are a consequence of TLR-induced secretion of inflammatory mediators such as TNFα and IL-1β. Accordingly, PAMPs such as LPS, lipopeptides, dsRNA and unmethylated DNA are among the strongest inducers of TNFα and IL-1β release *in vitro* and *in vivo* [5]. As most bacteria reside in the extracellular space, TLRs that detect bacterial PAMPs such as LPS and lipoproteins are located on the cell surface. Viral RNA and bacterial DNA but not host DNA are present in late endosome-lysosomes. Therefore, TLR3, TLR7 and TLR9 are located in these cellular organelles [12]. The restriction of TLR ligand recognition to specific cellular compartments such as the cell membrane or lysosomes not only increases chances to encounter specific PAMPs but also decreases the chance of TLRs to be exposed to and aberrantly activated by host molecules, thus adding an additional level of control to ensure proper TLR activation.

Regulation of bacterial translocation by the intestinal barrier

The intestinal microbiota hosts more than 99% of the bacterial mass in the body and is the principal source of bacterially derived PAMPs in health and many disease states. Several protective mechanisms ensure that only a minute amount of bacteria and bacterial products reaches the portal circulation under normal circumstances. These include a thick layer of mucins, secretion of IgA and antimicrobial factors, a tightly sealed epithelial surface and an active mucosa-associated lymphatic tissue (MALT) (see Figure 2) [30]. Accordingly, portal and systemic LPS levels are nearly undetectable in normal rats and healthy people, respectively [31,32,33]. In chronic liver disease, structural changes of the intestinal mucosa such as loss of tight junctions, widening of intercellular spaces, vascular congestion, and defects in the mucosal immune system promote the loss of barrier function and allow increased translocation of bacteria and bacterial PAMPs [30]. Whereas the upper gastrointestinal tract is only sparsely populated, microbial density gradually increases distally with about 10^5 colony-forming units/mL in the jejunum to 10^8 in distal ileum and cecum, and up to 10^{12} in the colon [34]. Although, intestinal anaerobic bacteria outnumber aerobic bacteria by a ratio of 100:1 to 1,000:1 [34], virtually all translocating bacteria are aerobic [30]. In fact, anaerobic bacteria suppress colonization and growth of potentially invasive microbes and thereby exert an important role in maintaining gastrointestinal health and in reducing the translocation of potentially harmful microbes [35]. Accordingly, selective elimination of anaerobic bacteria promotes intestinal bacterial overgrowth and translocation [35]. Gram-negative bacteria such as Escherichia coli, Klebsiella pneumoniae, enterococci and streptococci not only represent the species that are most proficient at translocation, but also cause the large majority of infections in patients with cirrhosis [36,37]. In cirrhosis, overgrowth of bacteria, especially in locations with low bacterial counts such as the proximal small intestine, and overgrowth of strains with a higher

translocation capacity may occur, possibly due to changes in the intestinal motility and the decreased luminal levels of bile acid, a suppressor of bacterial growth [30]. Bacterial overgrowth together with the above described changes in the intestinal mucosal barrier result in an increased rate of bacterial translocation and endotoxemia.

Damage associated molecular patterns (DAMPs)

Several TLRs not only have the ability to recognize more than one ligand, but often recognize ligands with completely different chemical structures [38]. The best examples for the high promiscuity of TLRs are TLR2 and TLR4. TLR4 recognizes lipids such as the lipid A portion of LPS as well as proteins from respiratory syncytial virus, vesicular stomatitis virus and mouse mammary tumor virus [39,40,41]. TLR2 recognizes a wide range of ligands including lipoteichoic acids, various proteins including lipoproteins and glycoprotein's, zymosan, and peptidoglycan as well as lipopolysaccharides from specific bacterial strains [11,38]. The ability of TLRs to recognize ligands that are chemically unrelated is believed to be the basis for the activation of TLRs by endogenous ligands. This activation does not occur under normal circumstances but only when there is a change in the environment that either leads to the release of endogenous ligands from a cellular compartment that is usually not in contact with TLRs, or to the modification of endogenous mediator that gives them the ability to activate TLRs [38,42]. Due to the association of many endogenous ligands with tissue injury, the nomenclature of damage associated molecular pattern (DAMP) has been suggested. The best characterized DAMPs include HMGB1, hyaluronan, S100 proteins, the alternatively spliced extra domain A of fibronectin and heat shock protein 60. Most of these ligands act as agonists of TLR2 or TLR4, or both receptors. However, there is still ongoing controversy whether these DAMPs indeed are bona fide TLR ligands since many of these ligands have either been purified in bacterial systems or have a high affinity to bacterial products suggesting that bacterial products or other TLR activating substances such as lipids or DNA rather than the ligands themselves mediate their TLR activating effect [43]. Further studies including genetic inactivation of candidate molecules are required to characterize DAMPs as bona fide TLR ligands, and it is likely that only some of the above mentioned candidates will be confirmed as TLR ligands. Furthermore, it has been suggested that endogenous DNA can act as a TLR9 agonist to promote autoimmune reactions [5]. HMGB1 and hyaluronan from eukaryotic sources have been shown to activate TLRs thus largely excluding bacterial contaminants as mediators of TLR signals [44,45]. Moreover, HMGB1 and hyaluronan are elevated in liver disease and HMGB1 plays a potential role in the pathophysiology of liver disease [46,47]. For these reasons, we will limit further discussion on TLR-activating DAMPs to HMGB1 and hyaluronan.

Release of DAMPs

Release of DAMPs into the extracellular space is achieved by a number of different mechanisms that involve (i) leakage from necrotic cells, (ii) increased synthesis and post-translational modification in response to inflammation, and (iii) degradation of inactive precursors into TLR-mimetic degradation products in inflammatory environments.

HMGB1

HMGB1 is a DNA-binding protein that induces bends in the helical DNA structure to facilitate multiple physical interactions of DNA with transcription factors, recombinases and steroid hormone receptors, and to thus allow transcription and other nuclear transactions to take place [48]. In addition to this transcription factor-like function, HMGB1 also has cytokine-like effects that require its presence in the extracellular space [48,49]. Release of HMGB1 into the extracellular space is mediated by two mechanisms: (i) Acetylation of many of its 43 lysine residues that lie in proximity to its two nuclear-localization signals thus reducing interaction

with the nuclear importer protein complex, preventing nuclear re-entry and promoting secretion of HMGB1 [50]. This active HMGB1 secretion process seems to occur predominantly in inflammatory cells. (ii) The passive diffusion of HMGB1 from cells that undergo necrosis [51]. Importantly, HMGB1 release does not occur from apoptotic cells, presumably because HMGB1 is tightly bound to cruciform DNA and hypo-acetylated proteins within the apoptotic-cell nucleus whereas it is only loosely bound to DNA in necrotic cells [49]. Thus HMGB1 has been suggested to be a signature DAMP that signals the presence of necrosis, and subsequently triggers inflammation [51].

Hyaluronan

Hyaluronan is a negatively charged high molecular weight glycosaminoglycan, which is ubiquitously distributed in the extracellular matrix and a component of the basement membrane. At sites of inflammation and tissue destruction, high molecular weight hyaluronan can be broken down to lower molecular weight hyaluronan fragments via oxygen radicals and enzymatic degradation. In contrast to high molecular weight hyaluronan, low molecular weight hyaluronan has cytokine-like properties, and induces inflammatory gene expression in epithelial cells, endothelial cells, fibroblasts, dendritic cells (DCs), and macrophages [52]. In the extacellular space, hyaluronan is bound by the CD44 receptor which mediates some of its proinflammatory effects. However, hyaluronan is able to stimulate chemokine production in the absence of CD44 [45]. Since the disruption of basement membranes is typically associated with injury, it has been suggested that the recognition of low molecular weight hyaluronan by TLRs and other receptors is part of an injury recognition system.

III. TLR expression in the liver

Due to its anatomical links to the gut, the liver is constantly exposed to gut-derived bacterial products, and functions as a major filter organ and a first line of defense. 80% of intravenously injected endotoxin is detected in the liver within 20–30 minutes [53,54]. Moreover, the liver is an important site for bacterial phagocytosis and clearance as it hosts more then 80% of the body's macrophages. Kupffer cells, the resident macrophages of the liver, are able to efficiently take up endotoxin and phagocytose bacteria carried through the portal vein and are considered to play a major role in the clearance of systemic bacterial infection [55,56]. The healthy liver contains low mRNA levels of TLRs such as TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, TLR10 and signaling molecules such as MD-2 and MyD88 in comparison to other organs [57,58,59], suggesting that the low expression of TLR signaling molecules may contribute to the high tolerance of the liver to TLR ligands from the intestinal microbiota to which the liver is constantly exposed.

Kupffer cells

Kupffer cells, the resident macrophages of the liver, play a crucial role in host defense which is linked to their ability to phagocytose, process and present antigen, and secrete various proinflammatory mediators including cytokines, prostanoids, nitric oxide, and reactive oxygen intermediates [55,56,60]. Kupffer cells are among the first cells in the liver to be hit by gut-derived toxins such as LPS and orchestrate inflammatory responses within the liver. Accordingly, Kupffer cells express TLR4 and are responsive to LPS [61]. Although some studies have demonstrated that Kupffer cells are involved in the uptake and hepatic excretion of LPS [62,63], others have shown that Kupffer cell depletion does not reduce LPS clearance [64]. Moreover, Kupffer cells can inactivate LPS by deacetylation [65]. Following stimulation with LPS at concentrations between 0.1 and 1000 ng/ml, Kupffer cells produce TNFα, IL-1β, IL-6, IL-12, IL-18 and several chemokines [66]. Notably, Kupffer cells mediate the majority of cytokine and chemokine expression in liver after LPS injection (25 μg i.p.) as demonstrated by depletion experiments [67]. Kupffer cells also functionally express TLR2,

TLR3 and TLR9 [68,69,70,71]. In comparison to peripheral blood monocytes, Kupffer cells express low levels of CD14 [58]. Moreover, freshly isolated human Kupffer cells secrete the anti-inflammatory cytokine IL-10 in response to stimulation with LPS which contributes to the downregulation of proinflammatory cytokines [72]. Thus, Kupffer cells may have a higher LPS tolerance to adapt to the special circumstances in their anatomical location that is frequently hit with low levels of LPS even under normal conditions.

Hepatocytes

Hepatocytes fulfill metabolic and detoxifying functions in the liver, and are important mediators of the acute phase response. Hepatocytes express TLR4 receptors and are responsive to LPS, but this response is fairly weak with only two-fold elevated levels of serum amyloid A (SAA) and a less than 2-fold induction of most upregulated genes in a microarray after LPS. Moreover, doses of 100 ng/ml and higher were required to see significant effects in hepatocytes [73,74]. Similarly, stimulation with TLR2 ligands induces NF-κB activation and a weak induction of SAA [74]. The expression of TLR2 in hepatocytes is upregulated by LPS, TNFα, bacterial lipoprotein and IL-1β in an NF-κB-dependent manner indicating that hepatocytes become more responsive to TLR2 ligands under inflammatory conditions [74]. In contrast, TLR4 expression in hepatocytes is not upregulated by proinflammatory mediators [75]. Hepatocytes play a major role in the uptake of LPS and its removal from the systemic circulation by secreting LPS into the bile [62,64]. Clearance of LPS occurred at a similar rate in rats which had been depleted of Kupffer cells by gadolinium chloride indicating that hepatocytes are the principal mediators of this process [64]. A recent study demonstrated that TLR4, CD14 and MD-2 are required for the uptake of LPS by hepatocytes [76]. Interestingly, TLR4 signaling is not required for this process as hepatocytes from TLR4-deficient C3H/HeJ mice were as efficient as those isolated from TLR4-sufficient C3H/HeOuJ mice to take up LPS [76]. Although this study clearly demonstrated the ability of hepatocytes to take up LPS, the uptake of LPS in vivo through this TLR4-dependent mechanism remains to be proven.

Hepatic Stellate Cells

Following liver injury, hepatic stellate cells undergo an activation process and become the predominant extracellular matrix-producing cell type in the liver [77]. Activated human hepatic stellate cells express TLR4 and CD14 and respond to LPS with the activation of IKK/NF-κB and JNK as well as the secretion of proinflammatory cytokines [78]. Activated mouse hepatic stellate cells express TLR2, TLR4 and TLR9 and respond to LPS, lipoteichoic acid, N-acetyl muramyl peptide and CpG-DNA with an upregulation of Erk phosphorylation and IL-6, TGFβ1 and MCP-1 secretion [47,79,80,81]. Quiescent murine hepatic stellate cells express as much TLR4 as in vivo-activated hepatic stellate cells and are highly responsive to LPS, even at doses as low as 1 ng/ml [47]. Moreover, quiescent HSCs activate NF-κB in response to LPS injection (0.1 mg/g intraperitoneally) *in vivo* [47]. Notably, LPS downregulates the TGFβ pseudoreceptor Bambi in quiescent hepatic stellate cells to promote TGFβ signaling and stellate cell activation [47].

Biliary epithelial cells

Biliary epithelial cells line the biliary tree which connects the liver with the intestinal lumen to deliver bile to the intestine. Mouse biliary epithelial cells express CD14, MD-2 and TLR2, TLR3, TLR4 and TLR5 [82] and display NF-κB activation and TNFα production after LPS (1 μg/ml) stimulation [82], and an increase in CDX2 and MUC2 following TLR2 or TLR4 stimulation [83]. Human biliary epithelial cells express TLR1–10 [84,85]. In an *in vitro* model of biliary cryptosporidiosis, *C. parvum* recruits TLR2 and TLR4 to the host-cell-parasite interface, and induces NF-κB activation, IL-8 and human β-defensin 2 expression [84].

Sinusoidal endothelial cells

Sinusoidal endothelial cells form the fenestrated lining of the hepatic sinusoids and thus have an important function in hepatic perfusion and nutrient supply. Sinusoidal endothelial cells constitutively express TLR4 and CD14 as well as TLR9, and show an increase in NF-κB activation after LPS (10 ng/ml) stimulation [86,87]. Moreover, mRNA for TLR1–9 was detected in sinusoidal endothelial cells and functional expression of TLR3 has been demonstrated by the ability of supernatants from poly-IC treated sinusoidal cells to reduce HBV replication in immortalized hepatocytes [88]. After repetitive LPS challenges, sinusoidal endothelial cells show reduced NF-κB activation, CD54 expression and a reduced ability to promote leukocyte adhesion [86]. In sinusoidal endothelial cells, LPS tolerance is not regulated at the level of TLR4 surface expression, but appears to be linked to prostanoid expression [86]. Although some authors propose sinusoidal endothelial cells to be involved in the hepatic uptake of LPS, other studies have not found such a role [62,64].

Hepatic dendritic cells

Hepatic dendritic cells (DCs) are the professional antigen-presenting cells of the liver. During inflammation, dendritic cells are recruited into the liver sinusoids from where they can migrate to periportal and pericentral areas. Like other DCs, hepatic DCs express TLR2 and TLR4 [57,89]. While one study found a lower expression of TLR4 on hepatic DCs in comparison to their splenic counterparts [57], another study reported a higher expression of TLR2 and TLR4 on hepatic cDCs and increased TNF-α and IL-6 after PGN and LPS (10 μg/ml) stimulation [89]. However, both studies reported a weaker stimulation of naive allogeneic CD4+ T cells by hepatic DCs in comparison to splenic DCs [57,89].

IV. Role of TLRs in chronic liver disease

Alcohol-induced liver injury

Acute and chronic ingestion of alcohol lead to a strong elevation of portal and systemic levels of endotoxin in animal models and humans [31,32,33]. Whereas healthy controls display only 2.5 pg/ml of endotoxin in peripheral blood, patients with alcoholic fatty liver, alcoholic hepatitis and alcoholic cirrhosis display 14 pg/ml, 16 pg/ml and 19 pg/ml endotoxin in peripheral blood [31]. Interestingly, acute ingestion of alcohol in rats leads to significantly higher levels of portal endotoxin than systemic levels with concentrations of 30–80 pg/ml in the portal vein 2 hours after gavage [33,90]. Thus, peripheral measurements are likely to underestimate the amount of endotoxin that targets the liver, most likely due to the efficient clearance of endotoxin by the liver. Endotoxin is a crucial mediator of liver injury in alcoholic liver disease as demonstrated by the significant reduction of alcoholic liver injury following elimination of the gram-negative microbiota by antibiotics [91], and the sensitization to LPS-induced liver injury following long-term ethanol exposure [92]. The elevation of endotoxin appears to be predominantly caused by two mechanisms (see Figure 3): (i) Alcohol consumption leads to changes in the intestinal microbiota with upper gastrointestinal tract bacterial overgrowth being more than six times more frequent in alcoholics than in non-alcoholic [93,94]. However, the effects of alcohol on the composition of the intestinal microbiota have not been studied in detail. (ii) A large body of literature has clearly documented that alcohol ingestion disrupts the intestinal epithelial barrier causing enhanced permeability [95,96,97] thus allowing increased levels of LPS to enter the portal circulation. It has been suggested that the intestinal microbiota converts ethanol into acetaldehyde which in turn disrupts tight junctions and increases paracellular permeability [98,99].

Kupffer cells have been established as a crucial cellular target of LPS in ethanol-induced liver injury as demonstrated by a strong reduction of alcoholic liver injury following depeletion of Kupffer cells with gadolinium chloride [100]. Moreover, the activation of Kupffer cells in

alcoholic liver disease largely depends on TLR4 since TLR4-mutated C3H/HeJ mice as well as TLR4-deficient mice display strongly reduced levels of proinflammatory mediators in the liver and blunted liver injury despite elevated endotoxin levels [101,102]. The TLR4-mediated signal in alcoholic liver injury is mediated through a MyD88-independent pathway [102], most likely through the adapter molecule Trif (see Figure 3). NADP(H) oxidase is a crucial downstream mediator of TLR4 in Kupffer cells during alcohol-induced liver injury, as mice deficient in $p47^{phox}$, the main cytosolic component of NADP(H) oxidase, show an absence of free radical production, NF-κB activation, TNFα mRNA induction and liver pathology after ethanol treatment [103].

Non-alcocoholic fatty liver and steatohepatitis

There is accumulating evidence that the intestinal microbiota plays an essential part in the promotion of hepatic fat accumulation. Ob/ob mice as well as obese humans have a different composition of their cecal microbiota in comparison to lean controls [104,105]. One mechanism by which the altered composition of the intestinal microbiota in ob/ob mice as well as in humans promotes obesity and fatty liver is TLR-independent through an increased bacterial capacity for energy harvest [106]. A second mechanism by which the altered intestinal microbiota may promote obesity and fatty liver is an increase in LPS-containing microbiota [107] and increased intestinal permeability to LPS [108]. Accordingly, a 4-week high-fat diet increased plasma LPS concentration two to three times [107]. Notably, subcutaneous infusion of a low dose of LPS resulted in excessive weight gain, insulin resistanceand increase liver triglycerides in mice [107]. Vice versa, selective intestinal decontamination results in decreased endotoxin levels in mice on a high-fat diet [109], and improved glucose tolerance and reduced hepatic triglycerides in mice with diet-induced obesity as well as in ob/ob mice [110].

In addition to its role in hepatic fat accumulation, LPS is also involved in the development of non-alcoholic steatohepatitis (NASH). A crucial role for LPS signaling in NASH was first demonstrated by the increased hepatic sensitivity of genetically obese Fa/Fa rats and ob/ob mice to low doses of LPS [111]. This finding has been further confirmed in TLR4-mutant mice that display decreased injury and lipid accumulation following methionine-choline-deficient (MCD) diet, a model of non-alcoholic steatohepatitis [112]. In contrast, TLR2-deficient mice are not protected from steatohepatitis after MCD diet [113]. The predominant role of TLR4 signaling in NASH is further emphasized by the ability of the TLR4 ligand LPS but not the TLR2 ligand peptidoglycan to exacerbate liver injury in mice treated with a methionine-choline-deficient diet [113]. Notably, probiotics reduce hepatic injury and inflammation in ob/ob mice [114] suggesting that modulation of the gut microbiota by probiotics may represent a feasible approach for the prevention or treatment of NASH.

Hepatic fibrosis and cirrhosis

Chronic liver injury leads to the development of hepatic fibrosis, the increased accumulation of extracellular matrix in the liver, and hepatic cirrhosis, an advanced stage of hepatic fibrosis in which functional liver tissue is largely replaced by extracellular matrix and regenerating nodules. The development of hepatic fibrosis and cirrhosis occurs in virtually any type of chronic hepatic injury including viral hepatitis, alcohol, autoimmune and metabolic disease [77]. There is abundant data demonstrating that LPS is elevated in experimental models of hepatic fibrosis [47,115,116] and in patients with cirrhosis [31,117,118]. Whereas healthy subject displayed endotoxin levels of less than 3 pg/ml, patients with Child-Pugh class A, B and C had endotoxin levels of 4.9 pg/ml, 7.9 pg/ml and 10.2 pg/ml, respectively [118]. It is believed that changes in intestinal motility, subsequent alterations of the intestinal microbiota, decreased mucosal integrity and suppressed immunity in hepatic fibrosis contribute to a failure of the intestinal mucosal barrier and cause increases in bacterial translocation and LPS levels in later stages of hepatic fibrosis and cirrhosis [30,119,120,121,122,123]. Studies from the

1950s have shown that antibiotics prevent hepatic injury and fibrosis induced by CCl_4 treatment or a choline-deficient diet, and that endotoxin enhances hepatic fibrosis induced by a choline-deficient diet [124,125]. Recent studies using TLR4-mutant as well as gut-sterilized, CD14- and LBP-deficient mice have demonstrated the crucial role for the LPS-TLR4 pathway in hepatic fibrogenesis [47,126]. TLR4-mutant mice display a profound reduction in hepatic fibrogenesis in three different experimental models of biliary and toxic fibrosis [47]. The crucial role of LPS is supported by the finding that LBP-deficient as well as gut-sterilized mice also have a marked reduction of hepatic fibrosis [47,126]. In addition to LPS, endogenous TLR ligands such as hyaluronan and HMGB1 are also elevated in murine fibrogenesis [47]. However, in view of the crucial role of the intestinal microbiota in hepatic fibrogenesis, their contribution is at best moderate. Accordingly, the inhibition of HMGB1 signaling by a blocking antibody did not improve biliary fibrogenesis (RFS, unpublished results). In contrast to TLR4, TLR2-deficient mice did not show a profound reduction in hepatic fibrosis following bile duct ligation [47]. TLR4 is expressed on two key mediators of hepatic fibrogenesis, Kupffer cells and hepatic stellate cells [47,61,78]. Kupffer cells initiate fibrogenesis by secreting proinflammatory and profibrogenic cytokines, while hepatic stellate stells are the predominant source of extracellular matrix production in the fibrotic liver [77]. Although Kupffer cells express the highest levels of TLR4 in the liver and are considered a prime target of LPS, TLR4 expressed on quiescent and activated hepatic stellate cells is the main mediator of fibrosis as demonstrated in TLR4-chimeric mice [47]. LPS directly targets hepatic stellate cells *in vivo* to upregulate chemokines and attract Kupffer cells. At the same time, TLR4 activation induces a downregulation of the TGFβ pseudoreceptor BAMBI on hepatic stellate cells. These two mechanisms work hand in hand to promote the activation of hepatic stellate cells by Kupffer cell released TGFβ, and subsequently hepatic fibrosis (see Figure 4) [126]. Notably, a recent study has identi ed a single nucleotide TLR polymorphism that results in a T399I substitution and confers a signi cantly reduced risk for brosis progression in patients with chronic hepatitis C virus infection [127]. This polymorphism is associated with a reduced TLR4 responsiveness thus confirming the profibrogenic role of TLR4 in a clinically relevant setting. Two recent studies also demonstrated that hepatic stellate cells express TLR9, and that TLR9-deficient mice display decreased hepatic fibrosis [80,128]. The decrease in hepatic fibrosis was linked to the ability of TLR9-expressing hepatic stellate cells to recognize apoptotic hepatocytes resulting in stellate cell activation [128].

TLRs may also modulate several of the complications of hepatic fibrosis. Probiotics and selective intestinal decontamination decrease hepatic encephalopathy in rodent models as well as in patients [129,130,131]. However, it is not clear whether this effect is mediated through TLR-dependent mechanisms as probiotic treatment led not only to a significant reduction of endotoxin but also of the ammonia, a principal mediator of encephalopathy [132]. Moreover, LPS has been implied in the regulation of blood pressure in cirrhosis. In two studies, selective intestinal decontamination significantly decreased endotoxin levels and improved the hyperdynamic circulatory state of cirrhosis but did not significantly reduce the hepatic venous pressure gradient [133,134]. In another study, there was no decrease in hepatic venous pressure gradient in patients with significant portal hypertension but none of the involved patients had detectable serum endotoxin levels [135].

Hepatocellular carcinoma

The liver is probably the best example for the link between chronic inflammation and cancer that was postulated by Rudolf Virchow more than 100 years ago [136]. Almost 80% of hepatocellular cancers in the western world develop as a consequence of chronic inflammation and arise in fibrotic or cirrhotic livers [137]. The involvement of TLR signaling in fibrosis-associated HCC has not yet been investigated. However, there is a high probability that fibrosis-associated HCC is mediated by TLR signaling as TLR4 and MyD88 are required for the

development of fibrosis [47], a predisposing condition for HCC development. An important role for the TLR signaling molecule MyD88 was shown in DEN-induced hepatocellular carcinoma (HCC) in male mice [138], but this tumor-promoting pathway is mediated by IL-1R, another upstream activator of MyD88 [139]. Therefore, it is possible that DEN-induced non-fibrotic HCC and fibrosis-associated HCCs may both be MyD88-dependent but mediated by different signaling cascades.

HCV infection

Hepatitis C affects approximately 3.2 million people within the United States and 170 million people worldwide [140]. About 30% of patients chronically infected with Hepatitis C virus (HCV) show signs of active hepatic inflammation, and are at risk of developing fibrosis, cirrhosis and hepatocellular carcinoma. Accordingly, Hepatitis C continues to be one of the leading indications for liver transplantation in the United States [141].

Patients with chronic HCV infection display more than 10-fold-increased serum levels of endotoxin with <5 pg/ml in healthy controls and > 50 pg/ml in chronic HCV patients with mild to moderate hepatic inflammation and less than 25% incidence of hepatic fibrosis [142]. Moreover, several HCV ligands act as TLR agonists [143,144]. However, HCV has developed strategies to avoid activation of anti-viral pathways by TLRs and their ligands: HCV selectively impairs innate immune pathways that limit HCV replication such as type I interferons (see Figure 5) while at the same time generating a chronic inflammatory state that causes persistent liver injury [145,146]. When double-stranded RNA from HCV binds to TLR3 within the endosome, the expected response would be activation of TRIF and subsequent upregulation of IFN-β through TBK-1/IKKε and IRF-3. However, the IFN-β response is strongly inhibited by a mechanism that involves the cleavage of TRIF by nonstructural HCV protein NS3/NS4A [147]. Furthermore, NS3 binds directly to TBK-1, disrupting the activation of IRF-3 and the upregulation of IFN-β [148]. Thus, HCV inhibits the ability of the TLR-3 pathway to upregulate IFN-β and hinders viral clearance by two distinct mechanisms. In addition to disrupting the function of TLR3, HCV also affects the function of other TLRs integral to the viral and immune response. In vitro studies utilizing a murine macrophage line that stably express HCV proteins, demonstrate that expression of HCV proteins inhibits the signaling pathways of TLR2, TLR4, TLR7 and TLR9. Specifically, these studies showed that the NS5A inhibits TLR signaling by binding to the adaptor protein MyD88 and inhibiting the recruitment of IRAK-1 leading to a decrease in MyD88-dependent signals [149]. Myeloid dendritic cells from patients with chronic HCV display an increased expression of TLR2 and TLR4, but a decrease in the cytokine response to TLR agonists [150] thus confirming the impairment of TLR signaling by HCV in primary cells. Cell culture produced HCV impairs TLR9-induced IFNα production in plasmacytoid dendritic cells, but has no effect on TLR3- and TLR4- myeloid and monocyte derived dendritic cells suggesting that HCV may specifically target plasmacytoid dendritic cells [151]. Accordingly, TLR ligand-dependent activation of naive CD4 T cells by plasmacytoid dendritic cells is impaired in hepatitis C virus infection [152]. Thus restoration or boosting of TLR signaling pathways that are related to HCV eradication may be a promising treatment strategy. Accordingly, one week of treatment with the TLR7 agonist isatoribine caused a significant reduction of plasma HCV RNA, an increase in the levels of OAS, a marker of antiviral immunity, and an increase in the levels of the chemokine IP-10 and neopterin, a marker of macrophage activation [153].

While certain TLR pathways, especially those that are related to viral clearance, are inhibited by HCV, other TLR signaling pathways may be activated by HCV and thus promote chronic inflammation. For example, Hepatitis C core protein and NS3 were found to activate TLR signaling via TLR2, a response that was abrogated in monocytes isolated from MyD88 or TLR2 deficient mice [143]. Another study utilizing B cells from patients infected with HCV as well

as an immortalized lymphoma cell line, demonstrated increased TLR4 expression and higher levels of IFN-β and IL-6 in HCV cells in comparison to controls [154]. HCV core and NS3 proteins have also been shown to produce both pro and anti-inflammatory cytokines (TNF-α and IL-10) in human macrophages through the TLR2/1 and TLR2/TLR6 heterodimers [144]. Moreover, macrophages from HCV patients do not display tolerance to LPS after pretreatment with different TLR ligands, leading to persistent macrophage activation and cytokine release [142]. In summary, the current data supports the hypothesis that there is a decreased activation of TLR signaling pathway related to virus eradication but an increased activation of TLR signaling pathways related to inflammation. It is likely that this constellation promotes virus expansion, inflammation and potentially the progression to fibrosis and cirrhosis.

Hepatitis B

Hepatitis B virus (HBV) causes a chronic infection in about 10% of adults exposed to the virus affecting about 1.25 million within the United States and 370 million people worldwide [155]. One study reported a 24-fold induction in endotoxin levels in acute HBV infection, and a 72-fold induction of endotoxin levels in chronic HBV infection [156]. Moreover, it has been suggested that HBV contains TLR ligands [157]. Several TLRs block HBV replication through their ability to upregulate interferons. In HBV transgenic mice, activation of TLR3 by poly-I:C leads to an IFN-dependent inhibition of HBV replication [158]. These findings have been extended in two recent studies which demonstrated reduced HBV replication after injection of TLR3, TLR4, TLR5, TLR7 and TLR9 ligands into HBV transgenic mice [159], and the ability of supernatants from non-parenchymal cells stimulated with TLR3 and TLR4 agonists to repress HBV replication [88]. In a recent clinical study, TLR 1–10 receptor expression was quantified in patients with chronic Hepatitis B and compared to healthy controls. This study demonstrated that TLR1, TLR2, TLR4 and TLR6 were down regulated in HBV infected peripheral blood monocytes, and these cells also had a decreased cytokine response to TLR2 and TLR4 ligands [160]. Interestingly, HBeAg-positive chronic hepatitis B decreases TLR2 expression in peripheral monocytes, Kupffer cells and hepatocytes whereas HBeAg-negative chronic hepatitis B is associated with increased TLR2 expression suggesting a direct effect of the HBeAg on TLR2 expression [161]. Thus, it appears that HBV has developed abilities to downregulate TLRs and thus avoid anti-viral pathways, but that prolonged infection and loss of HBeAg may upregulate TLR signaling pathways such as TLR2 that are not involved in anti-HBV responses but promote hepatic inflammation and disease progression. Independently of their effect on HBV replication, TLR7 and TLR9 agonists may also have a role as adjuvants in vaccines in Hepatitis B as they booster edcellular and humoral responses to HBsAg as single agents or in combination in mice [162,163,164]. However, these results still require confirmation in humans.

Hepatic autoimmune diseases

The liver is known to be a classical immunoprivileged site. Notably, activation of TLR3 is required for infiltration of CD8+ T cells and liver disease after injecting mice with activated liver-specific effector CD8+T cells, suggesting that TLR signals may represent an important trigger to overcome this immunoprivilege and to induce hepatic autoimmune disease [165].

Primary biliary cirrhosis (PBC) is an autoimmune disorder usually affecting middle aged adult women which causes severe injury to the interlobular bile ducts [166]. Biliary epithelial cells are known to express TLRs, but under normal conditions are tolerant to antigenic exposures [85]. Although biliary epithelial cells isolated from livers from patients with PBC express similar levels of TLRs compared to controls, they secreted higher levels of chemokines when stimulated with TLR3 agonist poly I:C and co-cultured with liver-infiltrating mononuclear cells [167]. Moreover, TLR3 and IFN-α/β have been found to be elevated in the portal tracts and liver parenchyma of patients with PBC when compared to control patients with

autoimmune hepatitis and Hepatitis C [168]. In addition, peripheral blood monocytes isolated from patients with PBC have been found to be hyperresponive to TLR ligands [169,170]. TLRs may also play a role in B cell proliferation in PBC. Peripheral blood monocytes taken from PBC patients and stimulated with the TLR9 ligand, CpG, have been shown to induce IgM-producing B cells and an increased expression of TLR9 on these cells [171,172]. Although high IgM levels are a typical feature of PBC, the relevance of the induction of IgM-producing B cells by CpG in PBC needs to be further investigated. There is only limited data on the role of TLRs in primary sclerosing cholangitis (PSC), an autoimmune disease that leads to fibro-obliterative changes within the intrahepatic and extrahepatic biliary tract. Stimulating isolated biliary epithelial cells with anti-biliary epithelial cell antibodies from patients with PSC leads to upregulation of TLR4 and TLR9, and a dramatically increased secretion of inflammatory cytokines under baseline conditions, and in the presence of LPS or CpG DNA [173].

V. Targeting TLRs in chronic liver disease

In recent years, a number of different approaches have been developed to modulate TLR signaling. These approaches include modulation of TLR ligand release from the intestinal microbiota by probiotics and antibiotics, activation of TLR signaling by synthetic TLR ligands and inhibition of TLR activation by small molecule inhibitors. Many of these recently developed agents have undergone evaluation in tissue culture experiments, mouse models and clinical trials, but their efficacy and clinical usefulness have not been proven beyond doubt in large sets of patients with liver disease.

TLR4-MD2 antagonists

Several small molecule inhibitors of TLR4 have been discovered and are currently being tested in human studies. Some of these inhibitors are lipid A mimetics which bind to the TLR4-MD2 complex but lack intrinsic activity, and thus prevent binding of the lipid A portion of LPS and subsequent TLR4 activation. The crystal structure of the TLR4-MD2 complex with bound “TLR4 antagonist” E5564 (Eisai Co., Ltd.) was recently published suggesting that the mechanism of action of E5564 is binding through a large internal pocket in MD-2 [17]. Thus, lipid A mimetic small molecule inhibitors should actually be classified as MD-2 inhibitors. In vitro, E5564 dose-dependently inhibited LPS-mediated activation of primary cultures of human myeloid cells and mouse tissue culture macrophage cell lines as well as human or animal whole blood at nanomolar concentrations as measured by production of tumor TNF- and other cytokines. In vivo, E5564 blocked induction of LPS-induced cytokines and LPS or bacterial-induced lethality in primed mice, and cytokine induction and symptoms after endotoxin injection in healthy volunteers [174,175]. CRX-526 (Corixa Corporation) is another lipid A mimetic antagonist of TLR4 signaling that has been shown to inhibit IL-6 and MIP-1α production after LPS stimulation *in vitro*, and to almost completely suppress LPS-induced TNFα release *in vivo* [176]. Moreover, CRX-526 reduced colitis in the dextran sodium sulfate and MDR1a-deficiency models of colitis [176]. TAK-242 (Takeda Pharmaceutical Company Ltd.) represents a second class of TLR4 antagonists [177]. TAK-242 exerts its inhibitor effects at the intracellular domain of TLR4 as demonstrated by a study in which the extracellular domain of TLR4 was replaced by CD4 to create a constitutively active receptor chimera [178]. Activity of this receptor chimera was inhibited by TAK-242 providing evidence that its inhibitory effect does not require the presence of the TLR4 extracellular domain [178]. TAK-242 prevented increases in serum levels of wide range of cytokines in mice injected with LPS, and protected mice from LPS-induced lethality [179]. Interestingly, TAK-242 showed beneficial effects even when administered after LPS challenge [179]. Both E5564 and TAK-242 are currently tested in phase III clinical trials in patients with septic shock [180]. To the best of our knowledge, none of the TLR4/MD-2 inhibitors have been tested in chronic liver disease. Based on the involvement of TLR4 in fibrogenesis, alcoholic liver injury and NASH,

small molecule inhibitors of TLR4 might be attractive candidates for the treatment or prevention of these diseases.

TLR7 agonists

The guanosine analogue isatoribine (Anadys Pharmaceuticals, Inc) is a small molecule ligand of TLR7 [181]. In a recent phase 1 trial in patients with chronic HCV infection, isatoribine induced a significant reduction of plasma HCV [153]. Eight out of 12 patients treated with 800 mg intravenous injection of isatoribine once daily for 7 days displayed a decrease of more than 0.5 $\log_{10}$ units with a mean change of -0.76 log in all 12 patients [153]. The reduction of viral load was correlated with an induction of markers of a heightened immune antiviral state, including a 7.6-fold induction of 2-, 5- oligoadenylate synthetase levels. This upregulation is comparable to that reported during the first week after the start of treatment with pegylated interferon alpha-2b. Side effects of isatoribine were mild to moderate and similar to those observed in patients treated with interferon-based therapies. Thus, TLR7 agonists such as isatoribine may represent good candidates for anti-viral therapy, potentially in combination with other anti-viral compounds. Recently, an oral prodrug of isatoribine, ANA975, was developed and may thus represent a novel oral treatment approach for patients with chronic HCV infection [182]. In contrast to isatoribine, the TLR7 and TLR8 agonist resiquimod appears to lack antiviral effects in HCV-infected patients as reported in a preliminary study [183].

TLR9 agonists

CPG 10101 (Actilon; Coley Pharmaceutical Group, Inc) is a synthetic oligodeoxynucleotide (ODN) that has been optimized to stimulate human TLR9. In a multicenter Phase 1b trial involving 60 patients with HCV infection, CPG 10101 induced a decrease in HCV RNA a greater than 1 log reduction in 22 of 40 patients who received more than 1 mg CPG 10101, and was thus similar to that reported for pegylated interferon monotherapy [184]. Moreover, CPG 10101 dose-dependently induced surrogate markers of anti-viral immunity such as (IFN)-gamma-inducible protein 10 (IP-10) and IFN-alpha as well as a sustained increase 2′5′-oligoadenylate synthetase [185]. CPG 10101 was well tolerated, and adverse events were consistent with CPG 10101's mechanism of action. Although data from this study suggests CPG 10101 to be a good candidate for further studies in chronic HCV infection, development of CPG 10101 as therapy for HCV in conjunction with PEG-IFN and/or ribavirin has been temporarily suspended for unknown reasons [185].

Probiotics and Antibiotics

Modulation of the intestinal microbiota is an emerging strategy to reduce bacterial translocation and circulating endotoxin levels, and potentially those of other bacterially derived TLR agonist. A number of different approaches have been taken to achieve this goal. Modulation of the enteric microbiota by probiotics or synbiotics, a combination of probiotics with prebiotics, has been demonstrated to reduce bacterial translocation [186,187,188], circulating endotoxin levels in animal models [132], and bacterial infection, a surrogate marker for bacterial translocation, in patients with hepatic cirrhosis [189,190]. In liver cirrhosis, probiotics have shown positive effects on several parameters including the improvement of liver function, prevention of infection, improvement of the hyperdynamic circulation and prevention of hepatic encephalopathy [132]. Other areas in which probiotics have positive effects on liver injury and ALT levels are (i) NASH with positive effects on ALT levels and liver histology in one mouse and one human study [114,191], (ii) alcoholic liver injury [192] and (iii) LPS-induced liver injury [187,188]. One problem is that a number of studies are relatively small and many of these are uncontrolled studies. The large number of probiotic strains and combinations of strains represents a second important problem, and it will require additional studies to confirm and ideally compare the efficacy of these probiotic strains or combinations for specific liver

diseases. Among the most commonly used strains and combinations of strains are: (i) VSL#3, a combination of *Streptococcus thermophilus*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei* and *Lactobacillus bulgaricus* has shown efficacy in reducing liver injury in patients with forms of chronic liver disease [191], in a mouse model of NASH [114], in a mouse model of LPS-induced liver failure [187]. (ii) Synbiotic cocktail 2000, a combination of Pediococcus pentosaceus, Leuconostoc mesenteroides, Lactobacillus paracasei F19, L. plantarum 2592 plus betaglucan, inulin, pectin and resistant starch that has been shown to improve Child-Turcotte-Pugh classification and reduce encephalopathy [132], and to reduce bacterial infections after orthotopic liver transplantation [190]. (iii) L. plantarum 299V that has been demonstrated to prevent infection after orthotopic liver transplantation in combination with fiber [189]. A second approach to reduce TLR ligands is the treatment with antibiotics to achieve selective intestinal decontamination of gram-negative bacteria, the predominant source of LPS. Selective intestinal decontamination has been shown to reduce bacterial translocation in many but not all studies performed in rats [193,194,195]. Importantly, norfloxacin administration reduced the 1-year probability of developing spontaneous bacterial peritonitis, hepatorenal syndrome, and improved the 3-month and 1-year probability of survival compared with placebo [196]. While the reduction of spontaneous bacterial peritonitis in norfloxacin-treated patients is a direct consequence of reducing bacterial strains in the microbiota responsible for spontaneous peritonitis, some of the positive effect on mortality are likely SBP-independent and related to reducing bacterial translocation and circulating levels of TLR ligands [196,197]. However, in view of the severe consequences of long-term antibiotics treatment, these treatment regimens will be reserved for selected high-risk patients with hepatic cirrhosis, and are not suited to reduce TLR agonists in patients with early stages of liver disease.

V. Conclusion

There is increasing evidence that TLRs play a key role in the pathophysiology of many chronic liver diseases. The possibility of targeting TLR signaling at different levels such as the intestinal microbiota, the level of TLRs, co-receptors such as MD2 and CD14 and downstream signaling molecules will open up a new therapeutic options for the treatment of chronic liver disease. However, a number of issues regarding the role of the TLRs and their ligands in liver disease need to addressed before TLRs can seriously be considered as pharmacological targets in liver disease: (i) The role of endogenous TLR ligands and their role in chronic liver disease need to be investigated more thoroughly. Well-designed *in vitro* and *in vivo* studies using mice in which the release of these ligands is blocked or induced by genetic methods are required to avoid any issues with contaminating bacterial ligands. (ii) As a large number of cell types in the liver express TLRs, a better understanding of cell-specific functions is required, e.g. by using cell-specific knockout strategies. (iii) The majority of studies on the role of TLRs in liver disease is based on animal models. Further translational research is required to firmly establish the role of TLRs in human liver disease. (iv) The potential therapeutic effect of TLR agonists, TLR antagonists and probiotics needs to be further assessed in well-controlled animal and human studies. It is possible that targeting of TLRs by small molecule antagonists may have immunosuppressive effects, and they may have to be restricted to selective patient groups and exclude patient groups with advanced liver disease and immunosuppression. Therefore, probiotics appear to be ideal candidates for the treatment of chronic liver disease such as alcoholic liver disease, NAFLD and NASH and liver fibrosis due to their high tolerability and limited side effects. However, it needs to be established whether the old pharmacologic teaching that “no effect is to be expected from a drug that does not have side effects” is a rule with exceptions. Moreover, it would be interesting to determine whether the suppression of TLR signaling pathways by HCV can be blocked pharmacologically, and whether TLR agonists may have some effects on HBV and HCV that differ from those of interferon, and could thus provide a useful addition to current interferon-based therapies. We can anticipate

that further research on TLR signaling in chronic liver disease will help to shape new concepts with the intestinal microbiota, TLRs and their downstream signaling mediators as pharmacological targets.

Summary Box 1

- Toll-like receptors are a cornerstone of the innate immune system and provide an almost instant anti-microbial response to fight pathogens.
- Toll-like receptors are pattern recognition receptors that detect the presence of minute amounts of signature molecules present in pathogens (pathogen associated molecular patterns=PAMPs).
- Activation of Toll-like receptors activate anti-viral and pro-inflammatory signaling pathways.
- Toll-like receptors signal through the adapter molecules MyD88, Trif or both to activate the “MyD88-dependent” and “MyD88-independent” signaling pathways.
- It has been suggested that Toll-like receptors may also be activated by endogenous ligands. Many of these endogenous ligands are associated with injury and inflammation and belong to a group of molecules termed damage associated molecular patterns (DAMPs). However, none of these molecules has been proven a bona fide TLR ligand beyond doubt.

Summary Box 2

- The liver is a target of bacterial TLR ligands due to its anatomic connection to the intestine.
- Under normal circumstances, the liver is exposed to small amounts of bacterial PAMPs but does not show signs of inflammation due to its higher tolerance to PAMPs and its ability to efficiently excrete PAMPs such as LPS.
- In many types of chronic liver disease, levels of PAMPs are elevated. Most research has focused on LPS, and has shown increased LPS levels in chronic viral hepatitis, liver fibrosis and cirrhosis, and alcoholic liver disease.
- LPS promotes liver injury and fibrogenesis under many circumstances. Blocking LPS release from the intestinal microbiota, or inhibiting activation and signaling of the LPS receptor TLR4 may therefore represent a feasible strategy for the prevention or treatment of chronic liver disease.
- TLRs and downstream signaling molecules play a role in chronic viral hepatitis. Chronic HCV infection leads to a downregulation of anti-viral signaling pathways. Activation of specific TLR signals may booster anti-viral immunity, and therefore represent a novel treatment approach for chronic viral hepatitis.

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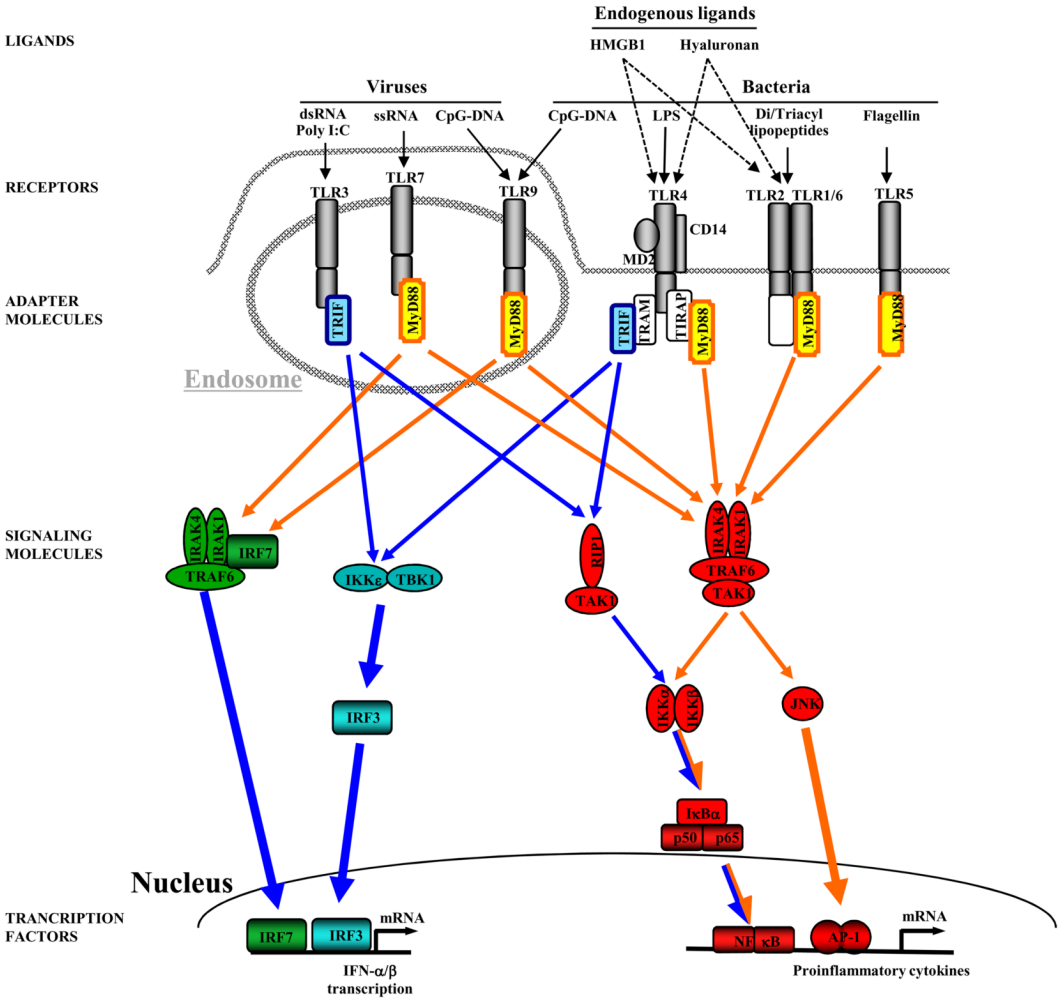

Figure 1. TLR signaling

TLRs that are predominantly activated by viral PAMPS are located within the endosome whereas TLRs that are predominantly activated by bacterial PAMPS are located on the cell surface. In addition to PAMPs, several endogenous mediators including hyaluronan and HMGB1 have been suggested to activate TLR2 and TLR4. TLRs mediate their signaling through two adapter molecules, MyD88 and Trif to induce up-regulation proin ammatory and antiviral genes. MyD88-induced signals (marked in orange) predominantly activate NF-κB, IRF-7 and JNK, Trif-dependent signals (marked in blue) predominantly activate NF-κB and IRF-3 (adapted from Schwabe et al, Gastroenterology 130:1886–900).

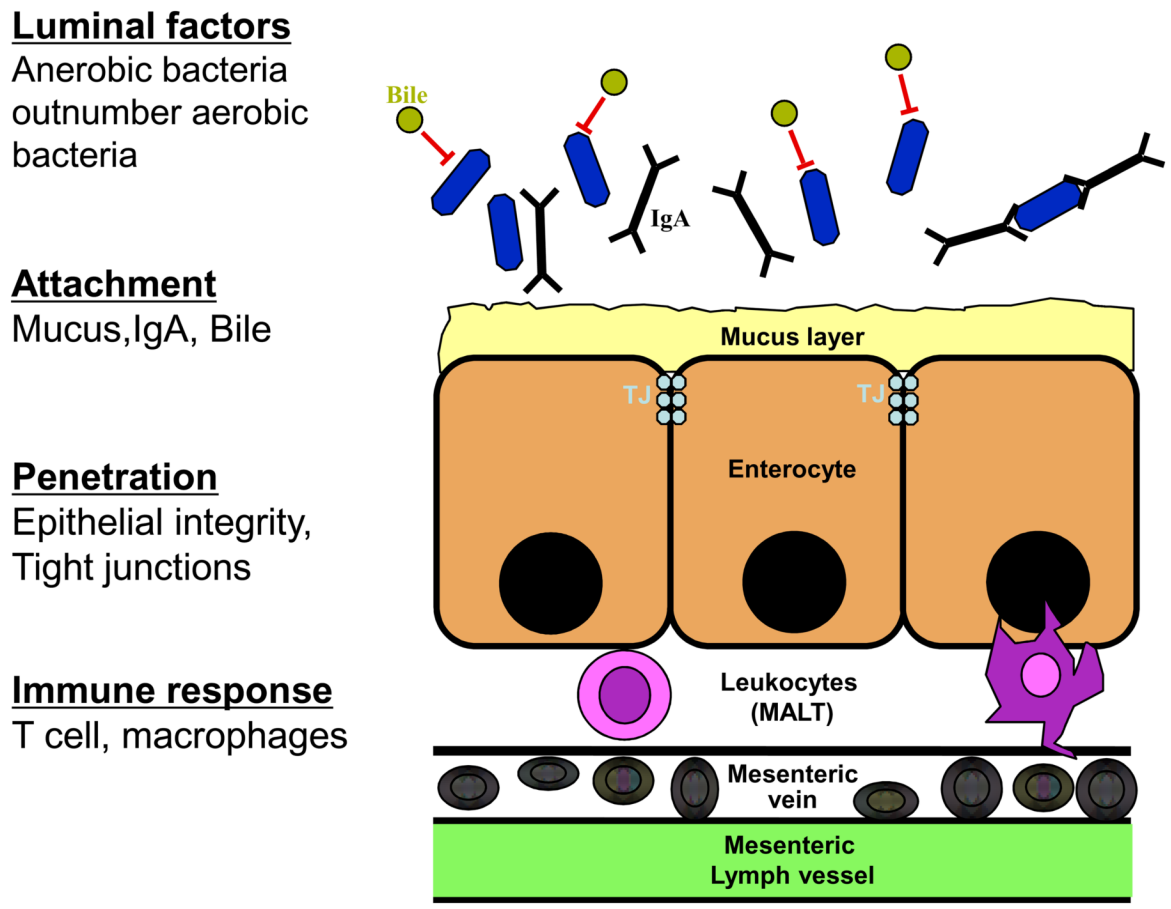

Figure 2. Prevention of bacterial translocation by the intestinal epithelial barrier

Under normal circumstances, a number of protective mechanisms at different levels ensure that only a minimal amount of bacterial transloction occurs: (i) Luminal factors such as the predominance of anaerobic bacteria which limit the growth and translocation of aerobic and facultative anaerobic bacteria; (ii) bile inhibits bacterial overgrowth; (iii) IgA prevents microbial entry and transports IgA-bound microbes from the lamina propria back to the lumen (iv) a thick mucus layer prevents bacterial contact and attachment (v) intact tight junctions prevent paracellular penetration (vi) the mucosa-associated lymphatic tissue (MALT) phagocytoses translocating bacteria. (adapted from Wiest et al., Hepatology 2005; 41:422–33.)

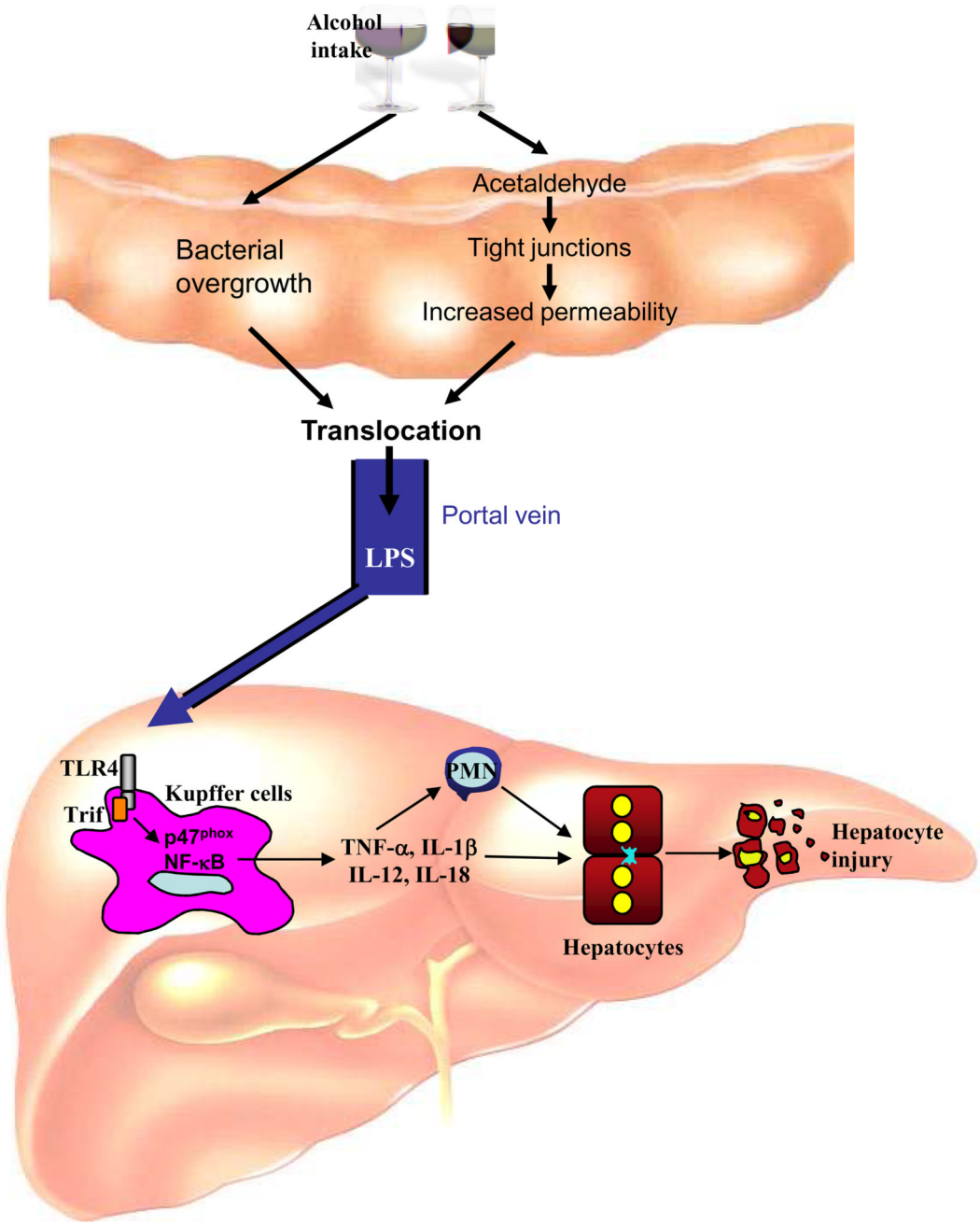

Figure 3. Promotion of alcoholic liver injury by an LPS-TLR4 signaling cascade

Orally ingested alcohol increase intestinal permeability leading to increased levels of LPS in the portal vein. In the liver, LPS binds to TLR4 on Kupffer cells to activate NF-κB, and NADPH oxidase, through a MyD88-independent pathway. Release of cytokine by Kupffer cells promotes hepatocyte injury through the recruitment of neutrophils through direct effects on hepatocytes.

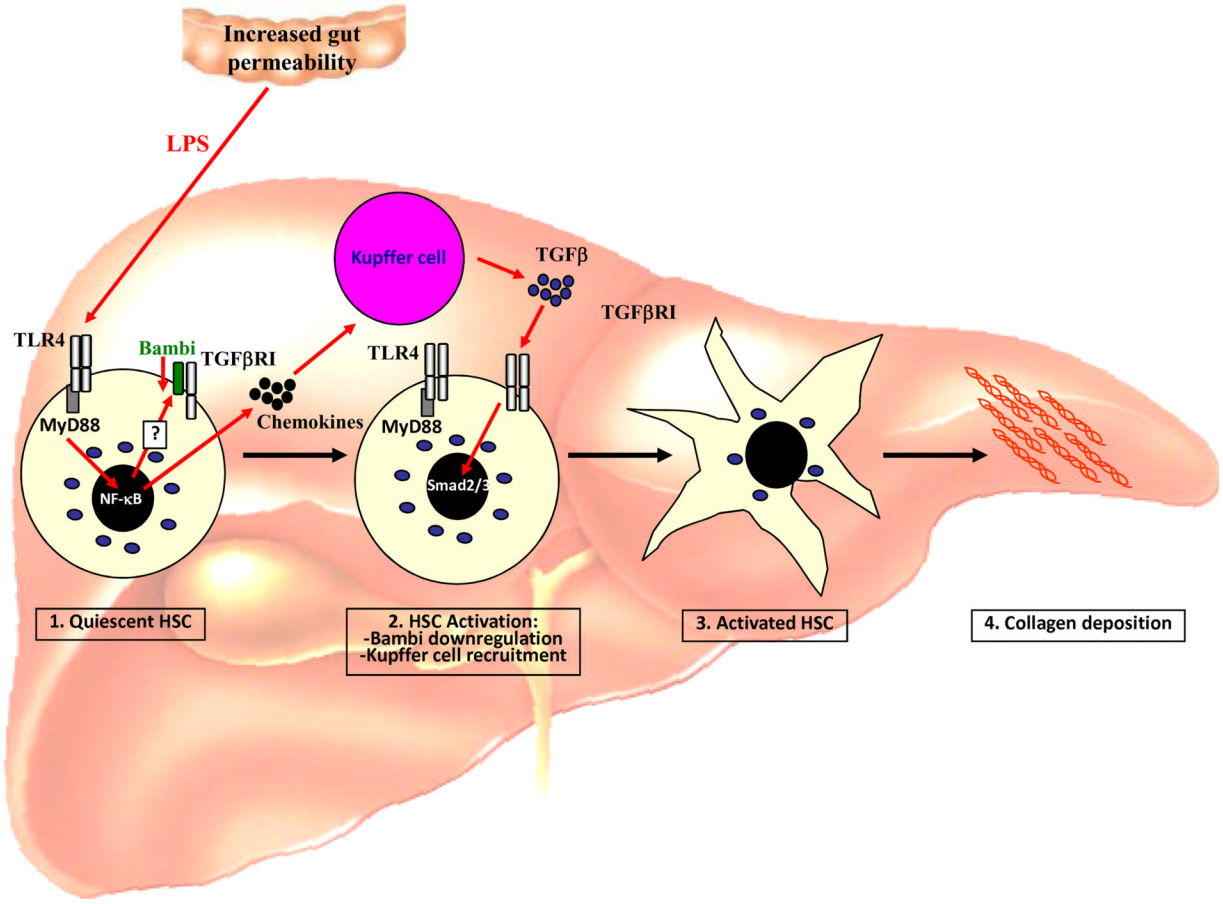

Figure 4. Promotion of hepatic stellate cell activation and fibrosis by LPS

Following liver injury, alterations in the bacterial microbiota and the intestinal mucosal barrier cause an increase in the translocation of LPS. LPS directly targets quiescent hepatic stellate cells resulting in (i) downregulation of the TGFβ pseudoreceptor Bambi and (ii) upregulation of chemokines. These two signals complement each other to promote hepatic stellate cell activation: 1. Downregulation of Bambi through a TLR4-MyD88-NF-κB signaling cascade sensitizes hepatic stellate cells towards the effect of TGFβ. 2. Chemokines induce Kupffer cells, a main source of TGFβ in the injured liver, to migrate towards hepatic stellate cells. Together, these two mechanisms allow TGFβ-dependent activation of hepatic stellate cell by Kupffer cells resulting in increased deposition of extracellular matrix and liver fibrosis.

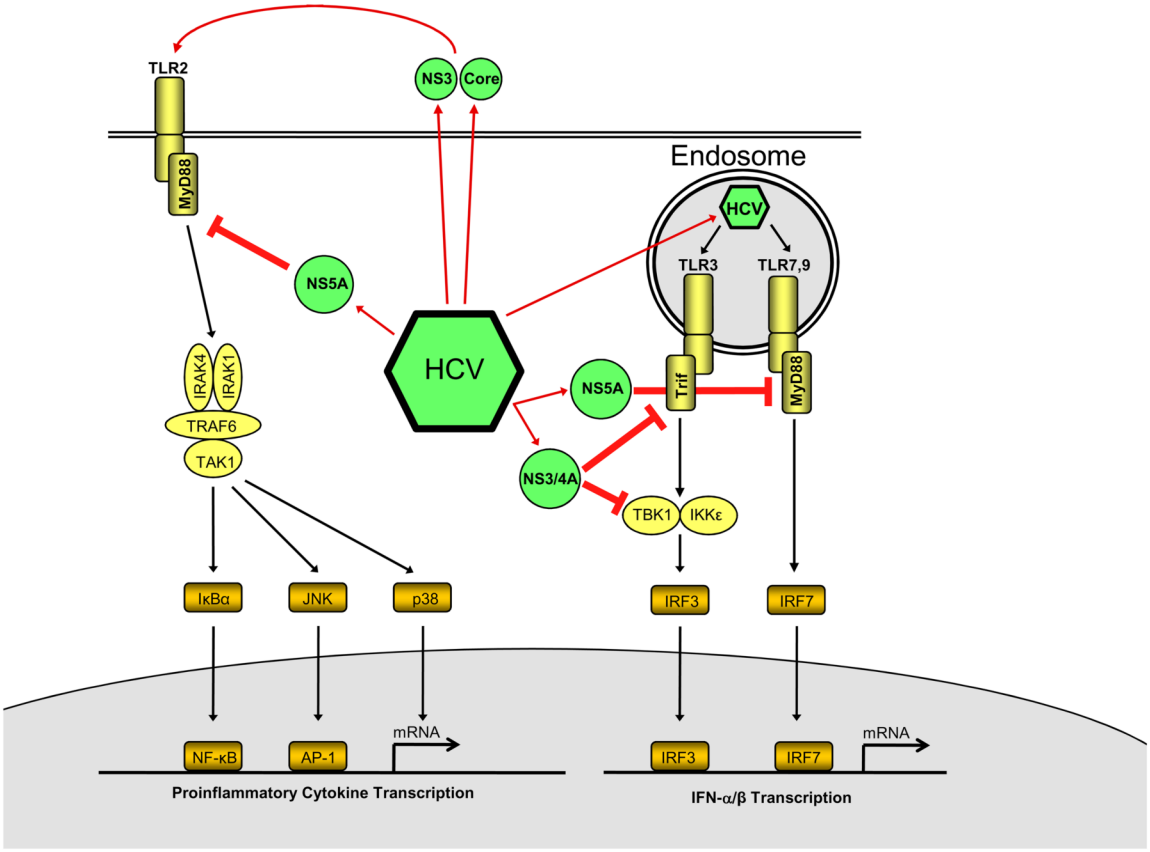

Figure 5. Positive and negative regulation of TLR signaling by HCV

Double-stranded RNA from HCV binds to TLR3 within the endosome. However, efficient TLR3 signaling is prevented by 2 mechanisms: (i) Degradation of Trif by HCV NS3/4A and (ii) by NS3 by binding to TBK1 and blocking the association between TBK1 and IRF3. Moreover, HCV NS5A also blocks TLR9-induced levels at the level of MyD88. The interference of HCV with these anti-viral pathways HCV prevents eradication of HCV by the immune system. At the same time, some HCV proteins promote inflammatory signals through TLR2 and TLR4. These may be dampened by inhibition of MyD88 signaling by HCV NS3 but are likely to contribute to chronic inflammation and potentially the progression to fibrosis and cirrhosis. (Figure based on [8]).