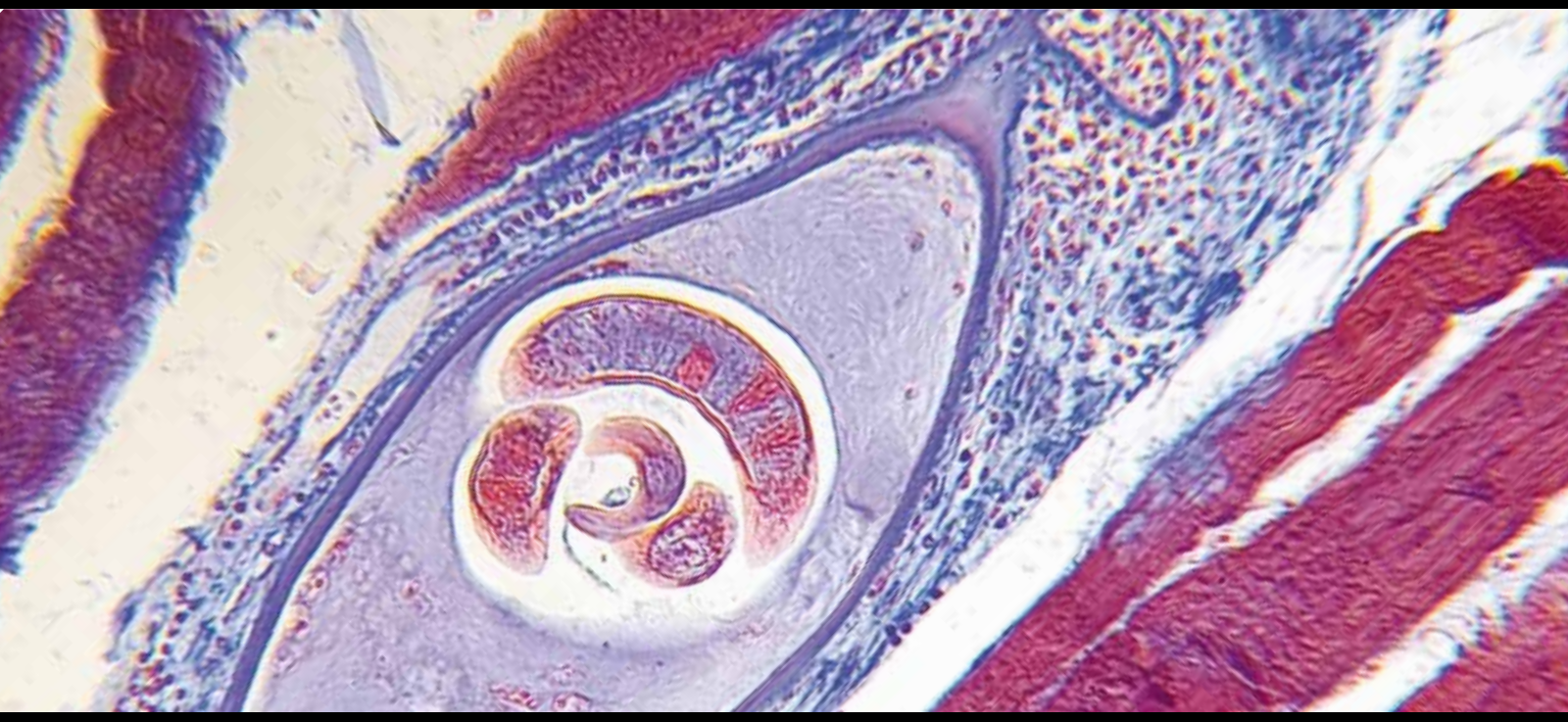
Gastroenterology Research and Practice

Toll–Like Receptor Signaling in Liver Diseases

Guest Editors: Ekihiro Seki, Ian Nicholas Crispe, Timothy R. Billiar, Gyongyi Szabo, and Keigo Machida



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Editorial Board

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Editorial

Toll-Like Receptor Signaling in Liver Diseases

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Drosophila Toll was initially discovered as the factor responsible for determining dorsoventral polarity in *Drosophila* embryo, and the subsequent studies revealed its antifungal functions. In the late 1990s, mammalian homologs of Toll were determined as Toll-like receptors (TLRs). Early studies on TLRs attempted to identify their specific ligands, new family member of TLRs, and their intracellular signal transduction pathways. Intracellular signal transductions of TLRs share common elements with IL-1 receptor downstream intracellular signaling. Subsequent studies for TLRs have focused on infectious diseases, since TLRs recognize pathogen-associated molecular patterns (PAMPs) and induce strong responses for host defense. Recently, endogenous TLR ligands released from dying and/or damaged cells were identified. These studies have extended the idea that TLR signaling is also activated in the absence of exogenous pathogens and in general sense "danger signals" to alert the host of either exogenous or endogenous "danger."

Liver has a unique anatomy that is closely associated with the intestines through the portal vein and bile ducts. Even when intestinal barrier functions are intact, liver is constantly exposed to low levels of microbial products derived from commensal microflora through portal vein blood. Liver contains not only parenchymal hepatocytes, but also nonparenchymal immune cells and nonimmune cells. Hepatic immune cells include Kupffer cells (resident liver tissue macrophages), T cells, B cells, dendritic cells, NK cells, and NKT cells. These cells produce a broad array of cytokines upon activation of TLRs. nonimmune cells in the liver include endothelial cells, biliary epithelial cells, and hepatic stellate cells. Hepatic nonimmune cells also express TLRs and respond to TLR ligands to induce innate immune responses including cytokine and type I IFN production. However, the liver in normal condition prevents spontaneous inflammation induced by microbial products through TLRs due to the specific barrier functions in the liver and intestines or the "liver tolerance" regulated by intercellular and intracellular mechanisms. Upon breakdown of this regulation, inflammation is induced through innate immune responses including TLR signaling in the liver. Moreover, sterile inflammation-associated danger signals may also trigger liver inflammation through TLRs. Thus, acute and chronic liver diseases are highly associated with triggering TLR signaling by intestine-derived microbial products and sterile insult-associated products from damaged cells.

In the present special issue of *Toll-Like Receptor Signaling in Liver Diseases*, the most recent advances in TLR research in the liver are reviewed by worldwide authorities of liver TLR research. The first part of this issue overviews TLR signaling in general. In the first paper, Drs. M. Yamamoto and K. Takeda reviews the current views of TLR signaling including their discovery of the functions of all four TLR adaptor proteins by generating knockout mice. In the second paper, Drs. A. E. Bigorgne and I. N. Crispe review the area of hepatic intercellular crosstalk mediated by TLRs. In the third paper, the research group of Drs. M. Fujimoto and T. Naka have cloned SOCS-1 and reported that SOCS-1 negatively regulates TLR signaling by SOCS-1. They summarize the negative regulation of TLRs by SOCS proteins and the previous reports studying the SOCS family in human

liver disease. In the next paper, Dr. H. Tsutsui and her colleagues summarize their studies that have identified TLR-mediated IL-1β and IL-18 processing through activation of the inflammasome in the liver. In the fifth paper, Dr. M. Gale Jr. and his research group have uncovered the intracellular innate immune signaling against HCV infection. He and his colleague review the biological host response against HCV and how HCV escapes from RIG-I-dependent innate immune response for sustaining HCV infection.

The next sections address specific liver functions and diseases. In the sixth paper, Dr. K. Machida summarizes his previous work studying the association between HCV and TLR4, and now his research is extending to the study of HCV-mediated tumorigenesis in which cancer stem cells could be crucial, and its possible cross-talk with alcohol. In the following paper, Drs. Y. Iimuro and J. Fujimoto review molecular mechanisms triggering liver regeneration after partial hepatectomy. TNF-α and IL-6 are known to trigger liver regeneration. They discuss the role of TLR/MyD88-dependent signaling upstream TNF-α and IL-6 in liver regeneration. They also describe the possible interaction between NF-κB and JNK/c-Jun in liver regeneration. In the eighth paper, cutting-edge research on TLRs in ischemia-reperfusion injury has been done by the research group led by Drs. A. Tsung and T. Billiar. They were the first to identify that HMGB-1 released from damaged cells is an endogenous ligand for TLR4 and that HMGB-1 triggers ischemia-reperfusion liver injury through TLR4. The interaction between alcoholic liver disease and TLR signaling has been studied for more than ten years. Dr. Thurman's research group demonstrated the role of TLR4 and gut microflora in alcoholic liver disease using intragastric ethanol infusion model. Currently, Dr. Szabo's research group advances the studies in this field. In ninth paper, she and her colleagues concisely summarize the importance of TLRs and adaptor molecules in alcoholic liver disease. The importance of TLRs has been demonstrated not only in alcoholic liver disease, but also in nonalcoholic liver disease. In the tenth paper, Drs. K. Miura and D. Brenner outline the recent advance of TLRs and nonalcoholic liver disease. In the next paper, Dr. E. Seki and his colleagues have demonstrated the interaction of the TLR4 and TGF-β signaling pathways in hepatic stellate cells. They discuss the signaling of TLR4 and the other TLRs in the activation of hepatic stellate cells and liver fibrosis. The proportion of NK cells and NKT cells in liver is much greater than that in the other organs. These cells are major targets for TLR3 ligand poly I:C. Dr B. Gao's research group is focsing on this signaling in liver physiology and in liver disease models. In the twelfth paper, they summarize their recent findings of TLR3 signaling in liver disease. In the following paper, Dr. S. Maeda demonstrated the role of hepatocyte NF-κB, JNK, and MyD88 using an animal model of liver cancer. He discusses the role of TLRs, NF-κB, and JNK signaling in liver cancer. Modification of gut microflora might be a potential target for liver disease because of the unique anatomical association between the liver and intestines. In the fourteenth paper, Dr. I. Hines and his colleague review the molecular mechanisms by which gut microflora contributes to the development of liver disease. In the fifteenth paper, Drs. T. Hayashi and K. Suzuki highlight their previous work on TLR4-mediated expression of protein S and C4b binding protein in the liver. The role of TLR4 signaling in the development of insulin resistance is intensively being studied. In the last paper, Drs. J. J. Kim and D. D. Sears summarize the recent advances in the field of innate immunity and insulin resistance.

In this special issue, we gather 16 articles highlighting our current knowledge of TLR signaling in the liver. TLR signaling induces the initial response in inflammation. This response may then induce intestinal barrier destruction and subsequent bacterial translocation as well as the release of endogenous ligands from damaged cells. These events could cause a secondary activation of TLR signaling. Thus, targeting either the initial or secondary responses induced by TLR signaling might become effective therapy for liver diseases. While we could not include discussion of the potential of TLR agonists for the therapy of liver disease in this special issue due to page limitations, this might be an attractive approach for some liver diseases, such as liver fibrosis and cancer. We trust that the articles in this special issue will help in understanding the TLR-related mechanisms underlying liver diseases and stimulate new ideas for targeting TLRs and their signaling pathways to develop new therapeutic applications for liver disease.

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Review Article

Current Views of Toll-Like Receptor Signaling Pathways

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On microbial invasion, the host immediately evokes innate immune responses. Recent studies have demonstrated that Toll-like receptors (TLRs) play crucial roles in innate responses that lead not only to the clearance of pathogens but also to the efficient establishment of acquired immunity by directly detecting molecules from microbes. In terms of intracellular TLR-mediated signaling pathways, cytoplasmic adaptor molecules containing Toll/IL-1R (TIR) domains play important roles in inflammatory immune responses through the production of proinflammatory cytokines, nitric oxide, and type I interferon, and upregulation of costimulatory molecules. In this paper, we will describe our current understanding of the relationship between TLRs and their ligands derived from pathogens such as viruses, bacteria, fungi, and parasites. Moreover, we will review the historical and current literature to describe the mechanisms behind TLR-mediated activation of innate immune responses.

1. Introduction

Innate and adaptive immunities play important roles in the elimination of various pathogens such as viruses, bacteria, and parasites in mammals [1–3]. The adaptive immune system exerts highly specific responses to microbes by producing antibodies from B cells or through the generation of killer or helper T lymphocytes, resulting in life-long immunological memory. This process may take weeks, or even months, to establish sufficient levels of immunity. On the other hand, the innate immune system promptly responds to the invasion of microbes and acts as the first line of defense, whereby innate immune cells such as macrophages or dendritic cells (DCs) play a central role in the production of proinflammatory cytokines or nitric oxide.

Almost 20 years ago, Janeway proposed that pattern recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs) specific to each pathogen, are expressed on innate immune cells and discriminate self or nonself structures [4]. However, the existence of PRRs had not been elucidated until 1996, when Hoffman's group identified "Toll" in *Drosophila*, a mutant defective in antifungal defense [5]. Subsequently, a mammalian homolog of Toll was discovered using a bioinformatics approach, and its overexpression in mammalian cells was shown to induce proinflammatory cytokines and induce expression of costimulatory molecules to stimulate acquired immunity [6]. This study opened new avenues for the identification of additional members of this family of proteins, now known as Toll-like receptors (TLRs), in humans and mice [1–3]. To date, more than 10 members of the TLR family have been reported in mammals and function as PRRs, recognizing a variety of PAMPs, such as lipopolysaccharide, lipoprotein, nucleic acids, amongst others [1–3].

Signaling molecules that participate in *Drosophila* Toll pathways, such as Dorsal, Cactus, or Pelle, had been previously identified by the isolation of the corresponding insect mutants in the 1980s [7]. In 1991, the amino acid sequence of the cytoplasmic portion of interleukin-1 (IL-1) was reported to resemble that of *Drosophila* Toll [8]. Since activation of IL-1R signaling pathways activated a transcription factor, nuclear factor kappa B (NF-κB), a mammalian homolog of *Drosophila* Dorsal, the counterpart of *Drosophila* Toll had been first considered to be IL-1R at that time [8]. After identification of mammalian TLRs, TLRs and the IL-1R were demonstrated to possess a highly conserved intracellular domain, now known as the Toll-IL-1R (TIR) domain. Ligand recognition of TLRs causes dimerization of the cytoplasmic

TIR domains, culminating in activation of downstream intracellular signaling. Similar to *Drosophila* Toll signaling and mammalian IL-1R signaling, TLR signaling also activates NF-κB as well as mitogen-activated protein kinases (MAPKs) to stimulate gene expression, including proinflammatory cytokines and costimulatory molecules [9]. In addition, the mammalian TLR system establishes antiviral immune responses predominantly through the production of type I interferon (IFN) [10].

In this paper, we will discuss the current view of mammalian TLR pathways, focusing on the molecular basis of extracellular and intracellular signaling events.

2. PAMPs and TLRs

So far, there are 10 members of the human and 13 members of the mouse TLR family that have been identified [1–3]. TLR1-TLR10 are conserved between humans and mice, although TLR10 is not functional in mice because of a retroviral insertion. In addition, TLR11-13 are not present in humans. Thus, despite some species-specific receptors, most members are conserved in mammals.

Among the TLRs, the ligand of TLR4 was first identified by genetic studies. The C3H/HeJ mutant mouse strain is hyporesponsiveness to lipopolysaccharide (LPS), a cell wall component mainly found in Gram-negative bacteria, and possesses a recessive autosomal mutation in the Lps locus [11, 12]. Positional cloning of the Lps locus revealed a point mutation in the TLR4 gene. TLR4-deficient mice also showed a similar hyporesponsiveness to LPS [13]. C3H/HeJ-type TLR4 fails to activate NF-κB in response to LPS, indicating that TLR4 is essential for the recognition of LPS *in vivo*.

In addition to LPS, bacterial lipoprotein moieties are recognized by TLR1, TLR2, and TLR6. TLR1 plays an important role in the recognition of a synthetic lipoprotein, *N*-palmitoyl-*S*-dipalmitoylglyceryl (Pam_3) Cys-Ser-$(Lys)_4$ (CSK_4) (Pam3CSK4), and the outer-surface lipoprotein of the pathogen *Borrelia burgdorferi*, outer surface protein A (OspA) [14, 15]. TLR6 participates in the recognition of macrophage-activating lipoprotein 2 kD (MALP-2) derived from mycoplasma [16]. Both TLR1 and TLR6 require dimerization with TLR2 to be functional [16]. TLR2 is also essential for the recognition of peptidoglycan, lipoarabinomannan, porins, *Trypanosoma cruzi* Glycosylphosphatidylinositol-anchored mucin-like glycoproteins (tGPI-mucin), or Hemagglutinin (HA) proteins from not only bacteria but also viruses or parasites [1].

Genomic nucleic acids from bacteria and viruses, or their analogs, stimulate the production of proinflammatory cytokines and type I IFN. Among them, immunostimulatory bacterial DNA was first identified in Calmette-Guerin bacilli, which are capable of promoting antitumor activity and inducing type I IFN (IFN-α/β) and type II IFN (IFN-γ) in human peripheral blood leukocytes [17, 18]. Among the TLR family members, TLR9 is responsible for the recognition of unmethylated CpG DNA. TLR9 also recognizes genomic DNA from DNA viruses such as HSV-1, HSV-2, or MCMV [1]. As well as nucleic acids, hemozoin, a malaria-derived insoluble crystal, is a ligand for TLR9 [19].

RNA is also a TLR ligand. TLR3 recognizes a synthetic double-stranded RNA (dsRNA) analog, polyinosinic-polycytidylic acid (poly I:C), and dsRNA derived from Reovirus, EMCV, RSV, or West Nile virus (WNV) [1]. In contrast to dsRNA recognition by TLR3, guanosine-rich and uridine-rich single-stranded RNAs (ssRNAs) derived from HIV or influenza virus are ligands for TLR7 [20, 21]. In addition, low molecular weight compounds of the nucleoside analog imidazoquinoline, known as Imiquimod (Aldara, R-837, S-26308), and R-848 (resiquimod, S-28463) are synthetic TLR7 ligands [22, 23].

TLR5 and TLR11 recognize protein moieties from bacteria or parasites. TLR5 is essential for the recognition of a component of bacterial flagella, flagellin. The highly conserved central portion of flagellin that is pivotal for bacterial motility is bound by TLR5 [24]. TLR11 recognizes a parasite-derived profilin-like molecule that is a potent inducer of IL-12 and known as soluble Toxoplasma antigen. It plays an important role in parasite motility and invasion into host cells [25, 26]. TLR11 might also recognize PAMPs from uropathogenic bacteria, since TLR11-deficient mice are highly susceptible to the pathogen. However, the natural ligand for TLR11 from uropathogenic bacteria has not yet been identified [27]. Thus, TLRs recognize a number of PAMPs from various microbes. (See Figure 1).

3. Molecular Basis of TLRs Structure and Ligand Recognition

TLRs are type I transmembrane proteins that consist of three major domains: (1) a leucine rich extracellular domain; (2) a transmembrane domain; (3) a cytoplasmic TIR domain. Ligand recognition by TLRs is mediated by the extracellular domain that harbors a leucine rich repeat (LRR) composed of 19–25 tandem copies of the "xLxxLxLxx" motif [28]. So far, the crystal structure of TLR1, TLR2, TLR3, TLR4, and TLR6 with or without their ligands has been resolved, and these studies predict that the extracellular domain of the TLRs forms a horseshoe-like structure [28]. Notably, structural analysis and biochemical studies indicate that all TLRs form hetero- or homodimers (e.g., TLR1/TLR2, TLR2/TLR6, TLR3/TLR3, and TLR4/TLR4), which probably facilitates dimerization of the cytoplasmic TIR domain to activate intracellular signaling [28]. In the case of the homodimers of TLR3 or TLR4, direct or indirect interactions by ionic and hydrogen bonds with their ligands are essential for the recognition [29, 30]. On the other hand, TLR2 forms a heterodimer together with either TLR1 or TLR6 to recognize triacyl or diacyl peptides in internal protein pockets through hydrophilic interactions [31, 32].

4. Localization of TLRs: Extracellular and Intracellular TLRs

The TLR family can be largely divided into extracellular and intracellular members. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 are largely localized on the cell surface to recognize PAMPs. On the other hand, TLR3, TLR4, TLR7, TLR8,

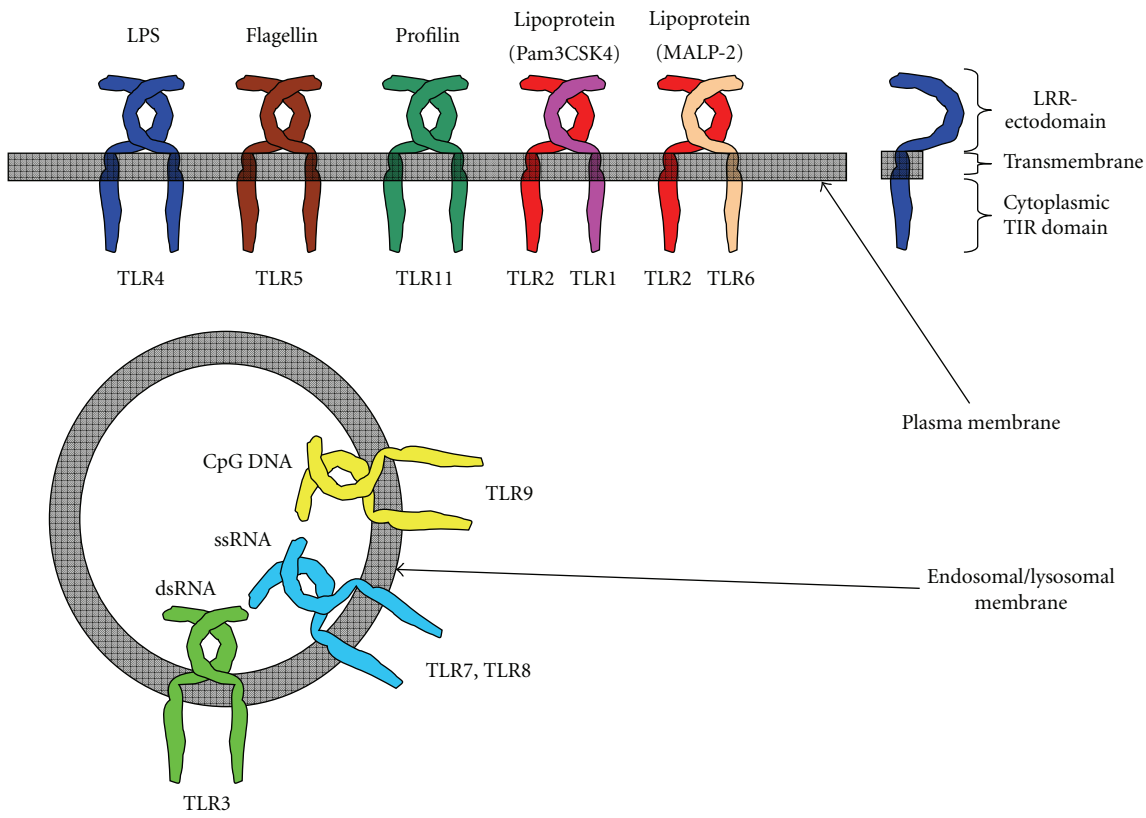

Figure 1: Extracellular and intracellular TLRs. TLRs can be divided into extracellular and intracellular TLRs. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 recognize their ligands on the cell surface. On the other hand, TLR3, TLR7, TLR8, and TLR9 are intracellularly localized.

and TLR9 are intracellularly expressed in endosomal- or lysosomal-compartments and the endoplasmic reticulum (ER).

TLR4 is tightly bound to MD-2 on the cell surface [33]. In addition, CD14 and LPS-binding protein (LBP) also participate in the recognition of LPS by TLR4/MD-2. CD14 efficiently transfers an LBP-LPS complex to TLR4/MD-2 for cellular activation. In addition, TLR4 is internalized into endosomal compartments in response to LPS stimulation [33]. For TLR2 ligand recognition, TLR1, TLR6, and a host on non-TLR receptors, such as CD36 or Dectin-1, form heterodimers with TLR2 and are involved in the recognition of most TLR2 ligands or β-glucan, respectively [34, 35]. The intracellular TLRs such as TLR3, TLR7, TLR8, and TLR9 are localized on the ER membrane in resting cells. However, upon stimulation, they are trafficked to the endosomal compartment [36, 37]. The intracellular localization is regulated by the ER membrane protein UNC93B, which directly interacts with the intracellular TLRs [38]. Mice bearing a point missense mutation of this gene are defective in the trafficking of TLR3, TLR7, and TLR9 and fail to activate cellular signaling [38]. Moreover, processing of the ectodomain of TLR9 by cathepsins in the endolysosomal compartments is required for compartment-specific activation [39, 40]. Thus, the localization of TLRs responsible for detecting foreign (nonself) nucleic acids is tightly regulated to avoid a response to self-DNA on the cell surface [41]. (See Figure 1).

5. NF-κB and MAPK Activation in TLR-Mediated Intracellular Signaling

As discussed above, the cytoplasmic portion of TLRs shows high similarity to that of IL-1R family members and is called the TIR domain. Activation of TLR signaling culminates in NF-κB and MAPKs that regulate gene expression of various immune and inflammatory mediators [9]. In TLR signaling, NF-κBs are sequestered by cytoplasmic IκB proteins including nuclear inhibitor of NF-κB (IκB)α, and IκBβ in unstimulated cells. Stimulation by TLR ligands mediates degradation of these IκBs by the proteasome, a process dependent on their phosphorylation by the IκB kinase (IKK) complex composed of IKKα, IKKβ, and NEMO (also known as IKKγ), allowing the nuclear translocation of NF-κB [42]. Transcriptional activity is controlled by a variety of nuclear proteins such as Akirin2 or the family of nuclear IκBs, IκBζ, IκBNS, and Bcl-3 [43–45].

The activation of the IKK complex is regulated by several MAPK kinase kinases (MAP3K) including TAK1, MEKK3, Tpl2, and ASK1 [1]. Among them, TAK1 has been most studied in view of its molecular role in TLR/IL-1R-mediated IKK activation [46]. TAK1 participates in IKK activation in a complex with TAK1-binding protein 1 (TAB1), TAB2, and TAB3, at least *in vitro* [46]. However, the roles of TAB protein family members in TLR/IL-1R-mediated signaling remain controversial, since neither TAB1 nor TAB2-deficient

mice showed any abnormalities of TLR/IL-1R signaling pathways [47–49]. The activation of the TAK1-TABs complex is regulated not by protein phosphorylation, but by lysine 63 (K63)-linked ubiquitination [50]. In contrast to K48-linked ubiquitination that mediates proteasome-dependent protein destruction, K63-linked ubiquitination is involved in cellular processes such as DNA repair, in addition to activation of the TAK1-TABs complex [51]. The formation of K63-linked polyubiquitin chains is catalyzed by the E2 ubiquitin conjugating enzyme complex Ubc13 and Uev1A [52]. The role of Ubc13 in TLR-mediated NF-κB activation remains controversial, since mice lacking Ubc13 exhibit almost normal NF-κB activation and IκB degradation in response to TLR ligands or IL-1 [53]. The TAK1 complex also regulates activation of MAPKs, such as ERK1/2, p38, and JNK, to control mRNA expression or the stability of mRNA for inflammatory genes by mediating phosphorylation of AP-1 transcription family proteins [54, 55].

Upstream of the TAK1 complex, a RING finger-containing E3 ligase, TRAF6, is involved in K63-linked ubiquitination-mediated signaling [1]. The activity of TRAF6 is regulated by the family of death domain containing-IL-1R-associated kinases (IRAKs), IRAK1, IRAK2, IRAK-M, and IRAK-4 [56, 57]. Among them, IRAK-1, IRAK-2, and IRAK-4 positively regulate TRAF6 activity, while IRAK-M limits TLR/IL-1R-medaited immune responses. Genetic studies using mice lacking IRAK-1, IRAK-2 or IRAK-4 have demonstrated that deficiency of both IRAK-1 and IRAK-2 is comparable to the lack of IRAK-4 alone, suggesting a central role of IRAK-4 in TLR/IL-1R-mediated signaling. Moreover, IRAK-4 interacts with an upstream adaptor molecule, MyD88, through a homophilic interaction of the death domain [58]. MyD88 plays a critical role in both TLR- and IL-1R-mediated signaling pathways [59, 60]. (See Figure 2).

6. Specific Participation of the TIR Domain-Containing Adaptor Molecules in TLR Signaling

MyD88 is a member of the family of cytosolic TIR domain-containing adaptor molecules. In addition to MyD88, the family includes TIRAP (also known as Mal), TRIF (also known as TICAM-1), TRAM (also known as TICAM-2), and SARM. As discussed above, the cytoplasmic portion of TLRs harbors a TIR domain, to which individual TIR domain-containing adaptors are selectively recruited to specific TLRs, generating signaling specificity for each TLR [9]. MyD88 is a master adaptor molecule that is utilized by not only all IL-1R family members, but also by almost all TLRs, with the exception of TLR3 [1]. TIRAP interacts with MyD88 through the TIR domain and selectively participates in TLR2- and TLR4-mediated MyD88-dependent signaling pathways [61, 62].

Whereas LPS, the TLR4 ligand, fails to stimulate the production of proinflammatory cytokines in MyD88-deficient cells, it still activates NF-κB and MAPK and induces gene expression of type I IFN, indicating the presence of MyD88-independent pathways in TLR4 signaling [63, 64]. Moreover, TLR3 activates MyD88-independent signaling, suggesting the existence of other TIR domain-containing adaptor molecules that function in the TLR3- and TLR4-mediated pathways [65]. TRIF plays a critical role in the TLR3- and TLR4-mediated MyD88-independent pathways [63, 64]. Although TRIF is bound to TLR3 through the TIR domain, TLR4 utilizes TRAM to activate TRIF-dependent signaling [66–68]. Thus, TLR4 utilizes MyD88 and TIRAP for the MyD88-dependent pathway to induce mainly proinflammatory cytokines, or TRIF and TRAM for the MyD88-independent pathway to induce type I IFN and IFN inducible genes (IRGs) [9]. Moreover, internalization of TLR4 is shown to be required for proper activation of the TRIF-dependent pathway [69]. In addition, a very recent study demonstrates a two-stage activation mechanism for TLR4-mediated signaling pathways, in which assembly of a multiprotein complex including MyD88, TRAF6, Ubc13, IKKγ, cIAP1/2, TAK1, and TRAF3 induces K63-linked ubiquitination of cIAP1/2 that leads to degradation of TRAF3, subsequently resulting in MyD88-signaling complex inducing its translocation from membrane to the cytosol and TAK1 activation [70].

Regarding SARM, a previous biochemical report has suggested that human SARM is required for negative regulation of the TLR3-mediated MyD88-independent pathway by inhibiting the interaction of TRIF with TLR3 [71]. However, mice lacking SARM do not show any abnormalities in TLR3-mediated, or other TLR-mediated, signaling, indicating the minor role of SARM in TLR signaling pathways [72].

7. Signaling Pathways for TLR-Mediated Type I IFN Production

TLR3/TLR4-mediated MyD88-independent (TRIF-dependent) signaling induces the expression of type I IFN and IRGs [9]. In addition, TLR7- and TLR9-mediated MyD88-dependent pathways also lead to type I IFN production, especially in a subtype of dendritic cells called plasmacytoid dendritic cells (pDCs) [73]. Thus, TLR-mediated type I IFN production is mediated by the TLR3/TLR4-mediated TRIF-dependent or the TLR7/TLR9-mediated MyD88-dependent pathways [1]. Expression of type I IFN is largely controlled by IRF transcription factors, which consist of 9 members. Among them, IRF1, IRF3, IRF7, and IRF8 are involved in TLR-mediated type I IFN production [74].

Downstream of TRIF, two TRAF proteins, TRAF3 and TRAF6, participate in type I IFN production [75, 76]. Although deficiency in TRAF3 has resulted in severe abnormalities in TRIF-mediated type I IFN production as well as IL-10 production, it is largely normal in TRAF6-deficient cells, suggesting that TRAF3 rather than TRAF6 plays the critical role in the TLR3/TLR4-mediated TRIF-dependent pathway [75, 76]. Signaling from TRAF3 activates IRF3, predominantly leading to IFN-β expression [75, 76]. Phosphorylation of the C-terminal serine/threonine rich portions of IRF3 by IKK-related kinases, TBK1 (also known as T2K or NAK) and IKK-i (also known as IKKε), is required for this activation [77, 78]. Phosphorylated IRF3 forms a homodimer and translocates into the nucleus

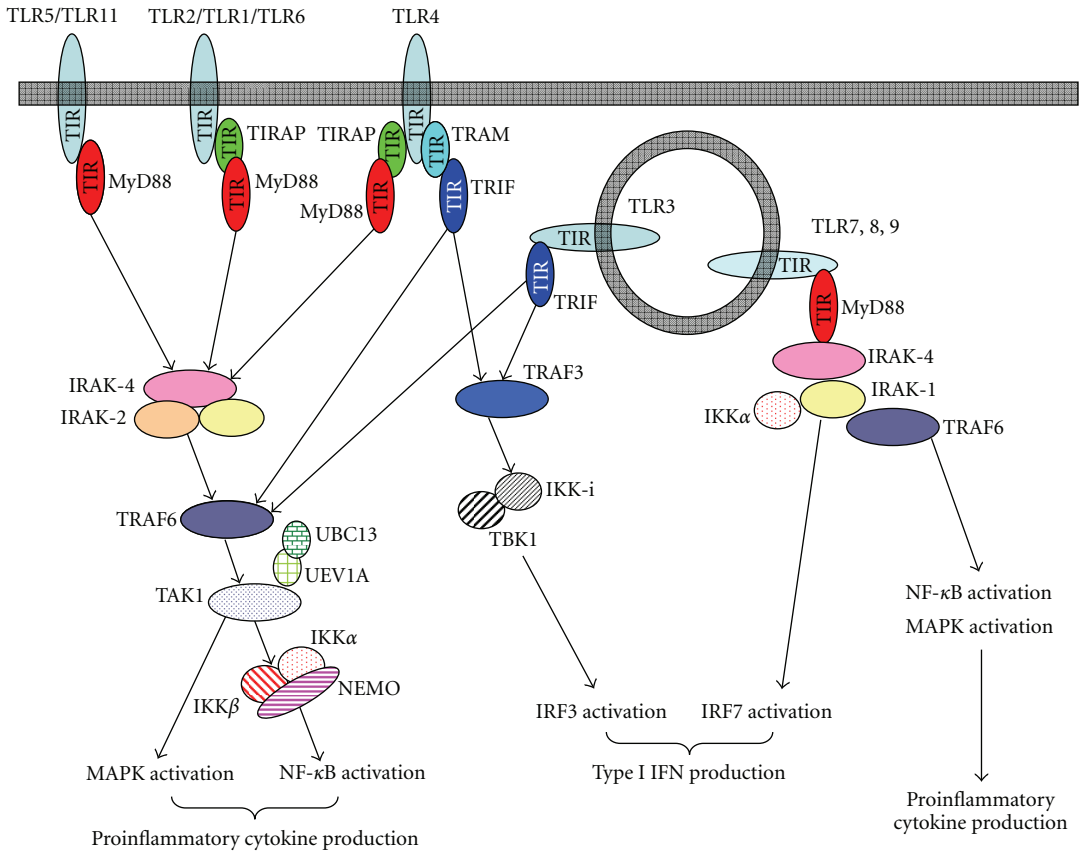

Figure 2: TLR-mediated MyD88-dependent or TRIF-dependent pathways. TIR domain-containing adaptors MyD88, TIRAP, TRIF, and TRAM define TLR-mediated signaling. MyD88 and TIRAP are adaptors for the MyD88-dependent pathways, which mainly activate proinflammatory cytokine production. On the other hand, TRIF and TRAM are adaptors for IRF3 activation, resulting in production of type I IFN by TLR3- or TLR4-mediated TRIF-dependent pathways. In pDCs, TLR7/TLR9-mediated MyD88-dependent pathways induce IRF7 activation, leading to IFNα production.

where it binds to the DNA and interacts with the nuclear coactivator proteins p300 and CBP to positively regulate the transcription of the IFNβ gene [79]. IKK-*i* and TBK1 are bound to TANK (also known as I-TRAF), NAP1 (also known as AZ2) and TBKBP1 (also known as SINTBAD) [80–82]. Although the molecules regulate the kinase activities of TBK1 and IKK-*i*, much like NEMO, which critically controls IKKα and IKKβ, their physiological roles in TLR3/TLR4-mediated type I IFN production remain to be elucidated [1].

In vivo stimulation by TLR7 or TLR9 ligands in mice leads to the production of high levels of IFNα, mainly from pDCs [1]. TLR7- and TLR9-dependent IFNα production requires MyD88, but not TRIF [83, 84]. MyD88-dependent expression of IFNα is mediated by IRF7 [83, 84], a protein structurally related to IRF3 that plays a master role in TLR7- and TLR9-mediated IFNα production [74]. Like IRF3, which is phosphorylated by IKK-*i* and TBK1 [85, 86], IRF7 is also phosphorylated in response to TLR7 and TLR9 ligands in pDCs, although by IRAK-1 and IKKα [83]. Moreover, PI3K, osteopontin, TRAF3, and TRAF6 regulate IRF7 activation [87]. In addition to IRF7, IRF1 participates in IFNβ gene expression in conventional DCs (cDCs) [88]. IRF1 as well as IRF7 is directly bound to MyD88 and activated in response to TLR9 ligands [89]. Moreover, IRF8 also plays an important role in IFNα and IFNβ, as well as IL-12 p40, production by pDCs and other DCs [90]. Another IRF member, IRF5, is indispensable for proinflammatory cytokine production, rather than type I IFN, in the MyD88-dependent pathways mediated by almost all TLRs [91]. Thus, IRFs are divergent regulators of the production of not only type I IFN, but also inflammatory cytokines. (See Figure 2).

8. Conclusions

A variety of PAMPs derived from a wide range of pathogens, including viruses, bacteria, fungi, and parasites, are recognized by TLRs. After ligation of the PAMPs, TLRs initiate intracellular signaling pathways to activate immune responses via the TIR domain. The TIR domain of the receptor interacts with the intracellular TIR domain-containing adaptors MyD88, TIRAP, TRIF, TRAM, and SARM, which generate the specificity of the downstream signaling and unique outputs of each TLR. The MyD88-dependent pathways mainly regulate proinflammatory cytokine production and IRF1- or IRF7-mediated type I IFN production in DCs. On the other hand, TLR3- and

TLR4-mediated TRIF-dependent pathways control IRF3-mediated IFNβ production. Large bodies of the past, current, and future literature on TLR signaling pathways can be applied not only to liver immune cells, such as Kupffer cells, but also nonimmune cells including hepatocytes and other liver cells.

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Review Article

TLRs in Hepatic Cellular Crosstalk

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Toll-like receptors (TLRs) are expressed on all major subsets of liver cells. Both exogenous ligands derived from pathogens, and endogenous ligands that are products of cellular injury, engage these receptors and activate aspects of innate immunity. These receptors play a role in viral and parasitic infections of the liver, in ischemia-reperfusion injury, and in toxic liver damage, promoting antipathogen immunity but also hepatocellular injury and fibrogenesis. However, TLRs may also participate in negative feedback that limits tissue injury. In the complex environment of the liver, TLRs participate in pathologic cascades involving multiple cell types, manifesting their effects both through cell-autonomous actions, and via cellular crosstalk. In this paper we survey the involvement of TLRs in these diverse processes.

1. Introduction

The Toll-like receptors (TLRs) form a multigene family that is well conserved between human and murine species. These receptors are cell surface or intracellular receptors for molecular signatures characteristic of viruses, bacteria, and parasites, including features of their nucleic acids, proteins, and lipid and carbohydrate components. The pathogen-associated molecular patterns (PAMPs) engaged by TLRs are basic features of these microorganisms that cannot readily be modified by genetic mutation, thus that are features of entire categories of microorganisms. A prime example is the lipopolysaccharide (LPS) endotoxin of the cell walls of Gram-negative bacteria, which engages a cell surface member of the TLR family, TLR4, activating multiple downstream signaling pathways that result in the synthesis of cytokines and interferons. TLRs share functional similarities, and downstream effector mechanisms, with other pathogen recognition systems such as the RIG-I like proteins that detect viral nucleic acids, and the NOD-like receptors that respond to bacterial cell wall elements.

All of the known TLRs are expressed in the liver, and this is likely to be biologically important since the liver receives blood from the intestine, which is an internal body surface exposed to PAMPs derived from harmless commensal bacteria in the gut lumen as well as potentially antigenic components of the diet and from time to time, invasive microorganisms.

Hepatic injury is associated with an increase of liver exposure to bacterial products, but the healthy liver is able to develop a tolerance towards bacterial products coming from the gut. Specifically, the exposure of liver sinusoidal endothelial cells (LSECs) to low levels of LPS results in the loss of their TLR4 expression, resulting in LPS insensitivity [1]. This effect is not limited to homologous ligand, since the administration of the TLR3 ligand, poly I:C, also downregulates LPS sensitivity on Kupffer cells (KCs) [2]. In hepatocytes, this mechanism depends on SOCS-1 which interacts with TIRAP in the TLR signaling pathway [3].

In the liver, immune responses are complicated by the immune competence of many populations of cells, including an unusual assembly of lymphocytes in which Natural Killer (NK) cells and CD8+ T cells are unusually abundant, as well as Dendritic Cells (DCs), KCs, LSECs, hepatic stellate cells (HSCs), hepatocytes, and bile duct cells. Any or all of these cell types may respond to TLR signals, and any of them may act as antigen-presenting cells (APCs) that can engage T cells. Inflammatory or immune pathologies that converge on the hepatocyte (such as hepatocellular injury and regeneration), or the HSC (fibrosis, cirrhosis), very likely involve other cell types. For example, innate immune signals may activate KCs, the KCs may elaborate cytokines, and these cytokines may act

on HSCs, either promoting or suppressing fibrogenesis. Here we address the issue of how TLRs may be involved in such cellular cross-talk in liver immunopathology.

The analysis would be more straightforward if each liver cell type expressed a characteristic set of TLRs. However, there is very little segregation of TLR expression: studies with both purified cell cultures and cell lines support the idea that all liver cell populations express essentially all TLRs at the mRNA level. Comprehensive studies of the responsiveness of individual cell types to a full range of TLR ligands are few. At present, no specific liver cell population can be identified as central in TLR-mediated pathologies. Furthermore, the effects of TLR ligation vary from cell to cell.

While TLRs can initiate innate immune cascades through the recognition of exogenous PAMPs, they also recognize endogenous signals released by damaged cells. Thus, dying cells release RNA, which can engage TLR3; nuclear DNA that can engage TLR9; and HMBG1 (high mobility group box protein 1) that can engage TLR4. This gives endogenous injury signals, like exogenous PAMPs, access to both the MyD88 and the TRIF signaling pathways. This raises the possibility that immunopathology, initiated by a TLR-mediated response to a PAMP, can result in tissue damage that sustains itself through other TLR-mediated mechanisms. In the liver, TLRs are involved in infections (HCV, HBV), but also in "sterile injury" models including Concanavalin A (ConA)-induced hepatitis, bile duct ligation, partial hepatectomy, acetaminophen toxicity, and ischemia-reperfusion injury. TLRs and particularly TLR4 play a key role in liver regeneration, in alcoholic liver injury, in the development of steatosis, and in the recruitment of activated $CD8^+$T cells to the hepatic sinusoids [4]. In addition, tissue culture models reveal some pathways of cell-cell interaction triggered by TLR engagement. In our own experiments, TLR2, TLR3, and TLR4 activation of human Kupffer cells resulted in differential secretion of IL-18 and IL-10, which coordinately regulated the activation and IFN-γ synthesis of cocultured human liver NK cells [5].

Downstream effects of TLR signaling in diverse models include hepatocellular injury, measured most often as an increase in serum alanine aminotransaminase (ALT); fibrogenesis, measured by the upregulation of timp-1, collagen, and α-smooth muscle actin expression; steatosis quantified mainly by hematoxylin eosin saffron (HES) histochemical staining of lipid droplets; liver regeneration assessed *via* hepatocyte mitotic activity and the restoration of liver mass; and anti-pathogen effects such as suppression of HCV replication or malaria parasite load. The detailed articulation of these numerous interacting pathways is still some way in the future. We can, however, explore some structuring ideas through the current literature, and try to discern some common themes in TLR involvement in liver pathology.

2. TLRs Responding to Endogenous Ligands

While it is clear that TLRs may respond to molecules released by injured cells, there are also experimental models induced by mechanical or toxic stresses that do involve *bona fide* bacterial products, and these cases need to be distinguished from the involvement of TLRs in "sterile injury" where the ligands are truly endogenous. Bacterial endotoxemia contributes directly to liver injury in apparent unrelated hepatic pathologies such as cholestasis, chronic alcoholic hepatitis, or steatohepatitis. In bile duct-ligated animals, pretreatment with intraperitoneal LPS upregulates the expression of TLR4 and MyD88 [6], as well as CD14 and CD68 [7], and confers hypersensitivity to LPS in fibrotic livers. The increase in proinflammatory cytokines (TNF-α and IL-6) secretion was observed as early as two hours after LPS administration in BDL rats. These models of liver injury in apparently unrelated hepatic pathologies.

In other models, TLRs are involved but the source of their ligands is less clear. For example, after hemorrhagic shock, endogenous damage-associated molecular patterns (DAMPs) are released from cells during traumatic injury allowing them to interact with TLRs. In particular, TLR4 expression is required on bone marrow-derived cells and on liver parenchymal cells to detect systemic and remote organ response to hemorrhagic shock [8]. However, it is unclear if this model also results in LPS translocation across the gut.

In nonalcoholic steatohepatitis, a direct link exists between TLR4 and Kupffer cells in the pathogenesis of liver injury. In a methionine choline deficiency (MCD) diet-induced model of steatohepatitis, histological evidence of liver inflammation, portal endotoxemia and enhanced TLR-4 expression occurs in wild-type mice fed the MCD diet. In contrast, injury and lipid accumulation markers were significantly lower in TLR4 mutant mice. Targeted destruction of Kupffer cells with clodronate liposomes blunted histological evidence of steatohepatitis and prevented increases in TLR4 expression, induced increases in the production of TNF-α, ICAM-1, and significantly impaired the development of hepatic injury. In support of the idea that the expression of TLR4 on Kupffer cells is essential for injury, destruction of these cells also prevented increases in TLR-4 expression [9].

In alcoholic liver disease, low expression of the anti-inflammatory factor GILZ (Glucocorticoid Induced Leucin Zipper) in monocytes contributes to liver inflammation and hypersensitization to LPS. GILZ messenger RNA (mRNA) levels were lower in the livers of patients with AH versus those without AH. A treatment with glucocorticoids enhances GILZ expression and abrogates macrophage sensitivity to LPS and subsequent proinflammatory cytokine secretion [10].

In this model, TLR4 is required for liver injury but MyD88, its principal downstream effector, is not necessary for injury. Alcohol feeding results in a significant increase in liver injury in wild-type (wt), TLR2−/−, and MyD88−/− but not in TLR4−/− mice [11]. The expression of inflammatory mediators (TNF-α and IL-6) and the TLR4 coreceptors (CD14 and MD2) significantly increases in livers of alcohol-fed wt, TLR2−/−, or MyD88−/−, but not in TLR4−/− mice, compared to controls. Alcohol feeding also induces nuclear factor-kappaB activation in a TLR4-dependent, but MyD88-independent manner. This shows that while TLR4 deficiency was protective, MyD88 deficiency failed to prevent alcohol-induced liver damage and inflammation by implication suggesting that the alternative TLR4 signaling

pathway involving the adapter protein TRIF, and also IRF-7, was involved.

A very recent report shows that inflammation occurs after both major trauma and infection injury [12]. Mitochondrial DAMPs (MTDs) include formyl peptides and mitochondrial DNA. Mitochondrial DNA activates human polymorphonuclear neutrophils (PMNs) through TLR9. The assessment of MMP8 protein expression—a marker of neutrophil infiltration—was increased in whole livers of rats injected intravenously with mitochondrial DAMPs whereas control rats showed no evidence of hepatic inflammation.

After tissue trauma, mitochondrial DAMPs that express at least the two molecular signatures, formyl peptides, and mtDNA, act on pattern recognition receptors recognizing bacterial PAMPs. These activate PMN in the circulation rather than at specific targets, inciting nonspecific attack on multiple organs, including the liver, while suppressing chemotactic responses to infective stimuli.

Other intracellular "alarmins" may similarly be important after injury, and other immune cells probably respond to MDTs. Injury-derived MDTs, however, are clearly recognized by innate immunity using pattern recognition receptors that alternatively sense bacteria. This novel model may explain why responses to these ancient "enemies within" released by injury can mimic sepsis.

3. TLRs May Mediate Injury-Limiting as well as Injury-Promoting Pathways

One good example of this "injury limiting effect" is the response of conventional DCs to TLR9 ligation by DNA released from cells during ischemia-reperfusion injury. By secreting IL-10, these DCs respond to damage-associated patterns released by injured cells, providing the host with protection from progressive damage, potentially limiting tissue injury in the presence of dying cells [13].

Similarly, the ligation of TLR9 inhibits NF-κB binding activity in T cells, and increases survival of mice in a model of ConA-induced hepatitis. Liver injury—as measured by circulating ALT levels—decreases after pretreatment with CpG oligodeoxynucleotides sequences that can engage TLR9 [12]. However this is not a property of TLR9 in general, since this receptor promotes liver injury in acetaminophen injury [14], in ischemia/reperfusion [13], and in nonalcoholic steatohepatitis [15].

In acetaminophen injury, acetaminophen treatment results in hepatocyte death and the free DNA released from apoptotic hepatocytes activates TLR9. This triggers a signaling cascade that increases transcription of the genes encoding pro-IL-1β and pro-IL-18 in LSECs. TLR9 antagonists and aspirin reduced mortality from acetaminophen hepatotoxicity [16]. In ischemia/reperfusion injury, TLR4, but not TLR2, is specifically required to initiate the tissue-damaging cascade, as manifested by liver function (serum ALT levels), pathology, and local induction of proinflammatory cytokines/chemokines (TNF-α, IL-6, and CXCL10) [14].

In a model of liver injury induced with acetaminophen, there was MyD88-dependent recruitment of neutrophils into the liver, as assayed by the abundance of the neutrophil enzyme myeloperoxidase (MPO) [17]. This was due not to TLR signaling, but required the IL-1R expressed on non-bone marrow-derived cells. Sterile neutrophilic inflammation is thought to contribute to the pathogenesis of acute ischemia-induced liver injury and to impair healing. Blocking such sterile inflammation is a potentially attractive strategy to limit the damage of acute sterile inflammation and to stop the ongoing damage in chronic inflammation from progressing to liver injury.

In a model of segmental liver ischemia-reperfusion injury, the treatment of wt mice with an inhibitory cytosine-guanosine dinucleotide (iCpG) sequence reduced significantly the serum ALT and inflammatory cytokines after liver ischemia-reperfusion injury, and the same was seen in TLR9-deficient mice. Liver damage was mediated by bone marrow-derived cells because wt mice transplanted with TLR9−/− bone marrow were protected from injury. Injury in wt mice partly depends on TLR9 signaling in neutrophils, which enhanced production of ROS, IL-6, and TNF-α. *In vitro*, DNA released from necrotic hepatocytes increased cytokine secretion in liver nonparenchymal cells and neutrophils through a TLR9-dependent mechanism. Inhibition of both TLR9 and HMGB1 caused maximal inflammatory cytokine suppression in neutrophil cultures and conferred even greater protection from ischemia-reperfusion injury *in vivo* [13].

In diet-induced obesity, TLR9−/− mice show less steatohepatitis and liver fibrosis than wt mice. Among inflammatory cytokines, IL-1β production is suppressed in TLR9−/− mice. Kupffer cells produce IL-1β in response to CpG oligodeoxynucleotides leading to steatosis and inflammation [15]. Similarly, CpG DNA promotes liver injury in the presence of D-Gal, promoting apoptotic death in hepatocytes [18]. Taken together, these data do not yield a simple model for the involvement of TLR9 signaling in modulating liver injury. The most straightforward interpretation is that TLR9 has both pro- and anti-injury effects, depending on the cell types concerned and their interactions.

Two single nucleotide polymorphisms (SNPs) of the TLR4 gene emerge as conferring protection from fibrosis progression compared to wt. The study of the functional linkage of this SNP to HSCs responses show that both HSCs from TLR4−/− mice and a human HSC line reconstituted with either TLR4 D299G and/or T399I cDNAs were hyporesponsive to LPS stimulation compared to those expressing wt TLR4, as assessed by the expression and secretion of LPS-induced inflammatory and chemotactic cytokines (MCP-1 and IL-6). The conclusion is that TLR4 D299G and T399I SNPs that are associated with protection from hepatic fibrosis reduce TLR4-mediated inflammatory and fibrogenic signaling and lower the apoptotic threshold of activated HSCs. These findings provide a mechanistic link that explains how specific TLR4 SNPs may regulate the risk of fibrosis progression [19].

Conversely, when TLRs do not intervene in injury-limiting pathways, they can promote liver failure through direct interaction with mediators promoting injury. Although the receptor for advanced glycosylation end products (RAGEs) has been shown to interact with HMGB1, the recent

identification of direct recognition of HGMB1 with different TLRs has confirmed the wide range of possible interactions. The HMGB proteins have been described to play a role as late mediators of lethality in sepsis as well as in cells undergoing necrosis, but not in cell death due to apoptosis nor from cells exposed to inflammatory cytokines. HMGB1 is a nuclear factor, which is released from injured cells, including hepatocytes. It is argued that HMGB1 interacts with TLR4, since TLR4 defective (C3H/HeJ) mice exhibits less damage in the hepatic ischemia-reperfusion model than wt C3H/OuJ mice [20].

TLR9 sits at the interface of microbial and sterile inflammation by detecting both bacterial and endogenous DNA. Released in the extracellular compartment during acute inflammatory responses, HMGB1 also interact with TLR2 [21] and TLR9 [22]. Unlike LPS, which primarily increased the activity of IKK-β, HMGB1 exposure resulted in activation of both IKK-α and IKK-β. Kinases and scaffolding proteins downstream of TLR2 and TLR4 were involved in the enhancement of NF-κB-dependent transcription by HMGB1. Transfections with dominant negative constructs show that TLR2 and TLR4 were both involved in HMGB1-induced activation of NF-κB. Interactions of HMGB1 with TLR2 and TLR4 may provide an explanation for the ability of HMGB1 to generate inflammatory responses that are similar to those initiated by LPS [21]. TLR3 may also act to modulate aspects of liver inflammation by activating NKT cells that can eliminate gamma-delta T cell through apoptosis [23], thus changing the cellular makeup of inflammatory infiltrates.

Stellate cells are subject to regulation both through their own TLRs and via cross-talk. Thus, stellate cells are maintained in an undifferentiated state by interaction with fresh LSEC acting via VEGF and NO [24].

As mammalian cells undergo apoptosis, genomic DNA undergoes significant modifications, which include caspase-mediated cleavage but also aberrant methylation and oxidative damage. Such changes may result in enrichment in CpG sequences in comparison to random DNA from the human genome. As a sensor for cell injury, HSCs phagocytose apoptotic hepatocyte bodies with subsequent regulation of TGF-β and collagen-α1 mRNA [25]. The consequence is TLR9-dependent HSC differentiation as well as a stop signal to retain HSC at sites of hepatocyte apoptosis [26]. Other groups have shown that TLR4 downstream activation can have important functional consequences on hepatic stellate cells. TLR4 activation in hepatic stellate cells sensitizes HSCs to TGF-β-induced signals and upregulates chemokine secretion and induces chemotaxis of Kupffer cells [27, 28]. In particular, LPS induces signal transduction and upregulates chemokines (IL-8, CCL2) and adhesion molecules (VCAM-1 and ICAM-1) in activated human HSCs from patients with hepatitis C virus induced cirrhosis [27].

4. TLRs in Host Defense against Hepatocellular Pathogens

TLRs were first identified in *Drosophila melanogaster* as a genetic element, the lack of which predisposed adult flies to lethal fungal infection [29]. Recognition of the significance of such nonrearranging receptors led Medzhitov and Janeway to search for homologous molecules in the mouse and human genomes, [30], while a convergent line of research using positional cloning identified the receptor for LPS as a TLR [31]. While additional roles of these molecules continue to emerge, the principal phenotypes of TLR-deficient mice are associated with increased susceptibility to infection. The TLRs also play this role in the liver.

Viruses interacting with host cells can modulate expression and function of TLRs. In chronic hepatitis C virus (HCV) infected patients, expression of TLR4, 7 and 8 is increased in peripheral $CD14^+$ cells together with circulating levels of TNF-α, IL-6, and IL-12p35. The incubation of PBMC with HCV core protein triggers the expression of TLR2 and suppresses TLR4 and TLR7 [32]. Activation of nonparenchymal cells such as KCs and LSECs with TLR ligands leads to the secretion of IFN-β, which powerfully suppresses HCV replication [33]. In the same way, while the expression of messenger RNA encoding all TLRs is detectable in HSCs, the spectrum of TLR ligands, which are capable to induce secretion of antiviral cytokines, is restricted to TLR3 in human HSCs. Moreover, such ligation results only in IFN-β sufficient to suppress the replication of either LCMV or HCV, but not in the synthesis of relevant amounts of other IFNs (i.e., neither IFN-γ nor IFN-α) [34].

These host defense-promoting effects of TLR engagement are not only antiviral. TLR2, 3, 4, and 9 ligands can reduce the liver load of parasites in murine *Plasmodium yoelii* infection. In particular, CpG as a TLR9 ligand causes an 88% decrease in hepatic parasite load, and in mice challenged with 100 sporozoites, results in complete suppression of parasitemia for at least 14 days [35]. This effect of CpG was accompanied by increases in hepatic IL-12 and TNF-α as well as IFN-γ, and decreases in IL-10 and TGF-β1. The effect of Kupffer cell depletion is to abrogate these effects and restore parasite load. It therefore seems likely that CpG was eliciting a three-way cross-talk among liver cells: the activation of Kupffer cells resulting in IL-12 and TNF-α; the activation of either NK cells or T cells since these are the main sources of IFN-γ; and hepatocytes, the cells in which the parasite develops or fails to develop under conditions stimulating TLR2, 3, 4 or particularly TLR9. *Plasmodium berghei* infection induces IL-12 through MyD88-dependent pathways, but not TLR2, 4, and 6 [36]. This secretion of IL-12 induces hepatocyte killing through hepatic CD1d-independent DX5+ T cells through a perforin-dependent mechanism [37]. Hepatic *Listeria monocytogenes* infection induces IL-12 and IL-18 production in Kupffer cells through TLR/MyD88 signaling, which stimulates NK cells to produce IFN-γ, and this is critical for eradication of *Listeria* organisms from the host [38, 39].

The evolutionary history of TLRs, and their role in directly binding to PAMPs, may suggest that the primary TLR function is to initiate the innate immune response to pathogens and to condition accessory cells in ways that promote the induction of adaptive immunity. However, TLR activation can also be a mechanism through which responses are sustained. One example comes from the response to an

Adenovirus vector, in which both the innate and adaptive immune response depend on TLR2 and TLR9 [40]. The key point is that Adenovirus was able to activate signaling through ERK1/2 in Kupffer cells, but that such activation was independent of TLR2 and MyD88. However, the sustained activation of ERK1/2 required both TLR2 and MyD88. With respect to NF-kB activation, early activation required MyD88 but not TLR2, while sustained activation required both MyD88 and TLR2. Cytokine and chemokine responses also required MyD88 and TLR2. It is not entirely clear how this works, but the most likely model is that the innate immune responses, initiated by direct activation of ERK1/2, release endogenous ligands that signal via a receptor coupled to MyD88, and for full immune activation also act via TLR2. Such ligands have not yet been identified.

A strikingly similar role for TLR signaling in sustaining an innate response was found in a very different experimental model. In poly-microbial septic peritonitis, in the absence of any exogenous viral stimulus, the initial cytokine and chemokine burst was increased in TLR3−/− mice; however this response was more rapidly curtailed, and these mice were protected from the lethal effects of sustained inflammation [41]. The same was found in an ischemic gut injury model. Furthermore, the investigators found that RNA released from apoptotic neutrophils could activate macrophages from wt, but not from TLR3−/− mice, suggesting that a positive feedback loop was acting through neutrophil recruitment and apoptosis, activation of macrophages by released RNA, and macrophage activation leading to sustained inflammation.

Integrating these elements, we arrive at a picture where TLRs have two distinct roles in responses to exogenous infection. First, they may act directly as sensors of PAMPs synthesized by pathogens, and in this role they are likely to initiate the first steps in an immune response. But in addition, TLRs may amplify or sustain an immune response by signaling in response to exogenous molecular products of tissue damage, such as DNA (TLR9), HMGB1 (TLR4), and RNA (TLR3). In the early days of the recognition of the importance in immune activation of diverse signals apart from T and B cell receptor ligation, it is argued that lymphocytes are responding not to "nonself" molecules but to "danger", which can only be construed as a metaphor for tissue injury [42]. Alternatively, it was counter-argued that such "danger" could be better understood as molecular signatures characteristic of pathogens, the signals that were subsequently named PAMPs [43]. Now, through an analysis of the diverse roles of TLRs in infectious disease in the liver and elsewhere, we can see that both positions were correct. What is more, "danger" and PAMPs activate and sustain inflammation through the same family of receptors.

5. TLR Signals Are Transmitted via Cell-Cell Crosstalk

Cross-talk is when a TLR acts on one cell but the biological effect is transmitted to a different cell type. Few examples of TLR-driven cross-talk have been explored in the liver, but the examples that exist implicate several major cell types in interactions of this kind.

We have studied the impact of TLR signaling in human Kupffer cells, obtained from fresh human liver lobes sampled during the process of living donor liver transplantation. This procedure yields intrahepatic leukocytes that are rich in human KC and human liver NK cells, and these were used in cross-talk experiments *ex vivo*. Engagement of TLR3 resulted in powerful NK cell activation in KC-NK cell cocultures, but not when the two cells were separated in a Transwell tissue culture system [5]. Stimulation via TLR2 or TLR4 resulted in less dramatic NK cell activation, but the full activation of NK cells was restored by blocking IL-10. Thus the TLR ligands that activated the MyD88 signaling pathway induce IL-10 along with proinflammatory cytokines, and inhibit NK cells; the TLR3 ligand, working only via the TRIF pathway, does not induce IL-10. Conversely, the main NK cell-activating signal is IL-18, synthesized in response to all three TLR ligands [5]. Thus, Kupffer cells are integrating signals from different TLRs, and integrating them to modulate NK cell activity via cross-talk.

While these experiments in human cells emphasize the effects of IL-18 on NK cells, we have also explored IL-18 action in murine NK-T cells. In these cells, also, it is a powerful activating factor [44]. Hepatitis C virus-encoded core protein is able to interfere with TLR-mediated activation of human Kupffer cells. Specifically, core binds to TLR2, and this can induce diverse proinflammatory cytokines [45, 46], but also IL-10. We also show that HCV core acting on human Kupffer cells can suppress other antiviral mechanisms, including type 1 IFN and the upregulation of TRAIL [24]. Thus, viruses can exploit TLRs to disable immunity.

One important negative regulatory effect of cross-talk is the recently discovered action of LSEC in suppressing the transdifferentiation of HSCs via nitric oxide [24]. The key point is that only resting LSECs have this effect, since endothelial cells harvested from a liver undergoing capillarization do not suppress HSC trans-differentiation. The LSECs are influenced in their capacity to make nitric oxide by VEGF, which therefore acts as an antifibrotic factor. While there is no evidence relating to the influence of TLRs in NO synthesis by LSECs, there is a strongly analogous case. In neurovascular endothelium, NO synthesis was induced by *Neisseria meningitidis,* and this effect was blocked by antibodies to TLR2 and TLR4—but not to TLR9 [47]. Extrapolating to the liver, where LSECs are constitutively exposed to LPS concentration ranging from 100 pg/mL to 1 ng/mL, it seems likely that such low level TLR4 engagement supports NO synthesis and maintains HSCs in their resting state.

TLR4 signaling enhances TGF-β1 in HSCs by downregulating the TGF-β1 pseudo-receptor Bambi, thus stimulating fibrosis. Quiescent hepatic stellate cells are the predominant targets through which TLR4 ligands—but not TLR2 ligands—are required to promote fibrogenesis. In quiescent HSCs, TLR4 activation upregulates chemokine secretions induces chemotaxis of KCs, and allows for unrestricted activation by KCs [28].

We can therefore see signals that are initiated by TLRs participating in cross-talk between KCs and NK cells, and between KCs and HSCs, and we can envisage mechanisms

through with TLRs modulate cross-talk between KCs and NK-T cells, and between LSECs and HSCs. Since the expression of TLRs is almost universal among liver cell subsets, the documentation of such TLR-driven cell-cell interactions is sure to increase.

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Review Article

SOCS1, a Negative Regulator of Cytokine Signals and TLR Responses, in Human Liver Diseases

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Toll-like receptor (TLR) signaling pathways are strictly coordinated by several mechanisms to regulate adequate innate immune responses. Recent lines of evidence indicate that the suppressor of cytokine signaling (SOCS) family proteins, originally identified as negative-feedback regulators in cytokine signaling, are involved in the regulation of TLR-mediated immune responses. SOCS1, a member of SOCS family, is strongly induced upon TLR stimulation. Cells lacking SOCS1 are hyperresponsive to TLR stimulation. Thus, SOCS1 is an important regulator for both cytokine and TLR-induced responses. As an immune organ, the liver contains various types of immune cells such as T cells, NK cells, NKT cells, and Kupffer cells and is continuously challenged with gut-derived bacterial and dietary antigens. SOCS1 may be implicated in pathophysiology of the liver. The studies using SOCS1-deficient mice revealed that endogenous SOCS1 is critical for the prevention of liver diseases such as hepatitis, cirrhosis, and cancers. Recent studies on humans suggest that SOCS1 is involved in the development of various liver disorders in humans. Thus, SOCS1 and other SOCS proteins are potential targets for the therapy of human liver diseases.

1. Introduction

Proper and coordinated activation of immune signal pathways is required for immune responses, including eradication of invading pathogens. Toll-like receptor (TLR)- and cytokine receptor-mediated signaling are involved in innate and subsequent adoptive immunity. Aberrant and/or sustained activation of immune signal pathways may result in serious disorders such as septic shock, autoimmunity, and cancer. Thus, immune signals must be tightly regulated for preventing overactivated immune responses. A number of regulatory mechanisms on immune signaling pathways have been reported. A family named suppressor of cytokine signaling (SOCS) represents a negative regulator for various cytokine signaling (Table 1) [1]. SOCS proteins play important roles in maintaining organ homeostasis by preventing the harmful cytokine responses in various organs [2]. In this paper, we will focus on SOCS1, a member of SOCS family, which plays a key role in the negative regulation of both cytokine receptor- and TLR-mediated signaling. We will further discuss the importance of SOCS1 in the pathogenesis of liver diseases.

2. Regulation of Immune Signal Pathways by Suppressor of Cytokine Signaling (SOCS)

2.1. Inhibition of Cytokine Signaling by SOCS. Cytokine receptor-mediated signaling critically regulates cellular functions including proliferation, differentiation, and survival. SOCS proteins are originally discovered as cytokine-induced proteins that negatively regulate cytokine receptor signaling (Table 1) [1]. This regulation by SOCS proteins prevents the harmful overactions mediated by cytokine signaling. The physiological roles of SOCS proteins have been extensively investigated by the studies using knockout animals [1, 2].

In mammals, eight members of SOCS proteins (SOCS1 to SOCS7 and CIS) have been reported [1]. These proteins consist of two conserved motifs, a central SH2 domain and a C-terminal SOCS box (Figure 1) [1]. SOCS1 and SOCS3 possess a kinase-inhibitory region (KIR) domain that is

Table 1: Inducing factors of SOCS family proteins and suppressed signaling by SOCS family proteins (see [1, 3, 4]).

SOCS family	Inducers	Suppressed Signaling*
CIS	IL-2, IL-3, EPO, GM-CSF, GH, Prolactin	IL-2, IL-3, EPO, GH, Prolactin
SOCS-1	IL-2, IL-3, IL-4, IL-6, IL-7, IL-9, IL-10, IL-13, IL-15, IL-21, IFN-α/β, IFN-γ, LIF, TNF-α, TGF-β, EPO, G-CSF, SCF, GH, TSH, Prolactin, CNTF, Cardiotrophin, Insulin, LPS, CpG DNA	**IL-2**, **IL-4**, IL-6, **IL-7, IL-12, IL-15**, **IL-21**, **IFN-α/β**, **IFN-γ**, LIF, **TNF-α**, EPO, TPO, TSLP, SCF, GH, **Prolactin**, **Insulin**, **LPS**, **CpG DNA**
SOCS-2	IL-2, IL-6, EPO, GH, Prolactin, Insulin, CNTF,	IL-6, **GH**, **IGF-I**
SOCS-3	IL-1, IL-2, IL-3, IL-4, IL-6, IL-9, IL-10, IL-11, IL-12, IL-22, IL-23, IL-27, IFN-α/β, IFN-γ, LIF, TNF-α, TGF-β, EPO, TPO, G-CSF, GH, TSH, Prolactin, Leptin, CNTF, Cardiotrophin, EGF, Insulin	IL-1, IL-2, IL-4, **IL-6**, IL-9, IL-11, **IL-27**, IFN-α/β, IFN-γ, **LIF**, OSM, EPO, **G-CSF**, GH, Prolactin, **Leptin**, CNTF, Cardiotrophin, **Insulin**, LPS
SOCS-4	EGF	EGF
SOCS-5	EGF	IL-4, IL-6, EGF
SOCS-6	SCF, Insulin	SCF, Insulin
SOCS-7	Insulin, IGF-1	**Insulin**

* Signaling shown in red is demonstrated in the studies using knockout mice.

critical for inhibition of kinase activity [1]. The SH2 domain of SOCS proteins is a crucial component for association of SOCS proteins with phosphorylated tyrosine residues on tyrosine kinases or cytokine receptors [1]. This association inhibits cytokine signaling by suppressing kinase activity or by masking docking sites for adaptor molecules on the receptors. In addition, the SOCS box recruits a complex containing elongin B, elongin C, cullin-5, RING-box-2, and E2 ligase via its subdomains, a B/C box and a Cull5 box, and mediates ubiquitination of SOCS-bound proteins for proteasomal degradation [5].

Among SOCS proteins, SOCS1 is a prototype molecule. A number of studies indicate that SOCS1 preferentially binds to JAK kinases via its SH2 domain and inhibits signaling by shutting down JAK kinase activity via the KIR domain as well as by promoting JAK degradation via the SOCS box [2]. Given the role of JAK kinases in signaling of cytokine receptors, mice lacking SOCS1 exhibit hypersensitivity to a variety of cytokines, including IFN-α, IFN-γ, IL-2, IL-4, IL-7, IL-12, and IL-15 (Table 1) [1, 2]. SOCS1 deficient mice are born normally, but die within three weeks of age due to lymphocyte-dependent multiorgan inflammatory disease [1, 2].

2.2. SOCS1 Negatively Regulates TLR Signaling. The expression of SOCS1 is induced by various cytokines, including IL-4 and IFN-γ, and also by TLR ligands, such as LPS and CpG-DNA (Table 1) [3]. TLR ligands induce SOCS1 expression directly through the activation of early growth response-1 (Egr-1) [6] and/or indirectly through cytokines, including IL-6 and IFN-β induced by initial TLR signaling [3]. This finding raises the possibility that SOCS1 regulates TLR signaling. In accordance with this, enforced expression of SOCS1 results in reduced response of cells to TLR ligands. More importantly, SOCS1 deficient mice are hypersensitive not only to cytokines but also to TLR ligands [7, 8]. Upon stimulation with LPS, a TLR4 ligand, or CpG-DNA, a TLR9 ligand, SOCS1 deficient macrophages produce an increased amount of inflammatory cytokines, including TNF-α, IL-6, and IL-12. Furthermore, LPS tolerance, refractoriness to second challenge with LPS after initial LPS exposure, is not induced in SOCS1 deficient mice. Although IFN-γ/STAT1 pathway is the major target for SOCS1, SOCS1 deficient cells lacking IFN-γ or STAT1 still exhibited enhanced response to LPS [7, 8]. This suggests that the hypersensitivity of SOCS1 deficient cells to LPS is largely due to their dysregulated response to TLR signaling, but not IFN signaling secondarily induced by TLR signaling. Thus, SOCS1 directly induces the negative regulation of TLR signaling [3, 5].

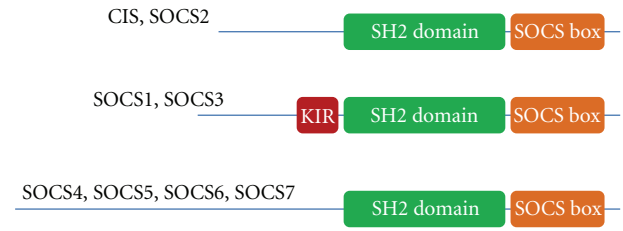

Figure 1: Schematic structure of SOCS proteins. SOCS proteins are structurally characterized by a central SH2 domain, a docking motif to tyrosine-phosphorylated proteins, and a c-terminal SOCS box that recruits Elongin B/C complex. SOCS1 and SOCS3 also possess a KIR domain, which plays an important role in inhibition of JAK kinase activity. The length of N-terminal domain varies between SOCS proteins and SOCS4-7 possesses relatively long N-terminal domain.

3. Mechanisms of Negative Regulation of TLR Signaling by SOCS1

SOCS1-mediated negative regulation of TLR signaling has been reported by a number of studies [3]. Although the major mechanism by which SOCS1 regulates TLR-mediated

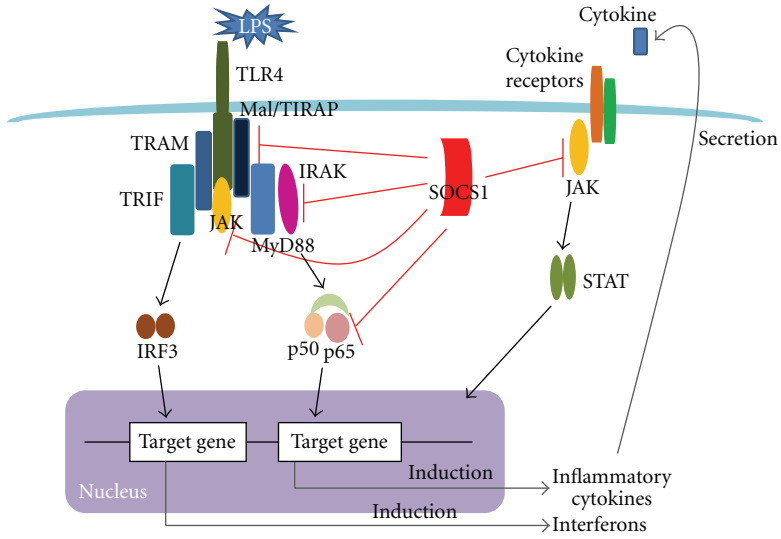

Figure 2: Proposed inhibitory mechanisms of SOCS1 on TLR signaling. The absence of SOCS1 results in heightened response to TLR ligands. Possible mechanisms of action of SOCS1 proposed so far are outlined here. Upon induction, SOCS1 binds to Mal/TIRAP and mediates its degradation via proteasomal pathway (1). SOCS1 binds to IRAK and may modulate its activity (2). SOCS1 binds to p65 subunit of NFκB and targets it for proteasomal degradation (3). SOCS1 inhibits JAK2 activated directly after TLR stimulation (4). SOCS1 regulates TLR-mediated response indirectly by inhibition of TLR-induced cytokines such as IFN-β (5).

responses remains a matter of debate, several mechanisms have been proposed as outlined below (Figure 2).

3.1. Regulation of TLR-Mediated Cytokine Signaling by SOCS1. The activation of TLR signaling results in the induction of inflammatory cytokines such as TNF-α, IL-6, and IFN-α/β. Given the role of SOCS1 in inhibition of cytokine-mediated signaling, one of the most representative functions of SOCS1 is to regulate intracellular signaling activated by cytokines which are induced by initial TLR signaling. Indeed, several reports demonstrated that the signaling of IFN-β induced by the first TLR stimulation is the critical target of SOCS1 [9–11]. In accordance with this, SOCS1 deficient cells are highly sensitive to type I IFNs, and lethal disease in SOCS1 deficient mice is partly mediated by the dysregulation of IFN-α/β signaling [12].

3.2. Association of IRAK with SOCS1. Two independent studies have shown that LPS-induced proinflammatory cytokine production and NF-κB activation are inhibited in cells overexpressing SOCS1 [7, 8]. Among signaling molecules that bridge between TLR and NF-κB, IL-1 receptor-associated kinase 1 (IRAK1) is a target of SOCS1. SOCS1 directly associates with IRAK1 via its SH2 domain [7].

3.3. Regulation of Mal/TIRAP Protein Expression by SOCS1. The TLR adaptor molecule Mal/TIRAP is also a target of SOCS1 [13]. TLR stimulation induces Btk-dependent tyrosine-phosphorylation of Mal and generates a binding site for SH2 domain of SOCS1. Subsequent interaction between SOCS1 and Mal induces ubiquitination and proteasomal degradation of Mal, resulting in abrogation of TLR/Mal-dependent NF-κB activation [13]. A recent report has shown that SOCS1 also regulates the intracellular protein levels of Mal in hepatocytes, which inhibit intracellular uptake of LPS by hepatocytes [14].

3.4. Regulation of NF-κB Activation by SOCS1. SOCS1 also regulates TLR-induced NF-κB activation by direct interaction with p65 subunit of NF-κB upon stimulation with LPS. SOCS1 downregulates p65 protein levels by ubiquitin-mediated degradation of p65 [15].

3.5. Regulation of TLR-Induced JAK2 Activation by SOCS1. Several reports demonstrated that JAK2 in macrophages contributes to the induction of proinflammatory cytokines by LPS stimulation [16, 17]. Since JAK2 is a major target of SOCS1, it is likely that the inhibitory effect of SOCS1 on LPS signaling is partially due to the binding of SOCS1 to JAK2 in TLR4/JAK2 pathway [17].

4. SOCS1 in Liver Pathophysiology

SOCS1 expression is induced in hepatocytes by a variety of exogenous stimulation such as IL-6 and IFN-γ during liver pathobiology. SOCS1 regulates various intracellular signaling pathways in hepatocytes, which may modulate liver pathophysiology (summarized in Table 2).

4.1. Protective Roles of SOCS1 in Hepatitis. SOCS1 deficient mice display a fatal neonatal disease, which is characterized by aberrant activation of T cells and multiple organ injury. Hepatic inflammation accompanied by fatty degeneration and hepatocyte necrosis is the major cause of the death in SOCS1 deficient mice. This fulminant hepatitis in SOCS1 deficient mice is due to exacerbated activation of hepatic

Table 2: Proposed involvement of SOCS1 in liver pathophysiology.

	Mechanism for SOCS1 dysregulation	Pathogenic consequences in liver
Increased SOCS1	(1) Excessive stimulation with cytokines/TLR ligands	Insulin resistance
		Reduced liver regeneration
Reduced SOCS1	(1) CpG methylation	Hepatitis
	(2) Promoter polymorphism?	Liver fibrosis
	(3) Genetic mutation?	Hepatocellular carcinoma

lymphocytes, including NKT cells [18], and increased sensitivity of hepatocytes to inflammatory cytokines such as IFN-γ [19].

SOCS1 expression is also important for suppression of hepatitis in adult mice. Concanavalin A- (ConA-) induced hepatitis is a murine model of T cell-mediated acute hepatitis. In this model, IFN-γ/STAT1 pathway accelerates liver injury, while IL-6/STAT3 ameliorates disease [20]. Both SOCS1 and SOCS3 are induced in the liver after ConA injection [20] and provide reciprocal regulation in IFN-γ/STAT1 and IL-6/STAT3 pathway [21]. Compared to wild type mice, mice with specific deletion of SOCS1 in hepatocytes (hepatocyte-specific SOCS1 conditional KO mice) exhibit severe ConA-induced hepatitis with increased mortality [22]. Conversely, SOCS3 conditional KO mice exhibit reduced liver injury [23]. Collectively, SOCS1 expression in the liver prevents fatal hepatitis via the suppression of exacerbated liver inflammation.

4.2. Protective Roles of SOCS1 in Liver Fibrosis and Carcinoma. Dimethylnitrosamin (DMN) treatment induces liver fibrosis in mice. SOCS1 heterozygous mice exhibit enhanced liver damage, severe liver fibrosis, and increased mortality after the treatment with DMN, compared to wild type mice. After treatment with diethylnitrosamin (DEN), a chemical agent that induces hepatocellular carcinoma (HCC), SOCS1 heterozygous mice developed more tumors than wild type mice [24]. These findings suggest that endogenous SOCS1 prevents liver fibrosis and hepatocarcinogenesis.

4.3. SOCS Proteins Regulate Liver Regeneration. MyD88-dependent innate immune signaling induces inflammatory cytokines, including TNF-α and IL-6, to initiate liver regeneration after partial hepatectomy (PH) [25, 26]. SOCS genes are induced through MyD88 during liver regeneration [26, 27]. Produced IL-6 during liver regeneration activates STAT3, which is an important component to induce hepatocyte proliferation as well as a crucial target of SOCS1 and SOCS3 in hepatocytes. Enforced SOCS1 or SOCS3 expression in hepatocytes inhibits STAT3 activation and hepatocyte proliferation induced by IL-6, hepatocyte growth factor (HGF), and epidermal growth factor (EGF), which results in the suppression of liver regeneration [28, 29]. Mice lacking SOCS3 in hepatocytes exhibit enhanced STAT3 activation, hepatocyte proliferation, and liver weight restoration after PH [30]. Thus, SOCS molecules negatively regulate physiological proliferation of hepatocytes after PH.

4.4. Role of SOCS Proteins in Metabolic Syndrome. Proinflammatory cytokines are key factors to develop metabolic syndrome, including insulin resistance. Several lines of evidence suggest that inflammatory cytokines exacerbate insulin resistance via SOCS protein induction in the liver [31]. The expression of SOCS1 and SOCS3 is increased in a murine model of obesity [32]. *In vitro* experiments using cells overexpressing SOCS suggest that members of the SOCS family, such as SOCS1, SOCS3, and SOCS6, inhibit insulin signaling directly by interacting with insulin receptor and/or IRS [31]. *In vivo*, Adenoviral overexpression of SOCS1 or SOCS3 in the liver enhances insulin resistance and fatty acid synthesis [32]. In addition, cells lacking SOCS1, SOCS3, or SOCS7 exhibit increased sensitivity to insulin [33–36]. These results suggest that the induction of SOCS1 and other SOCS proteins in hepatocytes plays an important role in hepatic metabolism and pathogenesis of metabolic syndrome.

5. SOCS1 in Human Liver Disease

Studies using animal models suggest that inadequate induction or impaired expression of SOCS1 may induce liver diseases in humans. Thus far, although inactivating mutations in SOCS1 gene were detected in a subset of lymphoma cells [2], such mutations have not been observed in hepatic disorders. Nevertheless, previous studies have shown that SOCS1 CpG islands in human primary hepatocellular carcinomas (HCCs) are frequently methylated, suggesting that the epigenetic silencing of SOCS1 participates in tumor growth of HCCs [37, 38]. Interestingly, SOCS1 methylation is highly prevalent in HCC with HCV infection but not with HBV infection [39]. In addition, SOCS1 gene methylation is also detected in patients with hepatitis C [24]. These results suggest that endogenous SOCS1 is a tumor suppressor of HCCs and epigenetic modification of SOCS1 expression by HCV infection leads to the progression of hepatic inflammation and the progression of HCCs.

On the other hand, genetic polymorphism on SOCS1 promoter region may impair SOCS1 induction. Indeed, a recent study demonstrated that the SOCS1 polymorphisms in Caucasians are associated with alteration in body mass index [40]. Although it remains unclear whether SOCS1 polymorphisms modify SOCS1 expression in the liver and SOCS1 polymorphisms influence insulin sensitivity and lipid metabolism, SOCS1 polymorphisms might be associated with metabolic syndrome in humans.

6. SOCS3 in TLR Signaling and Human Liver Diseases

Overexpression studies have shown that both SOCS1 and SOCS3 exhibit similar inhibitory functions on various cytokine signaling *in vitro* (Table 1). However, SOCS1 and

SOCS3 are not functionally interchangeable. Interestingly, SOCS3 deficient mice are embryonic lethal, due to placental insufficiency as a result of hypersensitivity to LIF [1]. Additional studies using SOCS3 conditional KO mice revealed that SOCS3 is required for negative regulation of IL-6, leptin, and G-CSF signaling [3]. These results indicate that SOCS3 has nonredundant physiological inhibitory functions in cytokine signaling, including IL-6, LIF, leptin, and G-CSF signaling.

Several reports have shown that SOCS3 regulates TLR signaling. LPS treatment strongly induces SOCS3 expression in macrophages and hepatocytes [41]. In addition, like SOCS1, SOCS3 inhibits TLR-mediated responses [2]. However, the action of SOCS3 in TLR signaling may be complex, because there are conflicting reports describing that SOCS3 has little or even enhancing effect on TLR response [11, 42]. Moreover, mice lacking SOCS3 in myeloid cells are resistant to LPS-induced lethal shock [22]. Nevertheless, it is of interest to elucidate the precise function of SOCS3 in TLR response, since accumulating evidence suggests that SOCS3 is important for the pathology of hepatic diseases.

As described earlier, SOCS3 is induced in the liver during liver regeneration. Hepatocyte proliferation and hepatic weight restoration are enhanced in hepatocyte-specific SOCS3 knockout mice [30]. ConA or DMN treatment induces excessive fibrosis in hepatocyte-specific SOCS3 knockout mice, although hepatic inflammation is ameliorated in these mice [43]. In addition, hepatocyte-specific SOCS3 knockout mice develop more liver tumors than wild type mice after DEN treatment [23, 30]. Stimulation of cytokines such as IL-6 induces constitutive activation of STAT3 in SOCS3 deficient hepatocytes. Given the crucial role of IL-6/STAT3 pathway in HCC development [44, 45], this might be the mechanism of enhanced tumorigenesis in hepatocyte-specific SOCS3 knockout mice. These findings suggest that SOCS3 in hepatocytes regulates hepatocyte homeostasis, including survival and proliferation, which further contributes to the regulation of fibrosis and carcinogenesis in the liver. Notably, SOCS3 in hepatic T cells may have a different role in the regulation of hepatic inflammation. Conditional deletion of SOCS3 in T and NKT cells exacerbates ConA-induced hepatitis [46], while transgenic overexpression of SOCS3 in T and NKT cells [46, 47] or intraperitoneal administration of cell-penetrating SOCS3 in mice [48] prevents ConA-induced liver injury.

In humans, SOCS3 is implicated in HBV- and HCV-induced pathology of the liver. In contrast to the observation in SOCS1, SOCS3 expression is upregulated in the livers of HBV [49] and HCV patients [23, 50]. HBV X protein enhances SOCS3 expression in hepatocytes [51]. HCV core protein induces SOCS3 expression in hepatoma cells [52, 53], while it inhibits SOCS1 expression [54]. Enhanced SOCS3 expression may prevent HCC development, but may induce insulin resistance [53], immune dysfunction [49], and refractoriness to IFN therapy [52]. Interestingly, in the liver of HCV patients with HCC, SOCS3 expression is increased in noncancerous region but remained low in HCC region [23, 30]. This observation suggests that SOCS3 in hepatocytes is somehow silenced during tumorigenesis in HCV patients. Thus, while further studies are required, SOCS3 could be an attractive target for the therapy of human liver diseases.

7. Conclusion

Although the mechanism by which SOCS1 inhibits TLR signaling is not fully understood, accumulating evidence indicates that the absence of SOCS1 strongly enhances TLR responses. Since SOCS1 is induced in the liver by multiple factors including cytokines, TLR ligands, and insulin, SOCS1 should regulate both cytokine and TLR signaling under various physiological and/or pathological conditions. In accordance with this, mice lacking SOCS1 are sensitive to a variety of liver diseases including hepatitis, liver cirrhosis, and HCC. Moreover, silenced SOCS1 expression due to CpG methylation is involved in pathogenesis of hepatitis C, HCC, and many other types of human cancers [2]. It is also possible that impaired SOCS1 expression may underlie the pathogenesis of other human liver diseases induced by infection, autoimmunity, drugs, and alcohol. In contrast, it has been shown that an excessive induction of SOCS1 by TLR ligands and cytokines contributes to the alteration of insulin sensitivity in metabolic syndrome. Future studies on SOCS1 and other SOCS proteins will provide insight into the pathobiology of human liver diseases and develop new strategies for the treatment of acute and chronic liver diseases.

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Review Article

The TLR4/TRIF-Mediated Activation of NLRP3 Inflammasome Underlies Endotoxin-Induced Liver Injury in Mice

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Administration of heat-killed *Propionibacterium acnes* renders mice highly susceptible to LPS. After LPS challenge *P. acnes*-primed mice promptly show hypothermia, hypercoagulation (disseminated intravascular coagulation), elevation of serum proinflammatory cytokine levels, and high mortality. The surviving mice develop liver injury. As previously reported, IL-18 plays a pivotal role in the development of this liver injury. Many cell types including macrophages constitutively store IL-18 as biologically inactive precursor (pro) form. Upon appropriate stimulation exemplified by TLR4 engagement, the cells secrete biologically active IL-18 by cleaving pro-IL-18 with caspase-1. Caspase-1 is also constitutively produced as a zymogen in macrophages. Recently, NLRP3, a cytoplasmic pathogen sensor, has been demonstrated to be involved in the activation of caspase-1. Here, we review the molecular mechanisms for the liver injuries, particularly focusing on the TLR4/NLRP3-mediated caspase-1 activation process, with a brief introduction of the mechanism underlying *P. acnes*-induced sensitization to LPS.

1. Introduction

TLR, topics of this issue, is an extracellular sensor family of pathogen-associated molecular patterns (PAMPs) [1, 2]. As described by Yamamoto et al. in this special issue, TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 are expressed on the cell surface, while TLR3, TLR7, TLR8, and TLR9 are expressed on the membrane of endosome, which is a transport vesicle originated from the cell membrane to trap and transport the extracellular macromolecules into the inside of the cells. Besides, mammalian host possess cytoplasmic sensors consisting of at least two families, RIG-I-like receptor (RLR) and Nod-like receptor (NLR) families [3–5]. After sensing intracellular, virus-derived double-stranded (ds) RNA, RLR members relay a signal to activate inflammatory responses for viral clearance via induction of proinflammatory cytokines and type 1 IFNs [6, 7]. Some of the NLR family members are associated with the cytoplasmic formation and activation of inflammasome. Inflammasome is a multiple protein complex and is regarded as the platform for activation of caspase-1 [8, 9]. Caspase-1 is produced as enzymatically inactive precursors (pro) and requires appropriate cleavage to become active. Macrophages including Kupffer cells constitutively produce procaspase-1 and accomplish caspase-1 activation in the inflammasomes after being stimulated [10–12]. Caspase-1 cleaves biologically inactive pro-IL-1β and pro-IL-18, leading to extracellular release of the corresponding active forms. Many cell types including Kupffer cells produce and store pro-IL-18 in the steady state, while they start to produce pro-IL-1β only after activation with appropriate stimuli [13–15]. Thus, the inflammasomes contribute to the secretion of IL-18 and IL-1β via activation of caspase-1.

Inflammasome is composed of certain member of NLR and procaspase 1 [5, 8, 9] (Figure 1). NLR family members are divided into two groups. One is an NLRP group possessing pyrin domain (PYD), and the other is an NLRC group lacking PYD but possessing caspase recruitment domain (CARD) [19]. NLRP1 (Nalp1), NLRP3 (Nalp3), and NLRC4 (Ipaf) have been demonstrated to nucleate the

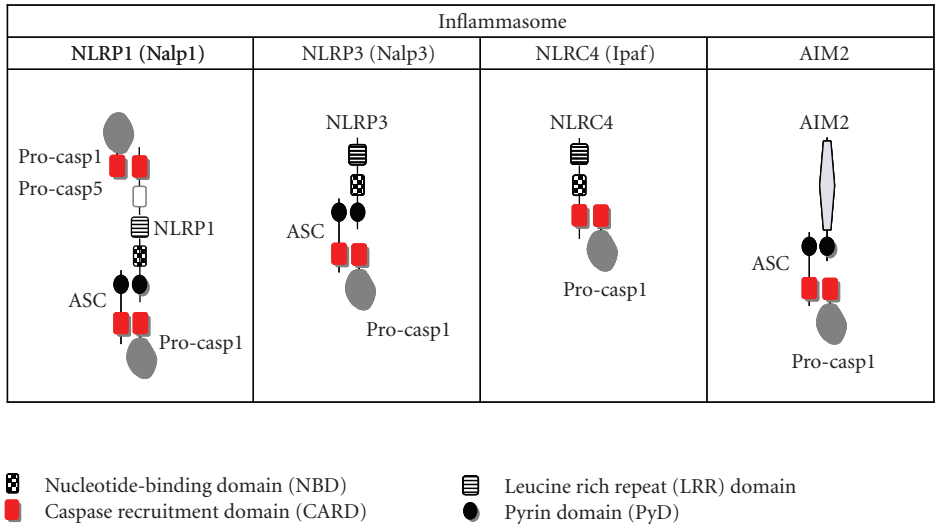

Figure 1: Inflammasome constituents. There have been reported at least four types of inflammasomes, NLRP1 (Nalp1), NLRP3 (Nalp3), NLRC4 (Ipaf), and AIM2 inflammasomes. NLR is subdivided into two groups, PYD-possessing group, namely, NLRP and PYD-lacking group NLRC. After exposure of cells to the corresponding stimuli, these NLRs and AIM2 are believed to be self-oligomerized. As it contains CARD at an N-terminus and PYD at a C-terminus, self-oligomerized NLRP1 can recruit procaspase (casp)-1 and procaspase-5 by action of its CARD and also assembly procaspase-1 with help from ASC that possesses both PYD and CARD. Self-oligomerized NLRP3 recruits procaspase-1 by interposing ASC between them. In contrast, self-oligomerized NLRC4 recruits procaspase-1 by directly interacting CARD of procaspase-1 with its CARD. AIM2, belonging a different protein family, is also believed to be self-oligomerized after recognition of double-stranded DNA and recruits procaspase-1 with help from ASC. NLR, Nod-like receptor; CARD, caspase recruitment domain; PYD, pyrin domain.

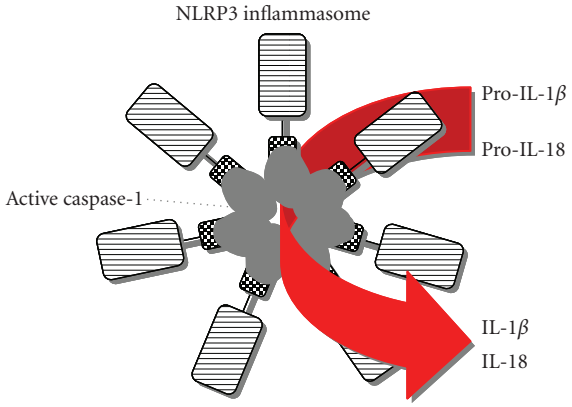

Figure 2: A possible schema for IL-18/IL-1β processing by the inflammasome. After appropriate stimulation, the NLRP3 inflammasome is activated to induce active caspase-1, which eventually results in conversion of pro-IL-18/pro-IL-1β into biologically active IL-18/IL-1β.

inflammasomes [5, 19]. In the inflammasome these NLRs are believed to sense cytoplasmic PAMPs exemplified by LPS presumably via their leucine rich repeat (LRR) domain. LRR domain of NLRP3 can recognize all of the TLR agonists except for TLR5 agonist, flagellin of bacterial flagellum, while that of NLRC4 senses flagellin [20]. After being stimulated the NLRs are self-oligomerized by binding each other using their nucleotide-binding domain (NBD). Self-oligomerized NLRP1 directly recruits procaspase-1 by homophilic protein-protein interaction between its N-terminal CARD and CARD of procaspase1 and/or procaspase-5 [8]. ASC consisting of PYD and CARD is regarded as an adapter protein for caspase-1 activation. The NLRP1 can bind to PYD of ASC by its PYD domain at C-terminus, and CARD of ASC eventually recruits procaspase-1 by CARD-CARD interaction (Figure 1). The same scenario can be sketched for the recruitment of procaspase-1 around the oligomerized NLRP3-ASC complexes (Figure 1). NLRC4 has CARD but not PYD. Upon appropriate stimulation of NLRC4, procaspase-1 is recruited onto NLRC4 directly by CARD-CARD interaction (Figure 1). Recently, AIM2, belonging to a different protein family namely PYHIN, was reported to activate caspase-1 by sensing cytoplasmic ds-DNA [7, 21–25]. After recognition of ds-DNA by HIN 200 domain of it, AIM2 might be self-oligomerized for recruitment of procaspase-1 by similarly interposing ASC between these two proteins (Figure 1). Recruitment of procaspase-1 into these inflammasomes is likely to activate caspase-1, leading to conversion from pro-IL-18 and pro-IL-1β into active IL-18 and IL-1β [26] (Figure 2).

As previously reported, mice having received heat-killed *Propionibacterium acnes* are highly susceptible to LPS [27–30]. *P. acnes*-primed mice suffer from liver injuries after LPS challenge. However, administration of neutralizing anti-IL-18 antibodies (Abs) just before LPS challenge can prevent *P. acnes*-primed mice from the liver injury [17]. Besides, *Il18*$^{-/-}$ mice are resistant to the *P. acnes*/LPS treatment [18]. Thus, IL-18 is important for the development of liver injuries.

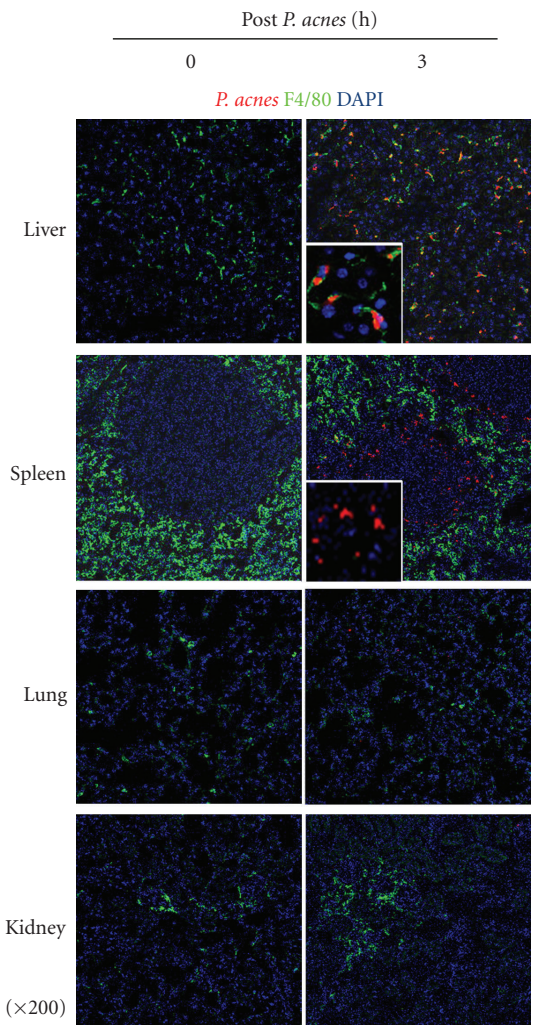

FIGURE 3: Kupffer cells promptly capture heat-killed *P. acnes*. Cy3-labeled, heat-killed *P. acnes* (red) were administered into naïve wild-type mice through a tail vein, and at 3 h tissue specimens were sampled. Frozen tissue slices were incubated with anti-F4/80 mAb (green) and DAPI for detecting macrophages and cell nuclei, respectively. F4/80$^+$ cells (Kupffer cells) capture *P. acnes*, while both F4/80$^+$ and F4/80$^-$ cells ingest them in the spleen. In contrast to live and spleen, both lung and kidney rarely contain *P. acnes*. Magnification of insets is x800.

Here, we review the mechanisms for the *P. acnes*/LPS-induced liver injuries, particularly focusing on those how active IL-18 is released. Prior to addressing this, we would like to introduce the cellular and molecular mechanisms by which pretreatment with *P. acnes* render mice highly susceptible to LPS.

2. Endotoxin Shock Syndrome in *P. acnes*/LPS-Treated Mice

Hypothermia, hypercoagulation (disseminated intravascular coagulation; DIC), high lethality, and tissue injuries are major clinical manifestations of endotoxin shock syndrome [30–35]. After challenge with a subclinical dose of LPS, naïve wild-type (WT) mice do not show these signs (Table 1). In contrast, mice having received heat-killed *P. acnes* 7 days before are highly susceptible to LPS. *P. acnes*-primed mice, but not naïve mice, show obvious and gradual reduction of rectal temperature, serum elevation of proinflammatory cytokines including IL-6, IFN-γ, and TNF-α, high mortality and liver injuries after challenge with the same subclinical dose of LPS [18, 30, 32]. LD_{50} to LPS in *P. acnes*-primed mice is a thousandth or less of that in naïve mice [30]. Furthermore, they exhibit severe hypercoagulation status, which is monitored by plasma levels of coagulation indicator, thrombin antithrombin complexes (TAT), and anti-fibrinolytic protein, plasminogen activator type 1 (PAI-1) that potently inhibits fibrinolysis by blocking conversion from plasminogen into fibrinolytic plasmin [32, 36]. *P. acnes*-primed mice, but not naïve mice, tremendously increase plasma levels of TAT and PAI-1 after LPS challenge [32]. Thus, *P. acnes* treatment powerfully sensitizes mice to LPS.

3. Kupffer Cell Ingestion of Heat-killed *P. acnes*

P. acnes, a Gram-positive bacterium, is often detectable on human skin and has been believed to be relevant to various inflammatory diseases, such as synovitis, acne, pustulosis, hyperostosis, and osteitis (SAPHO) and sarcoidosis [37]. What happens in mice treated with heat-killed *P. acnes*? To address this, we labeled heat-killed *P. acnes* by Cy3, injected them into WT mice through a tail vein, and sampled various tissue specimens at 3 h. We examined tissue distribution of Cy3$^+$ particles by confocal microscopic analyses. Expectedly, heat-killed *P. acnes* are accumulated in the liver and spleen, whereas they were almost absent in the lung and kidney (Figure 3). F4/80$^+$ cells principally capture *P. acnes* in the liver, while both F4/80$^-$ cells and F4/80$^+$ cells ingest them in the spleen (Figure 3).

At day 7 after *P. acnes* treatment tremendous hepatosplenomegaly is observed (Figure 4(a)). The liver doubles its normal weight, whereas weight of spleen achieve more than 5 times (Figure 4(b)). In contrast to the liver and spleen, weight of kidney or lung remains unchanged. In the liver, the dense granulomas primarily consisting of F4/80$^+$ macrophages develop, in the center of which *P. acnes*-ingested F4/80$^+$ Kupffer cells are localized (Figure 4(c)), suggesting that *P. acnes*-ingested F4/80$^+$ Kupffer cells might recruit many F4/80$^+$ macrophages. Immunostaining using rhodamine-conjugated anti-F4/80 mAb followed by counterstaining with hematoxylin reveals that abundant F4/80$^+$ cells are present in the hepatic granulomas [38]. In contrast to the liver, obvious accumulation of F4/80$^+$ cells around *P. acnes* is absent in the spleen (Figure 4(c)). Many dendritic cells were reported to compose the hepatic granulomas as well [39, 40]. *P. acnes* treatment increases hepatic F4/80$^+$ cell number to 30 times and more of that in naïve mice, while the splenic F4/80$^+$ cell number reaches only less than 5 times (Figure 4(d)). Furthermore,

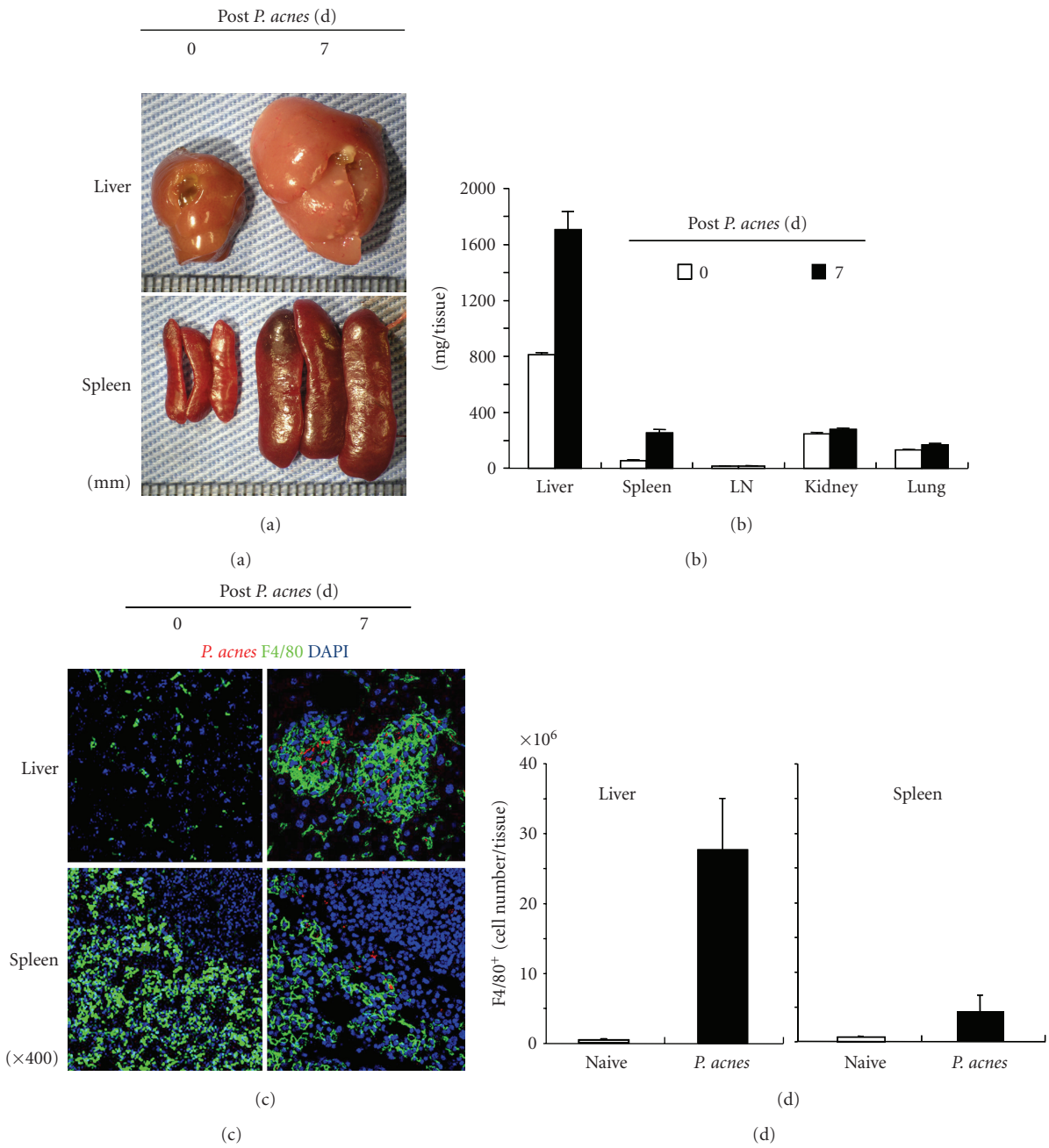

Figure 4: Hepatosplenomegaly and hepatic granulomas in *P. acnes*-primed mice. Wild-type mice received Cy3-labeled, heat-killed *P. acnes*, and at day 7 various tissues were removed and weighed (a, b). The liver slices were stained with anti-F4/80 mAb and DAPI for detecting macrophages and cell nuclei, respectively (c). Kupffer cells and splenocytes were prepared from *P. acnes*-primed (closed) or naïve mice (open). After staining with anti-F4/80 mAb, proportion of $F4/80^+$ cells were determined by FACS, and total $F4/80^+$ cell number was counted.

splenic macrophages from *P. acnes*-primed mice produce much higher levels of proinflammatory cytokines including TNF-α in response to LPS than do those from naïve mice [32]. This is also the case for Kupffer cells. Thus, *P. acnes* treatment induces both numerical increase and qualitative alteration of macrophages in the liver and spleen. This may implicate the importance of macrophages for the accomplishment of the LPS sensitization by *P. acnes* treatment.

4. Requirement of Macrophages for the Sensitization to LPS Induced by *P. acnes* Treatment

Depletion of macrophages rescues *P. acnes*-primed mice from the liver injury and high mortality induced by the subsequent challenge with LPS [38] (Table 1). This clearly demonstrates the indispensability of macrophages for the *P. acnes*-induced sensitization to LPS. Intravenous injection of

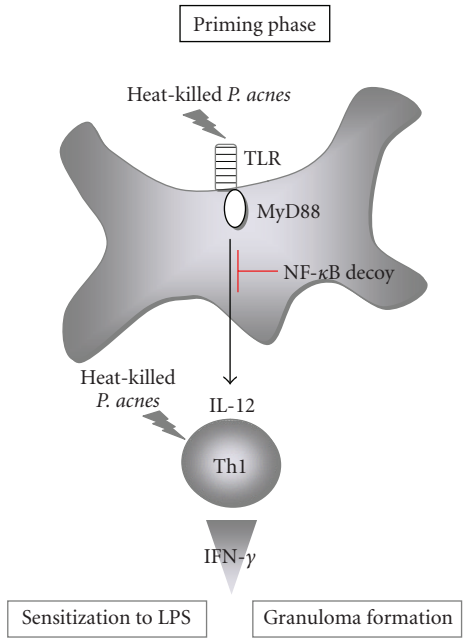

Figure 5: Molecular and cellular mechanisms underlying *P. acnes*-induced sensitization to LPS. After recognition of heat-killed *P. acnes*, cytoplasmic domain of TLR9 recruits MyD88 and relays a signal for nuclear translocation of NF-κB, eventually resulting in various gene expressions including IL-12 production. IL-12 is involved in the development of *P. acnes*-specific Th1 cells, which produce robust IFN-γ in response to *P. acnes*. IL-12 also activates hepatic NK cells to release IFN-γ. IFN-γ derived from Th1 cells and NK cells sensitize mice to LPS and induce their dense hepatic granuloma formation. Administration of NF-κB decoy profoundly inhibits both the LPS sensitization and the hepatic granuloma formations [16].

clodronate liposome depletes macrophages in mice, while control PBS liposome do not affect them [41]. These two groups of mice are treated with *P. acnes*, followed by LPS challenge at day 7. The *P. acnes*-primed mice depleted of macrophages show phenotypes similar to naïve mice after LPS challenge [38]. They lack liver injury and 100% survive (Table 1). *P. acnes*-primed mice receiving PBS liposome, however, show the susceptibility to LPS similar to that in *P. acnes*-primed mice [38]. Thus, macrophages are necessarily required for the *P. acnes*-induced sensitization to LPS.

5. Importance of MyD88-IL-12-IFN-γ Axis for the Sensitization to LPS

It is well established that IFN-γ can potently prime macrophages to efficiently respond to LPS [42]. IFN-γ-primed macrophages produce much larger amounts of TNF-α and IL-6 than naïve cells [32]. Furthermore, Th1 cell differentiation occurs both in the liver and spleen after *P. acnes* treatment in a manner dependent on IL-12, a prototype cytokine for Th1 cell differentiation [32, 43]. Splenocytes and splenic $CD4^+$ T cells from *P. acnes*-primed WT mice produce a large amount of IFN-γ but entirely not IL-4 in response to heat-killed *P. acnes* and immobilized anti-CD3 mAb, respectively [18, 32]. Besides, splenic $CD4^+$ T cells from *P. acnes*-primed $Il12p40^{-/-}$ mice do not differentiate into Th1 cells [18, 44]. Hepatic $CD4^+$ T cells differentiate toward Th1 cells as well, which is totally inhibited by the treatment with neutralizing anti-IL-12 monoclonal antibody (mAb) [45]. IL-12 directly activates hepatic NK cells to produce IFN-γ [46, 47]. Furthermore, hepatic NK cells are numerically increased and acquire the high responsiveness to LPS during *P. acnes* priming phase [48]. From these observations together, one may assume the importance of IL-12-IFN-γ axis for the development of LPS sensitization via induction of Th1 cells. Expectedly, *P. acnes*-primed $Ifn\gamma^{-/-}$ mice, $Il12p40^{-/-}$ mice or mice with inherited unresponsiveness to IL-12 are resistant to LPS, in terms of lack of hypothermia, hypercoagulation or high mortality [32, 49] (Table 2). In addition, neither $Ifn\gamma^{-/-}$ nor $Il12p40^{-/-}$ mice form dense hepatic granulomas after *P. acnes* treatment [18, 50] (Table 2). Thus, IL-12-IFN-γ axis is critical for the LPS sensitization.

As they cannot actively enter into inside of cells, heat-killed *P. acnes* are likely to be recognized by extracellular sensor TLR. As expected, MyD88, which is a key signal adapter molecule of the major TLR signal pathway [2], is essentially required for the development of hepatic granulomas after *P. acnes* priming, strongly suggesting critical role of TLR/MyD88 pathway in the development of *P. acnes*-induced LPS sensitization. $Myd88^{-/-}$ mice lack hepatic granuloma formation after *P. acnes* treatment, and after LPS challenge *P. acnes*-primed $Myd88^{-/-}$ mice do not suffer from the mortality or liver injuries [38, 51] (Table 2, Figure 5). The MyD88-mediated pathway activates nuclear translocation of NF-κB [2]. It is intriguingly to note that administration of NF-κB decoy during *P. acnes* priming phase completely abrogates the hepatic granuloma formation and the sensitization to LPS in WT mice [16]. This strengthens further the importance of the MyD88-mediated pathway for the LPS sensitization. Among TLR members, TLR9 that senses bacterial unmethylated CpG DNA, but not TLR2 that recognize bacterial cell wall product peptidoglycan, was clearly verified to be required for the LPS sensitization by *P. acnes* priming [52, 53]. Indeed, *P. acnes*-primed $Tlr2^{-/-}$ mice are comparably susceptible to LPS as *P. acnes*-primed WT mice, although *P. acnes* possess abundant TLR2 ligands in their cell walls (52). In contrast, *P. acnes*-primed $Tlr9^{-/-}$ mice, like $Myd88^{-/-}$mice, fail to develop hepatic granulomas and become susceptible to LPS [53]. This suggests that unmethylated CpG-DNA of *P. acnes* is pivotal for the sensitization to LPS at least by *P. acnes*-priming. Taken together, these observations strongly suggest that the MyD88-IL-12-IFN-γ axis plays a pivotal role in the hepatic granuloma formation and sensitization to LPS (Figure 5).

Upon challenge with TNF-α instead of LPS, *P. acnes*-primed WT mice show the manifestations/signs similar to those of endotoxin shock syndrome [29, 30, 32], indicating that TNF-α is an effector cytokine and that *P. acnes* treatment tremendously facilitates responsiveness to TNF-α. TNF-α-challenged, *P. acnes*-primed mice, but not naïve mice,

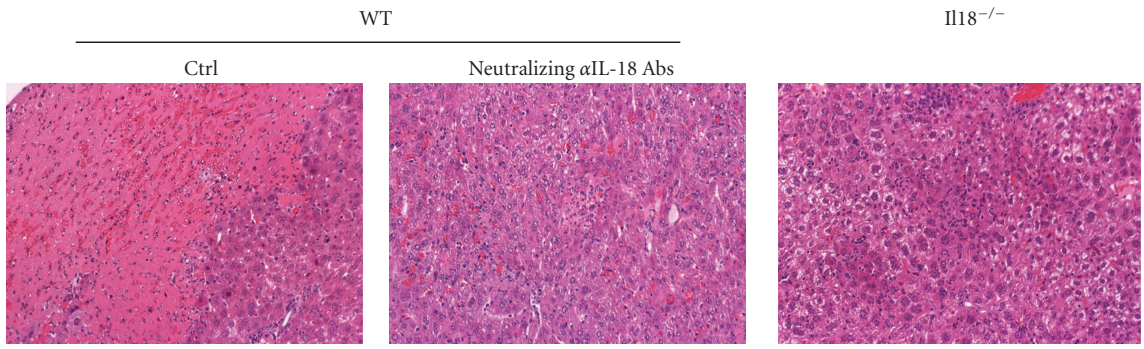

Figure 6: Importance of IL-18 for the development of *P. acnes*/LPS-induced liver injury. *P. acnes*-primed wild-type (WT) showed liver necrosis after LPS challenge. However, treatment with neutralizing anti-IL-18 just before LPS challenge could protect against this liver injuries [17]. Furthermore, *Il*18$^{-/-}$ mice were resistant to the sequential treatment with *P. acnes* and LPS [18].

Table 1: Clinical manifestations upon LPS challenge. Naïve mice, *P. acnes*-primed mice, and *P. acnes*-primed mice depleted of macrophages (Mø) were challenged with LPS, and hypothermia, hypercoagulation, lethality and liver injuries were monitored by measurement of rectal temperature[a], measurement of plasma TAT/PAI-1 levels[b] and histological analyses[d].

Mice		Systemic alterations			Liver injury[d]
		Hypothermia[a]	Hypercoagulation[b]	Lethality (%)[c]	
Naïve		−	±	0	−
P. acnes-primed	Mø-sufficient[e]	+++	+++	100	+++
	Mø-ablated[f]	ND	ND	0	−

[a]+++ indicates more than 5°C reduction of rectal temperature after LPS challenge; − indicates less than 1°C of it.
[b]+++ indicates more than 10 μg/ml of plasma TAT levels; − indicates normal range of them (< 50 ng/ml); ± indicates less than 200 ng/ml.
[c]Mice were monitored for 48 h after LPS challenge.
[d]+++ indicates more than 300 IU of serum ALT levels; − indicates normal range of them (< 50 IU).
[e] *P. acnes*-primed mice were treated twice with PBS liposome.
[f] *P. acnes*-primed mice were treated twice with clodronate liposome to deplete macrophages.
ND; not done

suffer from hypothermia with exceptionally high plasma levels of plasma TAT and PAI-1 and show high mortality. Consistently, *P. acnes*-primed *Ifnγ*$^{-/-}$ mice are resistant to TNF-α as well [32]. Thus, *P. acnes* treatment renders mice highly susceptible to LPS for TNF-α production and also to TNF-α itself via induction of IFN-γ production.

6. Importance of IFN-γ for the Systemic Endotoxin Shock Manifestations after LPS Challenge

Administration of neutralizing anti-IFN-γ mAb just before LPS challenge could partly rescue *P. acnes*-primed mice from hypothermia, hypercoagulation, and high mortality [32], demonstrating the importance of endogenous IFN-γ for the accomplishment of LPS phase as well. Taken together, IFN-γ is a master regulator of the systemic endotoxin shock syndrome by induction of the sensitization to LPS and activation of the LPS phase.

7. IL-18 Is Necessary and Sufficient for the Development of Liver Injuries

Upon LPS challenge many *P. acnes*-primed WT mice shortly died, and the surviving mice suffer from liver injuries later (Figure 6). Blockade of IL-18 or genetic depletion of *Il18* can protect against the liver damages [17, 54] (Figure 6). Upon LPS challenge *P. acnes*-primed *Il*18$^{-/-}$ mice having normally dense hepatic granulomas develop the endotoxin shock syndrome comparably as *P. acnes*-primed WT mice [18]. In contrast, the surviving *Il*18$^{-/-}$ mice evade the liver injuries [18] (Figure 6). These results indicate that IL-18 is necessary for the development of this liver injury. Furthermore, administration of IL-18 causes liver injuries in *P. acnes*-primed WT mice but not naïve mice [55]. Therefore, IL-18 is necessary and sufficient for *P. acnes*/LPS-induced liver injury.

IL-18 is capable of inducing hepatocytotoxic TNF-α directly in many cell types [13]. NK cells and Th1 cells, but not naïve CD4$^+$ T cells, express IL-18R [47, 56]. During *P. acnes* priming phase, naïve CD4$^+$ T cells differentiate into *P. acnes*-specific Th1 cells as described above. Therefore, IL-18 activates both NK cells and *P. acnes*-specific Th1 cells to produce robust IFN-γ, which in turn might fully activate Kupffer cells and hepatic macrophages to further produce TNF-α [54]. In addition, IL-18 has potent capacity to induce and upregulate Fas ligand expression on NK cells enough to kill Fas-expressing hepatocytes [54]. Thus, endogenous IL-18 participates in the liver injuries through induction of proinflammatory cytokines and cell death-inducing protein.

Table 2: Importance of IL-12-IFN-γ axis for in vivo LPS sensitization by *P. acnes* treatment. Mice with various genotypes were sequentially administered with *P. acnes* and LPS. At day 7 after *P. acnes* priming, hepatic granuloma formation was determined by histological analyses.

P. acnes-primed mice	Sensitization phase	LPS phase				
	Granuloma formation[a]	Hypothermia[b]	Hypercoagulation[c]	Serum TNF-α[d]	Lethality[e]	Liver injury[f]
WT	+++	+++	+++	+++	100	+++
$Il12p40^{-/-}$	−	−	−	−	0	−
$Ifn\gamma^{-/-}$	−	−	−	−	0	−

[a]+++ indicates that 20% and more area of the liver section is occupied by granulomas; − indicates no granulomas.
[b]+++ indicates more than 5°C reduction of rectal temperature after LPS challenge; − indicates less than 1°C of it.
[c]+++ indicates more than 10 μg/mL of plasma TAT levels; − indicates normal range of them (< 50 ng/mL); ± indicates < 50 ng/mL and > 200 ng/mL.
[d]+++ indicates more than 5 ng/mL; − indicates less than 0.1 ng/mL.
[e]Mice were monitored for 48 h after LPS challenge.
[f]+++ indicates more than 300 IU of serum ALT levels; − indicates normal range of them (< 50 IU).

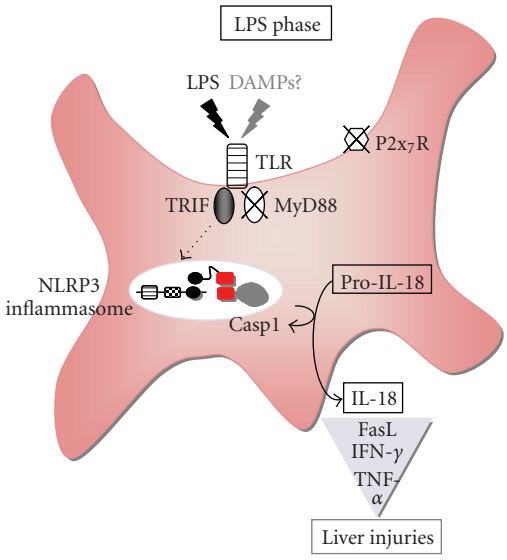

Figure 7: Molecular and cellular mechanisms for LPS phase. After challenge of *P. acnes*-primed wild-type mice with LPS, TLR4 is activated via TRIF for activation of NLRP3 inflammasome, which eventually leads to cleavage of procaspase-1 into its active form caspase-1. Active caspase-1, then, processes pro-IL-18 into active IL-18 for extracellular release. IL-18 upregulates both hepatotoxic Fas ligand (FasL) expression and TNF-α production. DAMPs and alarmin released from the injured hepatocytes might further activate the Kupffer cells, eventually resulting in the acceleration of inflammatory responses. DAMPs, damage-associated molecular patterns.

8. Kupffer Cells Secrete IL-18 in a Manner Dependent on TRIF and the NLRP3 Inflammasome

Many investigators use peritoneal exudate cells (PEC) prepared from the mice administered intraperitoneally with thioglycorate or bone marrow-derived macrophages (BMM) by incubation of bone marrow cells with recombinant monocyte colony-stimulating factor as conventional murine macrophages. These two types of macrophages cannot secrete IL-1β or IL-18 after stimulation with LPS alone. However, LPS-primed PEC or BMM can secrete robust IL-1β and IL-18 upon subsequent stimulation with exogenous ATP in a TLR4- and caspase-1-dependent fashion [57, 58]. From these observations the following possibilities have been believed. First, TLR4-mediated signal pathway cannot activate caspase-1. Second, ATP signaling via its cell surface receptor P2x$_7$R is a central event required for the caspase-1 activation in LPS-stimulated macrophages. Third, the TLR4-mediated signal pathway is only required for induction of proIL-1β production. We also confirmed the absence of IL-1β or IL-18 release from LPS-activated PEC or BMM. In contrast to these PEC and BMM, WT Kupffer cells can release substantial amounts of IL-1β and IL-18 in response to LPS or synthetic lipid A (active center of LPS) alone [15, 38, 54, 59] (Table 3), strongly suggesting involvement of the TLR4 signaling in release of IL-1β and IL-18. As LPS-stimulated caspase-1-deficient Kupffer cells do not secrete IL-1β or IL-18 [11, 12], caspase-1 is an essential processing enzyme of such IL-1β and IL-18. In fact, western blotting analyses reveal active form of caspase-1 in the lipid A-stimulated WT Kupffer cells [38] (Table 3). Expectedly, $Tlr4^{-/-}$ Kupffer cells fail to activate caspase-1 upon stimulation with lipid A [38]. Thus, Kupffer cells seem to be different from PEC or BMM in the ability to activate caspase-1 upon TLR4 engagement. However, it is still to be elucidated how Kupffer cells acquire the potential to activate caspase-1 in response to TLR4 agonists alone.

The TLR4 signaling is relayed by the MyD88- and TRIF-mediated pathways [2]. $Myd88^{-/-}$ Kupffer cells stimulated with TLR4 agonists show normal caspase-1 activation [38]. As the $Myd88^{-/-}$ Kupffer cells cannot produce pro-IL-1β, eventually resulting in lack of mature IL-1β secretion [38] (Table 3). In contrast to proIL-1β, pro-IL-18 is constitutively stored in $Myd88^{-/-}$ Kupffer cells as well as WT cells [15]. Therefore, it is convincing that $Myd88^{-/-}$ Kupffer cells cultured with LPS can secrete IL-18 [15, 38] (Table 3). $Trif^{-/-}$ Kupffer cells show the reverse phenomena. Despite of their normal production of pro-IL-1β and pro-IL-18, $Trif^{-/-}$ Kupffer cells cannot release IL-1β or IL-18 due to their inability to activate caspase-1 [38]. These results demonstrate a pivotal role of TRIF but not

Table 3: Requirement of MyD88 and TRIF for LPS sensitization and Caspase-1 activation, respectively. Mice with various genotypes were sequentially treated with *P. acnes* and LPS, and liver specimens and sera were sampled for histological analyses and measurement of IL-18/IL-1β levels by ELISA, respectively. Kupffer cells were incubated with LPS for 4 h, and each supernatant was collected for western blotting analyses and ELISA. Naïve mice have no hepatic granulomas or injuries, and their serum IL-18 and iL-1β were undetectable. Naïve Kupffer cells released no IL-18 and IL-1β.

Genotype	Sensitization phase	Response to LPS				
		In vivo		LPS-stimulated Kupffer cells		
				Western blotting analyses		ELISA
	Granuloma formation[a]	Liver injury[b]	Serum IL-18/IL-1β[c](ELISA)	ProIL-1β production[d]	Active Casp1[e]	IL-1β/IL-18 release[f]
WT	+++	+++	+++	+++	+++	+++
Tlr4$^{-/-}$	+++	–	–	–	–	–
Myd88$^{-/-}$	–	–	–	–	+++	++[g]
Trif$^{-/-}$	+++	–	–	+++	–	–
Casp1$^{-/-}$	+++	–	–	+++	–	–
Asc$^{-/-}$	+++	–	–	+++	–	–
Nlrp3$^{-/-}$	+++	–	–	ND	ND	–
P2x7r$^{-/-}$	+++	+++	+++	+++	+++	+++

[a]+++ indicates that 20% and more area of the liver section is occupied by granulomas; – indicates no granulomas.
[b]+++ indicates more than 300 IU of serum ALT levels; – indicates normal range of them (< 50 IU).
[c]+++ indicates more than 1000 and 50 pg/mL of serum IL-18 and IL-1β levels, respectively; – indicates normal range of them.
[d]+++ indicates 10 times and more pro-IL-1β density in cell lysates; – indicates the absence of pro-IL-1β.
[e]+++ indicates the presence of active Caspase-1 (Casp1) in supernatant; – indicates the absence of it.
[f]+++ indicates more than 100 pg/mL; – indicates undetectable levels.
[g]++ indicates more than 50 pg/mL of IL-18, but undetectable IL-1β.
ND; not done

MyD88 in the TLR4-mediated caspase-1 activation (Table 3, Figure 7).

Asc$^{-/-}$ Kupffer cells have the phenotype similar to *Caspase*1$^{-/-}$ cells [38, 60], suggesting that NLRP3 or AIM2 inflammasome or unidentified one that needs ASC protein (Figure 1) is involved in the caspase-1 activation. Lipid A-stimulated *Nlrp3*$^{-/-}$ Kupffer cells fail to secrete IL-18 or IL-1β [38]. Therefore, the NLRP3 inflammasome activation is necessary for the TLR4-mediated casapse-1 activation (Table 3, Figure 7).

These results cannot exclude the possibility that the TRIF-mediated pathway might cause extracellular release of ATP and that this self-derived ATP might activate the NLRP3 inflammasome in LPS-stimulated Kupffer cells [57, 58]. Unexpectedly, *P2x7r*$^{-/-}$ Kupffer cells show normal caspase-1 activation and normal release of IL-1β and IL-18 [38]. These results demonstrate the dispensability of endogenous ATP/P2x$_7$R-mediated pathway for the TLR4/TRIF/NLRP3-mediated caspase-1 activation (Table 3, Figure 7).

Although we now know the importance of the NLRP3 inflammasome, the precise mechanisms by which the TRIF-mediated signal pathway activates the NLRP3 inflammasome is unclear. It is also unknown whether NLRP3 protein directly recognizes TLR4 agonists. If so, how do the TLR4 agonists translocate into the inside of Kupffer cells? Alternatively, does TRIF-mediated pathway trigger synthesis of cytoplasmic NLRP3 agonist? If so, what is the NLRP3 agonist? And, how about the molecular mechanisms for the TRIF-induced NLRP3 agonist? We need further extensive studies to address these key queries.

9. Requirement of NLRP3 Inflammasome Activation for the Liver Injury

The capacity to activate caspase-1 reflects on the development of liver injury [38, 60]. Expectedly, *P. acnes*-primed *Tlr4*$^{-/-}$ mice, although manifesting normal levels of hepatic granuloma formation, can avoid the liver injury after LPS challenge accompanied by lack of serum elevation of IL-18 (Table 3, Figure 7). Since they fail to develop hepatic granulomas, *P. acnes*-primed *Myd88*$^{-/-}$ mice lack the production of robust IL-18 after *P. acnes* priming, presumably resulting in escape from the liver injury (Table 3, Figure 7). This demonstrates again requirement of MyD88 for the *P. acnes*-induced LPS sensitization. Conversely, *P. acnes*-primed *Trif*$^{-/-}$ mice, *Caspase*1$^{-/-}$ mice, *Asc*$^{-/-}$ mice and *Nlrp3*$^{-/-}$ mice all have normal dense granulomas in their livers, but fail to develop liver injury after LPS challenge, concomitant with the absence of the serum IL-18 increase (Table 3, Figure 7). *P2x7r*$^{-/-}$ mice have comparable phenotype as WT mice (Table 3, Figure 7), demonstrating dispensability of endogenous ATP/P2x$_7$R pathway for the liver injuries. Thus, the TLR4/TRIF-mediated activation of NLRP3 inflammasome is critical for the development of the *P. acnes*/LPS-induced liver injuries via activation of caspase-1 for maturation and release of IL-18.

10. Methods

10.1. Mice. C57BL/6 mice were purchased from Clea Japan (Osaka, Japan). Female mice (8–12-week-old) were used for this study. Mice were maintained under specific pathogen-free conditions, and received humane care as outlined in the Guide for the Care and Use of Experimental Animals in Hyogo College of Medicine.

10.2. Reagents. Monoclonal antibody (mA) against F4/80 of mouse macrophage was purchased from BMA (Augst, Switzerland). DAPI was from KPL (Gaitherburg, MD).

10.3. Treatment with P. acnes. Heat-killed *P. acnes* were labeled with or without Cy3 (GE, Buchinghamshire, UK) according to the manufacture's instruction and were injected into mice through a tail vein. At the indicated time points various tissues and tissue specimens were sampled for weighing and analysis of the cellularity by confocal microscopy, respectively.

10.4. Confocal Microscopic Analysis. Frozen sections of various tissues were incubated with mAb against F4/80, biotinylated antirat IgG, and then Alexa Fluor 488-conjugated streptavidin (Molecular Probes). Nuclei were stained by DAPI. The immunostaining of each section was evaluated using a laser scanning confocal microscopy [61, 62].

10.5. Flowcytometry [61]. Spleen cells and Kupffer cells were isolated from variously treated WT B6 mice [55]. Cells were incubated with APC-conjugated anti-F4/80 mAb.

11. Closing Remarks

PAMPs evoke innate immune responses by activating pattern recognition receptors (PRRs), such as TLR, NLR, and RLR. Similarly, injured host cells release endogenous "damage"-associated molecular patterns (DAMPs) that induce similar responses via recognition by PRRs [63–65]. For example, high-mobility group box1 protein (HMGB1) that is localized in the nuclei of various cell types in the steady state becomes to be extracellularly released upon stimulation of the cells with death stress. HMGB1, then, initiates innate immune responses via activating TLR4 [66]. Mitochondria are endosymbionts derived from certain bacteria during the evolution of life. Therefore, it is plausible that mitochondria possess DAMPs homologous to its ancestral PAMPs. Very recently, this was verified [67]. Mitochondrion possesses unmethylated CpG-DNA and formyl peptides similar to bacterial N-formylated proteins, which are recognized by PRRs expressed on neutrophils, TLR9, and formyl peptide receptor, respectively. Intravenous injection of the mitochondrial DAMPs causes systemic inflammatory responses and lung injuries [67]. As trauma patients have elevated serum levels of these mitochondrial DAMPs, sterile injury-induced systemic inflammatory response syndrome (SIRS), often occurring after severe trauma, might undergo in response to endogenous mitochondrial DAMPs derived from the injured cells [67]. In addition to DAMPs, self-derived "alarmin" is proposed as another potent inflaming molecules. "Alarmin" is compartmentalized in certain organelle in the steady state. Once damaged, cells begin to actively secrete "alarmin", which in turn triggers inflammatory responses. Intraperitoneal injection of dying cells was reported to be able to trigger peritonitis with dense neutrophil recruitment in an IL-1α/IL-1R-dependent manner [68]. Furthermore, administration of acetoaminophen, a common antipyretic, causes massive liver injury with sterile neutrophilic inflammation in a manner dependent on IL-1α presumably derived from the damaged hepatocytes as well [68]. Thus, dying cell-derived IL-1α is regarded as alarmin. Like IL-1α, IL-33 is localized in the cell nuclei in the steady state and is believed to be secreted after stimulation of the cells with death stress [69]. Histone proteins derived from cell nuclei play a role as alarmin as well [70]. These endogenous DAMPs and alarmin might accelerate liver injuries induced by exogenous PAMPs and might become novel therapeutic targets for severe sepsis with organ failures.

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Review Article

Hepatitis C Virus Evasion from RIG-I-Dependent Hepatic Innate Immunity

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Exposure to hepatitis C virus (HCV) usually results in persistent infection that often develops into chronic liver disease. Interferon-alpha (IFN) treatment comprises the foundation of current approved therapy for chronic HCV infection but is limited in overall efficacy. IFN is a major effector of innate antiviral immunity and is naturally produced in response to viral infection when viral pathogen-associated molecular patterns (PAMPs) are recognized as nonself and are bound by cellular pathogen recognition receptors (PRRs), including Toll-like receptors (TLRs) and the RIG-I-like receptors (RLRs). Within hepatocytes, RIG-I is a major PRR of HCV infection wherein PAMP interactions serve to trigger intracellular signaling cascades in the infected hepatocyte to drive IFN production and the expression of interferon-stimulated genes (ISGs). ISGs function to limit virus replication, modulate the immune system, and to suppress virus spread. However, studies of HCV-host interactions have revealed several mechanisms of innate immune regulation and evasion that feature virus control of PRR signaling and regulation of hepatic innate immune programs that may provide a molecular basis for viral persistence.

1. Introduction

In response to virus infection, signaling pathways within mammalian cells direct a variety of intracellular events that generate an antiviral state directly within the infected cell. This antiviral innate immune response represents our very frontline of immune defense against virus infection. If this response is successful, exposure to the virus will turn into an abortive, self-limiting infection, and the virus will be cleared. It is the hepatic innate immune response that imposes initial antiviral defenses against HCV infection [1]. This response is triggered when the infected cell recognizes a molecular signature or PAMP within the invading virus through the actions of cellular PRRs [2, 3]. The PAMP/PRR interaction initiates signaling cascades that induce the expression of antiviral effector genes [4, 5]. Viral protein and/or nucleic acid products comprise an array of PAMP signatures that can engage specific PRRs, including TLRs or the RLRs, RIG-I, and MDA5 [6–9].

Upon virus infection and PAMP recognition, RLRs and TLRs operate through independent signaling cascades separated in part through the localization of the distinct PRRs. RLRs are cytosolic whereas TLRs are typically localized within endosomes or on the cell surface. Nevertheless, both sets of PRR signaling cascades can lead to the activation of PAMP-driven transcription factors, IFN gene expression and protein secretion, and ISG expression that results in the immediate induction of the intracellular innate immune response both within the infected cell and within bystander cells that respond locally or systemically to the secreted IFN [10]. Interferon regulatory factor- (IRF-) 3 and nuclear factor-kappa B (NF-κB) are the first transcription factors activated in response to HCV infection of hepatocytes (Figure 1(a)). During HCV infection, their activation proceeds through RIG-I signaling, with likely contributions from TLRs, whose signaling pathways promote IRF-3 and NF-κB nuclear translocation and transactivation functions. Other IRF family members, including IRF-1, IRF-5, and IRF-7, contribute to innate immune responses and should be

considered important for immunity against HCV infection [11, 12].

2. Toll-Like Receptors Mediate Endosomal PAMP Recognition of HCV

Toll was first identified as a transmembrane receptor regulating insect morphogenesis [13]. Toll mutation also results in increased susceptibility to fungi in Drosophila [14]. Ten members of human Toll-like receptors (TLRs) were later identified as sensing receptors of various pathogen-associated molecular patterns (PAMPs). Human TLRs are expressed in a tissue-specific manner, and many are expressed in dendritic cells (DCs) and macrophages [15]. Although each TLR detects a distinct set of PAMPs, a common extracellular leucine-rich repeat (LRR) motif is responsible for PAMP sensing. When the LRR engages a PAMP, the TLR transmits a signal through the cytoplasmic domain of the receptor to drive a signaling cascade that results in the production of various cytokines that serve to define the innate immune response and initiate immune cell recruitment.

This signaling drives macrophages and DCs to differentiate into full-blown antigen-presenting cells to initiate antigen-specific adaptive immunity. In humans, at least three major TLRs are important in virus infection and immunity: TLR3 [16–18], TLR7 [19–24], and TLR9 [25, 26], which are typically expressed within endosomes. Double-stranded RNA (dsRNA) PAMPs are detected by TLR3 whereas TLR7 recognizes a specific uridine-rich ribonucleotide motif within a single-strand RNA [27], and TLR9 recognizes DNA PAMP motifs encoding CpG nucleotides [28, 29]. It has also been reported that TLR2 and TLR4 are also involved in inflammation responses during HCV infection [30–32]. Thus, TLR3 and TLR7 specifically recognize PAMPs that accumulate during RNA virus infection, and each has been shown to be important for innate immunity against HCV infection either directly in hepatocytes (TLR3) or indirectly through PAMP signaling of antigen-presenting cells (TLR3 and TLR7) that accumulate HCV products through their phagocytic activity [15, 33, 34].

3. RLRs Mediate Cytosolic RNA PAMP Recognition

Retinoic acid-inducible gene-I (RIG-I) is the prototypical member of the RLR family, which also includes melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). The RLRs have a C-terminal RNA helicase domain with RNA-binding activity [35]. RIG-I and MDA5 contain N-terminal tandem caspase activation and recruitment domains (CARDs), but these are not present within LGP2 (Figure 1(b)). Both RIG-I and LGP2 are regulated by a C-terminal repressor domain, which remains unidentified in MDA5 [36, 37] (Figure 1(b)). Recent studies have revealed that RIG-I and MDA5 detect different RNA viruses, with RIG-I being essential for detection of HCV [9, 38]. The mechanism of PAMP recognition by RIG-I has been best characterized among the RLRs (presented below). The nature of MDA5 PAMP ligands was recently defined as long-stable double-stranded (ds) RNA that are distinct from RIG-I ligands [39].

4. The Role of RIG-I in Nonself Recognition and Antiviral Innate Immunity

Early studies previously defined an association between retinoic acid and ISG expression wherein it was reported that the expression of certain ISGs could be in part induced by retinoic acid [40, 41]. Indeed, in addition to its expression being induced by retinoic acid, RIG-I is an ISG and its PRR function may serve to connect IFN and retinoic acid signaling events that modulate antiviral immunity. RIG-I is the best characterized of the RLRs and was implicated as a PRR through functional cDNA screening that identified human RIG-I as positive regulator of ISG expression [42] and as a PRR of HCV [43]. Structure-function studies have identified the RIG-I CARDs as the signaling domain, which interacts with a downstream molecule, IPS-1, to relay signaling to IRF-3 and NF-κB. Indeed, overexpression of the tandem CARD alone is sufficient to activate downstream signaling and subsequent type I IFN production [35], and the tandem CARD domains are necessary for RIG-I function [36]. In an interferon-cured HCV replicon cell, Huh 7.5, the amino acid T55 within the first CARD domain was found to be mutated to an isoleucine, which associated with loss of innate immune induction by RNA virus infection and increased permissiveness to HCV RNA replication compared to RIG-I wild-type parental cells [43]. This mutation in RIG-I (T55I) has recently been shown to abrogate RIG-I interaction with the E3 ubiquitin ligase, TRIM25, to ablate TRIM25-mediated RIG-I ubiquitination that otherwise serves to enhance RIG-I signaling activation and interaction with the IPS-1 adaptor protein [44].

Understanding the nature of RIG-I ligand/PAMP RNA continues to be a major focus of the innate immunity research field. Our current understanding of RIG-I ligand biology centers on the role of exposed 5′ triphosphate (5′ppp) as the central feature of a nonself PAMP ligand of RIG-I. Various studies have shown that RIG-I can bind single-stranded (ss) or double-stranded (ds) RNA but in each case PAMP recognition is dependent upon the RNA harboring a 5′ppp. For dsRNA, RIG-I preferentially recognizes RNA longer than 25-base pairs with an ssRNA overhang region containing a 5′-triphosphate (5′ppp) motif [35, 38]. While RIG-I does not bind to DNA, it selectively binds to poly(rI:rC) and poly(rA:rU) dsRNA, and poly-U/UC ssRNA, the later identified as a PAMP motif within the HCV genome [42, 43]. These studies showed that cytoplasmic ssRNA containing a 5′ triphosphate and uridine- or adenosine-rich viral RNA motifs of a variety of viruses are well recognized by RIG-I, and that PAMP RNA binding and innate immune signaling are governed by the C-terminus repressor domain (RD) of RIG-I [45].

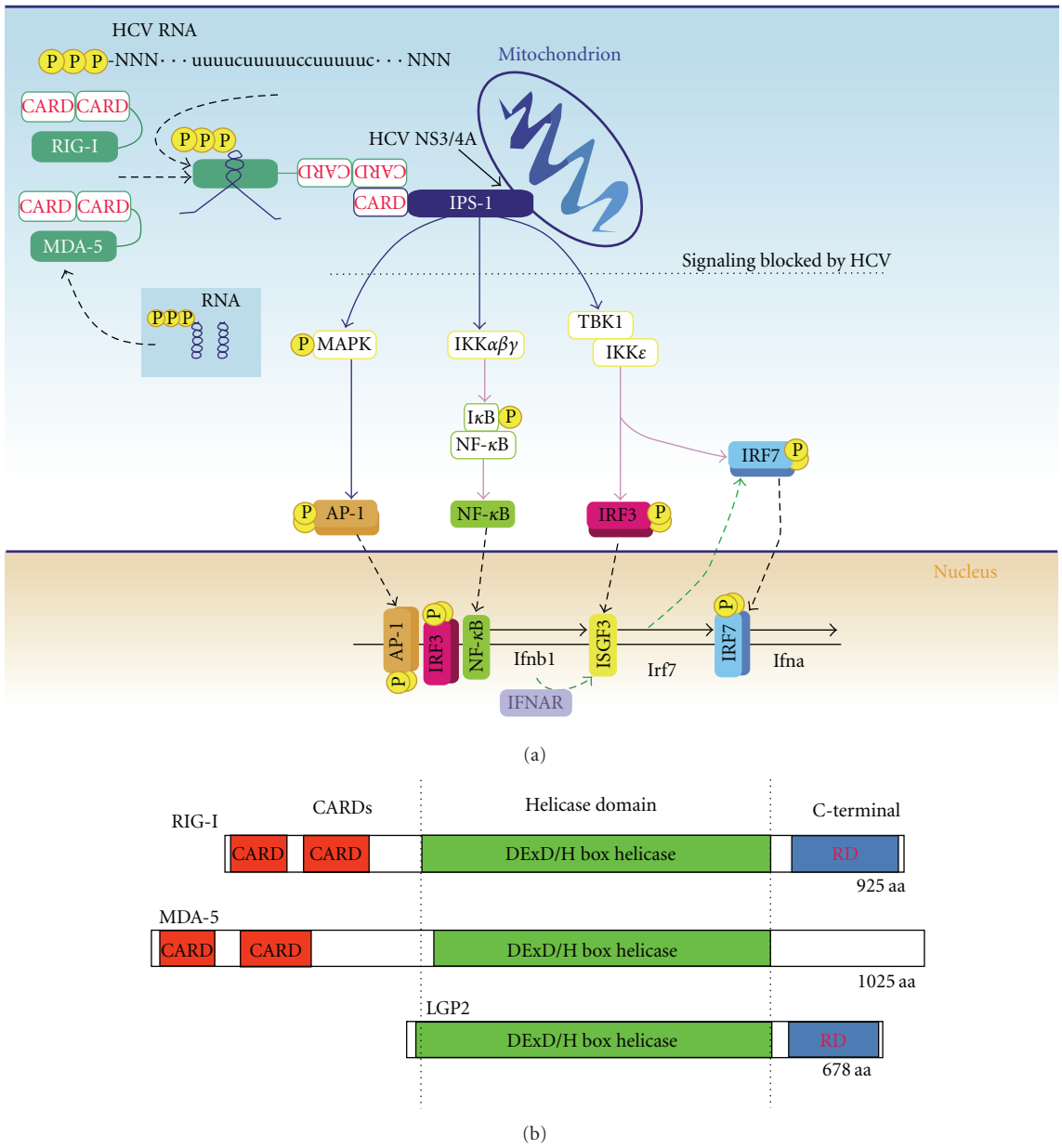

FIGURE 1: (a) The innate immune induction pathway through RLR activation. HCV 5′ppp RNA with poly-U/UC motif is shown as the RIG-I ligand RNA; 5′ppp dsRNA is depicted beneath MDA5 and could serve as either RIG-I or MDA5 ligand RNA. The site of NS3/4A targeting of IPS-1 within the RLR pathway during HCV infection is indicated by the arrow, and the resulting signaling blockade is indicated by the broken line. (b) The RLR family members. CARD, helicase, and C-terminal domains, including the repressor domain (RD), are indicated. Numbers refer to amino acid (aa) length.

How are endogenous "self" and viral "nonself" RNA species actually discriminated by RIG-I? As shown in Figure 2, host RNA synthesis occurs in the nucleus. Unprocessed cellular RNA transcripts contain 5′-triphosphate. However, the 5′-triphosphate is modified or processed before the transcripts arrive to the cytoplasm; the mRNA acquires a 7-methylguanosine CAP structure at its 5′ end; tRNA undergoes 5′ cleavage and a series of nucleotide base modifications; ribosomal RNA, which does possess 5′ppp, is readily complexed with ribosomal proteins to form ribonucleoprotein that masks the 5′ppp from RIG-I recognition. Indeed, 5′-OH or a 5′-methylguanosine capped RNA do not bind to RIG-I and therefore cannot promote the conformational change required for RIG-I activation [36, 45–48]. Endogenous, self RNA species thus avoid detection by RIG-I by the presence of a 5′ cap, specific RNA processing, or compartmentalization as an RNP. Viral RNA, however, either freshly introduced by infection or produced during viral replication, contains the essential nonself marker 5′-triphosphate paired with other nonself motifs such as dsRNA or ssRNA poly-U/UC

PAMP motifs. Recently, it is reported that cytosolic poly(dA-dT) DNA motifs are converted into 5′-triphosphate RNA by RNA polymerase III within the host cell cytosol and can thus induce innate immune programs through nonself recognition of the RNA product by RIG-I [49, 50]. This pathway may be important in the sensing of Epstein-Barr virus-encoded small RNAs, which are transcribed by RNA polymerase III. These findings suggest that viral and possibly even certain undefined cellular pol-III transcripts should be considered as possible PAMP RNA ligands of RIG-I.

The overall structural features of the RLRs define each as a DEx/D box RNA helicase. In terms of RIG-I, the helicase domain and C-terminal RD mediate nonself RNA recognition and binding of viral PAMP RNA [42]. RIG-I activation is dependent on PAMP RNA binding and the actions of the RD such that ectopic expression of full-length RIG-I will not render signaling of ISG expression unless the RD engages a specific RNA PAMP. Functional analyses revealed that RIG-I is maintained in an autorepressed state through the RD mediating intermolecular inhibitory interactions with the CARD and helicase domains [36], and that signaling activation occurs upon PAMP RNA binding that repositions the RD and CARDs into a signaling-ON state [36, 51]. RD repositioning is dependent on ATP hydrolysis activity of RIG-I, which also serves to drive RIG-I translocation along a bound RNA to survey for PAMP motifs [52]. By this model, RIG-I signaling activation proceeds once RIG-I has engaged a PAMP motif within a bound RNA to thus confer RD repositioning and release of signaling autorepression. An important feature of this model is that RIG-I would be constantly binding and translocating along an RNA until it encounters a PAMP motif defining specific ligands as nonself or until it releases (self) RNA lacking PAMP features. Thus RIG-I and the RLRs in general may survey the cytosolic environment for nonself, PAMP RNA. In this sense, RIG-I is a constant sentinel poised to rapidly detect HCV infection within the hepatocyte.

5. Triggering the Innate Immune Response to HCV Infection

As noted above, the nature of the host cell PRR that serves to detect HCV RNA as nonself and to trigger the innate immune response to HCV infection was revealed through studies of the Huh7-derived cell line, termed Huh7.5. This cell line does not exhibit an intracellular innate immune response to RNA virus infection and was found to be highly permissive to supporting HCV RNA replication [43, 53]. cDNA complementation studies identified RIG-I as a PRR for HCV RNA wherein RIG-I was first thought to bind to HCV dsRNA motifs located within the viral genome 3′ nontranslated region (NTR). These studies revealed that RIG-I was essential for triggering the activation of IRF-3 and NF-κB in response to RNA virus infection in hepatocyte-derived cells, resulting in IFN-β expression and onset of the intracellular innate immune response [43]. Furthermore, in cultured cells the HCV NTRs present dsRNA PAMP structures that may serve as potent agonists of TLR3 signaling [33], though this has not been formally proven *in vivo*. Together, these observations suggest that during HCV infection various RNA motifs are recognized and engaged by RIG-I and possibly TLR3 to trigger antiviral defenses [43].

The ability of HCV RNA to trigger innate immune signaling in hepatoma cells and in the liver within *in vivo* mouse model was evaluated using molecular approaches to define the specific PAMP motifs responsible for immune triggering. The outcome of these studies revealed that RIG-I was essential for innate immune signaling in hepatocytes and for hepatic innate immunity triggered by HCV RNA *in vivo*. The 3′ NTR of the HCV genome was identified as the primary HCV PAMP region that activates RIG-I signaling [54]. Importantly, this region is critical for HCV replication [55–58] and consists of three parts: a variable region containing two stem loops, a poly-U/UC-rich region that is single stranded and of variable length from 30 to more than 100 nt, and a conserved "X" region, which contains three stem loops (Figure 3). These components of the HCV 3′ NTR are present in all viral genotypes. It was expected that dsRNA or RNA with secondary structure located within the HCV RNA 3′ NTR would be the primary HCV PAMP for RIG-I. However, neither the highly structured X region nor the variable region dsRNA motifs of the HCV genome can activate RIG-I signaling. Remarkably, the poly-U/UC region, in conjunction with the essential 5′ppp, were identified as the HCV PAMP that serves to define the HCV RNA as nonself through recognition by RIG-I [54]. Importantly, while the 5′ppp was found to be absolutely necessary for RIG-I recognition of HCV RNA, it was not sufficient but specifically required the second nonself element, the poly-U/UC domain or its replication intermediate (the poly-A/AG domain of the negative-strand RNA), for stable binding to RIG-I that drives the conformation change of the RD and innate immune signaling activation [46]. Thus, nonself recognition of RNA as a PAMP likely requires multiple motifs of recognition by RIG-I that marks an RNA substrate as nonself or as a PAMP ligand to specifically stimulate innate immunity.

RIG-I signaling activation upon PAMP engagement results in RIG-I interaction with the downstream adaptor protein, IFN-beta promoter stimulator 1 (IPS-1). RIG-I/IPS-1 binding is mediated by the CARDs of each protein, This CARD-CARD interaction takes place on intracellular membranes and is anchored by IPS-1, leading to recruitment of a large signaling complex. In hepatocytes, this IPS-1 "signalosome" drives the activation of IRF-3 and NF-κB by the IKK and/or Tank-binding kinase 1 (TBK1), protein kinases, and associated signaling partners. The exact mechanism by which interaction of RIG-I with IPS-1 activates signal transduction through IPS-1 remains to be determined, especially how RIG-I effectively relocalizes to interact with IPS-1 membrane-associated signaling domains. Moreover, recent studies now show that NLRX1 and the C1q receptor, gC1qR, can impart negative regulation to IPS-1-dependent signaling, indicating that RIG-I signaling activation must overcome this negative regulation in order to impart innate immunity [59–61]. However, the mechanisms of this regulation and how RIG-I may dominate to drive

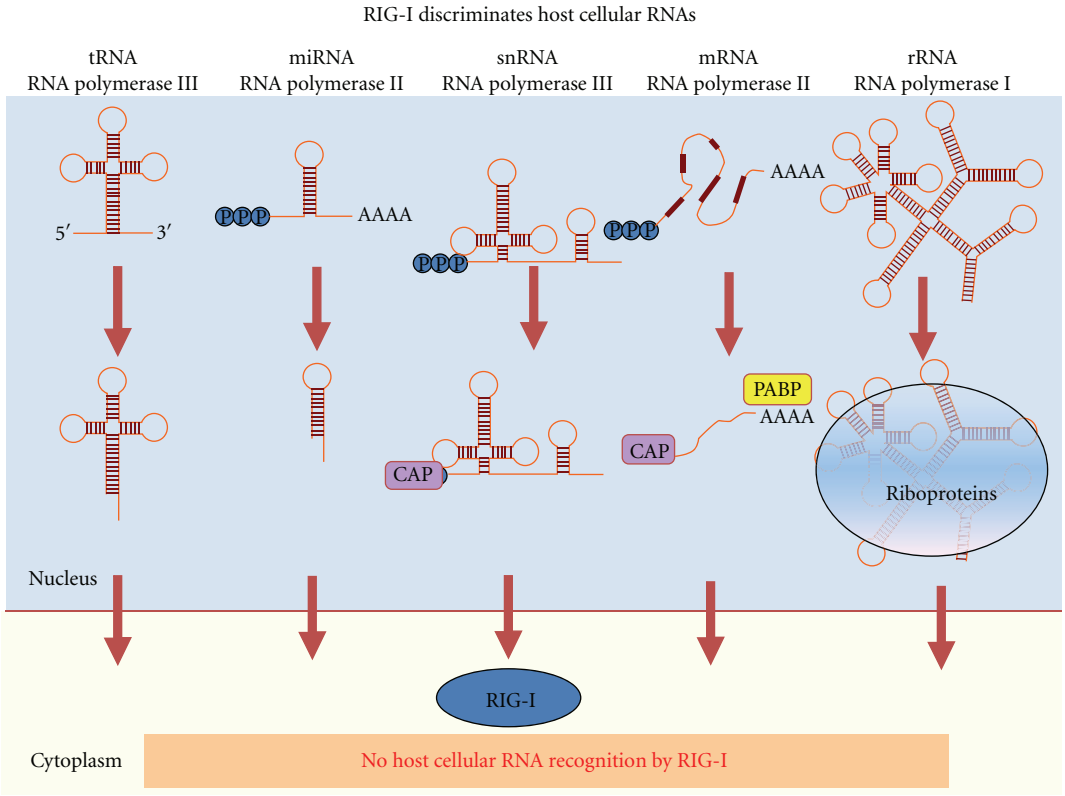

Figure 2: RIG-I discrimination of self and nonself RNA. The primary transcripts of cellular mRNA are produced in the nucleus and are modified before export to the cytoplasm; 5′ppp is replaced by a 5′ cap structure on mRNAs while it is processed from tRNAs; microRNAs are processed to a length insufficient for RLR recognition. Cellular RNA-binding proteins can prevent RIG-I detection of 5′-triphosphatae in rRNAs though masking as an RNP.

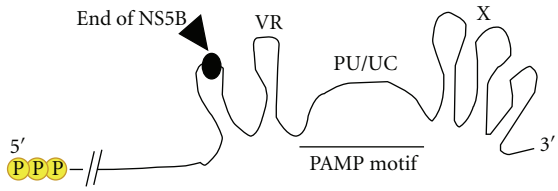

Figure 3: Domains of the HCV genome featuring the 3′ NTR. Details are described in the text. 5′ppp and the poly-U/UC region of the HCV genome or the genome replication intermediate RNA are the major PAMP determinants of HCV that are recognized by RIG-I. Arrow head marks the end of the HCV protein-coding region. The PAMP motif is indicated.

positive signaling are not yet known. Moreover, recent studies also show that mitochondria dynamics change during virus infection to impart enhancement of RIG-I signaling, but how this influences HCV infection and immunity is not defined [62, 63].

6. Evasion of the RIG-I Pathway by HCV

Despite the fact that HCV RNA can trigger RIG-I signaling of antiviral innate immunity, approximately 70–80% of all HCV-infected patients become chronically infected. Several studies have revealed that HCV can effectively evade various arms of the innate immune programs that are induced during infection, and that these outcomes are linked with an overall adaptive immune deficit that supports persistent virus replication and chronic infection.

HCV has evolved to counteract the RIG-I pathway [64], and this regulation serves to establish the infected hepatocyte as a platform for the propagation of chronic HCV infection. HCV evasion of RIG-I is mediated by the essential viral protein, termed NS3/4A. NS3/4A is a complex of the NS3 and NS4A proteins of HCV and serves to process the HCV nonstructural proteins from the precursor HCV polyprotein. NS3 interacts with its cofactor, NS4A, to anchor the NS3/4A complex to intracellular membranes and to facilitate complete activation of the NS3 protease domain [65, 66]. During the early stages of HCV infection and viral RNA replication, NS3/4A accumulation supports the targeted cleavage of IPS-1 by the serine protease activity of the NS3/4A complex [67–69]. Cleavage of IPS-1 by NS3/4A takes place near the IPS-1 C-terminal transmembrane domain, thus revealing IPS-1 and the IPS-1 signalosome from their membrane substrate (see Figure 1(a)). It has been shown that at early hours of HCV infection, the hepatocytes were able to relocalize IRF-3 from the cytoplasm to the nucleus; however, once cleavage of IPS-1 by NS3/4A occurs, which usually can be detected 24 hours after infection, none of the cells were

found with nuclear IRF-3 [67]. As a result, IPS-1 can no longer signal downstream to activate IRF-3 and NF-κB, and the infected cell no longer produced IFN-beta nor expressed ISGs. IPS-1 cleavage by NS3/4A completely disrupts RLR signaling and serves to block signaling through the RIG-I pathway during HCV infection [67, 69–71]. Indeed, IPS-1 mutation at the cleavage motif or NS3/4A protease inhibitor restores the RIG-I signaling pathway to stimulating the ISG expression and limit HCV infection [67, 72]. Recent studies have demonstrated that IPS-1 oligomerization is required for activation of antiviral innate immune signaling. Thus, it appears that disrupting IPS-1 oligomerization through NS3/4A proteolysis could be a contributing mechanism of RIG-I signaling repression [73, 74]. NS3/4A has also been shown to cleave the Toll/interleukin-1 receptor/resistance domain-containing adaptor-inducing IFN (TRIF) protein, the signaling adaptor molecule for TLR3, to prevent TLR3-mediated antiviral signaling [75, 76]. While TRIF proteolysis by NS3/4A would render the infected cell refractory to TLR3 signaling after PAMP ligation, a role for this process *in vivo* remains to be demonstrated [77, 78]. Of note is that TLRs, including TLR3, are themselves ISGs and would be expected to be induced as a result of RIG-I signaling. In this sense, the TLR3 axis may represent an important amplification loop that drives and diversifies the antiviral innate immune response to HCV infection.

7. Future Prospective

Our current understanding of RIG-I regulation by HCV is that the viral NS3/4A protease is the major inhibitor of RIG-I signaling through its ability to target and cleave IPS-1. Of high interest is that NS3/4A protease inhibitors are currently under development as antiviral therapies for HCV [79]. We have found that these protease inhibitors not only block the maturation of HCV NS proteins, but also can block the ability of NS3/4A to cleave IPS-1 and TRIF to restore innate immune signaling in HCV-infected cells [75, 76]. These features of HCV protease inhibitors should offer new treatment options with the effect of suppressing viral replication while enhancing the innate immune response to infection. Moreover, these data provide a proof of concept that therapeutic strategies aimed at enhancing RIG-I signaling could prove beneficial in the clinic for treating chronic or even acute viral infections. In addition to suppressing virus infection directly, the innate immune response serves to drive further inflammatory responses and adaptive immune programs that ultimately control infection and provide long-lasting immunity against further viral challenge. In this case, it will be important to fully understand RIG-I immune regulation in the context of the global immune response with the goal of defining sites of immune interaction that can offer therapeutic benefit through the development and use of immune-modulator drugs to treat HCV and other viral infections.

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Review Article

TLRs, Alcohol, HCV, and Tumorigenesis

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Chronic liver damage caused by viral infection, alcohol, or obesity can result in increased risk for hepatocellular carcinoma (HCC). Ample epidemiological evidence suggests that there is a strong synergism between hepatitis C virus (HCV) and alcoholic liver diseases (ALD). The Toll-like receptor (TLR) signaling pathway is upregulated in chronic liver diseases. Alcoholism is associated with endotoxemia that stimulates expression of proinflammatory cytokine expression and inflammation in the liver and fat tissues. Recent studies of HCC have centered on cancer-initiating stem cell (CSC), including detection of CSC in cancer, identification of CSC markers, and isolation of CSC from human HCC cell lines. Synergism between alcohol and HCV may lead to liver tumorigenesis through TLR signaling.

1. HCV, Alcohol, and HCC

Chronic liver damage caused by viral infection, alcohol, or metabolic syndrome can result in increased risk for HCC which is the third most common cancer in the world [1]. This virus is a major cause of HCC, which is the fifth most common cancer in the world. HCC has a low five-year survival rate due to the lack of therapeutic options and is highly prevalent in the world, especially in Africa and Asia [1]. Clearly, understanding the molecular mechanisms of HCV-induced hepatocarcinogenesis is required for the eventual development of improved therapeutic modalities for this disease [2]. In particular, chronic infection with HBV or HCV represents a major risk factor for HCC [1]. HCV affects more than 170 million people worldwide [1, 3, 4].

Ample epidemiological evidence suggests that there is a strong connection between hepatitis C virus (HCV) and ALD. First, the prevalence of HCV is significantly higher among alcoholics than in the general population; for example, while the HCV positive rate in the general population of the U.S. is roughly 1%, it is 16% for alcoholics and nearly 30% for alcoholics with liver diseases [5]. Second, the presence of HCV infection correlates with the severity of the disease in alcoholic subjects, that is, HCV-infected patients with ALD develop liver cirrhosis and HCC at a significantly younger age than uninfected ALD patients, suggesting that alcohol and HCV work synergistically to cause liver damage [6]. Many studies also support synergistic interactions between HCV and alcoholism in hepatocarcinogenesis [7–11]. Heavy alcohol consumption and viral hepatitis synergistically increase the risk for HCC among blacks and whites in the U.S. [10]. HCC odds ratio increases to 48.3-fold and 47.8 from 8.1 and 8.6 by having concomitant alcohol abuse in HBV or HCV infected patients, respectively [10]. Indeed, our recent result demonstrates that the incidence of spontaneous HCC induction in the HCV core transgenic mice is increased 2-fold by chronic alcohol intake.

Recent studies with mice expressing HCV proteins have shed pivotal insights into the mechanisms underlying this synergism. The HCV core protein causes overproduction of reactive oxygen species which appears to be responsible for mitochondrial DNA damage [3, 12, 13]. The core protein also inhibits microsomal triglyceride transfer protein activity and VLDL secretion [14], which may underlie the genesis of fatty liver. The core protein also induces insulin resistance in mice and cell lines, and this effect may be mediated by degradation of insulin receptor substrates (IRS) 1 and 2 via upregulation of SOCS3 [15] in a manner dependent on PA28γ 73, or via IRS serine phosphorylation [16]. Thus, these core-induced perturbations such as oxidant stress and

insulin resistance, which are also known risk factors for ALD, may underlie the synergism reproduced in alcohol-fed core transgenic mice [17]. TLR2 and TLR4 are markedly upregulated in hepatocytes, Kupffer cells, and peripheral monocytes of patients with chronic hepatitis C. TLR2-mediated activation by hepatitis C is linked to the proinflammatory cytokine induction [18]. TLR-mediated signals result in liver disease associated with hepatitis B, hepatitis C, alcoholicnonalcoholic steatohepatitis, and hepatic fibrosis [19]. The most devastating consequence of the synergism between viral hepatitis and alcohol is HCC [7–11]. The risk of developing HCC as assessed by odds ratio increases from 8∼12 to 48∼54 by having concomitant alcohol abuse in HCV/HBV infected patients [9, 10]. The HCV core and NS3 protein activate TLR2/TLR1 and TLR2/TLR6 on monocytes to produce inflammatory cytokines [19]. The aforementioned effects of the core protein may contribute to the mechanisms of the synergism. However, the more direct mechanistic evidence has recently been attained by our research using mice expressing the HCV nonstructural protein NS5A in a hepatocyte-specific manner. These mice, when fed alcohol for 12 months, develop liver tumors in a manner dependent on TLR4 induced by NS5A [20]. This NS5A-induced TLR4 is activated by endotoxemia associated with alcohol intake, leading to accentuated TLR4 signaling which in turn upregulates the stem cell marker Nanog required for TLR4-dependent liver oncogenesis. This finding on the NS5A-TLR4-Nanog axis in synergistic oncogenesis, is beginning to shed a novel insight into molecular mechanisms for HCC in alcoholic HCV patients.

HCV contains a 9.5-kb single-stranded positive-sense RNA genome, which encodes a polyprotein that is, processed into multiple proteins by cellular and viral proteases. Nonstructural protein NS5A may interact with an interferon-induced, double-stranded RNA-activated protein kinase PKR [21], thus accounting for the resistance of most HCV strains to interferon treatment. NS5A has also been shown to have a cryptic trans-acting activity for some cellular gene promoters [22]. The core protein deserves special mention because, in addition to being a viral structural protein, it serves multiple regulatory functions, including the activation or suppression of various cellular and viral gene promoters. Furthermore, it binds to LTβR, TNF receptor and several other cellular proteins, including apolipoprotein AII [23]. The immune- and cytokine-mediating roles of the HCV core protein may play a key role in the synergistic effects of alcohol liver disease (ALD) on HCV-associated liver damage.

2. TLRs Signaling in HCC

The TLR signaling pathway is upregulated in chronic liver diseases. Many different cell types in the liver express TLRs [19]. Hepatocytes express TLR1 through TLR9. Stellate cells express TLR2, 3, and 4. Bile duct epithelium expresses TLR2, 3, 4 and 5. Kupffer cells express TLR2, 3, and 4. Chronic alcohol consumption activates other TLRs, such as TLR1, 2, and 6–9, which further increases the TNF-α response to LPS in mice [19]. Human monocytes exposed to ethanol for a week develop hypersensitivity to LPS through decreased IRAK-M expression, which activates mitogen-activated protein kinase (MAPK) and NF-κB through TLR4 signaling, leading to activation of NF-κB, AP-1, and ERK [24].

Hepatocyte-specific deletion of TAK1 in mice causes spontaneous hepatocyte death, inflammation, fibrosis, and carcinogenesis partially mediated by TNFR signaling, indicating that TAK1 is an essential component for cellular homeostasis in the liver. In a NASH mouse model, TLR9 signaling induces production of IL-1β by Kupffer cells, leading to steatohepatitis, inflammation, and fibrosis via induction of IL-1β [25]. Furthermore, modulation of TGF-β signaling by a TLR4-MyD88-NF-κB pathway links between profibrogenic and proinflammatory signals [26].

Several possible mechanisms may explain the high prevalence rate of HCV among alcoholics and the increased severity of liver diseases in these patients. First, alcohol may enhance the replication of HCV and thus increase the expression of viral RNA and proteins, resulting in more severe HCV-induced liver injury, independent of the damage induced by alcohol alone. Indeed, HCV titer has been shown to exhibit a correlation with the amount of alcohol consumption [27]. This enhanced effect on HCV replication could be caused directly by the metabolites of ethanol, such as acetaldehyde and free radicals, which may stimulate HCV replication and gene expression. It could also be caused indirectly through alcohol-induced inhibition of the antiviral immune response. Indeed, HCV replication is more active in immunodeficient patients, such as HIV-infected patients [28], and ethanol consumption can cause immunosuppression [29].

Another potential mechanism is the involvement of cytokines. Both ALD and HCV cause enhanced secretion of TNF and other cytokines, such as IL-1, IL-6, and IL-8, [30]. TNF is particularly interesting because there is a tight correlation between the serum TNF concentration and the severity of ALD [31, 32], and TNFR1 deficiency ameliorates experimental ALD [33]. TNF may cause cell death through the activation of the TRADD/FADD signal transduction pathway. Oxidative stress may also contribute to TNF cytotoxicity [34]. On the other hand, a variety of factors can modulate the effects of TNF; for examples, NF-κB [35–37], manganous superoxide dismutase (MnSOD) [38], and GSH inhibit TNF-induced cytotoxicity [39]. In experimental ALD, the mitochondrial pool of GSH is depleted and the hepatocytes become hypersensitive to TNF [39]. We have recently shown that HCV core protein binds to lymphotoxin-β receptor and TNF receptor [40] and that the expression of this protein in several cell lines sensitize them to TNF-induced cytolysis [41]; therefore, HCV-infected cells are particularly sensitive to TNF. It is interesting to note that HCV core protein also sensitizes cells to apoptosis mediated by Fas [42], which shares with TNF receptors signal transduction molecules such as FADD. These observations suggest that HCV-infected hepatocytes are very sensitive to TNF and possibly other cytokines as well. This enhanced sensitivity, coupled with the increased secretion of TNF in ALD, may account for the synergistic effects of ALD on HCV.

Table 1: Markers for liver cancer stem cells.

Gene name	Other name	Function	Species	Organ	References
CD133	Prominin 1 (PROM1)	Glycoprotein, membrane protrusions	Human, mouse	Liver, brain	[74, 78, 79, 87–89]
CD49f	Integrinα chain α6 (ITGA6)	Cell adhesion, cell signaling	Mouse	Liver	[74, 78]
CD90	Thy-1	Glycophosphatidylinositol (GPI) anchor	Mouse	Liver	[74]
CD44	Hyaluronic acid receptor	Cell adhesion and migration, metastasis	Mouse	Liver, breast	[74, 90]
CD117	KIT	C-kit receptor cytokine receptor	Mouse	Liver	[74]
CK19	Cytokeratin 19	Biliary lineage marker	Mouse	Liver	[91, 92]
OV-6	Oval cell marker	Early progenitor cells	Human	Liver	[92]
CD34	Glycoprotein	Cell-cell adhesion factor	Mouse	Liver, leukemia	[93]
AFP	α-fetoprotein	Fetal counterpart of serum albumin	Mouse	Liver	[94]

3. TLR4-Mediated AP1 Activation and HCC

Alcoholism is associated with endotoxemia that stimulates expression of proinflammatory cytokine expression and inflammation in the liver and fat tissues [43]. Development of liver cancer in the HCV core mice is associated with inflammation [44]. Expression of the proinflammatory cytokine TNF-α and IL-1β is induced by HCV-infected human B cells (16) and by its core protein in the transgenic mouse model [45, 46]. Recently, we have shown that HCV infection, through NS5A protein expression, upregulates TLR4 expression and proinflammatory cytokines [47], providing a potential explanation for increased inflammation in HCV-infected livers. Further accentuation of TLR4 signaling in HCV would be expected if combined with alcohol abuse. This may serve as part of the synergistc mechanism when superimposed by other key patholophysiological events common in these comorbidities such as CYP2E1 induction. CYP2E1 induction impairs hepatic insulin signaling [48, 49], induces oxidative DNA damage [50], primes macrophages to increase LPS-induced TNF-α production [51], sensitizes hepatocytes to TNF-α-mediated cell death via c-jun [52, 53], and more importantly, leads to marked potentiation of endotoxin-induced oxidant liver injury (35). Therefore, saturated fatty acids which are implicated in obesity and diabetes could serve as additional ligands to enhance signaling via TLR4 which is already upregulated by HCV NS5A [54]. Polyunsaturated fats actually result in greater rather than less liver injury [55]. These interactive and synergistic mechanisms involving TLR4 in HCV and alcohol most likely contribute to increased incidence of HCC via oxidant stress and inflammation and are the focal points of my proposed research.

c-Jun-deficient mice die between embryonic days E12.5 and E13.5 from massive apoptosis of hepatoblasts, erythroblasts, and other cell types [56, 57]. To overcome this problem, mice harboring a "floxed" c-jun allele that can be deleted in designated cell types upon expression of the Cre recombinase have been developed. Using this system, c-Jun expression was shown to be essential for proper proliferation in postnatal hepatocytes [58]. Moreover, the deletion of c-Jun in hepatocytes compromises the ability of these cells to enter the cell cycle and undergo rapid proliferation after partial hepatectomy [58]. A well-accepted model of HCC utilizes a chemical carcinogen DEN (diethylnitrosamine) as a tumor initiator and phenobarbital as a promoter. By using this model and tissue-specific knockout mice, the loss of JNK1 in the liver was shown to reduce DEN-induced HCC development [59]. The requirement for c-*jun* was restricted to an early stage of tumor development in chemically induced HCC in mice [60]. In our study, c-jun knockout dramatically reduced the incidence of spontaneous and DEN-induced HCC in HCV core transgenic mice, supporting the role of c-jun in this model of hepatocarcinogenesis (Hepatology in press). Alcoholic liver disease patients have increased levels of hepatic RANTES/CCL5. Ethanol augments RANTES/CCL5 expression in rat liver sinusoidal endothelial cells and human endothelial cells via activation of NF-κB, HIF-1α, and AP-1 [61]. *In vitro* studies using liver-derived cell lines have demonstrated rapid activation of AP-1 by HBV or HCV proteins [62], and this mitogenic effect is implicated in hepatocytes' susceptibility to liver cell transformation via fixation of genetic mutations caused by oxidant stress. Indeed, Further c-Jun prevents apoptosis by antagonizing p53 activity as another contributing factor for HCC development [60, 63]. Ectopic expression of HCV core protein constitutively activates AP-1 via JNK [64]. Activation of JNK is also implicated in ASH and NASH [49, 52]. Therefore, activation of JNK and c-jun most likely plays pivotal roles in synergistic induction of liver cancer by HCV and alcohol.

3.1. Cancer Stem Cells and HCC. Stem cells have three major characteristics: self renewal, asymmetric and multiple cell division (clonality), and plasticity. The liver has a high regenerative potential, and hepatic small oval progenitor cells around the peripheral branches of the bile ducts, the canals of Hering, can differentiate into biliary epithelial cells and hepatocytes [65]. These oval liver progenitor cells share molecular markers with adult hepatocytes (albumin, cytokeratin 7 [CK7], CK19, oval cell markers (OV-6, A6, and OV-1), chromogranin-A, NCAM (neural cell adhesion molecule)) and fetal hepatocytes (α-fetoprotein) (Table 1) [65, 66]. They are also positive for more common stem cell markers such as CD34+, Thy-1+, c-Kit+, and Flt-3+ (FMS-like tyrosine kinase 3) [67]. Thus, it currently remains unclear whether these stem cells are derived from the bone marrow and just migrate to this periportal niche

or whether they represent true resident liver stem/progenitor cells. Binding of stroma-derived factor-1α (SDF-1α) to its surface receptor CXCR4 activates oval hepatic cells [68]. Forty percent of HCC have clonality, and thus are considered to originate from progenitor/stem cells [66, 69–71]. Recent studies of HCC have centered on CSC, including detection of CSC in cancer, identification of CSC markers, and isolation of CSC from human HCC cell lines. CSC were identified as a CD117+/CD133+ hepatic precursors in regenerating liver tissue [72] and a CD45−/CD90+ subpopulation of tumor cells in HCC [73]. The CD90+ cells are not present in the normal liver and, when injected into immunodeficient mice, create tumors repeatedly. In human HCC and HCC cell lines, specifically, CD133+ cells, not CD133− cells, had the ability to self-renew, create differentiated progenies, and form tumors [74]. This coincided with the expression of genes associated with stem/progenitor status, such as β-catenin, NOTCH, BMI, and OCT3/4. When compared to CD133− cells, the CD133+ cells isolated from the HCC cell lines showed higher expression of CD44 and CD34, but both CD133 subpopulations displayed similar expression for CD29, CD49f (integrin α6), CD90 and CD117 [74]. Furthermore, CD133+/CD49f+ cells in liver tumors correlate with tumorigenecity and "stemness" genes, such as Wnt/β-catenin, Notch, Hedgehog/SMO, Bmi, and Oct3/4 [75–77]. CD133+/CD49f+ HCC cancer stem cells confer resistance to chemotherapy, and this presents a major obstacle for the treatment of HCC [78]. One potential reason for this chemoresistance may lie in the plasticity of cancer stem cells with dysregulated signaling and gene expression. Several oncogenic signaling pathways in cancer stem cells of HCC, have been described including activated PI3K/AKT [79], signal transducer and activator of transcription 3 (STAT3) [80, 81], Notch [82], Hedgehog [83, 84], and transforming growth factor-beta (TGF-β) [85, 86].

Nanog is one of the core transcription factors found in pluripotent embryonic stem cells (ESCs) [95]. It is essential for maintaining self-renewal and pluripotency of both human and mouse embryonic stem cells [96–99]. Overexpression of Nanog induces and maintains the pluripotency and self-renewing characteristics of ESCs under what normally would be differentiation-inducing culture conditions [100]. Recently, Nanog expression has been reported in human neoplasms, including germ cell tumors [101–104], breast carcinomas [104], osteosarcoma [105], and HCC [79]. Ectopic expression of Nanog induces an oncogenic potential in NIH3T3 [106].

4. Nanog-Positive Cancer Stem Cells Induced by HCV and Alcohol

Alcohol synergistically enhances the progression of liver disease and the risk for liver cancer caused by HCV. Toll-like receptor 4 (TLR4) is induced by hepatocyte-specific transgenic (Tg) expression of the HCV nonstructural protein NS5A, and this induction mediates synergistic liver damage and tumor development by alcohol-induced endotoxemia [20]. The stem/progenitor cell marker, Nanog, is upregulated as a novel downstream gene by TLR4 activation and the presence of CD133/Nanog-positive cells in liver tumors of alcohol-fed NS5A Tg mice [20]. Transplantation of p53-deficient hepatic progenitor cells transduced with TLR4 results in liver tumor development in mice following repetitive lipopolysaccharide (LPS) injection, but concomitant transduction of Nanog short-hairpin RNA abrogates this outcome [20]. Despite the common understanding that TLR4 is one of the pattern recognition receptors expressed predominantly by innate immune cells such as macrophages and lymphocytes, our study demonstrates that hepatocytes can be the primary cellular site of both TLR4 upregulation and its pathologic consequences in the context of HCV infection. Therefore, the TLR4-dependent mechanism synergizes liver disease by HCV and alcohol and is partly dependent on Nanog, a TLR4 downstream gene.

Nanog transduction alone is not as effective as TLR4 activation in liver tumorigenesis, as shown by our cell transplantation experiment [20]. We believe that TLR4 activation induces other tumor-driver genes which cooperatively work with Nanog to cause liver oncogenesis. Thus, Nanog is still essential for TLR4-dependent oncogenesis, but it alone is poorly oncogenic. In our previous work using a cell line, we demonstrated that TLR4 promoter up-regulation by NS5A is mediated by PU.1, Oct-1, and AP-1 elements [47]. The similar transcriptional mechanism may underlie TLR4 induction in primary hepatocytes.

In summary, alcohol and HCV NS5A synergistically induce liver tumor development via induction and activation of TLR4 in mice. The importance of Nanog as a direct downstream gene of TLR4 in this oncogenesis has also been identified. Pharmacologic inhibition of TLR4 signaling may become a novel therapeutic strategy for HCV-associated liver tumors.

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Review Article

TLRs, NF-κB, JNK, and Liver Regeneration

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While hepatocytes rarely undergo proliferation in normal livers, they quickly induce proliferation in response to loss of liver mass by toxin or inflammation-induced hepatocyte injury, trauma, or surgical resection, leading to a restoration of liver mass to its original size. Recent studies suggest that Toll-like receptor (TLR) signaling participates in this regenerative response. Myeloid differentiation factor (MyD88), a common adaptor molecule in the TLR, IL-1 and IL-18 receptor signaling, plays a key role, at least, in the early phase of liver regeneration. Currently, definite ligands which bind to TLRs and initiate this process are still unclear. TLRs stimulated by their corresponding ligands, as well as tumor necrosis factor (TNF) receptors (TNFRs), can activate downstream signal molecules, including transcription factor nuclear factor (NF)-κB and c-Jun N-terminal kinase (JNK). Previous studies have revealed the important role of TNF receptor signaling, NF-κB, and JNK in liver regeneration by using hepatocyte-specific gene-modified animals. This review will summarize the current knowledge of TLR signaling and their related molecules in liver regeneration. We will also discuss whether modulating these factors may become new therapeutic strategies to promote liver regeneration in various clinical situations.

1. Introduction

The liver is a unique organ that has a great ability to regenerate by itself [1, 2]. After loss of considerable liver mass, orchestrated biological responses are quickly activated to restore the loss of liver mass by hepatocyte regeneration until the liver reaches its original size [1–4]. In this regenerative process, mainly mature hepatocytes proliferate by the stimulation of the regenerative factors released from parenchymal and nonparenchymal liver cells. However, liver failure and massive hepatocyte death (apoptosis and necrosis) in fluminant hepatitis could stimulate liver stem cells (or oval cells), which contribute to liver regeneration [5]. Suppressed proliferative ability in mature hepatocytes caused by hepato-toxin exposures, hepatitis B or C virus infection, or excessive lipid accumulation in alcoholic steatohepatitis (ASH), and nonalcoholic steatohepatitis (NASH), also induces the activation of liver stem cells leading to liver regeneration [5]. Because liver injuries induced by inflammation or ischemia/reperfusion and their relation to toll-like receptors (TLRs) are being discussed elsewhere in this issue, we will focus on the role of TLR signaling in liver regeneration after partial hepatectomy (PH), which is a pure hepatic regeneration model. We will further discuss the potential role of TLRs and subsequent signaling pathways that activate nuclear factor (NF)-κB and c-Jun N-terminal kinase (JNK) in liver regeneration.

2. Immediate-Early Gene Expression and Signal Transduction after Partial Hepatectomy

Among a number of biological responses after PH, a group of genes, so called immediate-early genes, are quickly induced [6, 7], which lead to the transition of cell cycle from G0 to G1 in hepatocytes. In contrast, some group of genes are upregulated during the entire period of regenerative responses [8, 9]. Meanwhile, importance of several proinflammatory cytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-6 in liver regeneration has been debated during the last decade [2–4]. It is now accepted that these cytokines play significant roles in the priming and early stage of liver regeneration after PH. Although expression

of TNF-α and IL-6 is upregulated 1 hr after PH, activation of transcription factor NF-κB and AP-1 and expression of immediate-early genes, including c-jun, c-fos, and c-myc, are upregulated within 30 min after PH [7]. These findings suggest that PH induces very quick biological responses, which lead to activation of NF-κB and AP-1, and expression of immediate-early genes. On the other hand, quick upregulation of urokinase-type plasminogen activator (u-PA) and uPA receptor (uPAR) is induced in response to PH [10], implying significant roles in the interaction between u-PA and uPAR in the priming phase of liver regeneration. u-PA also activates several growth factors, including hepatocyte growth factor (HGF) and ligands for epithelial growth factor receptor (EGFR), which induce strong regenerative responses in the liver [11].

Many investigators have proposed that gut microflora-derived lipopolysaccharide (LPS) might trigger the initiation of liver regeneration [12–14]. Upon PH, gut-derived LPS might activate nonparenchymal liver cells, particularly Kupffer cells, in which NF-κB is activated. Then, activated Kupffer cells release regenerative cytokines, such as TNF-α and IL-6. IL-6 activates STAT3, resulting in the initiation of liver regeneration after PH [2]. After the discovery of TLRs, gut-derived lipopolysaccharide (LPS), a ligand for TLR4, was reconsidered as a trigger of the initiation of liver regeneration [15, 16].

3. Toll-Like Receptors and Liver Regeneration

Toll-like receptors (TLRs) were originally identified as homologs of *Drosophila* Toll that regulates dorsoventral embryonic polarity and antifungal immunity [17]. TLRs facilitate innate immune responses for the host defense against microorganisms by recognizing pathogen-associated molecular patterns (PAMPs) [18]. In addition, endogenous components derived from dying host cells, termed damage-associated molecular patterns (DAMPs), can bind and activate TLRs [19, 20]. After the stimulation with corresponding ligands, TLRs relay signals via myeloid differentiation factor (MyD) 88, a common signal adaptor molecule shared by the receptor for IL-1 and IL-18, and all members of TLRs except for TLR3 [18]. This signal transduction leads to activation of NF-κB and results in the production of various proinflammatory cytokines, including TNF-α and IL-6. The MyD88-dependent pathway also activates p38 and c-Jun N-terminal kinase (JNK) as well. The TLR/MyD88-mediated biological events are reminiscent of those observations in liver regeneration after PH. We and others have investigated the role of TLR/MyD88-mediated signal transduction in liver regeneration after PH [15, 16]. Both studies demonstrated that in MyD88-deficient mice, induction of immediate-early genes, including c-fos, c-jun, JunB, and c-myc, was greatly diminished [15], and NF-κB activation in Kupffer cells was also subnormal after PH. Production of IL-6 and TNF-α and phosphorylation of STAT-3 were completely suppressed in MyD88-deficient mice after PH [15, 16]. However, these studies showed contradictory roles of MyD88 in the regenerative response of hepatocytes after PH. DNA synthesis in hepatocytes significantly delayed in the early phase of liver regeneration in our investigation [15] implies the significant role of MyD88-dependent signaling in the early and priming phase of liver regeneration. Meanwhile, the MyD88-deficient mice showed no delay in hepatocyte replication in the other report [16]. To discuss this discrepancy, we might consider the breeding condition of animals in different institutions. Recent reports demonstrated that mice from different animal suppliers have different composition of microflora in the intestine [21]. In addition, MyD88-deficient mice are more sensitive to changes in the composition of microflora [22]. Here, we propose that there is a large contribution of intestinal microflora-derived components, as the ligands for TLRs, to liver regeneration after PH. The differences in the composition of gut microflora might lead to the different regenerative responses between different institutions. Nowadays, the concept that TNFα, IL-6, and NF-κB are crucial for PH-mediated liver regeneration is still supported by many investigators. Thus, we confidently propose the concept that the MyD88-dependent regenerative response after PH is important for initiating the priming of liver regeneration. Nevertheless, liver mass was finally restored in MyD88-deficient mice compared to WT mice even in our experiment, suggesting that MyD88-independent compensatory regenerative processes also contribute to liver regeneration.

Another interesting finding is that the intact liver regeneration is induced in *TLR2*$^{-/-}$, *TLR4*$^{-/-}$ [16], *TLR2*$^{-/-}$ *TLR4*$^{-/-}$, and *TLR9*$^{-/-}$ mice [15] after PH. This result suggests that the gut-derived LPS and its receptor TLR4 do not play a major role in triggering the priming phase of liver regeneration. In addition, both IL-1 and IL-18 receptor signalings also use MyD88 as a signal adaptor molecule [23]. Therefore, possible contribution of IL-1 and IL-18 to the priming of liver regeneration should be considered. However, *Caspase-1*$^{-/-}$ mice, which lack the capacity to convert proform of IL-1β and IL-18 to active form [24, 25], exhibit normal liver regeneration after PH, suggesting minor roles of IL-1β and IL-18 in liver regeneration [15]. What is the crucial trigger of the MyD88-dependent biological events in the priming of liver regeneration? We propose that various gut microflora-derived components and/or unidentified endogenous ligands for TLRs activate multiple TLRs including TLR2, 4, 5, and 9 that lead to triggering the priming of liver regeneration after PH. Further investigation is required for proving this hypothesis.

The studies by others and us suggest that TLR/MyD88 signaling participates in the process of liver regeneration, especially in the early and priming phase, after PH. However, definite TLR ligands responsible for the priming process are still unknown. Further investigations are required for addressing this issue. Notably, LPS injection suppresses liver regeneration after PH, indicating that excessive TLR signaling inhibits this regenerative process [26]. Thus, the TLR signaling has double-edge sword-like functions in liver regeneration, and an appropriate magnitude of TLR signaling is required for intact liver regeneration.

On the other hand, TLR3 utilizes another adaptor molecule, TRIF (TIR domain-containing adaptor-inducing interferon-β). Recently, the role of TLR3 signaling in liver

regeneration has been reported [27]. In *TLR3*$^{-/-}$ mice, initiation of liver regeneration is shifted to the earlier time point, suggesting that TLR3 signaling inhibits the initiation of liver regeneration. This inhibitory effect of TLR3 signaling is also supported by a previous report that activation of TLR3 by injection of its ligand, polyinosinic-polycytidylic acid (poly (I : C)), suppressed liver regeneration after PH [28]. Interestingly, NF-κB activation in hepatocytes was significantly suppressed up to 10 hrs, but its activation was relatively prominent in Kupffer cells in *TLR3*$^{-/-}$ mice after PH. These findings suggest that after PH, unknown ligands, probably endogenous ligands for TLR3, activate NF-κB through Rip1 in hepatocytes that inhibit hepatocyte replication.

Other components of the innate immune system, complements including C3 and C5, have been reported to participate in liver regeneration after PH [29]. C3- or C5-deficient mice exhibited high mortality, liver parenchymal cell damage, and impaired liver regeneration after PH. NF-κB activation was markedly reduced at 1 hr after PH in C3-deficient mice. Thus, the complement system plays a significant role in the priming phase as well as in the subsequent proliferative phase during liver regeneration.

4. Roles of NF-κB in Liver Regeneration after Partial Hepatectomy

NF-κB is an essential transcription factor for maintaining liver homeostasis, including cell survival and death [30]. As NF-κB p65 whole body knockout mice cause the embryonic death due to an extensive hepatocyte apoptosis, NF-κB plays a crucial role in preventing hepatocyte apoptosis during liver development [31].

A very quick increase in NF-κB DNA binding activity after PH has been reported in early 1990s [32, 33]. We are the first demonstrating the role of NF-κB in liver regeneration after PH. We introduced IκB α superrepressor, a potent NF-κB inhibitor, by adenoviral vectors (Ad-IκBsr) to inhibit NF-κB activation in the liver [34]. In this study, the rat livers introducing superrepressor of IκB α (IκBsr) demonstrated the prominent hepatocyte apoptosis and blunted early increase in NF-κB DNA binding activity in hepatocytes during the initial 24 hrs after PH, suggesting antiapoptotic role of NF-κB in hepatocytes after PH. Liver regeneration was significantly impaired in this study. A similar experiment employing Ad-IκBsr in mice was reported by Yang et al. [35]. In this paper, overexpression of IκBsr in mouse livers also inhibited liver regeneration after PH but did not induce hepatocyte apoptosis. Discrepancy between these reports might be due to different infectious abilities against adenoviral vectors between rats and mice. This might induce hepatocyte apoptosis only in rats. Since TNFα is produced in the early period after PH, this discrepancy also might be due to different sensitivity to TNFα-induced cell death between rats and mice. Thus, activation of NF-κB in hepatocytes is important as an antiapoptotic factor, at least in rats, but not in mice after PH.

A subsequent study further reported the specific role of hepatocyte NF-κB in liver regeneration after PH [36]. Transgenic mice expressing a hepatocyte-specific mutant IκBα exhibited the normal regenerative response and did not show hepatocyte apoptosis after PH. TNFα treatment induced prominent apoptosis in these animals. A use of adenoviral vector in our study, indeed, possibly made the issue complicated because adenoviral vectors could infect not only hepatocytes, but also nonparenchymal liver cells including Kupffer cells. In the study by Maeda et al. [37] to inhibit NF-κB activation, IKKβ (inhibitor of kappaB kinase β, also known as IKK2) was inactivated in both hepatocytes and hematopoietic-derived cells (Kupffer cells) by conditional knockout technique using Mx-1 Cre transgenic mice. These mice reduced the proliferative response after PH. Taken together, these findings suggest that NF-κB activation in Kupffer cells is more important than that in hepatocytes.

In a recent study [38], the modulation of hepatocyte NF-κB activity by inactivation of IKKβ demonstrated the controversial results from the studies by Maeda et al., [37], Yang et al., [35], and us [34]. Hepatocyte-specific IKKβ knockout mice showed that accelerated hepatocyte proliferation and early NF-κB activation in nonparenchymal liver cells including Kupffer cells were observed. In contrast, a weak and delayed NF-κB activation in hepatocytes was seen after PH, suggesting that this IKK and NF-κB activation in hepatocytes might be through IKKα. These findings further suggest that IKKα in hepatocytes is crucial in liver regeneration after PH. Addressing this issue, further investigation is needed. JNK activation in hepatocyte-specific IKKβ knockout mice is prolonged in the liver after PH [38]. Because Cyclin D is a JNK/AP-1 target gene, a strong and sustained JNK activation could be involved in the accelerated liver regeneration by inducing Cyclin D expression in hepatocyte-specific IKKβ knockout mice. This study also examined pharmacological inhibition of IKKβ, which enables to inhibit IKKβ activity in both hepatocytes and nonparenchymal cells. This treatment had little effect on the regenerative process [38], which corroborates the results from Chaisson et al. [36], but it does not support the results from Maeda et al., Yang et al., and us; NF-κB in Kupffer cells is critical in liver regeneration.

The crosstalk between NF-κB and JNK in the liver after PH has been demonstrated in the study using Gadd45β (growth arrest and DNA-damage-inducible gene 45β), an NF-κB target gene, knockout mice [39]. *Gadd*45$\beta^{-/-}$ mice exhibited decreased hepatocyte proliferation and increased programmed cell death during liver regeneration, in which JNK activity was increased and sustained. This study further supports the concept that an appropriate activation of NF-κB in hepatocytes is important for liver regeneration by regulating JNK activity through Gadd45β. This issue will be discussed below.

It is now almost acceptable that the activation of NF-κB in Kupffer cells is crucial for intact liver regeneration after PH, especially for the priming phase [3]. Activated NF-κB conducts sequential production of TNFα and IL-6, each of which plays a significant role in the priming phase of the regenerative process. Indeed, early activation of NF-κB in liver regeneration after PH was demonstrated to primarily occur in Kupffer cells using *cis*-NF-κB-EGFP transgenic mice

[35]. Moreover, inactivation of NF-κB in Kupffer cells as well as in hepatocytes impaired the proliferative response after PH as mentioned above [37].

5. JNK Activation after Partial Hepatectomy

Prompt expression of *c-jun* and activation of c-Jun N-terminal kinase (JNK) have been reported after PH as described above [40, 41]. Fetuses lacking *c-jun* die at midgestation with defects in heart morphogenesis and increased apoptosis of both hepatoblasts and hematopoietic cells in the fetal liver [42, 43]. Moreover, mice lacking the JNK kinase SEK1 exhibit a liver defect similar to *c-jun*$^{-/-}$ fetus [44, 45]. Roles of the transcription factor AP-1 (activator protein-1), including c-Jun, as a regulator of cell survival and death are well summarized in a previous paper [46]. The function of c-Jun at later stages of liver development or in adult liver remained to be elucidated.

The role of c-jun during liver regeneration after PH was first reported by using floxed *c-jun* alle (*c-jun*^{f}) mice crossing with Alfp-cre or Mx-cre transgenic mice [47]. In either Alfp-cre or Mx-cre-induced conditional c-jun knockout mice, prominent hepatocyte death and lipid accumulation in hepatocytes were observed after PH. These mice exhibited high mortality and impaired liver regeneration. These results indicate that *c-jun* is required for hepatocytes regeneration after PH. Interestingly, c-Jun N-terminal phosphorylation is not required for c-Jun function in liver regenerative response [47].

Important roles of JNK activation after PH have been reported [48]. Inhibition of JNK activity in the liver using a small molecule JNK inhibitor (SP600125) resulted in reduced c-Jun phosphorylation and AP-1 DNA binding activity. JNK inhibition suppressed cyclin D1 expression and delayed hepatocyte proliferation after PH, resulting in decreased survival but not hepatocyte apoptosis. This implied that JNK drives the transition of cell cycle from G0 to G1 transition in hepatocytes and that cyclin D1 is a crucial target gene of the JNK pathway in liver regeneration. However, these results were different from the data obtained from the study in which c-jun was genetically inactivated [47]. Potential explanation for this discrepancy is that JNK mediates its effects on liver regeneration after PH through other targets such as ATF2 or JunD [48]. As mentioned above, c-Jun N-terminal phosphorylation is not required for regeneration after PH [47], suggesting that increased JNK activity after PH has other significant roles rather than its ability to phosphorylate c-Jun N-terminal. Two isoforms of JNK are expressed in the liver; JNK1 and JNK2 [49, 50]. Distinct functions in these JNKs have been reported [51]. Namely, JNK1 induces cell proliferation with c-Jun phosphorylation. In contrast, JNK2 suppresses proliferation by degradation of c-Jun. Sabapathy and Wagner also reported an acceleration of liver regeneration in *JNK2*$^{-/-}$ mice in their perspective [52].

HGF and EGFR-ligands activate JNK. Mice expressing an inducible *Met* mutation by Mx-cre system in the liver showed blunted c-jun phosphorylation around 48 h after PH and a great suppression of Erk1/2 phosphorylation during the entire regeneration process [53]. Hepatocyte-specific EGFR deletion resulted in reduced cyclin D1 expression and delayed hepatocyte proliferation after PH. Surprisingly, this observation was accompanied by sustained activation of c-Jun and reduced NF-κB DNA binding activity [54]. This prolonged c-Jun activation after PH in the hepatocyte-specific EGFR-deleted mice might be independent of EGFR-mediated JNK activation, or there might be unknown negative regulatory mechanism on JNK/c-Jun activation in the EGFR signaling cascade. Nevertheless, it is suggested that prolonged activation of c-Jun inhibits liver regeneration after PH. Prolonged activation of JNK after PH has also been observed in *Gadd*45$\beta^{-/-}$ mice as mentioned above [39]. Why does prolonged JNK activation suppress liver regeneration after PH? Notably, a genetical ablation of JNK2 prevents impaired liver regeneration and increased hepatocyte cell death in *Gadd*45$\beta^{-/-}$ mice after PH. Although it is obvious that JNK pathway directly induces cyclin D1 upregulation to contribute to hepatocyte proliferation [39], however, prolonged JNK activation hampers liver regeneration after PH, at least, by enhancing programmed cell death.

TGF-β-activated kinase (TAK)-1 is a crucial component for activating both NF-κB and JNK. An adenoviral overexpression of dominant negative in the liver accelerated the proliferative response after PH [55]. This might be explained by the inhibitory role of IKKβ and JNK2 in liver regeneration in previous studies [38, 52]. An appropriate crosstalk between JNK and NF-κB is critical for intact liver regeneration after PH. An opposing role of NF-κB and JNK in hepatocarcinogenesis has been proposed. Loss of NF-κB activity in hepatocytes increases the sensitivity to hepatocarcinogenesis with increased JNK activation [37, 56–58] while JNK1 knockout mice exhibit decreased carcinogenesis after N-nitrosodiethylamine administration [59]. The mutual crosstalk between the NF-κB and JNK has been well documented in the previous reviews [60, 61].

In summary, the transient upregulation of the two downstream targets NF-κB and JNK in TLR/MyD88 and TNFR signaling is strictly organized in Kupffer cells and hepatocytes upon PH, which leads to normal liver regeneration (Figure 1).

6. Clinical Issues in Liver Regeneration

Understanding the mechanism of liver regeneration is important for managing several clinical conditions, such as acute liver failure and impaired hepatic functions after liver transplantation. An issue concerning small-for-size grafts in liver transplantation is a well-known clinical subject. An experiment using small-for-size graft in rats revealed that suppressed AP-1 DNA binding activity and reduced cyclin D1 expression resulted in impaired liver regeneration. This was attenuated by administration of a radical scavenger [62]. Impaired liver regeneration in patients with NASH (nonalcoholic steatohepatitis) or NAFLD (nonalcoholic fatty liver disease) is also a major clinical issue. Animal experiments revealed that impaired liver regeneration with suppressed NF-κB DNA binding activity and delayed and prolonged *c-jun* expression after PH in ob/ob mice [63]. Microarray

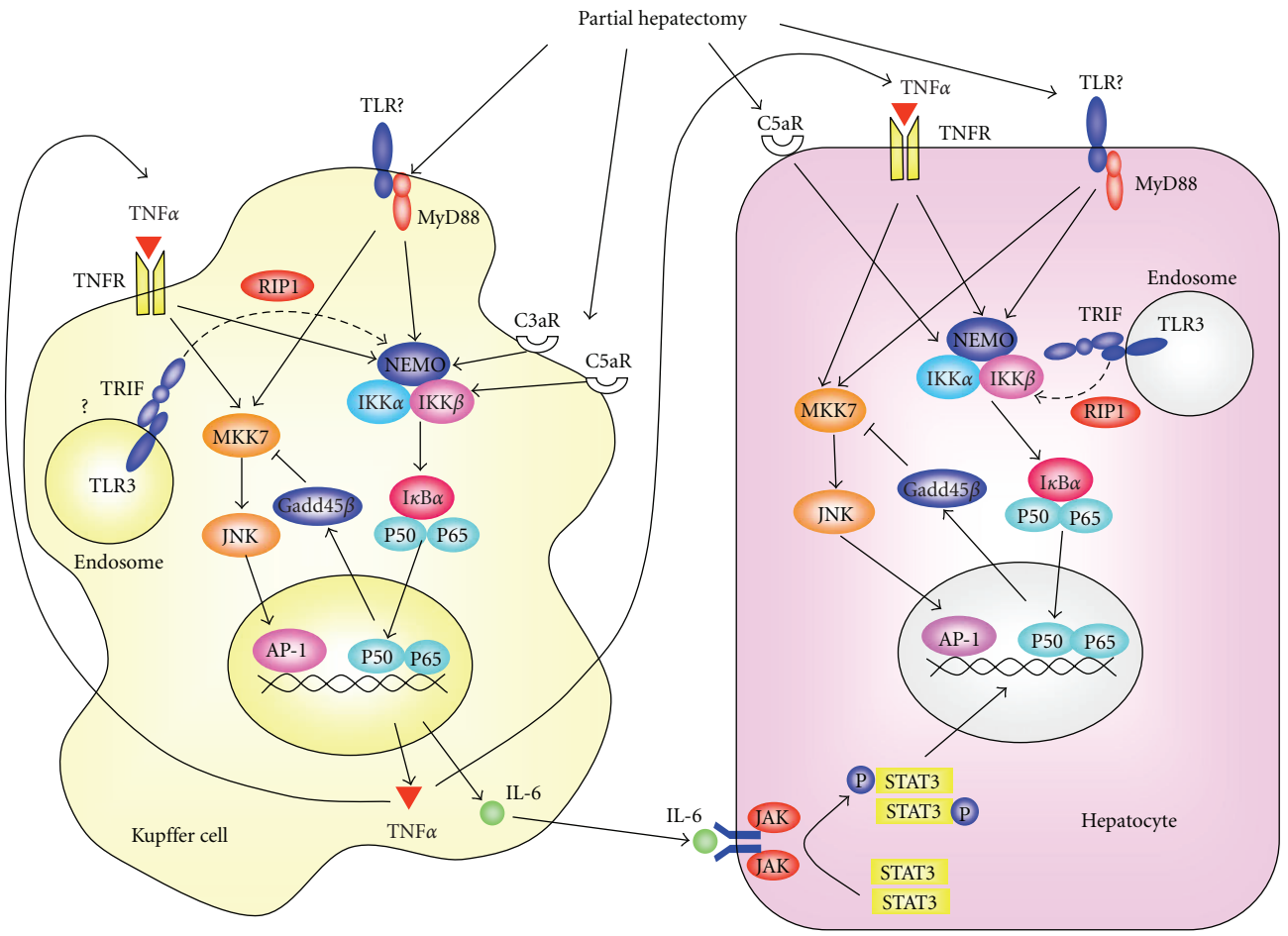

Figure 1: Signal transduction pathways during the priming phase of liver regeneration. Interactions between Kupffer cells and hepatocytes are also illustrated while other nonparenchymal cells are also possibly involved. AP-1, activator protein; C3aR, activated compliment 3 receptor; C5aR, activated compliment 5 receptor; Gadd45β, growth arrest and DNA-damage-inducible gene 45β; IKK, inhibitor of nuclear factor κB kinase; IL-6, interleukin-6; JAK, Janus-associated kinase; JNK, c-jun N-terminal kinase; MKK7, mitogen-activated protein kinase 7; MyD88, myeloid differentiation factor 88; NEMO, nuclear factor κB essential modulator; RIP, receptor interacting protein; TLR, toll-like receptor; TNFα, tumor necrosis factor α; TRIF, TIR-domain containing adaptor inducing interferon-β.

analysis in the patient livers with NASH revealed that mRNA expression of transcription factors such as v-Jun (oncogenic isoform of c-Jun) was significantly suppressed compared with nonobese control patients even without liver resection [64]. However, the most critical issue in clinical situation is impaired liver regeneration in patients with advanced liver fibrosis. A number of factors including deposition of excessive amount of extracellular matrix, existence of continuous inflammation, transformation of sinusoidal endothelial cells and hepatic stellate cells, and decreased portal blood flow may affect the regenerative ability in the fibrotic livers. In addition, increased JNK activity, as observed in animal models during fibrogenesis [65], may also account for the impaired regenerative process in these patients.

7. Summary

The ligands for TLRs are a strong candidate for triggering the initiation of liver regeneration. However, discovery of the real trigger which initiates liver regeneration is a challenging assignment for hepatologists because multiple ligands and multiple TLRs could contribute to this process. In addition, understanding of the interaction between the two major transcription factors; NF-κB and AP-1 (such as c-Jun), and the regulation of JNK activity by NF-κB seems to be critical to elucidate the well-orchestrated process. Further investigation of distinct roles between these factors in hepatocytes and nonparenchymal cells should be required employing cell-specific gene manipulation techniques. The management of the impaired balance between these factors in nonparenchymal cells and hepatocytes may provide insight into developing new strategies for inappropriate hepatic regenerative response in patients with chronic and acute liver diseases.

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Review Article

Toll-Like Receptors in Hepatic Ischemia/Reperfusion and Transplantation

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The family of Toll-like receptors (TLRs) function as pattern-recognition receptors (PRRs) that respond to a myriad of highly conserved ligands. These substrates include pathogen-associated molecular patterns (PAMPs) for the recognition of invading pathogens, as well as damage-associated molecular patterns (DAMPs) for the recognition of endogenous tissue injury. While the functions of TLRs are diverse, they have received much attention for their roles in ischemia/reperfusion (I/R) injury of the liver and other organs. The TLRs play central roles in sensing tissue damage and activating the innate immune system following I/R. Engagement of TLRs by endogenous DAMPs activates proinflammatory signaling pathways leading to the production of cytokines, chemokines and further release of endogenous danger signals. This paper focuses on the most recent findings regarding TLR family members in hepatic I/R injury and transplantation.

1. Introduction

The liver is a central integrator of the systemic immune response following acute traumatic or surgical insults. It is subject to injury and dysfunction following local insults such as ischemia-reperfusion (I/R), as well as systemic insults including hemorrhagic shock. Liver I/R is a pathophysiologic process whereby hypoxic organ damage is accentuated following return of blood flow and oxygen delivery. This process involves activation of the innate immune system, causing a proinflammatory response at the site of injury. Although the distal cascade of inflammatory responses resulting in organ damage after I/R injury has been studied extensively, the process by which initial cellular injury after an ischemic insult contributes to activation of the inflammatory response is poorly understood. Recently, Toll-like receptors (TLRs) have been shown to be critical for the full induction of the inflammatory response observed in experimental ischemia and reperfusion. The TLR receptors involved in alerting the innate immune system appear to be activated by damage-associated molecular pattern molecules (DAMPs) that are released during ischemic stress. In this paper, we will summarize the most recent findings regarding the role of TLRs in liver I/R.

2. Toll-Like Receptors

The family of Toll-like receptors are important components of the innate immune system responsible for recognizing a variety of exogenous and endogenous molecules [1]. In 1996 it was demonstrated that the *Drosophila* Toll protein is an essential part of the immune response to fungal infection in adult flies in addition to its established role in development [2]. The identification and characterization of the human Toll homologues soon followed [3]. A total of 13 TLRs have been identified in mammals: humans have 10 and mice 12 [4]. While all TLRs are transmembrane proteins, some reside at the cell surface, and some reside intracellularly. TLR1, TLR2, TLR4, and TLR6 are found at the cell surface, and all have an extracellular component comprised of luecine-rich repeat (LRR) domains. TLR3, TLR7, TLR8, and TLR9 are

intracellular, primarily located in the endoplasmic reticulum. All TLRs contain a conserved cytoplasmic Toll/IL-1 Receptor (TIR) domain that is shared by the receptors of the IL-1 and IL-18 families [5]. These features allow TLRs to signal through a group of adaptor molecules which also contain TIR domains. TLRs form heterodimers (TLR1 with TLR2 and TLR2 with TLR6, e.g.), or homodimerize (TLR4 and TLR9), and undergo conformational changes after ligand engagement which leads to association of individual TIR domains. Adaptor molecules are then recruited; these include MyD88, MyD88-adaptor-like (MAL, also referred to as TIR domain-containing adaptor protein (TIRAP)), TIR domain-containing adaptor-inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM), and sterile α- and armadillo motif-containing protein (SARM) [6]. Most TLRs utilize MyD88 to initiate intracellular signaling. The exceptions are TLR3, which interacts with TRIF exclusively, and TLR4 which is capable of utilizing both MyD88 and TRIF. MyD88 recruitment initiates activation of additional intermediate signaling molecules; these proteins include IL-1R-associated kinase 4 (IRAK4), which phosphorylates IRAK1, leading to the recruitment of TNFR-associated factor 6 (TRAF6) and TGF-β-activated kinase 1 (TAK1). These events culminate in activation of mitogen-activated protein kinases p38, c-Jun N-terminal kinase (JNK), and NF-κB through phosphorylation of I-κK. In contrast to MyD88-dependent signaling, the TRIF-dependent pathway recruits TRAF3, ultimately resulting in transcription of interferon-regulator 3 (IRF3) and production of IFN-β. While the MyD88 and TRIF-dependent signaling pathways are distinct, significant overlaps exist. For example, TLR4 signaling through TRIF can result in NF-κB activation, and signaling through MyD88 can induce activation of IRFs, particularly IRF1.

TLRs are expressed on several different cell types in the liver, including both parenchymal cells and nonparenchymal cells. Hepatocytes express low levels of TLR2, TLR3, TLR4, and TLR5 and are capable of responding to TLR2 and TLR4 ligands [7]. Similarly, biliary epithelial cells express TLR2, TLR3, TLR4, and TLR5. Kupffer cells, the liver's resident macrophages, are critical in the pathogenesis of I/R and express a number of different TLRs. Studies suggest that TLR4 signaling is critical in Kupffer cells because they are the first to be exposed to gut-derived endotoxin. When exposed to physiological levels of LPS, Kupffer cells secrete anti-inflammatory IL-10, suppressing activation of surrounding immune cells [8–10]. In addition to TLR4, Kupffer cells also express TLR2, TLR3, and TLR9. Most other hepatic nonparenchymal cells express various TLRs. Hepatic stellate cells express TLR4 and TLR9, sinusoidal epithelial cells express TLR4, and subsets of hepatic dendritic cells express TLR2, TLR3, TLR4, TLR7, and TLR9. The liver contains a high concentration of natural killer (NK) cells, and they express TLR1, TLR2, TLR4, TLR7, and TLR9 [11, 12]. It is worth noting that an exhaustive analysis of the functional expression of pattern recognition receptors on liver cell types has not been reported. However, we can conclude that activation of TLRs during I/R creates a diverse response in different cell types that respond to different TLR ligands.

3. Liver I/R

Ischemia/reperfusion injury is a phenomenon whereby tissues experience damage as a result of temporarily interrupted blood flow (ischemia) followed by its restoration (reperfusion). Clinically, liver I/R occurs in settings of elective liver surgery, trauma, shock, and transplantation. Two categories of hepatic I/R—warm and cold—are similar yet distinct processes that share a number of characteristics, both of which ultimately result in end-organ damage. Warm I/R commonly occurs during surgery, trauma, and low-flow states while cold I/R is experienced during organ preservation prior to transplantation. The pathophysiology of liver I/R injury includes both direct cellular damage as a result of the ischemic insult as well as delayed dysfunction following reperfusion resulting from activation of the immune system. The distal interacting elements in the cascade of inflammatory responses resulting in organ damage following hepatic I/R injury have been extensively studied. However, proximal events that initiate damage during I/R are less well characterized. The most recent work in this field points to a critical role for activation of TLRs after I/R as initiating events in the pathogenesis of I/R injury.

3.1. Warm Ischemia Reperfusion Injury. The process of warm I/R injury involves activation of immune pathways and is dominated by hepatocellular injury. While all cells types in the liver are involved in the process, Kupffer cells, the resident macrophages of the liver, are a key cell type involved in the earliest stages of I/R injury. Amongst their many functions, one of the most important is the production of reactive oxygen species (ROS). They were discovered to be an important source of ROS during I/R in the 1980s [13–15]. Normally, ROS production is useful in eliminating circulating pathogens and is the mechanism responsible for the "respiratory burst" observed when these cells are activated. However, excessive ROS after an ischemic insult is detrimental. While damage occurs directly from ischemia-induced oxidant stress on many cell types during the early phase of injury, ROS from Kupffer cells also contributes to the activation of inflammatory pathways that lead to neutrophil accumulation in the liver, resulting in additional, prolonged injury. Thus, Kupffer cell-derived ROS are involved in the pathogenesis of I/R injury through direct oxidant-mediated damage and by augmenting the local activation of proinflammatory pathways.

Another hallmark of I/R injury is the release of cytokines and chemokines from cells at the site of injury. Kupffer cells, in addition to releasing ROS, are also principally responsible for the release of cytokines during I/R. Both Tumor necrosis factor-alpha (TNFα) and Interluekin-1 (IL-1) are released from Kupffer cells within minutes following reperfusion [16, 17] and promote damage through a number of mechanisms. Briefly, these cytokines recruit neutrophils by promoting upregulation of neutrophil integrins and also activate and recruit CD4+ T cells to the site of injury [18–20]. Additionally, they promote the local production of key chemokine molecules which both attract and activate neutrophils [21–23]. Together, these events culminate in

hepatocellular damage and death, resulting in elevated serum transaminase levels and organ damage. While the extent of damage is dependent on a number of factors, including severity of the ischemic insult, all of the pathological factors contributing to I/R injury also share the commonality that they are, in part, dependent on TLR signaling. Thus, while multiple signaling networks are responsible for coordinating the inflammatory response during I/R injury, TLRs are critical in initiating and mediating these effects.

3.2. Cold Ischemia Reperfusion Injury. Cold I/R occurs in the setting of solid organ transplantation after a donor graft is harvested. Before cold storage, the organ is perfused with a preservation solution and remains ischemic until it is transplanted into the recipient. When the graft is reperfused cold I/R occurs. In contrast to warm I/R, cold I/R is dominated by damage to the sinusoidal endothelial cells and disruption of the microcirculation rather than damage to the hepatocytes. While cold storage times that occur in human transplantation vary greatly, animal studies focus on storage times of up to 18 hours, though some extend cold storage to 24 hours or more. In addition to cold I/R injury, liver transplantation involves additional factors such as immunologic tolerance and rejection [24, 25].

4. Role of Endogenous DAMPs in I/R

Acute, ischemic, and sterile tissue injury activates the innate immune system in a way similar to pathogenic infections from microbes, viruses, and fungi. This phenomenon occurs because pattern recognition receptors, including the TLRs, are capable of recognizing both PAMPs and DAMPs, leading to activation of similar downstream signaling cascades. Since the first report that an endogenous molecule, Heat Shock protein 60, could activate TLR4 signaling [26], a number of additional TLR-activating DAMPs have been discovered including hyaluronan, fibrinogen, heparin sulfate, High Mobility Group Box Protein 1 (HMGB1), and DNA [1]. During I/R, DAMPs come into contact with TLR-expressing cells through several different mechanisms. Direct cellular damage from oxidative stress during ischemia results in the passive release of DAMPs from necrotic cells; additionally, they are liberated from the cell matrix by proteases. Lastly, it appears that DAMP release from stressed cells may be a regulated mechanism. Recent reports show that DAMP-mediated TLR activation itself regulates the additional release of a number of danger signals, and therefore TLR signaling may function in an autocrine/paracrine fashion that culminates in excessive innate immune activation and organ damage.

5. TLR4 in Liver I/R

Amongst the most studied TLR in hepatic I/R is TLR4. Buetler et al. were the first to discover that TLR4 is a sensor for bacterial lipopolysaccharide (LPS) [27], and subsequent studies showed that TLR4 also plays a role in a number of acute sterile injury models, including liver I/R (Figure 1) [28].

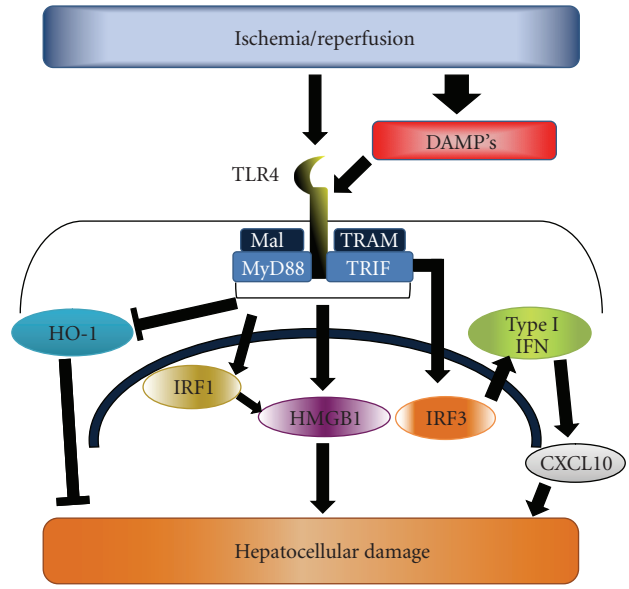

Figure 1: Role of TLR4 in hepatic I/R injury. Signaling through the pattern recognition receptor TLR4 mediates multiple inflammatory pathways following hepatic I/R. The activation of TLR4 signaling is dependent, in part, on circulating DAMPs. Downstream signaling events include the TRIF-dependent activation of IRF3, the production of Type I IFN, and upregulation of CXCL10. Additionally, TLR4 activation of IRF1 promotes release of the DAMP, HMGB1. Lastly, TLR4 activation is thought to suppress the cytoprotective HO-1 pathway.

5.1. Warm I/R. The involvement of TLR4 in warm I/R injury was first described by Wu and colleagues in 2004. This study used a model of partial hepatic I/R and showed that mice deficient in TLR4 signaling experienced significantly less liver damage compared to their wild-type counterparts. TLR4-deficient mice had significantly lower levels of serum aspartate transaminase levels (AST), as well as decreased levels of TNF-α mRNA and myeloperoxidase (MPO) after I/R [29]. Other groups have subsequently published similar findings, all of which show that TLR4-deficient mice experience less injury and inflammation after warm I/R [30–33]. These studies provided clues that TLR4 activation during I/R promotes damage through secretion of cytokines and recruitment of inflammatory cells to the liver. In addition to reduced secretion of proinflammatory mediators, Shen et al. also found that the protective Heme Oxygenase-1 (HO-1) pathway was upregulated in TLR4 deficient mice, suggesting that suppression of this pathway downstream of TLR4 activation is another damage-promoting mechanism during I/R [31]. Another model of sterile, ischemic injury is hemorrhagic shock (HS). HS results in systemic hypoperfusion and I/R-like damage to the liver. In addition to data from I/R models, we have shown that the liver damage induced by hemorrhagic shock is also strongly TLR4-dependent

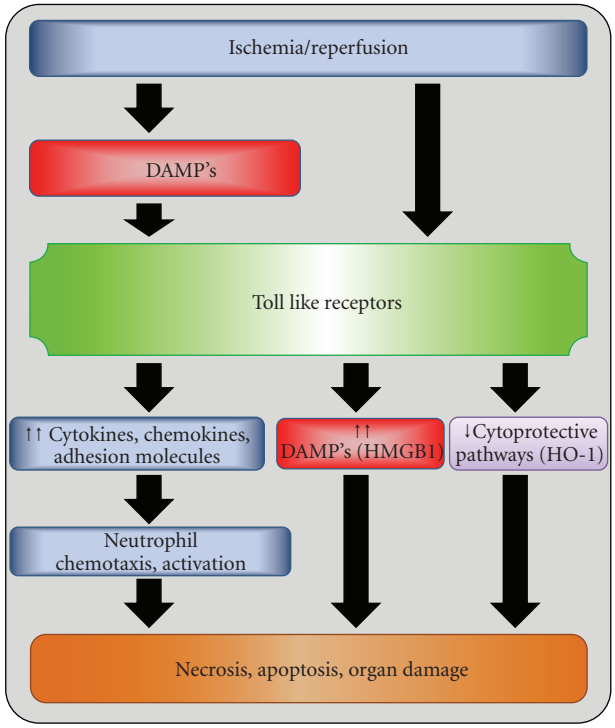

Figure 2: TLRs coordinate the response to hepatic I/R injury. Toll-like receptors are proximal to most elements of hepatic I/R injury. Following an ischemic insult, TLR activation by circulating DAMPs sets off signaling cascades in multiple cell types that coordinates the inflammatory response. Both TLR4 and TLR9 activation on hepatic nonparenchymal cells promotes production of proinflammatory cytokines, leading to additional neutrophil recruitment and organ damage. TLR4 activation has also been shown to augment the release of circulating DAMPs and may suppress protective cellular pathways.

[34], suggesting a common mechanism between these two ischemic insults.

One important question from early studies was the agent(s) responsible for activating TLR4 after I/R. While TLR4 is capable of recognizing a number of substrates, our laboratory has shown a key role for the endogenous nuclear molecule HMGB1 [32]. Administration of recombinant HMGB1 prior to I/R resulted in a significant increase in hepatocellular damage in TLR4 WT but not TLR4-deficient mice. Conversely, treatment with a neutralizing antibody to HMGB1 provided significant protection from I/R damage in WT mice but afforded no further protection from damage in TLR4-deficient mice. While this study showed that HMGB1 is capable of activating TLR4 in the setting of warm hepatic I/R, we subsequently found that TLR4 activation actively regulated the release of HMGB1 from hepatocytes. These studies showed that circulating levels of HMGB1 were significantly lower in TLR-defective mice after I/R, and we found this phenomenon to be dependent on TLR4-dependent production of ROS and Calcium/Calmodulin-Dependent Protein Kinase (CaMK) signaling [35]. Thus, while HMGB1 is an activator of TLR4, its release is also determined, in part, by TLR4 itself.

TLR4 is expressed in numerous cell types in the liver, including parenchymal hepatocytes and bone marrow-derived immune cells. In a study to delineate the role of TLR4 in different cell types of the liver, we generated TLR4 chimeric mice and found that hepatic injury after I/R is largely dependent on TLR4 expression on bone-marrow derived cells [36]. A chimeric mouse model in which recipient mice received lethal irradiation to eradicate bone marrow cells, followed by bone marrow transplantation, was used. This procedure permits reconstitution of bone marrow with syngeneic bone marrow from mice with either functioning or mutant TLR4 signaling. After 8–10 weeks, the immune cells within the liver are replaced with cells expressing the new phenotype while the long-lived parenchymal cells retain the host's phenotype. WT mice that lacked TLR4 on bone marrow-derived cells were protected from I/R injury similar to mice that lacked TLR4 in both parenchymal and bone marrow-derived cells. In contrast, transfer of WT TLR4 bone marrow cells to Mutant TLR4 mice resulted in significantly increased hepatocellular injury after I/R. In addition, WT mice, but not Mutant TLR4 mice, were protected from I/R injury following phagocytic cell depletion with gadolinium chloride while overexpression of dendritic cells with plasmid GM-CSF worsened damage in WT, but not Mutant, TLR4 mice [37]. Taken together, these data indicate that TLR4 signaling in NPCs, such as Kupffer and dendritic cells, is required for I/R-induced injury and inflammation. Similar results were reported in another study by Hui et al., who reported that TLR4 expression on both bone marrow and parenchymal cells was necessary for maximal damage after I/R. Chimeric mice that lacked TLR4 on either bone marrow-derived or parenchymal cells were protected from injury compared to mice with functional signaling in both cell populations. This study also showed that functional TLR4 signaling on nonbone marrow-derived cells (sinusoidal endothelial cells and hepatocytes) was necessary for the expression of ICAM-1, which adheres to circulating neutrophils recruited to the liver following damage. Neutrophil infiltration itself, however, was found to be dependent on bone marrow-derived cells [38].

Downstream TLR4 signaling pathways include both the MyD88 and TRIF signaling cascades, and TLR4-mediated hepatocellular damage appears to be independent of MyD88 signaling. This insight came from a study by Zhai et al. who used MyD88 KO and IRF3 KO mice. MyD88 KO mice were used to study one branch of the TLR4 signaling cascade while IRF3 KO mice were used to study TRIF-dependent signaling, since IRF3 is downstream of TRIF. They found that protection was not conferred to MyD88 KO mice during I/R while IRF3 KO mice were significantly protected [39]. These results suggested that TRIF-dependent signaling was critical in mediating warm I/R damage. Since TRIF-dependent signaling activates the interferon response, these authors undertook additional studies to delineate downstream mediators and found that both Type 1 Interferons and CXCL10 were critical for I/R damage in a TRIF-dependent fashion [40, 41]. This work suggests TLR4 mediated damage is MyD88 independent and involves activation of IRF3, type I interferons, and production of CXCL10.

Another member of the interferon regulatory factor family, IRF1, has also been shown to be downstream of TLR4 activation [42], and this molecule is important for I/R injury. IRF1 KO mice are protected from hepatocellular damage in warm I/R while *in vivo* adenoviral overexpression of IRF1 augments damage [43]. Furthermore, two studies using orthotopic liver transplantation have shown that IRF1 expression in hepatocytes is critical for mediating I/R injury [44, 45]. Unpublished work from our group shows that IRF1 expression during I/R is TLR4 dependent, as TLR4-defective mice have decreased levels of IRF1 expression after I/R. *In vitro*, IRF1 expression was decreased in TLR4-deficient hepatocytes, and adenoviral transfection of WT TLR4 into these cells restored IRF1 expression. We also found that IRF1 expression is critical for the release of the danger signal HMGB1. We observed that serum levels of HMGB1 in IRF1 KO mice were significantly lower than their wild-type counterparts, and IRF1 KO hepatocytes release significantly less HMGB1 into cell culture supernatants after nonlethal hypoxic stimuli. These novel findings connect three important mediators of I/R injury, TLR4, IRF1, and HMGB1.

5.2. Cold I/R. TLR4 has also been implicated in cold I/R injury during liver transplantation. Tsoulfas and colleagues showed that components of the LPS signaling pathway were inovolved in hepatic transplant preservation injury [46]. Subsequently, Shen et al. used an orthotopic liver transplant (OLT) model and found that absence of TLR4 signaling in donor livers protected recipients from hepatocellular damage. A similar pattern of protective changes observed in TLR4 KO grafts was observed in this model compared to similar studies in warm I/R. These changes include decreased serum ALT, CXCL10, ICAM-1, TNF-α, IL-1β, IL-2, and IFNγ and an increase in protective HO-1. These effects were observed regardless of the TLR4 genotype of the recipient [47]. In addition to murine studies, increased expression of TLR4 on monocytes of liver transplant patients has also been found correlate with acute rejection [48].

6. TLR2 in Liver I/R

Although the role of TLR2 in I/R is less well delineated, a number of studies have made important strides in determining its role. Classic TLR2 ligands include PAMPs from gram positive bacteria including peptidoglycan and lipoteichoic acid. Zhai and colleagues examined TLR2 KO mice in a model of warm I/R and found no significant difference in serum ALTs in TLR2 KO mice compared to WT controls [30]. Similar findings were noted by Shen and colleagues, who also showed that TLR2 KO mice were not afforded protection in warm hepatic I/R [31]. While no studies in hepatic I/R have identified a significant role for TLR2 in promoting or preventing damage, it has been implicated in other models of I/R, namely cardiac [49, 50], renal [51, 52], brain [53–55], and gut [56]. Taken together, these studies support the possibility that tissue and cell-type specific roles for TLRs exist.

7. TLR9 in Liver I/R

TLR9 is an intracellular molecule that functions as sensor for DNA. It was originally described by Hemmi et al. who showed that TLR9 KO mice failed to respond to bacterial CpG DNA [57]. Upon internalization of DNA from the plasma membrane, TLR9 translocates from the ER to endosomes and binds DNA. Although it was originally thought that TLR9 was specific for the recognition of bacterial DNA, it was subsequently discovered that host DNA is also capable of activating TLR9 [58–61]. In regard to liver I/R injury, Bamboat et al. found that TLR9 KO mice were protected from injury in warm I/R by a mechanism dependent on TLR9 expression on liver nonparenchymal cells. Using chimeric mice generated from adoptive bone marrow transfer, they reported that TLR9 expression on bone marrow-derived cells, but not liver parenchymal cells, to be important for damage after I/R [62]. *In vitro* experiments with hepatocytes and NPCs derived from TLR9 WT and KO mice suggested that DNA derived from necrotic hepatocytes was capable of activating TLR9-competent NPCs, causing increased production of proinflammatory cytokines. In contrast to its overall effect in liver I/R, a recent report from the same group showed that secretion of the anti-inflammatory cytokine IL-10 from conventional dendritic cells (cDCs) during liver I/R is TLR9 dependent. Depletion of cDCs resulted in increased liver damage after I/R, and this phenomenon could be reversed by exogenous transfer of WT cDCs prior to I/R. However, transfer of TLR9−/− cDCs failed to rescue mice from increased damage, suggesting that TLR9 activation in cDCs is protective [63]. These findings are in contrast to a report by our group, which demonstrated that DCs promote I/R injury through a TLR4-dependent pathway involving HMGB1 [37]. The seemingly conflicting roles of liver DC in liver I/R may be due to differences in the definition of DCs used in these studies. DC markers such as CD11c may be expressed in a wide variety of both intrahepatic leukocytes and nonhematopoietic cells [64, 65]. Thus, the role of various TLRs on DCs in modulating the hepatic microenvironment following I/R remains to be fully elucidated. These future studies will require stricter validation to determine that the cells being analyzed are indeed morphologically DCs.

8. Conclusion

Taken together, the results of the studies summarized in this paper provide convincing evidence demonstrating that TLR signaling is involved in the early activation of the innate immune system in the setting of I/R injury. Accumulating evidence points to endogenous molecules that are released from stressed or damaged cells or tissues during the course of I/R, as important triggers of the immune response in the setting of I/R (Figure 2). While much of the early work focused on the role of TLR4, new and future work will continue to delineate the roles of other TLRs in I/R injury. A better understanding of the molecular interactions involved in these processes, as well as greater knowledge of the intracellular pathways that mediate these signaling

cascades, may ultimately allow for the development of therapeutics aimed at ameliorating I/R injury and its adverse consequences.

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Review Article

Toll-Like Receptors in the Pathogenesis of Alcoholic Liver Disease

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In the multifactorial pathophysiology of alcoholic liver disease (ALD), inflammatory cascade activation plays a central role. Recent studies demonstrated that Toll-like Receptors, the sensors of microbial and endogenous danger signals, are expressed and activated in innate immune cells as well as in parenchymal cells in the liver and thereby contribute to ALD. In this paper, we discuss the importance of gut-derived endotoxin and its recognition by TLR4. The significance of TLR-induced intracellular signaling pathways and cytokine production as well as the contribution of reactive oxygen radicals is evaluated. The contribution of TLR signaling to induction of liver fibrosis and hepatocellular cancer is reviewed in the context of alcohol-induced liver disease.

1. Introduction

Alcohol abuse is a leading cause of morbidity and mortality worldwide [1] and alcoholic liver disease (ALD), ranging from steatosis, steatohepatitis to fibrosis and cirrhosis, affects over 10 million Americans [2]. Liver injury mediated by alcohol involves both liver parenchymal and nonparenchymal cells, including resident and recruited immune cells that contribute to liver damage and inflammation [3]. The concept of dysregulated innate immunity as an indispensable component of alcohol-induced liver disease dates back to the observations that patients with ALD have increased antibodies against *Escherichia coli* in plasma [4] and that chronic alcohol administration increases gut-derived endotoxin in the portal circulation, activating resident liver macrophages to produce several proinflammatory cytokines [5, 6]. Recognition of Toll-like receptors (TLR) as the key components involved in activation of the innate immune system enabled a substantial progress in understanding of the mechanisms mediating alcohol-induced liver injury.

2. Gut-Derived Bacterial Components Are Critical in the Pathogenesis of ALD

Due to its unique anatomy and blood supply the liver receives blood from the intestine, exposing hepatocytes and cells in the liver sinusoids not only to nutrients but also to gut-derived microbial products. The gut mucosal epithelium serves as an interface between the vast microbiota and internal host tissues [7]. Under normal circumstances, a normal balance of gut barrier function, gut permeability, and equilibrium of commensal and pathogenic microorganisms in the gut lumen is maintained and mostly prevents microbial translocation from the gut [8]. Lipopolysaccharide (LPS, endotoxin), a component of Gram-negative bacterial wall, and other components derived from bacteria in the intestinal microflora normally penetrate the mucosa only in trace amounts, enter the portal circulation, and are cleared by 80%–90% in the liver through uptake by Kupffer cells (resident liver macrophages) and hepatocytes in a manner that prevents cell damage or inflammation [9, 10]. These physiological uptake and detoxification are important for preventing systemic reactions to gut-derived bacterial components.

Multiple lines of evidence support the hypothesis that gut-derived endotoxin is involved in alcoholic liver injury Figure 1(a). First, it has been shown that excessive intake of alcohol increases gut permeability of normally nonabsorbable substances [11]. Second, intestinal Gram-negative bacteria, as well as blood endotoxin, are increased in acute [12, 13] and chronic [12, 14, 15] alcohol feeding models. Patients with alcoholic fatty liver, alcoholic hepatitis, and alcoholic cirrhosis have 5- to 20-fold increased plasma

endotoxin compared to normal subjects [8, 16] although it is unclear whether endotoxemia correlates with the extent of liver dysfunction [17, 18]. Third, intestinal sterilization with antibiotics [19] and displacement of Gram-negative bacteria with *Lactobacillus* treatment [20] prevented alcohol-induced liver injury. The mechanism underlying the disruption of the intestinal barrier appears to be multifactorial [21]. Disruption of tight junctions has been attributed to acetaldehyde [8] and liver-derived inflammatory cytokines, particularly TNF-α, that enter the systemic circulation and further disrupt tight junctions, thus perpetuating intestinal barrier dysfunction [22]. Gut permeability may be also increased by ethanol-induction of miR212, a microRNA that downregulates proteins of the zona occludens in intestinal cell culture and that was increased in colonic biopsy samples in patients with ALD [23].

Activation of Kupffer cells has been identified as one of the key elements in the pathogenesis of alcohol-induced liver damage. Kupffer cells are the largest population of tissue macrophages, predominantly distributed in the lumen of hepatic sinusoids, and exhibit endocytic activity against bloodborne materials entering the liver [10, 24]. Triggering of Toll-like receptor signaling drives Kupffer cells to produce inflammatory cytokines and chemokines and to initiate the inflammatory cascade [25]. Indeed, the essential role of Kupffer cells as a central component of the pathomechanism of ALD has been demonstrated in studies in mice and rats that show that inactivation of Kupffer cells with gadolinium chloride or liposomal clodronate can almost fully ameliorate alcohol-induced liver disease [26, 27].

3. Toll-Like Receptors Involved in ALD

The innate immune system recognizes conserved pathogen-associated molecular patterns, which are released during bacterial multiplication or when bacteria die or lyse [28], through pattern recognition receptors, including Toll-like receptors (TLRs) [29]. TLR4 recognizes endotoxin from Gram-negative bacteria, and TLR2 is essential for recognition of microbial lipopeptides, while TLR1 and 6 combined with TLR2 distinguish between triacyl- and diacyl-lipopeptides [30]. TLR3 recognizes viral double-stranded RNA, and TLR5 recognizes bacterial flagellin [31, 32]. TLR7 and TLR8 bind viral single-stranded RNA [33], and TLR9 recognizes prokaryotic CpG-rich DNA [34]. Kupffer cells express TLR4, TLR2, TLR3, and TLR9 [35–37], and hepatic stellate cells express TLR2, TLR4, and TLR9 [38, 39]. Liver sinusoidal endothelial cells express TLR4 [40, 41], and primary cultured hepatocytes express mRNA for all Toll-like receptors although they express very low levels of TLR2, TLR3, TLR4, and TLR5 and show weak responses *in vivo* [42, 43].

3.1. Role of TLRs in the Pathogenesis of Alcohol-Induced Liver Injury. Activation of Kupffer cells via TLR4-dependent mechanism plays a crucial role in the pathogenesis of alcohol-induced liver injury [6, 19, 44, 45]. LPS, a component of Gram-negative bacteria, is a potent activator of innate immune responses through its binding to the TLR4 complex and comprises three distinct parts: a carbohydrate (O-antigen), the oligosaccharide core region, and a lipid portion (Lipid A). Only the lipid A portion is immunogenic [46]. While TLR4 cannot directly bind LPS, the coreceptors CD14 and MD-2 bind LPS and upon LPS binding activate TLR4. CD14 is a GPI-anchored protein, which also exists in soluble form, and facilitates the transfer of LPS to the TLR4/MD-2 receptor complex that modulates LPS recognition [47]. MD-2 is a soluble protein that noncovalently associates with TLR4 and binds LPS directly to form a complex with LPS in the absence of TLRs [48]. The association between LPS and CD14 is facilitated by LPS-binding protein (LBP), which is a soluble shuttle protein [49]. TLR4, CD14, and LBP are critical in alcohol-induced liver injury. Alcoholic liver injury was prevented in C3H/HeJ mice [50], which have functional mutation in the TLR4 gene and have defective response to bacterial endotoxin [51]. Prevention of alcohol-induced liver inflammation and injury in C3H/HeJ mice was associated with decreased TNF-α expression, compared to wild-type mice. Similar protection from alcohol-induced liver injury was observed in mice deficient for LBP [52] and CD14 [53] whereas mice transgenic for human CD14 were hypersensitive to LPS [54].

Since disruption of intestinal barrier by ethanol increases permeability for macromolecular substances in general [8], it is likely that other bacterial components, in addition to LPS, are translocated to the portal blood in alcoholics. In particular, bacterial DNA was found in serum and ascites of patients with advanced liver cirrhosis leading to increased cytokine production in peritoneal macrophages [55–57]. Bacterial DNA, which is detected by TLR9, sensitizes the liver to injury induced by LPS via upregulation of TLR4, MD-2 and induction Th1-type immune response in the liver [58, 59]. Hepatic expression of TLR9 was increased in wild-type animals using the Lieber-DeCarli chronic alcohol feeding model, and alcohol feeding sensitized to TLR9 ligand CpG to enhance TNF-α production [60]. In patients with alcoholic cirrhosis, purified B cells stimulated with TLR9 ligand CpG *ex vivo* showed significant upregulation of immunoglobulin A, compared to B cells from control individuals [61], suggesting involvement of TLR pathways in alcohol-induced hyperimmunoglobulinemia [61–63]. Also, overexpression of TLR9, TLR4, and TLR2 was associated with impaired neutrophil function in alcoholic liver cirrhosis [64].

In addition to TLR4 and TLR9, increased expression of TLR1, 2, 6, 7, and 8 was observed in wild-type mice using the Lieber-DeCarli chronic alcohol feeding model, and feeding with alcohol resulted in sensitization to liver inflammation and damage because administration of TLR1, 2, 4, 6, 7, 8, and 9 ligands increased expression of TNF-α [60]. Interestingly, expression of these TLRs in mice on ethanol diet remained significantly increased in spite of concurrent administration of antibiotics that ameliorated liver injury [60]. Other investigations found that deficiency in TLR2 had no protective effect on alcohol-induced liver injury in a mouse model of chronic ethanol feeding [65] and that hepatic expression of TLR2 or TLR4 mRNA was

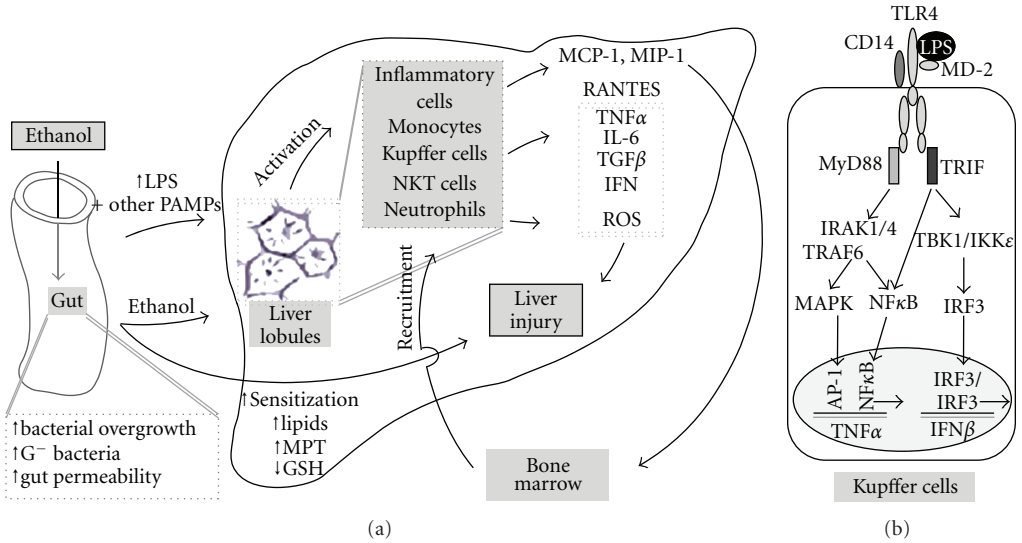

Figure 1: Pathophysiology of alcohol-induced liver injury. (a) Ethanol promotes translocation of LPS and other pathogen-associated molecular patterns (PAMPs) from the gut to the portal vein and to the liver. In the liver, LPS induces activation and recruitment of bone marrow-derived inflammatory cells. Activated bone marrow-derived cells synthesize inflammatory cytokines and reactive oxygen species that induce liver injury. Chronic ethanol per se contributes to sensitization of monocytes/macrophages to LPS and to sensitization of hepatocytes to the cytotoxic effect of inflammatory cytokines. The latter is brought about by accumulation of lipids, opening of mitochondrial permeability transition (MPT) pores, and depletion of glutathione (GSH). (b) In macrophages/Kupffer cells, TLR4 recognizes LPS in cooperation with its coreceptors, CD14 and MD-2. The signal is passed through MyD88-dependent or TRIF-dependent intracellular pathways, which activate various transcription factors, including AP-1, NFκB, and IRF3, and induces proinflammatory cytokine and Type I interferon genes.

not changed by chronic alcohol feeding or by acute ethanol administration [66], implying that the increased sensitivity of alcoholic fatty livers to LPS occurs without upregulation of TLR2 or TLR4 genes and may be related to an imbalance of proinflammatory/oxidative and cytoprotective mechanisms.

Taken together, it seems likely that sensitization to TLR ligands in alcohol-induced liver damage is regulated by multiple mechanisms, including those that are directly dependent on gut-derived bacterial components and TLR signaling, but also other mechanisms, such as lipid accumulation in hepatocytes [66, 67], histone acetylation in ethanol-exposed macrophages [68], or activation of Kupffer cells by C3 and C5 components of the complement pathway [69].

3.1.1. MyD88-Independent and Dependent TLR Pathways in ALD. TLR4 is unique among TLRs in its ability to activate two distinct signaling pathways Figure 1(b). One pathway is activated by the adaptors TIRAP (Toll/interleukin-1-receptor- (TIR-) domain-containing adaptor protein) and MyD88, which leads to activation of NF-κB and to the induction of proinflammatory cytokines. The second pathway (MyD88-independent) is activated by the adaptors TRIF and TRAM (TRIF-related adaptor molecule), which activates the TBK/IKKε kinase and interferon regulatory factor 3 (IRF3) to induce Type I IFNs as well as NF-κB activation [70, 71]. The two TLR4-dependent signaling pathways are induced sequentially, and the TRAM-TRIF pathway is only operational from early endosomes following endocytosis of TLR4 [72].

Recent evidence suggests that TLR4 downstream signaling in ALD is mediated predominantly through MyD88-independent pathway, rather than through the MyD88-dependent mechanism. Alcohol feeding with the Lieber-DeCarli diet [73, 74] resulted in significant steatosis and liver damage in MyD88-deficient mice compared to mice on pair-fed diet, and the extent of alcohol-induced changes was comparable in alcohol-fed MyD88-deficient and wild-type mice [65]. The involvement of the MyD88-independent TLR4 signaling pathway was indicated by upregulation of IRF7, an IRF3-inducible gene, in Kupffer cells [75]. In a different study it was reported that mice deficient in TRIF, which is a key TLR4 downstream adaptor in the MyD88-independent pathway, were protected against alcohol-induced liver disease, and it is likely that IRF3, a transcription factor downstream to TLR4/TRIF, binds to the TNF-α promoter resulting in induction of TNF-α [76]. These findings, together with the observation that mice deficient in IRF3 show protection against alcohol-induced liver injury (Szabo, unpublished data), demonstrate that TLR4-mediated signaling via MyD88-independent pathways is critical in induction of alcoholic liver disease.

3.1.2. Endotoxin Sensing and Loss of TLR Tolerance in ALD. Lipopolysaccharide is the most potent inducer of inflammatory cytokines, particularly TNF-α, in monocytes and macrophages. However, when LPS challenge is provided following an initial insult with LPS, induction of TNF-α is severely attenuated, a phenomenon called "LPS tolerance."

Recent studies demonstrated that upregulation of negative regulators of TLR signaling play a central role in TLR tolerance [77–79]. Experimental evidence suggests, however, that TLR tolerance can be broken by multiple sequential LPS administration *in vivo* and *in vitro* [80]. When mice were injected with a single dose of LPS, a second LPS challenge failed to induce significant serum TNF-α induction compared to the initial dose demonstrating TLR4 tolerance [81]. However, when LPS was given in 3-day intervals for 5 repeated times, the TLR tolerance was lost and serum TNF-α levels induced by the last dose of LPS were comparable to TNF-α induced by a single LPS administration [9]. The bimodal effects of LPS on TNFα production are reminiscent to the opposite modulation of inflammation by acute and prolonged alcohol use. At the cellular and molecular level, acute alcohol administration inhibited while chronic alcohol use increased production of proinflammatory mediators particularly when LPS was used as an inflammatory insult [82]. Increased TNF-α production and NF-κB activation were found in monocytes of patients with alcoholic steatohepatitis [83].

In vitro studies showed that prolonged alcohol exposure of monocytes for 4 days or longer *in vitro* augmented LPS-induced TNF-α production compared to alcohol-naïve cells [84]. The involvement of the TLR4 signaling pathway was suggested by increased IRAK-1 phosphorylation, increased IKK kinase activity, increased NF-κB nuclear translocation and DNA transactivation in human monocytes [84]. This upregulation of TLR4 signaling in the presence of diminished expression of IRAK-M in monocytes after prolonged alcohol treatment. Overexpression of IRAK-M prevented the increased LPS-induced TNF-α production in chronic alcohol-treated cells suggesting that loss of IRAK-M is likely to contribute to the loss of TLR tolerance in monocytes after prolonged alcohol exposure [84].

Previous studies have demonstrated that chronic alcohol use results in increased levels of LPS in the portal and systemic circulation that may mediate or amplify the loss of TLR4 tolerance after chronic alcohol treatment. The role of TLR tolerance and of the loss thereof may deserve further investigation [85].

4. Transcription Factors in ALD

The importance of molecular mechanisms culminating in nuclear events leading to activation of a wide array of transcription factors in various liver cell types is widely studied in progression of alcoholic liver injury. These transcription factors bind to the promoter regions in target genes resulting in induction of cytokines, chemokines, and various other mediators including kinases, adaptor proteins, and receptors. Studies using rodent models of alcoholic liver injury show that exposure to chronic alcohol increases expression of genes related to fatty acid synthesis and decreases fatty acid oxidation-related gene expression. Transcription factors such as sterol regulatory binding protein (SREBP) and peroxisome proliferator factor α (PPARα) play a pivotal role in fatty acid metabolism and are altered during chronic alcohol consumption. Liver-specific overexpression of SREBP-1α and SREBP-1c is observed in alcoholic liver injury leading to increased hepatic triglyceride content [86]. On the other hand PPARα expression and DNA binding activity were decreased in alcoholic livers resulting in decreased fatty acid oxidation [87]. Similar to alterations in transcription factors related to fatty acid metabolism, chronic alcohol-induced inflammatory mediators are also modulated by key transcription factors. The most studied transcription factor is NFκB, and alteration in its DNA binding activity has been observed in livers following chronic alcohol consumption [58] as well as in isolated monocytes/macrophages [88]. Chronic alcohol increased NFκB activity in monocytes and macrophages leading to an upregulation in various inflammatory cytokine and chemokine genes [89, 90]. Another transcription factor modulated by chronic alcohol exposure is AP-1 wherein increased expression and activity were observed in livers of chronic alcohol fed mice [91]. Activation of PPARγ, another transcription factor, was beneficial and prevented chronic alcohol-induced liver injury in mice [92]. While PPARγ is thought to be involved in anti-inflammatory cytokine production, its exact mechanism in alcoholic livers is not known. Furthermore Egr-1, another zinc finger transcription factor, is up-regulated in LPS-stimulated isolated Kupffer cells from chronic alcohol-fed mice and is dependent on ERK activation [93]. Egr-1 knockout mice were protective to alcoholic liver injury, indicating a role for the Egr-1-ERK pathway in the pathogenesis of alcoholic liver injury [94]. Thus, studies so far suggest that transcription factors play an important role in alcoholic liver injury, and future investigations are necessary to determine the complexity of regulation of the target genes in various liver types during alcoholic liver disease.

5. Proinflammatory Cytokine Induction in ALD

Alcoholic steatohepatitis is characterized by infiltration of various inflammatory cells in the liver, including monocytes, macrophages, neutrophils, and lymphocytes, which occurs as a consequence of activation of inflammatory mediators induced by TLR signaling [95, 96]. In humans with alcoholic steatohepatitis, serum TNF-α, IL-6, and IL-8 levels are increased, and their levels correlate with markers of the acute-phase response, liver function, and clinical outcome [97]. There is also evidence for activation of circulating monocytes in individuals with ALD, based on increased TNF-α production and increased NFκB activation [98–100].

Induction of TNF-α by TLR4 signaling and by reactive oxygen species in Kupffer cells has been identified as a major component in ALD [85, 101, 102]. The effect of TNF-α in hepatic inflammation and hepatocyte apoptosis is mediated through TNF receptor TNF-R1 [101]. Binding of TNF-α to TNF-R1 activates several signal transduction pathways [103], resulting in the activation transcription factors including NFκB and c-Jun-N-terminal kinase [104] and in activation of proapoptotic Fas-associated death domain [105].

Circulating levels of TNF-α and TNF-R1 are higher in patients with alcoholic steatohepatitis than in heavy drinkers with inactive cirrhosis, heavy drinkers who do not have

liver disease, and individuals with neither alcoholism nor liver disease [45, 83, 106]. High serum levels of TNF-α and TNF-R1 correlated with mortality in patients with acute alcoholic hepatitis [106–108]. Hepatic expression of TNF-R1 is enhanced in chronic ethanol consumption [109], and liver injury is substantially reduced when alcohol diet is administered in TNF receptor 1 (TNF-R1)—knockout mice or in rats that have been pretreated with anti-TNF-α antibodies or thalidomide, which reduces production of TNF-α [110, 111].

Under normal circumstances, hepatocytes are resistant to the proapoptotic effect of TNF-α; however, several conditions prime hepatocytes to TNF-α-mediated cell death in the setting of chronic alcohol consumption [112–115]. Hepatocytes from rats chronically fed alcohol have increased TNF-α induced cytotoxicity associated with mitochondrial permeability transition pore opening [114] and with a profound effect of alcohol on mitochondrial functional integrity [115, 116]. Also, decreased mitochondrial glutathione in alcohol-fed rats [117] or inhibition of hepatic transmethylation reactions by S-adenosylhomocysteine [113] has been shown to sensitize hepatocytes to TNF-α mediated cytotoxicity. Moreover, animal models of alcohol-induced liver injury show impaired function of proteasomes that increases hepatocyte sensitivity to TNF-α-mediated apoptosis [118]. Interestingly, although upregulation of TNF-R1 is observed in the livers of patients with alcoholic steatohepatitis [109], a recent *in vitro* study showed that free fatty acids sensitized HepG2 cells to TRAIL-mediated apoptosis, but not to cytotoxicity mediated by TNF-α [67].

In addition to the metabolic changes involved in sensitization to TNF-α cytotoxicity, the net effect of TNF-α on hepatocytes is influenced by other cytokines. For example, in mice that are deficient in IL-6, increased production of TNF-α induced by partial hepatectomy promotes death of hepatocytes instead of stimulating their proliferation [119]. Similarly, deficiency of IL-10, an anti-inflammatory cytokine inducible by adiponectin [120], exacerbates TNF-α-mediated liver injury in mice by alcohol [121]. Conversely, mice that are deficient in interleukin-12 [122], interferon-γ [123], or interleukin-18 [124] are protected against TNF-α-induced liver damage. The subtle balance between hepatocyte proliferation and apoptosis is also regulated by an autocrine cascade involving the pro-proliferative TGF-α and IL-1 receptor antagonist, and the antiproliferative IL-1β [125].

6. Toll-Like Receptors and Oxidative Stress in ALD

Cellular responses induced by oxidative stress play an important role in innate immune cell activation. Kupffer cells produce reactive oxygen species (ROS) in response to chronic alcohol exposure as well as endotoxin [126]. Interaction of NADPH with TLR4 is involved in LPS-mediated ROS generation and NFκB activation and production of inflammatory cytokines in neutrophils [127] and in human monocytes [128]. Pretreatment of chronic alcohol fed rats with inhibitor of NADPH oxidase diphenyleneiodonium (DPI) normalized ROS production, decreased LPS-induced ERK1/2 phosphorylation, and inhibited increased TNF-α production in Kupffer cells [126]. Inhibition of NADPH oxidase prevented steatosis, upregulation of TLR2, 4, 6, and 9 mRNA, and sensitization to respective ligand-induced liver injury [60], indicating a crosstalk between oxidative stress and TLR pathways in ALD. Protection from alcohol-induced liver injury was observed in p47 phox−/− mice, deficient in the main cytosolic component of NADPH oxidase, further supporting the important role of NADPH oxidase in alcohol-induced inflammatory response and liver injury [126].

7. TLR Signaling as Target for Therapy of ALD

Recently, a number of different approaches that modulate TLR signaling have been developed. These approaches include modulation of TLR ligand release from the intestine by probiotics [129, 130], activation of TLR signaling by synthetic TLR ligands [131–133], inhibition of TLR activation by small molecule inhibitors [134–136], and interference with cytokines induced by TLR signaling [137–139]. So far, probiotics and anticytokine therapeutic approaches have progressed into clinical trials in patients with ALD [129, 139, 140].

Modulation of intestinal microbiota using probiotics has been shown to reduce bacterial translocation [141, 142], circulating endotoxin levels in animal models [143], and bacterial infection, a marker for bacterial translocation, in patients with liver cirrhosis [144, 145]. Beneficial effects of probiotics have been reported in an animal model of alcohol-induced liver injury [20] and of LPS-induced liver injury [142, 146]. Patients with alcoholic liver cirrhosis treated with *Lactobacillus casei Shirota* three times daily for 4 weeks showed restoration of deranged neutrophil phagocytic capacity, compared to controls [130]. A recent open-label pilot trial showed that a 5-day administration of *Bifidobacterium bifidum* and *Lactobacillus plantarum* in alcohol-addicted psychiatric patients with mild alcoholic hepatitis ameliorated serum markers of liver injury to a significantly higher extent compared to control group treated with abstinence only [129]. These data suggest that modulation of the bowel flora may play a role in the pathogenesis and treatment of ALD and indicate a need for larger and rigorously designed clinical trials to support the use of probiotics in ALD.

While the role of TNF-α in the development of ALD has been well characterized [147], clinical investigations of the therapeutic efficacy of antibodies to TNF-α (e.g., infliximab) to treat patients with acute alcoholic hepatitis have generated variable results [139, 148]. There is particular concern about off-target effects of completely inhibiting TNF-α function. For example, since TNF-α is a critical component of immunity, infectious disease is a primary concern during TNF-α therapy [139, 149]. Moreover, TNF-α is required for normal liver regeneration as hepatocyte proliferation in response to injury is impaired in mice lacking TNF-α receptors [150]. Etanercept, a TNF-α neutralizing antibody,

appeared to increase short-term survival of patients with alcoholic hepatitis in a small pilot study [151] although a subsequent randomized, placebo-controlled trial conducted by the same investigators showed a worse 6-month survival rate in the group treated with etanercept than in the placebo group [152].

8. Alcohol: TLR Signaling and Liver Fibrosis

Alcohol-induced liver fibrosis is characterized by excessive deposition of extracellular matrix components due to increased matrix production and decreased matrix degradation [153]. Ethanol contributes to liver fibrosis in several aspects, including the upregulation of collagen transcription in hepatic stellate cells by acetaldehyde or reactive oxygen species from ethanol-exposed hepatocytes [154–157]. Also, phagocytosis of alcohol-induced hepatocyte apoptotic bodies activates hepatic stellate cells and Kupffer cells [158].

In addition, cytokines secreted by Kupffer cells activated by alcohol/LPS are of key importance in activation and transformation of hepatic stellate cells and induction of alcoholic liver fibrosis [153, 159, 160]. Recently, it has been shown that the crosstalk between Kupffer cells and hepatic stellate cells involves TLRs on both cell types [161]. Activated hepatic stellate cells express TLR4, CD14, and MD2. Stimulation of activated hepatic stellate cells with LPS resulted in a rapid activation of NF-κB, c-Jun N-terminal kinase and in upregulation of chemokines and adhesion molecules [162].

Interestingly, stimulation of hepatic stellate cells with LPS alone is not sufficient for their transformation into myofibroblasts. However, pretreatment with LPS strongly enhances response of hepatic stellate cells to TGF-β, which is a major profibrogenic cytokine derived predominantly from activated Kupffer cells [163]. The increased sensitivity of LPS-pretreated hepatic stellate cells to TGF-β has been linked to a TLR4-dependent downregulation of the TGF-β pseudoreceptor Bambi in HSCs, which is a negative regulator of TGF-β signaling [163]. Taken together, these findings suggest that LPS influences hepatic fibrosis via TLR4-dependent modification of TGF-β signaling in hepatic stellate cells and that hepatic stellate cells represent the primary liver cell compartment integrating inflammatory and fibrogenic pathways [164].

Additional components of the TLR system have been investigated as possible modulators of the fibrogenic process. Upon hepatocyte apoptosis, which is significantly increased in alcoholic liver disease, degradation of nuclear DNA activates immune cells via TLR9 [165]. Activation of TLR9 has been shown to modulate the biology of hepatic stellate cells, including inhibition of cell migration and upregulation of collagen production [38].

9. Alcohol: TLR Signaling and Hepatocellular Carcinoma

Alcoholic liver cirrhosis is a premalignant condition with approximately fourfold increase in the risk of hepatocellular carcinoma (HCC) [166]. The five-year cumulative incidence of HCC reaches 8% [167]. In addition, alcohol shows synergy with chronic hepatitis infection [168]. For example, the relative risk of developing HCC was 50-fold higher in heavy drinkers with chronic hepatitis C (HCV) whereas nondrinking HCV patients showed 15-fold increased risk, compared to abstaining controls without HCV [166].

Studies investigating the synergism between alcohol and HCV focused at the structural HCV core [169–171] and the nonstructural NS5A proteins [172, 173]. The HCV core protein causes overproduction of reactive oxygen species [169], induces insulin resistance [171], and inhibits very low density lipoprotein secretion from hepatocytes, contributing to steatosis [170]. However, although HCV core-transgenic mice fed with ethanol for 9 months have shown increased incidence of HCC, the mechanism of synergism between the HCV core protein and ethanol in hepatic carcinogenesis is not known [174].

Recently, the role of TLR4 in the synergism between alcohol and HCV nonstructural protein NS5A in hepatic oncogenesis has been proposed [175]. In a study with NS5A transgenic mice (NS5A Tg), it was reported that NS5A induces TLR4 expression in the liver. NS5A Tg mice developed fulminant hepatitis after administration of a single dose of LPS and showed aggravated alcoholic steatohepatitis after 4-week intragastric ethanol feeding [172]. Importantly, the adjuvant effect of NS5A was blunted in NS5A Tg mice who were deficient in TLR4 or who underwent gut sterilization with antibiotics, indicating the importance of endotoxin and TLR4 signaling in the synergism between alcohol/LPS and NS5A.

Furthermore, one-fourth of NS5A Tg mice fed Lieber-DeCarli ethanol diet for 12 months developed HCC, in contrast to no tumors found in WT or TLR4$^{-/-}$ NS5A mice, demonstrating that alcohol and NS5A synergistically induce liver tumors through TLR4 signaling [172]. Microarray analysis showed that NS5A Tg mice fed ethanol have increased liver expression of the stem/progenitor cell marker Nanog, which is involved in the genesis of CD133^{+} cancer stem cells. Nanog induction was dependent on NS5A and alcohol and was abrogated in TLR4$^{-/-}$ NS5A Tg mice fed alcohol. Further experiments demonstrated that Nanog is a novel downstream gene of TLR4 signaling.

Transplantation of p53-deficient hepatic progenitor cells transduced with Nanog or TLR4 resulted in spontaneous tumor development after 80 days or after repetitive LPS injections for 25 weeks, respectively. The tumor incidence caused by TLR4 transduction and LPS injections was reduced by coexpression of short hairpin RNA against Nanog, indicating that Nanog expression is involved in tumor formation and growth in this model [172]. Further experiments showed that Nanog-positive cancer stem cells did not upregulate TGF-β signaling after TLR4 activation [173]. Defective TGF-β pathway leads to spontaneous development of HCC [176].

Taken together, the recent data [172, 173] suggest that alcohol and HCV NS5A induce synergistic tumor development via induction and activation of TLR4 in mice and that this synergism involves the stem cell marker Nanog, which is

a TLR4-downstream regulated gene. These findings indicate that inhibition of TLR4 signaling may provide a therapeutic option for HCV-associated liver tumors.

10. Conclusion

In conclusion, there is clear evidence that alcohol consumption leads to increased intestinal permeability and endotoxemia, which results in activation of innate immunity via TLR4 signaling. Recent studies have contributed to the dissection of molecular mechanisms of TLR4 signaling in ALD, indicating the indispensable role of MyD88-independent pathway in mediating the effects of gut-derived endotoxin in ALD and suggesting the role of other TLRs in modulation of alcohol-induced liver injury. Moreover, novel data provide insight into the mechanisms of prolonged alcohol exposure on TLR4-induced inflammation and loss of LPS tolerance and the interplay between proinflammatory and anti-inflammatory cytokines mediating TLR-induced cytotoxicity. Further studies are needed to evaluate crosstalk between liver parenchymal and nonparenchymal cells. Understanding the cell-specific role of TLR signaling in ALD will further provide new insights into the pathogenesis of ALD and will reveal new targets for therapeutic intervention.

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Review Article

Role of Toll-Like Receptors and Their Downstream Molecules in the Development of Nonalcoholic Fatty Liver Disease

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Activation of innate immunity is associated with the development of liver disease, including non-alcoholic fatty liver disease (NAFLD). In the innate immune system, Toll-like receptors (TLRs) are sensors that recognize bacterial and viral components such as lipopolysaccharide, bacterial DNA, and peptidoglycan. Recent data have demonstrated that the liver is exposed to a high load of TLR ligands due to bacterial overgrowth and increased intestinal permeability in NAFLD. Upon stimulation by these TLR ligands, hepatic immune cells produce various mediators that are involved in host defense. On the other hand, these mediators alter lipid metabolism, insulin signaling, and cell survival. Indeed, some TLR-deficient mice demonstrate lesser degrees of NAFLD even though TLR ligands are increased. This paper will highlight the recent progress on the study of TLR signaling and their downstream molecules in the development of NAFLD.

1. Introduction

Nonalcoholic steatosis is a component of metabolic syndrome, and obese people with insulin resistance frequently have fatty liver disease [1]. Although steatosis is considered a benign liver disease, a subset of steatosis includes a progressive liver disease, nonalcohol steatohepatitis (NASH), that causes liver cirrhosis and cancer. In 1980, Ludwig et al. proposed the concept of "NASH", steatohepatitis without a history of excess alcohol intake [2]. Currently, the term "nonalcoholic fatty liver disease (NAFLD)" is more widely used because it is difficult to diagnose NASH at an early stage without histological examinations. Thus, NAFLD comprises a spectrum of disorders ranging from simple steatosis to advanced steatohepatitis and fibrosis. Since NAFLD has become the most common liver disease and the prevalence is estimated to be 14–24% of the population in developed countries [3–5], NAFLD is a growing public health concern worldwide.

Hepatic steatosis occurs when the amount of imported and synthesized lipids exceeds the export or catabolism in hepatocytes [6–8], including (1) increased lipid delivery to the liver, (2) increased lipid uptake in hepatocytes, (3) increased de novo lipogenesis in the liver, (4) failure of lipid export, and (5) impaired hepatic mitochondrial β-oxidation of fatty acids. Steatosis is present in most patients with insulin resistance, suggesting that dysfunction of insulin signaling is closely associated with excessive accumulation of lipid in the liver. However, hepatic inflammation and consequent fibrosis are not always observed in these patients, suggesting that additional factors are required for the development of NASH.

This paper highlights Toll-like receptors (TLRs) and their downstream targets, including inflammatory cytokines and chemokines, as emerging factors in the development of NAFLD. We further review the role of nuclear factor κB (NF-κB) and c-Jun N-terminal kinase (JNK), key molecules mediating TLR signaling in NAFLD. Since hepatic resident

macrophages, Kupffer cells, perceive various TLR ligands and produce inflammatory mediators through NF-κB and/or JNK activation [9–11], we will focus on TLR signaling in Kupffer cells.

2. The Gut-Liver Axis Is an Important Pathway in the Development of NAFLD

Gut microbiota, consisting of 15,000–35,000 species of bacteria, play a crucial role in nutrient absorption and energy storage [12–14]. Young conventionally reared mice have a 40% higher body fat and 47% higher gonadal fat content than germ-free mice, even though conventionally reared mice consume fewer calories. In addition, gnotobiotic mice exhibit a 60% increase in body fat within 2 weeks following transplantation of the gut microbiota from conventionally reared mice [15], indicating that gut microbiota contribute to nutrient acquisition. In particular, gut microbiota promote absorption of monosaccharides from the gut lumen, with resulting induction of de novo hepatic lipogenesis [15].

In addition to nutrient acquisition, gut microbiota are a source of bacterial products such as lipopolysaccharide (LPS), bacterial DNA, and peptidoglycan, which are delivered to the liver through the portal vein. In murine models of NAFLD, bacterial overgrowth is observed with compositional change as well as increased intestinal permeability by reducing the expression of tight junction proteins such as ZO-1 and occludin [16]. In human, the composition of gut microbiota differs between individuals with and without diabetes mellitus [17, 18]. Indeed, circulating bacterial components are elevated in NAFLD patients and in animal models [19–22]. As a result, liver cells are exposed to a high load of bacterial products that function as TLR ligands. Since TLR signaling is a key pathway to produce inflammatory cytokines and chemokines, the gut microbiota contribute to the development of NAFLD as a source of TLR ligands.

3. TLRs Are Associated with NAFLD

TLRs are associated with liver diseases including alcoholic liver injury, ischemia/reperfusion liver injury, liver fibrosis, and liver cancer [23, 24]. Among 13 TLRs identified in mammals, TLR2, TLR4, and TLR9 play a role in the development of NAFLD [20, 21, 25, 26]. To date, no information is available on the role of other TLRs in NAFLD. Results from gene-modified mice indicate that TLR4, and TLR9 signaling promote the progression of NAFLD.

Several groups have demonstrated that TLR4 signaling worsens NAFLD [19–21]. TLR4 is the receptor for LPS, a component of the Gram-negative bacterial cell wall. Serum LPS levels are increased in patients with hepatic steatosis caused by total parenteral nutrition or intestinal bypass [27–29]. Antibiotics treatment in these patients attenuates steatosis with decreased plasma levels of LPS [27–29]. Circulating LPS levels are elevated in most animal models of NAFLD induced by diets, including the high-fat (HF) diet, fructose-rich diet, methionine/choline-deficient (MCD) diet, and choline-deficient amino acid-defined (CDAA) diet [19–22]. Wild-type (WT) mice fed these diets show severe steatosis or steatohepatitis. In contrast, TLR4 mutant mice on these diets have less steatosis or steatohepatitis, although LPS levels are equivalent to those in WT mice. Even in mice on standard laboratory chow, continuous subcutaneous infusion of low-dose LPS results in hepatic steatosis, hepatic insulin resistance, and hepatic weight gain [30]. In addition, an intraperitoneal injection of LPS exacerbates liver injury in mice fed an MCD diet [31]. Eighty percent of intravenously injected LPSs molecules are detected in the liver within 20–30 min [32, 33]. These data indicate that the liver is the main target of LPS, and LPS-TLR4 is a key pathway for the progression of NAFLD.

TLR9 signaling contributes to the development of NASH [26]. TLR9 recognizes DNA containing an unmethylated-CpG motif that is highly expressed in bacteria-derived DNA [34]. Although bacterial DNA is detectable in blood and ascites in patients with advanced cirrhosis [35, 36], it remains unclear whether bacterial DNA is present at the early stage of liver disease and whether bacterial DNA contributes to NAFLD. We have recently demonstrated that bacterial DNA is detectable in the blood in a murine model of NASH, and that bacterial DNA binding to TLR9 contributes to the development of steatohepatitis [26]. WT mice on a CDAA diet showed severe steatohepatitis with insulin resistance. In contrast, TLR9-deficient mice had less steatohepatitis even though bacterial DNA was present in the blood [26]. In addition, TLR9-deficient mice demonstrated less insulin resistance and less fibrogenic response [26].

The role of TLR2 in NAFLD has not been well studied. TLR2 recognizes components of Gram-positive bacterial cell wall such as peptidoglycan and lipoteichoic acid [34]. At present, no studies have shown increased TLR2 ligands in NAFLD, which might be limited by current methodology. Blockade of TLR2 signaling prevents insulin resistance in HF diet-fed mice [37, 38]. In contrast, TLR2-deficient mice on an MCD diet exhibit equivalent levels of steatohepatitis but more severe steatohepatitis after LPS challenge compared to WT mice [25].

MyD88 is a key molecule in the development of metabolic syndrome including NAFLD [39, 40]. MyD88, an adaptor protein for all TLRs except for TLR3, is required for the expression of various inflammatory cytokines and chemokines [41]. MyD88-deficient mice are protected from metabolic syndrome including atherosclerosis [39, 40] and from liver injury induced by bile duct ligation or carbon tetrachloride [23, 42]. We have demonstrated that MyD88-deficient mice on a CDAA diet show less steatohepatitis with less insulin resistance compared with WT mice [26]. As expected, inflammatory cytokines and fibrogenic factors are also significantly suppressed in MyD88-deficient mice compared with WT mice fed a CDAA diet [26].

4. Endogenous TLR Ligands in NAFLD

Nonbacterial substances may function as TLR ligands; free fatty acids (FFAs) and denatured host DNA activate TLR2, TLR4 and TLR9 [43–46]. For instance, palmitate activates

WT macrophage but not TLR4-deficient macrophages [44]. Stearic acid and palmitic acid, potential TLR4 ligands, are rich in dietary fat, and circulating FFAs are elevated in patients with NAFLD [47]. These data demonstrate an association between TLR4 and FFAs. On the other hand, some reports have demonstrated that FFAs do not bind to TLR4 [48, 49]. LPS has a high affinity for lipids such as chylomicrons and fatty acids, suggesting that contaminated LPS in the lipids may be the actual TLR4 ligand. Although the LPS-lipids complexes still have affinity to TLR4, the toxic effect of LPS is decreased [50, 51]. Thus, the concept of lipids as endogenous TLR4 ligands is still unresolved. TLR4 also recognizes oxidized phospholipid [52] and HMGB-1 [53]. To date, the role of these TLR4 ligands has not been investigated in NAFLD.

Denatured host DNA is a candidate for a TLR9 ligand in liver injury. Apoptotic hepatocyte DNA induces type I collagen and TGFβ expression in hepatic stellate cells via TLR9 [45]. Denatured host DNA also stimulates sinusoidal endothelial cells to produce interleukin (IL)-1β via TLR9 [24]. In these studies, TLR9-deficient mice were resistant to carbon tetrachloride- or acetaminophen-induced sterile liver injury. If apoptotic host DNA functions as a TLR9 ligand, NASH livers are constantly exposed to TLR9 ligands because hepatocytes undergo apoptosis and necrosis in NASH. However, the unmethylated CpG-motif is uncommon in mammalian DNAs [54], and host DNA is recognized by other DNA sensors such as DNA-dependent activator of IFN-regulatory factors and the inflammasome which sense cytosolic DNA in TLR9-indendent manner [55, 56]. Although some FFAs and denatured host DNA are attractive candidates for TLR ligands, further investigations are necessary to determine whether these nonbacterial substances function as reliable TLR ligands in NAFLD.

5. Liver Cells That Perceive TLR Ligands

The liver is composed of various types of cells including hepatocytes, biliary epithelial cells, hepatic stellate cells, Kupffer cells, and sinusoidal endothelial cells. Most types of liver cells are reported to express TLRs and produce various inflammatory mediators in response to TLR ligands [10]. For instance, hepatic stellate cells and sinusoidal endothelial cells produce chemokines and inflammatory cytokines in response to a TLR4 ligand [23] and a TLR9 ligand [24], respectively. Among resident liver cells, Kupffer cells are well documented to respond to various TLR ligands such as peptidoglycan, double-stranded RNA, LPS, bacterial DNA, and probably other TLR ligands. In addition, Kupffer cells are a major source of inflammatory cytokines such as TNFα and IL-1β [9, 11]. These cytokines produced by Kupffer cells promote lipid accumulation and cell death in hepatocytes as described below in detail. These cytokines also induce hepatic stellate cells to produce profibrogenic factors such as TIMP1 and PAI-1 [26, 57, 58]. Thus, Kupffer cell-derived mediators through TLRs affect lipid metabolism, liver damage and liver fibrosis in NAFLD (Figure 1). Indeed, depletion of Kupffer cells ameliorates the progression of diet-induced steatohepatitis. Rivera et al. have reported Kupffer cell depletion delayed the development of steatohepatitis induced by an MCD diet [20]. We also have shown that depletion of Kupffer cells decreased inflammatory cytokines in mice on a CDAA diet, resulting in improvement of NASH [26]. These findings indicate that Kupffer cells play a pivotal role in the development of steatohepatitis. On the other hand, the roles of Kupffer cells in HF diet models, a simple steatosis model, are more complicated. While most of reports have shown that depletion of Kupffer cells ameliorates steatosis [59–62], one report shows an opposite effect [63]. This discrepancy may partially depend on the methodology to deplete Kupffer cells. Clodronate liposome was used to deplete Kupffer cells by intravenous injection [61] or intraperitoneal injection [62, 63]. Intravenous injection selectively depletes Kupffer cells and/or splenic macrophages but not visceral fat macrophages whereas intraperitoneal injection affects both Kupffer cells and visceral fat macrophages [23, 64]. Adipose tissue macrophages are activated in an HF diet model [63] and release various mediators such as TNFα and IL-6, which influence insulin signaling and lipid metabolism. These mediators could further activate Kupffer cells, and contribute to steatosis. Although further studies are necessary to determine the role of adipose tissue macrophages in the development of NAFLD, it is clear that Kupffer cells are important in the development of NAFLD.

6. NF-κB Activation in NAFLD

Activation of the transcriptional factor NF-κB, a downstream target for TLR-MyD88 signaling, is crucial for the inflammatory response in immune cells and is a key in the development of NAFLD [10, 11]. In NAFLD patients as well as animal models of NAFLD, NF-κB activation is observed in liver cells, including hepatocytes, hepatic stellate cells and Kupffer cells [23]. Hepatocytes respond minimally to TLR ligands in vivo, suggesting that other mediators activate NF-κB in hepatocytes [23, 65]. For instance, TNFα and IL-1β activate NF-κB in hepatocytes [26, 66]. On the other hand, TLR ligands directly activate NF-κB in Kupffer cells. TLR signaling triggers inflammatory cytokine and chemokine production in Kupffer cells through NF-κB activation [26, 67]. IKKβ activates NF-κB by the phosphorylation and subsequent degradation of Iκ-B, an essential inhibitor for NF-κB. Specific deletion of IKKβ in myeloid cells including macrophages results in suppression of inflammatory cytokine production, which prevents systemic insulin resistance induced by an HF diet [68].

It is unclear whether NF-κB activation in hepatocytes leads to steatosis. Hepatocyte-specific IKKβ overexpression induces steatosis [69]. In contrast, NF-κB essential modulator (NEMO) deficiency in hepatocytes results in spontaneous steatohepatitis [70]. NEMO deficiency completely blocks NF-κB activation, indicating that NF-κB activation in hepatocytes is not a primary cause of steatosis. We and others have recently demonstrated that hepatocytes increase their lipid content in response to TNFα and IL-1β [26, 71].

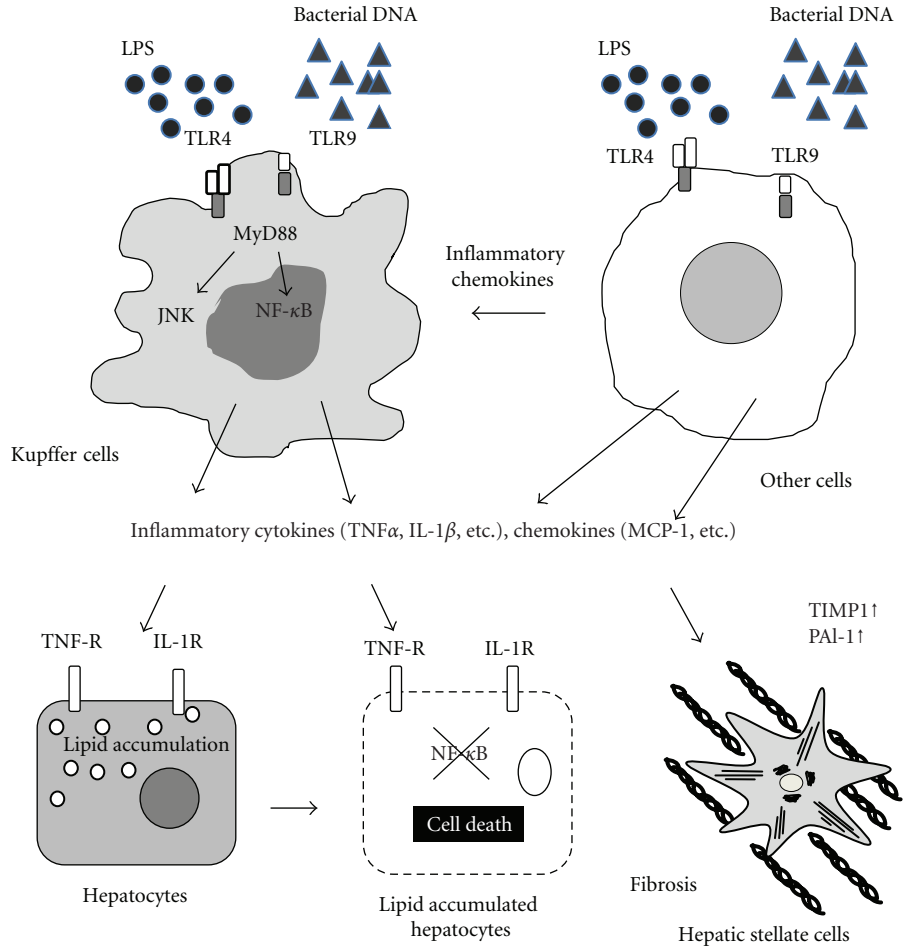

Figure 1: TLRs and downstream signaling in NAFLD. Kupffer cells respond to TLR ligands such as LPS and bacterial DNA through TLR4 and TLR9, respectively. Upon TLR ligation, MyD88, an adaptor molecule, is recruited to transmit the signals that activate NF-κB and JNK. Activated Kupffer cells produce inflammatory cytokines such as TNFα and IL-1β and chemokines such as MCP-1 (CCL2). These inflammatory cytokines and chemokines induce lipid accumulation in hepatocytes and cell death. In addition, TNFα and IL-1β promote liver fibrosis by activating hepatic stellate cells. Other cells including hepatic resident cells, infiltrated cells into the liver, and adipose tissue macrophages produce various mediators in response to TLR ligands. These pathways also contribute to the development of NAFLD.

In that process, TNFα and IL-1β activate NF-κB in normal hepatocytes. On the other hand, NF-κB activation by TNFα and IL-1β is blunted in lipid-laden hepatocytes [26]. NF-κB activation in hepatocytes may be required only for an initial step of lipid accumulation in the liver. Regardless of NF-κB activation in hepatocytes, inflammatory cell infiltrations and expression of F4/80, a marker for macrophage, are increased in both hepatocyte-specific IKKβ overexpressed mice and hepatocyte-specific NEMO-deficient mice [69, 70]. These data further support the concept that NF-κB activation in immune cells is a key event in the development of NAFLD.

7. JNK Activation in NAFLD

TLR-MyD88 signaling pathway activates JNK, a member of mitogen-activated protein kinases. JNK is an attractive target in the pathogenesis of NAFLD, because JNK activation plays a central role in the development of obesity and insulin resistance [72]. In patients and animals with NASH, JNK is activated in the liver, and JNK activation in immune cells results in inflammatory cytokine production [22]. Current research is analyzing the distinct roles of the JNK isoforms, JNK1 and JNK2, in the development of the metabolic syndrome including NAFLD. JNK1 promotes steatosis and inflammation in two different models of NAFLD [73]. In contrast, lack of JNK2 promotes liver injury [74]. However, it must be noted that the roles of JNKs are different in hepatocytes and Kupffer cells; JNK activation in hepatocytes is involved in cell death and insulin signaling whereas JNK activation in Kupffer cells induces inflammatory cytokine production. Recently, we and others have demonstrated the role of hematopoietic cells in the development of the metabolic syndrome including NASH [22, 75]. The results from chimeric mice generated by transplanting bone marrow cells lacking JNK1 or JNK2 into WT mice have shown that JNK1 in hematopoietic cells

contributes to developing metabolic syndrome by producing inflammatory cytokines. Thus, hematopoietic cells including Kupffer cells and recruited macrophages play a pivotal role in the development of NAFLD. On the other hand, JNK in hepatocytes is involved in cell death and insulin signaling. Thus, JNK plays multiple roles in multiple steps in NAFLD.

8. Inflammatory Cytokines and NAFLD

Inflammatory cytokines are important mediators in the development of NAFLD. Among inflammatory cytokines, TNFα and IL-1β have multiple functions including immune modulation, cell differentiation, proliferation, apoptosis, and energy metabolism. Indeed, expressions of TNFα and IL-1β are increased in NAFLD patients and animal models [76–79]. In contrast, most of TLR-deficient mice show decreased TNFα and IL-1β levels in NAFLD models [20, 26].

TNFα levels are increased in the liver, the adipose tissue, and the serum of NAFLD patients [76, 77]. Expression of TNF receptors is also increased in the liver of NAFLD patients [77]. Mice deficient in both TNF receptor type 1 and type 2 demonstrate less steatosis, inflammation, and liver fibrosis in a NASH diet model [80], indicating that TNF receptor signaling contributes to the development of NAFLD. So far, several mechanisms of TNFα-mediated functions are proposed: (1) insulin resistance, (2) release of fatty acids from adipose tissue, (3) regulation of lipid influx and efflux in hepatocytes, and (4) hepatocyte cell death. TNFα impairs insulin signaling by suppressing insulin receptors, insulin receptor substrate-1 and GLUT4 expressions [80], and by the expression of SOCS-3. As a result of insulin resistance, FFAs and glucose uptake are inhibited in adipocytes, whereas increased insulin levels promote FFA flux into hepatocytes and hepatic lipogenesis [81]. Moreover, TNFα increases fatty acid release from adipose tissue by promoting lipolysis, resulting in insulin resistance. In addition to impaired insulin signaling and FFA metabolism, TNFα promotes cholesterol accumulation in hepatocytes by inducing expression of LDL receptor and by inhibiting efflux of cholesterol [71]. Thus, TNFα promotes lipid accumulation in hepatocytes inducing insulin resistance, increased FFA levels, and lipid retention in the cells. Lipid-accumulated hepatocytes are vulnerable to various stimuli such as TNFα. In NASH patients, hepatocyte apoptosis and necrosis frequently occur. TNFα stimulation alone does not induce cell death in normal hepatocytes, because TNFα induces the upregulation of NF-κB-related antiapoptotic genes [66]. However, impaired lipid metabolism leads to hepatocyte apoptosis in the presence of TNFα. Hepatocytes laden with lipids have increased susceptibility to TNFα-induced cell death [82, 83]. Free cholesterol accumulation in hepatocytes depletes mitochondrial glutathione. This induces ROS generation in hepatocytes and then evokes cell death signaling [82]. In addition, lipid-accumulated hepatocytes increase the expression of ASK-1 and JNK in response to TNFα [83], which lead to cell death. These findings demonstrate that TNFα plays an important role in lipid metabolism as well as hepatocyte cell death in the development of NAFLD.

Increased IL-1β is recognized as a risk factor for the metabolic syndrome [84]. Indeed, expression of IL-1β as well as its receptor is increased in the adipose tissue of obese patients with type II diabetes [78, 79]. Single-nucleotide polymorphisms of IL-1β, which may elevate circulating IL-1β, are frequently observed in patients with metabolic syndrome including atherosclerosis [85, 86] and NASH [87]. In addition to these findings, blockade of IL-1β decreased the severity of atherosclerosis and insulin sensitivity in animal models [88, 89]. HF diet feeding, a diet model for obesity and hepatic steatosis, results in severe steatohepatitis in IL-1 receptor antagonist-deficient mice [90], suggesting that IL-1β plays an important role in NASH. The proposed functions of IL-1β are as follows: (1) lipid accumulation in hepatocytes [26, 71], (2) hepatocyte cell death [26], and (3) activation of hepatic stellate cells [26, 57, 58]. IL-1β promotes hepatic steatosis by activating PPARα [62] and diacylglycerol acyltransferase 2, an enzyme that converts diglyceride to triglyceride [26]. In addition, IL-1β promotes cell death in lipid-accumulated hepatocytes. Upon IL-1β stimulation, antiapoptotic genes are upregulated in normal hepatocytes. In contrast, proapoptotic genes such as Bax are induced in lipid-accumulated hepatocytes treated with IL-1β [26]. IL-1β induces the production of nitric oxide, generating peroxynitrite in the presence of superoxide radicals, which induces hepatocellular injury. In NAFLD, free radicals are generated by β-peroxidation of FFAs, and nitric oxide metabolites are increased in rats with NASH [91], which may further promote liver injury. Moreover, IL-1β contributes to liver fibrosis by activating hepatic stellate cells [26, 57, 58]. IL-1β induces the expression of TIMP-1 and TGFβ in hepatic stellate cells. We have shown that IL-1R-deficient mice are resistant to CDAA diet-induced liver fibrosis [26]. Thus, IL-1β is an important factor in the development of NAFLD.

9. Chemokines and NAFLD

Chemokines, strongly induced by TLR stimulation, play an important role in the development of metabolic syndrome including NAFLD. TLR4- and MyD88-deficient mice, which are resistant to metabolic syndrome, show reduced chemokine production compared with WT mice [39, 40]. MCP-1 levels are elevated in genetically obese diabetic (db/db) mice and in HF diet-fed mice. This suggests that MCP-1 and its receptor CCR2 contribute to the metabolic syndrome including obesity-related steatosis [92–95]. Indeed, MCP-1 overexpressing transgenic mice exhibit insulin resistance and hepatic steatosis as well as macrophage infiltration in adipose tissue. In contrast, MCP-1- or CCR2-deficient mice have attenuated HF diet-induced steatosis and macrophage infiltration. In these mice, inflammatory cytokine production is reduced, which could ameliorate steatohepatitis. Moreover, administration of a CCR2 antagonist improves insulin resistance. Clinical studies also demonstrate that MCP-1 levels in adipose tissue positively correlate with BMI, and patients with type II diabetes have higher serum MCP-1 levels than nondiabetes [96].

In addition to macrophage recruitment, MCP-1 promotes hepatic lipid accumulation by increasing lipid synthesis and by inhibiting lipid efflux from hepatocytes [97]. MCP-1 increases PEPCK level, resulting in de novo lipogenesis. MCP-1 decreases secretion of ApoB, which suppresses lipid efflux. Since hepatocytes do not express CCR2, hepatocytes may utilize other receptors such as CCR7 and CCR8 as the receptors for MCP-1. Thus, MCP-1 regulates lipid metabolism through macrophage recruitment and also directly on hepatocytes.

10. Perspectives

This paper summarized the role of TLRs and their downstream molecules in the development of NAFLD and showed that TLR signaling mediates steatosis, inflammation, and fibrosis. Thus, regulation of TLRs and their downstream molecules is potential targets for the therapy of NAFLD, in particular NASH. Several antagonists for TNFα, IL-1β, and CCR2 are used in NAFLD animal models [89, 93, 98–101]. In the future, these agents may be new tools for the therapy of human NAFLD. In addition to the blockade of TLR signaling, control of TLR ligands is another option for the therapy of NAFLD. Probiotics may suppress the growth of harmful intestinal bacteria and the generation of TLR ligands in the intestine. As a result, exposure to TLR ligands may be decreased in the liver. Beneficial effects of probiotics have been reported in animal NAFLD models [98, 102, 103]. Since their adverse effects are minimal in humans, randomized clinical trials of adequate size and methodology are needed for assessing the benefit of using probiotics on the NAFLD patients.

TLRs play multiple roles in multiple steps and in many hepatic cells in the development of NAFLD. In this review, we focused on the TLR signaling in Kupffer cells that produce key mediators in NAFLD. Other resident liver cells and recruited immune cells also produce many mediators that modulate the status of NAFLD in response to TLR ligands (Figure 1). Thus, better understanding of TLR signaling will provide new insight into the management and prevention of NAFLD.

Abbreviations

CCR: C-C chemokine receptor
CDAA: Choline-deficient amino-acid defined
CSAA: Choline-supplemented amino acid defined
FFA: Free fatty acid
HF: High fat
HOMA-IR: Homeostasis model assessment of insulin resistance
IL: Interleukin
IL-1R: IL-1 receptor
JNK: c-Jun N-terminal kinase
LPS: Lipopolysaccharide
MCD: Methionine and choline deficient
MCP-1: Monocyte chemoattractant protein-1
NAFLD: Nonalcoholic fatty liver disease
NASH: Nonalcoholic steatohepatitis
NEMO: NF-κB essential modulator
NF-κB: Nuclear factor κB
TNF: Tumor necrosis factor
TLR: Toll-like receptor
WT: Wild type.

Conflict of Interests

There is no conflict of interests to disclose for all authors.

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Review Article

Toll-Like Receptor Signaling and Liver Fibrosis

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Liver fibrosis occurs as a wound-healing scar response following acute and chronic liver inflammation including alcoholic liver disease, non-alcoholic steatohepatitis, hepatitis B and C, and autoimmune hepatitis. Myofibroblasts, mainly transdifferentiated from hepatic stellate cells, are pivotal cell types that produce fibrillar collagen. The activation of inflammatory cells, including Kupffer cells, is a crucial step for activating hepatic stellate cells. Toll-like receptors (TLRs) are pattern recognition receptors that sense pathogen-associated molecular patterns (PAMPs), which discriminate the products of microorganisms from the host. TLRs are expressed on Kupffer cells, endothelial cells, dendritic cells, biliary epithelial cells, hepatic stellate cells, and hepatocytes in the liver. TLR signaling induces potent innate immune responses in these cell types. The liver is constantly exposed to PAMPs, such as LPS and bacterial DNA through bacterial translocation because there is a unique anatomical link, the portal vein system between liver and intestine. Recent evidence demonstrates the role of TLRs in the activation of hepatic immune cells and stellate cells during liver fibrosis. Moreover, crosstalk between TLR4 signaling and TGF-β signaling in hepatic stellate cells has been reported. This paper highlights the role of TLR signaling in stellate cell activation and the progression of liver fibrosis.

1. Introduction

Liver fibrosis is a wound healing scar response following acute and chronic liver diseases including chronic hepatitis B and C, autoimmune hepatitis, nonalcoholic steatohepatitis, and alcoholic liver disease [1, 2]. The pathohistological findings of liver cirrhosis, the endstage of liver fibrosis, show hepatocellular death, a lobular inflammatory cell infiltrate, excessive deposition of extracellular matrix (ECM) protein, and the appearance of regenerative nodules that may result in liver failure, portal hypertension, and hepatocellular carcinoma [1, 2]. Thus, wound healing scar response in the liver represents a harmful response rather than a beneficial response in liver regeneration. Liver fibrosis is highly associated with chronic hepatocellular injury and subsequent inflammatory response that produces inflammatory cytokines and recruits inflammatory leukocytes into the injured site. This inflammatory circumstance in the liver drives the activation of hepatic stellate cells (HSCs) through various fibrogenic mediators including TGF-β and PDGF [1, 2]. Activated HSCs transdifferentiate into myofiboblasts, which then produce excessive ECM proteins, including collagen type I, III, and IV. This leads to an irreversible collagen deposition, resulting in liver fibrosis [1, 2].

Lipopolysaccharide (LPS, also known as endotoxin) levels in systemic and portal vein blood are increased in patients with cirrhosis [3, 4]. LPS is a Gram-negative bacterial cell wall component that binds to the pattern recognition receptor, Toll-like receptor (TLR) 4 with its coreceptors MD-2 and CD14, transmits the signals through adaptor proteins MyD88, TIRAP, TRIF, and TRAM to activate the kinases, IRAK1, IRAK4, TAK1, JNK, and IKK. These intracellular kinases lead to the activation of the transcription factors NF-κB, AP-1, and interferon regulatory factors (IRFs) resulting in the induction of potent innate immune responses [5]. Kupffer cells, resident macrophages in the liver, are known to respond to LPS through TLR4 to produce various inflammatory cytokines including TNF-α, IL-1β, IL-6, IL-12, IL-18, and chemokines in granulomatous liver disease, ischemia/reperfusion liver injury, nonalcoholic

steatohepatitis, and alcoholic liver disease [6–8]. HSCs, central cell types in liver fibrosis, also express high levels of TLR4 [9, 10]. A unique anatomical link, the portal vein system between the liver and intestines, may allow for the exposure of bacterial products, including LPS and bacterial DNA, to the liver [11]. However, the hepatic immune response is strictly regulated to avoid a harmful response in physiological conditions [11, 12]. In addition, sterile inflammation may provide endogenous TLR ligands for the activation of danger signals inducing fibrogenic response [7]. This paper summarizes the role of TLR signaling in HSC activation and liver fibrosis.

2. TLR Signaling in Hepatic Stellate Cells

HSCs are located in the space of Disse in the normal liver. Quiescent HSCs are primary cell types that store large amounts of Vitamin A-containing lipid droplets in the human body [1]. Activated HSCs are the major source of ECM protein in the fibrotic liver. Following liver injury, HSCs are activated by various fibrogenic stimuli, including TGF-β and PDGF, and inflammatory cytokines that are mainly produced from Kupffer cells [1, 2]. After activation, HSCs lose Vitamin A-containing lipid droplets and transdifferentiate into myofibroblasts that highly express α-smooth muscle actin (SMA). The excessive production and deposition of ECM proteins cause hepatic fibrosis [2]. We have demonstrated that activated human HSCs express TLR4 and its coreceptors MD-2 and CD14 [9]. LPS treatment induces the strong activation of NF-κB and JNK/AP-1 pathways in activated HSCs. LPS enhances expression of the adhesion molecules ICAM-1 and VCAM-1 on the cell surface, and induces the secretion of chemokines, IL-8 and MCP-1 in activated HSCs. LPS-induced IL-8 secretion is completely blocked by inhibiting NF-κB activation and partially inhibited by JNK inactivation, indicating the critical role of NF-κB and JNK in TLR4 signaling of HSCs. Recently, our study demonstrated that TLR4 upregulates the expression of various types of chemokine (MCP-1, MIP-1α, MIP-1β, RANTES, KC, MIP-2, and IP-10) and TLR2, and it downregulates the expression of bone morphogenetic protein (BMP) and activin membrane bound inhibitor (Bambi), a transmembrane suppressor of TGF-β signaling [10, 13]. Bambi is a type I TGF-β receptor that lacks an intracellular kinase domain and acts as an inhibitor of BMP, activin and TGF-β signaling. Overexpression of Bambi inhibits, while a dominant negative form of Bambi enhances, TGF-β signaling in HSCs [10]. Thus, TLR4-mediated Bambi downregulation augments TGF-β signaling in HSCs.

Although the ligands for TLR3 and TLR4 stimulate HSCs to induce IFN-β production through adaptor TRIF in macrophages [5], HSCs could produce IFN-β in response to the ligand for TLR3, but not TLR4, suggesting unique TLR3/TLR4-TRIF signaling pathways in HSCs, which might be distinct from those in macrophages [14].

HSCs express TLR2, a receptor for Gram-positive bacterial cell wall components, such as peptidoglycan and lipoteichoic acid [5, 15]. HSCs barely respond to TLR2 ligands. Pretreatment of TNF-α or IL-1β significantly upregulates TLR2 expression in HSCs. This primes HSCs to increase NF-κB activation and IL-8 production in response to TLR2 ligands [16]. LPS also upregulates TLR2 expression in HSCs [10], suggesting that the initiation by inflammatory mediators such as TNF-α, IL-1β, and LPS might be required for fulfilling TLR2 signaling in HSCs.

TLR9 that recognizes bacteria-derived, unmethylated CpG-containing DNA, is expressed in HSCs [17]. Watanabe et al. has demonstrated that host-derived denatured DNA from apoptotic hepatocytes induces a differentiation of HSC via TLR9 [17]. Apoptotic hepatocyte DNA induces fibrogenic responses with the elevation of mRNA levels of TGF-β and collagen type I in HSCs. In addition, apoptotic hepatocyte-derived DNA inhibits PDGF-induced HSC chemotaxis through TLR9 and MyD88 [17].

3. TLR4 Signaling in Liver Fibrosis

The activation of both HSCs and Kupffer cells that express TLR4 is associated with the progression of liver fibrosis. TLR4-mutant mice have less liver inflammation and fibrosis than TLR4-wild-type mice following bile duct ligation (BDL) and chronic treatment of carbon tetrachloride (CCl_4), or thioacetamide [10]. Mice deficient in CD14 and LPS-binding protein also show decreased cholestasis-induced liver fibrosis [18]. These results suggest a strong contribution of LPS-TLR4 interaction in the development of liver fibrosis. Indeed, systemic plasma LPS levels are significantly elevated in these three mouse models of experimental liver fibrosis [10, 19, 20], suggesting that intestinal microflora-derived LPS translocates into the liver through the portal vein by increased intestinal permeability following liver injury. We have tested the contribution of intestinal microflora in liver fibrosis. Mice were orally treated with a cocktail of nonabsorbable broad-spectrum antibiotics (ampicillin, neomycin, metronidazole, and vancomycin) for 4 weeks prior to induction of liver fibrosis [10, 21]. This antibiotic cocktail successfully reduced plasma LPS levels after BDL, leading to a significant attenuation of liver inflammation and fibrosis [10]. Thus, intestinal microflora-derived and translocated LPS participate in TLR4-mediated liver fibrosis, most likely due to increased intestinal permeability induced by intestinal dysbiosis, such as bacterial overgrowth, and disintegrity in the tight junction of intestinal epithelium. TLR4 is also activated by endogenous ligands, such as HMGB1, hyaluronan, and heat shock protein 60 [15, 22, 23]. Currently, we do not have strong evidence that endogenous TLR4 ligands are involved in liver fibrosis. Further investigation is needed.

Kupffer cells are well-known targets for TLR4 ligand LPS and produce various types of inflammatory and fibrogenic cytokines, which may activate HSCs [6, 7]. Quiescent and activated HSCs also express TLR4 [9, 10]. The specific roles of TLR4 in Kupffer cells and HSCs during liver fibrosis were unknown. To investigate these roles, we generated TLR4-chimeric mice by using bone marrow (BM) transplantation (BMT) [10]. Kupffer cells are known as radio-resistant

cells [24, 25]. Thus, a standard type of BMT with whole body irradiation insufficiently replaces Kupffer cells with donor BM-derived cells. To resolve this problem, we have established a new style of BM-chimera using a combination of whole body irradiation and BMT with specific deletion of Kupffer cells by liposomal clodronate injection [10]. These TLR4-chimeric mice have a successful replacement of endogenous Kupffer cells with donor BM-originated Kupffer cells, which contain TLR4-mutant BM-derived hematopoietic cells, including Kupffer cells, and TLR4-wild recipient-originated endogenous liver cells, including hepatocytes and HSCs. Few hepatocytes induce NF-κB nucleartranslocation in response to LPS in Kupffer cell-depleted mice, confirming that hepatocytes barely respond to the ligand for TLR4 compared with nonparenchymal liver cells [10, 26]. Recent exclusive studies have confirmed that HSCs are not BM derived [27, 28]. As mentioned above, Kupffer cells and HSCs are direct targets of LPS in vitro and in vivo [6, 9, 10]. The specific roles of TLR4 in Kupffer cells and HSCs were discriminated by this TLR4-chimera system. In this study, the mice with TLR4-mutant endogenous liver cells exhibited a significant reduction of liver fibrosis, and the mice with TLR4-wild endogenous liver cells had a sufficient degree of fibrosis in the liver after BDL [10]. These findings indicate that the recipient-originated endogenous liver cells, but not donor-derived BM cells including Kupffer cells, are crucial cell types that respond to TLR4 ligands in liver fibrosis.

4. Crosstalk between TLR4 Signaling and TGF-β Signaling in Stellate Cell Activation

There are at least two roles of TLR4 signaling in HSCs. First, TLR4-stimulated HSCs produce various chemokines and express adhesion molecules (ICAM-1, VCAM-1, and E-selectin) to recruit Kupffer cells and/or circulating macrophages by the site of HSCs. Indeed, conditioned medium produced from LPS-treated HSCs increased Kupffer cell migration and adhesion [10]. Second, the activation of TLR4 signaling enhances TGF-β signaling in HSCs [10]. HSCs isolated from collagen promoter-driven GFP transgenic (Coll-GFP) mice increase GFP intensity when collagen promoter activity is increased [29]. TGF-β treatment alone slightly increased collagen promoter activity in quiescent HSCs whereas LPS pretreatment further increased TGF-β-induced collagen promoter activity in HSCs. These findings suggested that TLR4 signaling enhances TGF-β signaling in HSCs [10]. We then tested the role of Kupffer cells in HSC activation by co-cultured Coll-GFP HSCs with Kupffer cells. Coll-GFP HSCs cocultured with Kupffer cells increased GFP expression, which was further augmented by LPS pretreatment, suggesting that TLR4 signaling enhances Kupffer cell-mediated HSC activation [10]. Coll-GFP HSCs co-cultured with TLR4-mutant Kupffer cells express a similar level of GFP expression to the HSCs co-cultured with wild-type Kupffer cells after LPS stimulation. Thus, TLR4 on Kupffer cells has a minor role for TLR4-mediated HSC activation, but Kupffer cells are required for HSC activation as the important source of TGF-β because HSC activation was completely abolished by treatment with a TGF-β inhibitor in co-culture of Kupffer cells and HSCs.

Comprehensive microarray analysis demonstrated that Bambi, a transmembrane TGF-β receptor inhibitor, was downregulated in HSCs after LPS stimulation, whereas other TGF-β signaling associated genes (TGF-β receptor, Smad family, SNoN, Ski, and Sara) were unchanged [10, 13]. Quiescent HSCs, but not Kupffer cells and hepatocytes, express high levels of Bambi in the liver. Importantly, Bambi expression is suppressed in in vivo activated HSCs isolated from mice after BDL or chronic CCl_4 treatment whereas HSCs isolated from bile duct ligated-TLR4-mutant mice have unchanged levels of Bambi expression [10]. We suggest that high levels of Bambi expression restrict TGF-β signaling in HSCs of normal livers. Upon TLR4 stimulation Bambi expression is quickly decreased. Then, TGF-β signaling becomes free from the restriction by Bambi to promote fibrogenic response. Interestingly, HSCs activated in culture do not downregulate Bambi expression. Thus, Bambi downregulation is an important feature of HSC activation in vivo. One study clearly demonstrated that human Bambi inhibits TGF-β-induced Smad3 phosphorylation, and silencing endogenous Bambi enhances TGF-β reporter activity [30]. The study further revealed that Bambi interacts with Smad7, interfering with the complex composed of type I and type II TGF-β receptors, and Smad3, resulting in inhibiting TGF-β signaling. In addition, Harada et al. demonstrated that Bambi expression in biliary epithelial cells is downregulated during epithelial-mesenchymal transition (EMT), suggesting an additional role of Bambi as a marker for EMT [31].

TLR4 signaling activates NF-κB and JNK/AP-1 pathways through MyD88 and TRIF [15]. TLR4-mediated downregulation of Bambi expression requires the activation of NF-κB and partially JNK through MyD88, but not TRIF, in HSCs. Indeed, Bambi expression was downregulated in WT and TRIF$^{-/-}$ HSCs, but not in MyD88$^{-/-}$, NF-κB, or JNK inactivated HSCs after LPS stimulation (E.S. unpublished observation) [10]. Similarly, MyD88$^{-/-}$, but not TRIF$^{-/-}$, mice demonstrated reduced fibrogenic gene expression at the early phase after BDL, whereas both MyD88$^{-/-}$ and TRIF$^{-/-}$ mice had reduced liver fibrosis at the late phase of liver fibrosis [10]. These findings suggest that MyD88 is crucial for the Bambi-regulated liver fibrosis, whereas TRIF regulates fibrogenic responses independently of Bambi, at least at the chronic stage. Taken together, TLR4 signaling is crucial in the activation of HSCs during liver fibrosis. Intestinal microflora is a major source of LPS as a ligand for TLR4 in liver fibrosis. TLR4 signaling in HSCs enhances the recruitment of inflammatory cells and downregulates Bambi for fibrogenic response Figure 1.

5. TLR4 Polymorphism, HSCs, and Liver Fibrosis

A recent genecentric functional genome scan in patients with chronic hepatitis C virus has identified seven single nucleotide polymorphisms (SNPs) that may predict the risk

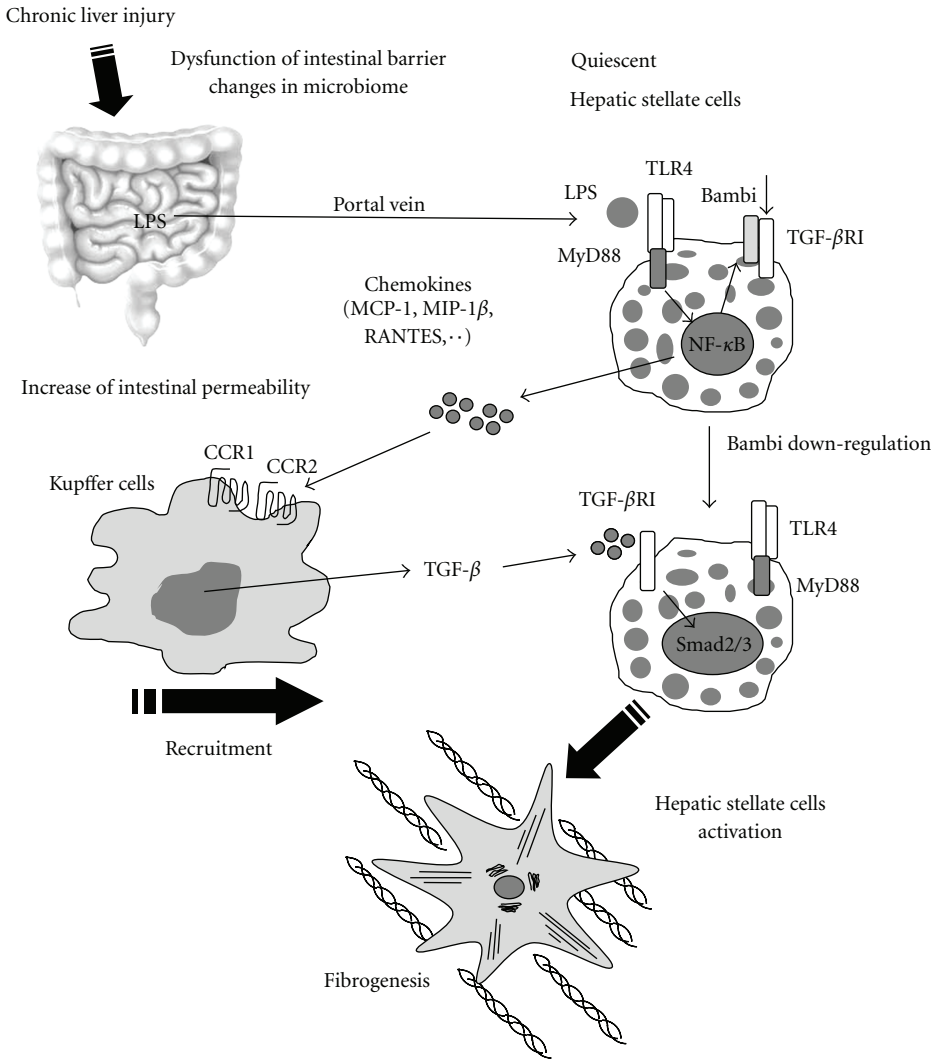

Figure 1: TLR4 signaling enhances TGF-β signaling in hepatic stellate cells. Upon liver injury, intestinal permeability is increased due to the intestinal dysbiosis and tight junction disintegrity, which allows microflora-derived LPS into the portal vein. This LPS stimulates TLR4 on hepatic stellate cells (HSCs). Quiescent HSCs express high levels of Bambi which restricts TGF-β signaling. TLR4 stimulation leads to the production of various chemokines (MCP-1, MIP-1β, and RANTES) in HSCs, recruiting Kupffer cells through their CCR1 and CCR2. Recruited Kupffer cells produce TGF-β which binds to TGF-β receptor type I in HSCs. Simultaneously, TLR4 signaling downregulates Bambi expression through MyD88 and NF-κB in HSCs. HSCs become free from restricted TGF-β signaling by the downregulation of Bambi, which eventually induces HSC activation.

of developing liver cirrhosis [32, 33]. Among these seven SNPs, a TLR4 T399I SNP is the second most predictive in protecting the progression of liver cirrhosis. TLR4 D299G is another TLR4 SNP. These two SNPs are associated with a blunted response to LPS [34]. These findings confirmed the relevance of TLR4 in a large group of patients with liver fibrosis. Based on this study, Guo et al. examined the response of HSCs with TLR4 D299G and T3991 SNPs to LPS. TLR4 D299G and/or T3991 SNPs were reconstituted into a human stellate cell line, LX-2 cells, and immortalized $TLR4^{-/-}$ mouse HSCs ($TLR4^{-/-}$mHSC) [35]. LX-2 cells or $TLR4^{-/-}$mHSCs expressing either the one or both SNPs displayed a marked reduction of NF-κB activity and cytokine production (MCP-1 and IL-6) and unchanged Bambi expression [35]. The TLR4 SNPs inhibited HSC growth and enhanced spontaneous HSC apoptosis [35]. These findings demonstrated the mechanistic function of the two TLR4 SNPs in HSC activation and the risk of fibrosis progression.

6. TLR3 in Liver Fibrosis

TLR3 recognizes double stranded RNA, such as polyinosinic-polycytidylic acid (poly I:C), to induce a potent innate immune response that includes the production of interferon type I and type II. TLR3 ligand poly I:C treatment attenuates liver fibrosis induced by the treatment of

3,5-diethoxycarbonyl-1,4-dehydrocollidine (DDC) diet and CCl_4 [36]. This fibrosis reduction by poly I:C is NK cell dependent and IFN-γ dependent. NK cells stimulated with poly I:C induce the cytotoxicity to activated HSCs, but not quiescent HSCs. Poly I:C directly, and indirectly through IFN-γ, enhances TRAIL expression in NK cells, and this increases the cytotoxicity of NK cells against activated HSCs, resulting in the suppression of liver fibrosis. The research group further demonstrated that poly I:C-dependent NK cell-dependent suppression of liver fibrosis is inhibited in ethanol-treated animals [37]. These findings suggest that the reduction of TLR3-mediated NK cell-dependent HSC killing is one of the mechanisms underlying the enhancement of liver fibrosis in alcoholic liver disease.

7. TLR9 in Liver Fibrosis

TLR9 recognizes bacteria-derived unmethylated CpG rich DNA [5]. A recent report from our group clearly suggests that liver fibrosis progression is associated with intestinal microflora-derived products and their translocation [10]. Previous studies have shown that patients and animals with cirrhosis have increased bacterial DNA levels in their plasma and ascites [38, 39]. On the other hand, Watanabe et al. demonstrated that denatured host origin DNA from dying hepatocytes stimulates HSCs through TLR9 in liver fibrosis. $TLR9^{-/-}$ mice showed a reduction of liver fibrosis after BDL and chronic CCl_4 treatment [17, 40]. These findings suggest that both bacterial DNA and host denatured DNA derived from dying cells participate in the progression of liver fibrosis as a ligand for TLR9.

Recently, Connolly et al. have demonstrated the contribution of TLR9 and CD11c-expressing cells to liver fibrosis [41]. CD11c is expressed on dendritic cells (DCs) and some monocytes and macrophages [42]. They used the model of liver fibrosis induced by thioacetamide plus leptin treatment as a similar pathophysiology to CCl_4 models. Depletion of CD11c-expressing cells using CD11c-DTR mice significantly reduced liver fibrosis, suggesting the importance of CD11c-expressing non-parenchymal liver cells in the progression of liver fibrosis [41]. CD11c-expressing cells from fibrotic liver increased the capacity to produce TNF-α, IL-6, and various chemokines, which were further increased by TLR9 ligand stimulation in CD11c-expressing cells of fibrotic livers, but not of normal livers. This increased sensitivity of fibrotic liver CD11c-positive cells to TLR ligands was induced only by the ligand for TLR9, not TLR3, TLR4, or TLR5. CpG-DNA-primed fibrotic liver CD11c-positive cells increased NK cell cytotoxicity and cytokine production, including IFN-γ in a TNF-α dependent manner [41]. CpG-DNA-primed fibrotic liver CD11c-positive cells induced HSC proliferation and production of inflammatory mediators, such as IL-1α, IL-6, and MCP-1. This study demonstrated the important features of TLR9 and DCs in liver fibrosis using CD11c-depleted mice. However, the studies for DCs in liver fibrosis are still incomplete. Further studies are needed to clarify the role of DCs in the progression of liver fibrosis.

8. TAK1 in Liver Fibrosis

TAK1 is a MAP3K which is activated by the signaling of TLRs, IL-1 receptor, TNF receptor, and TGF-β receptor [43, 44]. TAK1 is an upstream kinase of both IKK/NF-κB and JNK/AP-1 pathways [15]. The NF-κB pathway regulates the expression of antiapoptotic genes, such as Bcl-2, Bcl-xL, A20, iNOS, c-FLIP, IAPs, and TRAF family molecules, to block death receptor-mediated or mitochondria-mediated hepatocytes death [45]. NF-κB also prevents prolonged JNK activation. Prolonged JNK activation induces phosphorylation of the E3 ligase Itch and subsequent ubiquitination and degradation of caspase-8 inhibitor c-FLIP, which accelerates hepatocyte apoptosis [46]. Thus, NF-κB protects hepatocytes from apoptosis whereas JNK promotes apoptosis. Therefore, we could not predict whether TAK1 tends to induce or protect from hepatocyte apoptosis. As expected, neither NF-κB nor JNK activation following TNF-α stimulation occurred in $TAK1^{-/-}$ hepatocytes [47]. Surprisingly, $TAK1^{-/-}$ hepatocytes increased the sensitivity to TNF-α-induced cell death. More surprisingly, spontaneous hepatocyte death occurred in hepatocyte specific $TAK1^{-/-}$ mice [47]. These mice display spontaneous liver injury, inflammation, and fibrosis at the age of one month and develop hepatocellular carcinoma at the age of nine months [47, 48]. These results suggest that spontaneous persistent hepatocyte death occurs in hepatocyte specific $TAK1^{-/-}$ mice, and these dying hepatocytes release alarmins which stimulate both Kupffer cells and HSCs, resulting in liver inflammation and fibrosis Figure 2. These findings are evidence that liver fibrosis and carcinogenesis are associated with persistent hepatocyte injury and inflammation without any carcinogens. Hepatocyte specific $TAK1^{-/-}$ mice will be great animal models for addressing the role of the interplay between fibrosis and hepatocellular carcinoma.

9. Conclusion

While patients with mild to moderate liver fibrosis may not show clinical symptoms, liver cirrhosis is a major cause of morbidity and mortality. Recent advanced studies demonstrated the strong evidence of the role of TLR signaling in liver fibrosis. A number of issues concerning the role of TLRs for HSC activation and liver fibrosis still need to be addressed. First, although we have shown the evidence that intestinal microflora promotes liver fibrogenesis due to bacterial translocation, we still need to address the mechanism of bacterial translocation in liver fibrosis, which includes changes in microbiome composition and disintegrity of intestinal tight junction. Second, the studies for the endogenous TLR ligands which promote HSC activation have not been completed. Very recently, mitochondrial DNA has been reported as an endogenous TLR9 ligand [49], which might be an endogenous ligand to activate HSCs. Third, we need to address the mechanism by which TLR signaling regulates Bambi expression and by which Bambi regulates HSC activation. The research on TLRs and HSCs in liver fibrosis has just been started. The future basic and translational studies will uncover additional

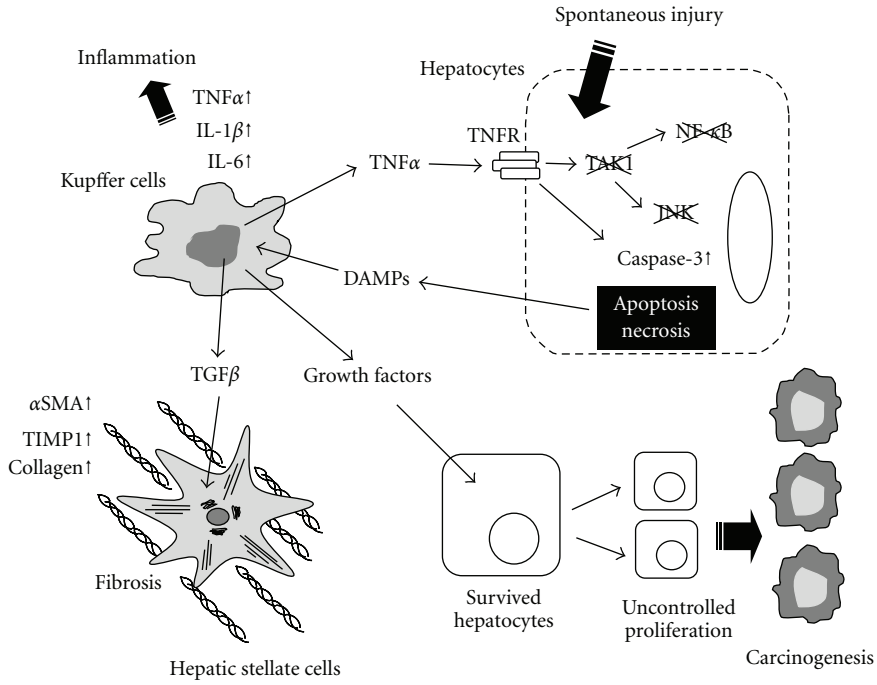

Figure 2: Ablation of TAK1 in hepatocytes induces spontaneous liver injury, inflammation, fibrosis, and cancer. Spontaneous hepatocyte death occurs in hepatocyte specific TAK1-deficient mice followed by the release of damage-associated molecular patterns (DAMPs) which stimulate Kupffer cells to produce TNF-α. This TNF-α further induces cell death in TAK1-deficient hepatocytes lacking activation of NF-κB and JNK. TNF-α, IL-1β, and IL-6 released from Kupffer cells cause liver inflammation. Kupffer cell-derived TGF-β stimulates hepatic stellate cells resulting in fibrogenesis. The persistent hepatocyte death and uncontrolled compensatory proliferation in the livers of hepatocyte specific TAK1-deficient mice induce the reactivation of onco-fetal liver genes that are associated with the initiation of hepatic carcinogenesis.

clinical relevance of TLRs and their related signaling in liver fibrosis. Clinically, hepatocellular carcinoma is strongly associated with liver fibrosis and cirrhosis. However, it is still unknown whether severe liver fibrosis promotes the initiation and/or progression of hepatocellular carcinoma. We wish to open this mysterious door in future studies using hepatocyte specific TAK1$^{-/-}$ mice.

Conflicts of Interest

There is no conflict of interest to disclose for all authors.

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Review Article

Toll-Like Receptor 3 in Liver Diseases

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Toll-like receptor 3 (TLR3) is a member of the TLR family that can recognize double-stranded RNA (dsRNA), playing an important role in antiviral immunity. Recent studies have shown that TLR3 is also expressed on parenchymal and nonparenchymal cells in the liver as well as on several types of immune cells. In this review, we summarize the role of TLR3 in liver injury, inflammation, regeneration, and liver fibrosis, and discuss the implication of TLR3 in the pathogenesis of human liver diseases including viral hepatitis and autoimmune liver disease.

1. Introduction

TLR3 is a member of the Toll-like receptor family that can recognize double-stranded RNA (dsRNA) from viruses, endogenous dsRNA from dying cells, or synthetic dsRNA polyriboinosinic:polyribocytidylic acid (poly I:C). TLR3 expression has been found in endosomal compartments (such as dendritic cells and macrophages) or at the cell surfaces (such as human fibroblasts). Binding of TLR3 and its ligand leads to conformational changes in the TLR3 cytoplasmic tail, followed by recruitment of TIR domain-containing adaptor inducing IFN-β (TRIF), and subsequent activation of the mitogen activated protein (MAP) kinase pathway, the NF-κB family of transcription factors, and the IFN regulatory factor (IRF) family of transcription factors, which then induce interferon (IFN) and inflammatory cytokine production [1, 2]. It is generally believed that TLR3 plays an important role in host response to viruses via recognizing dsRNA; however, its role in antiviral immunity has been questioned by some *in vivo* studies [3, 4]. The controversial reports on the role of TLR3 in the antiviral defense may be due to the difference in the type of viruses, the type of cells that are infected, the viral load, its model of infection (endoplasmic versus cytoplasmic), and stage of infection. Recent studies have shown that TLR3 also plays important roles in the pathophysiology of a variety of liver diseases [5–7], which may attribute to the wide expression of TLR3 on all types of liver cells, including hepatocytes [8–10], stellate cells [11], sinusoidal endothelial cells [12], Kupffer cells, biliary epithelial cells [13, 14], as well as immune cells such as NK cells, NKT cells [15], and liver lymphocytes [12]. In this review, we summarize the recent findings regarding the role of TLR3 in liver injury, inflammation, regeneration, fibrosis, viral infection, and autoimmune liver disease.

2. TLR3 in Liver Inflammation and Injury

It has been noticed for many years that injection of mice with the TLR3 ligand poly I:C induced significantly liver inflammation with a predominant accumulation of NK cells [16, 17]. Recent studies suggest that such NK cell accumulation is due to the recruitment of NK cells from the spleen, which is regulated positively by the expression of chemokines and the presence of T cells [18], but regulated negatively by the β2 integrin CD11b [19]. Treatment with poly I:C induced mild liver injury in normal mice via an NK cell-dependent manner [20] but induced massive liver necrosis in D-galactosamine (D-GalN)-sensitized mice [21, 22]. In the model of liver injury induced by poly I:C/D-GalN, it is believed that injection of these two reagents elevates the expression of retinoic acid early inducible-1 (Rae-1) on Kupffer cells. Upregulated Rae-1 acts as an NK

cell stimulating ligand to activate NK cells via targeting NKG2D receptor on NK cells. Activated NK cells then produce a large amount of IFN-γ, which acts in synergy with Kupffer cell-derived TNF-α to induce massive hepatocellular damage [22]. In addition, poly I:C treatment also induced significantly liver injury in transgenic mice with HBV surface antigen (HBs-B6) [23, 24]. It was shown that depletion of NK cells or blockage of IFN-γ but not depletion of Kupffer cells or neutralization of IL-12 diminished the poly I:C-induced liver injury in HBs-B6 mice, suggesting that NK cells/IFN-γ contribute to the pathogenesis of liver injury in this model. In contrast to the detrimental effect of poly I:C on liver injury, pretreatment with poly I:C had a beneficial effect to reduce the mortality and liver injury induced by lipopolysaccharide plus D-GalN in mice [25]. This protective effect of poly I:C seems to be mediated via poly I:C downregulation of TLR4 expression on Kupffer cells/macrophages and subsequent reduction of the responsiveness of Kupffer cells/macrophages to LPS stimulation. In addition, activation of TLR3 on Vα14 iNKT cells may also negatively regulate liver inflammation via preventing intrahepatic $\gamma\delta$ T cell accumulation [15].

Although the effect of poly I:C on liver injury has been extensively investigated, the role of TLR3 signaling in these effects remains obscure. TLR3-deficient mice had reduced response to poly I:C stimulation, reduced production of inflammatory cytokines stimulated by poly I:C, and resistance to poly I:C/D-GalN-induced mortality, suggesting a critical role of TLR3 signaling in poly I:C-mediated liver injury [21]. The important role of TLR3 in liver inflammation and injury has also been recently revealed in Concanavalin A- (Con A-) induced T cell hepatitis model by using TLR3-deficient mice [12]. Injection of Con A markedly increased TLR3 expression on liver lymphocytes and sinusoidal endothelial cells. Disruption of the TLR3 gene abolished Con A-induced liver injury. Finally, by using chimeric mice, Xiao et al. [12] demonstrated that TLR3 signaling in both nonhematopoietic and hematopoietic cells plays a critical role in the pathogenesis of Con A-induced T cell hepatitis. However, what the endogenous ligands are and how these ligands activate TLR3 in this Con A-induced T cell hepatitis model remain unknown.

3. TLR3 in Liver Regeneration

Liver regeneration is a very complicated process orchestrated with a series of signaling cascades induced by cytokines, growth factors and hormones [26]. Current knowledge from experimental studies of liver regeneration suggests that tissue loss or cell damage triggers innate immune responses and initiates liver regeneration. Among the multiple innate components, the role of TLR4 signaling in liver regeneration has been extensively investigated. However, the results on the role of TLR4 signaling and its adapter protein myeloid differentiation factor-88 (MyD88) in liver regeneration have been controversial [27, 28]. Early studies showed that TLR4/MyD88 pathway was critical for the initiation of liver regeneration [27]. Impaired liver regeneration was observed in MyD88$^{-/-}$ mice after partial hepatectomy (PHx). This phenomenon was associated with grossly subnormal induction of the expression of immediate early genes involved in hepatocyte replication and the phosphorylation of STAT3 in the liver, and the reduced production of TNF-α/IL-6 by the activation of NF-κB in the Kupffer cells. Surprisingly, a later report indicated liver regeneration was not suppressed in mice deficient in MyD88, TLR2, TLR4, or CD14 gene [28]. Although normal hepatocyte DNA replication was observed in Myd88 knockout mice, PHx-mediated induction of proinflammatory cytokines TNF-α, IL-6, and their downstream signaling pathways was reduced in MyD-88 knockout mice [28]. At present, the reasons for the discrepancy between these 2 studies are not clear [27, 28]. The different surgical techniques and animal facility environment may contribute to the different findings on liver regeneration in MyD88 knockout mice.

In contrast, a series of evidence show that TLR3/IFN-γ/STAT1 axis plays an inhibitory role in liver regeneration, suggesting that the innate immune system may play an important role in balancing liver regeneration [29–32]. We have previously demonstrated that after infection with murine cytomegalovirus (MCMV) or poly(I:C) injection, NK cells are activated and produce IFN-γ that in turn attenuates liver regeneration after PHx. Depletion of NK cells or disruption of either the IFN-γ gene or the IFN-γ receptor gene enhances liver regeneration and partially abolishes the negative effects of MCMV and poly(I:C) on liver regeneration. These results suggest that viral infection and the TLR3 ligand negatively regulate liver regeneration via activation of innate immunity (NK/IFN-γ) [31]. Consistent with these results of the inhibitory role of TLR3 in liver regeneration, TLR3$^{-/-}$ mice demonstrated earlier hepatocyte proliferation and an increase in liver regeneration following PHx [29]. In the absence of TLR3, hepatocyte proliferation was accelerated while the levels of IL-6 and soluble interleukin-6 receptor (sIL-6R) were reduced. TLR3 signaling was induced in hepatocytes at the early time points after PHx, resulting in enhanced NF-κB activation, the increase levels of Rip3 and activation of caspase-8, with no evidence of apoptosis. These findings suggest TLR3 signaling plays an important role in inhibiting liver regeneration [29].

We have further demonstrated that IFN-γ inhibits liver regeneration via activation of STAT1 and subsequent induction of IRF-1 and p21 [30, 32]. Disruption of the STAT1 gene abolished poly I:C suppression of liver regeneration and the inhibitory effect of poly I:C on liver regeneration was diminished in IRF-1$^{-/-}$ and p21cip1$^{-/-}$ mice. Treatment with IFN-γ *in vitro* inhibited cell proliferation of wild-type mouse hepatocytes, but not STAT1$^{-/-}$ hepatocytes. The inhibitory effect of IFN-γ on cell proliferation was also diminished in IRF-1$^{-/-}$ and p21cip1$^{-/-}$ hepatocytes, but enhanced in SOCS1$^{-/-}$ hepatocytes. Hepatocyte proliferation was unaffected by treatment with poly I:C alone, but when hepatocytes were cocultured with liver lymphocytes, hepatocyte proliferation was inhibited by IFN-γ/STAT1-dependent mechanisms. Moreover, in HCV-infected livers with cirrhosis, activation of STAT1 was detected and correlated positively with liver injury but correlated negatively with hepatocyte proliferation.

4. TLR3 in Liver Fibrosis

Liver fibrosis is a common response to virtually all forms of chronic liver injury and is characterized with hepatic stellate cell (HSC) activation and accumulation of extracellular matrix proteins [33, 34]. HSCs are generally believed to be the most important cells in producing collagens and contributing to the pathogenesis of liver fibrosis. Activation of HSCs is controlled by many cytokines and growth factors. Among them, TGF-β is considered the most important factor to induce HSC transformation while PDGF plays a critical role in stimulating HSC proliferation. Recent studies have also suggested that TLR4 plays an important role in promoting liver fibrosis via enhancing TGF-β signaling in HSCs [35]. Our laboratory has demonstrated that treatment of mice with the TLR3 ligand poly I:C markedly inhibits liver fibrosis [36, 37], which was confirmed later by another laboratory [38]. The studies from our laboratory as well as other laboratories suggest that multiple mechanisms contribute to poly I:C-mediated inhibition of liver fibrosis. First, poly I:C treatment induces NK cell activation, and activated NK cells then kill early-activated or senescence-activated HSCs that have increased expression of NK cell activating ligands [36, 38]; Second, poly I:C treatment induces NK cells to produce IFN-γ that subsequently induces HSC apoptosis and cell cycle arrest [37]. Recent studies from Dr. Schlaak's laboratory have shown that poly I:C treatment stimulates HSCs that express high levels of TLR3 to produce type I IFN-β [11]. Since type I IFN is known to inhibit HSC proliferation [39], it is plausible to speculate that activation of TLR3 may also directly block HSC proliferation via production of IFN-β, thereby contributing to suppression of liver fibrosis. Furthermore, we have demonstrated that chronic alcohol consumption suppresses poly I:C-mediated activation of NK cell activation and induction of cytotoxic mediators on liver lymphocytes [40] and poly I:C inhibition of liver fibrosis [41]. Alcohol inhibition of poly I:C-mediated activation of NK cells is probably mediated via suppression of poly I:C activation of TLR3 signaling on NK cells [42, 43]. Finally, abrogation of the antifibrotic effect of NK cells by alcohol may be an important mechanism contributing to alcohol acceleration of liver fibrosis in patients with viral infection [41]. We have previously reported that injection of poly I:C inhibits liver regeneration induced by partial hepatectomy [31]. Interestingly, a recent paper shows that injection of poly I:C also inhibits liver regeneration induced by administration of single dose of CCl4 [44]. However, it is not clear whether poly I:C also has inhibitory effect on liver regeneration in a model of liver fibrosis induced by chronic CCl4 treatment, and whether inhibition of liver regeneration by TLR3 also contributes to liver fibrogenesis.

5. TLR3 in Viral Hepatitis Infection

TLR3 is generally believed to play an important role in the innate immune response against viral infection, including viral hepatitis infection, although controversial results have been reported [4]. The antiviral effects of TLR3 signaling on viral hepatitis infection are likely mediated via stimulating of a variety of cells to produce type I IFN that subsequently inhibits HCV or HBV replication [11, 45–47]. These cells include HSCs [11], monocyte-derived dendritic cells [48], hepatocytes [47], Kupffer cells [46], sinusoidal endothelial cells [46], NK cells [49], and so forth. Recent studies showed that TLR3 can also directly sense HCV infection in human hepatocytes, acting independently of retinoic acid-inducible gene I (RIG-I), followed by activation of IRF-3 and ISGs that suppress HCV replication [10]. However, several lines of evidence suggest that TLR3 signaling is suppressed during viral hepatitis infection, which may contribute to the escape of hepatitis virus from the surveillance of innate immunity and lead to the chronic infection. First, expression of TLR3 and type I IFN was significantly decreased in monocyte-derived dendritic cells from patients with chronic HBV or acute-on-chronic HBV liver failure compared with normal healthy individuals. Such reduction correlated positively with severity of the disease [50]; Second, infection of hepatoma cells with HCV *in vitro* degraded TRIF protein, an essential TLR3 adaptor, and subsequently attenuated poly I:C-induced signaling [10]; Third, the NS3/4A serine protease of hepatitis C virus (HCV) can interrupt TLR3 and/or RIG-I-mediated signal transduction by proteolytic cleavage of TRIF and/or CADIF [51–53]. The TLR3 pathway plays an important role in influencing host innate immunity and viral clearance during viral hepatitis infection, and may represent a useful therapeutic approach for the treatment of viral hepatitis. However, a greater understanding of the specific cellular source of TLR3 signals and TLR3 pathway changes in different stages of viral infection may assist in the design of appropriate therapeutic interventions that target this TLR3 pathway in patients with chronic viral hepatitis infection.

The association between TLR3 gene polymorphisms and chronic HCV infection was also recently investigated [54]. Two single nucleotide polymorphisms (SNPs) were identified within the TLR3 gene: rs5743305 (T/A) is located within the promoter region; rs3775291 (C/T) is located within exon 4. Both SNPs were not found to be associated with TLR3 gene expression in peripheral blood mononuclear cells (PBMCs); however, a tendency of higher TLR3 gene expression in the liver was found for exon 4 TT genotypes. No association was found between both SNPs and the clinical parameters of disease progression of chronic HCV infection, but the TLR3 exon genotype was found to be related to resistance to HCV subtype Ia. These studies suggest that the TLR3 SNP associated with higher TLR3 expression in the liver might be related to the resistance to HCV subtype Ia infection but has no role in disease progression after a chronic infection is established.

6. TLR3 in Autoimmune Liver Disease

TLR3, together with other endosomal TLRs (TLR7 and TLR9), have been implicated in the pathogenesis of a variety of autoimmune diseases [1], including primary

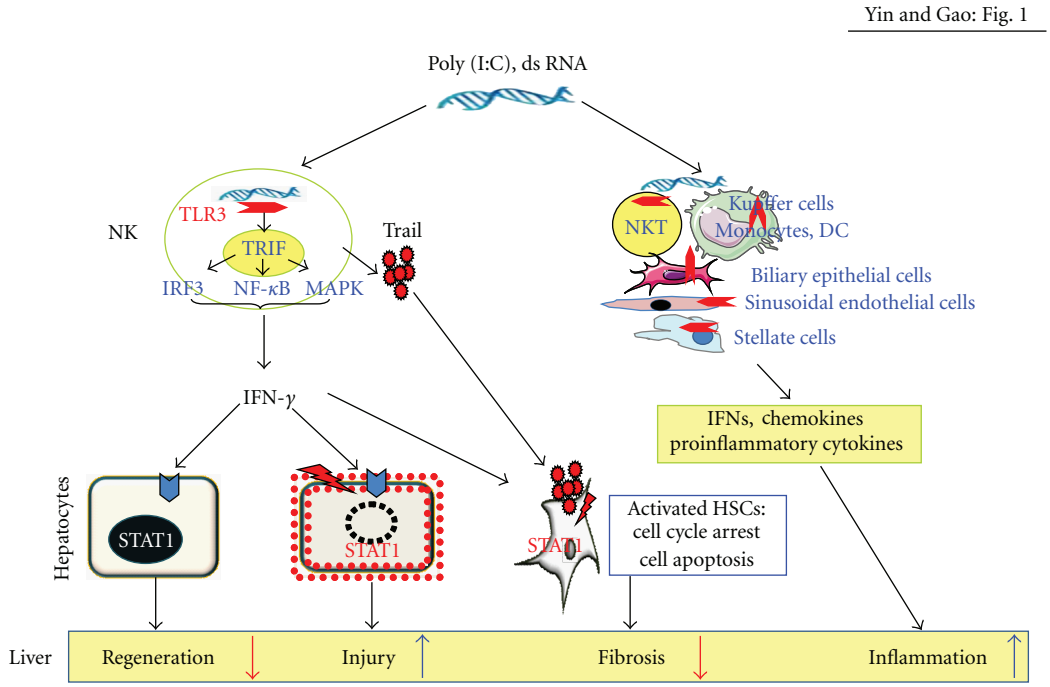

Figure 1: A model depicting the roles of TLR3 in liver injury, inflammation, regeneration, and fibrosis. Poly I:C or dsRNA binds TLR3 receptor in NK cells and stimulates NK cells to produce IFN-γ that induces hepatocyte cell cycle arrest and apoptosis via activation of STAT1. IFN-γ also induces stellate cell cycle arrest and apoptosis in an STAT1-depdent manner, resulting in inhibition of liver fibrosis. Poly I:C can also inhibit liver fibrosis via activation of NK cell killing of early activated stellate cells. In addition, poly I:C or dsRNA targets TLR3 in many other cell types, followed by production of IFNs, chemokines, and anti-inflammatory cytokines, resulting in liver inflammation.

biliary cirrhosis. The critical role of TLR3 in autoimmune hepatitis was clearly demonstrated in a model of hepatitis induced by infection of lymphocytic choriomeningitis virus (LCMV) [55]. This study suggests that poly I:C activation of TLR3 on antigen-presentation cells such as macrophages and dendritic cells produce type I IFN and TNF-α, which then trigger the release of CXCL9 by hepatocytes, Kupffer cells, endothelial cells, and so forth. CXCL9 then attracts CXCR3 positive self-reactive CD8+ T cells to kill hepatocytes, resulting in autoimmune hepatitis. However, the role of TLR3 in the pathogenesis of human autoimmune diseases is less clear. Immunohistochemistry analyses showed that the expression of TLR3 was markedly increased in biliary epithelial cells at sites of ductular reaction in primary biliary cirrhosis and autoimmune hepatitis [13, 14]. A strong positive correlation between the mRNA levels of TLR3 and type I IFN in the liver was found in the patients with primary biliary cirrhosis, suggesting TLR3 signaling is involved in the pathogenesis of primary biliary cirrhosis [14]. In animal models, injection of poly I:C induced primary biliary cirrhosis-like cholangitis (such as infiltration of mononuclear cells and elevation of AMA autoantibodies) in a genetically susceptible mouse strain of female C57BL/6 mice [56, 57]. At present, the mechanisms by which poly I:C treatment induces cholangitis remain obscure. It is believed that poly I:C activation of TLR3 signaling in biliary epithelial cells as well as hepatocytes and immune cells in the liver results in production of type I IFN, which subsequently contributes to the pathogenesis of primary biliary cirrhosis [13, 14, 58]. However, a recent study suggests that poly I:C induction of type I IFN in biliary epithelial cells is mediated via targeting RIG-I and melanoma differentiation-associated gene 5 (MDA5), not TLR3 [13]. Further studies are required to clarify the role of TLR3 in primary biliary cirrhosis.

In summary, TLR3 is expressed on parenchymal and nonparenchymal cells in the liver as well as in many types of immune cells including macrophages, dendritic cells, NK cells, NKT cells, and so forth (see Figure 1). In general, activation of TLR3 by dsRNA induces NK cell accumulation and activation in the liver, leading to liver inflammation and injury. TLR3 signaling also negatively regulates liver regeneration via stimulating NK cells to produce IFN-γ that subsequently induces hepatocyte death and cell cycle arrest via an STAT1-dependent manner. Activation of NK cells by the TLR3 ligand poly I:C inhibits liver fibrosis via killing of activated stellate cells and producing IFN-γ that subsequently induces stellate cell apoptosis and inhibits stellate cell proliferation. Clinical studies suggest that TLR3 may contribute to the resistance to HCV subtype Ia infection but seems to have no role in disease progression after a chronic infection is established. TLR3 has also been implicated in the autoimmune liver disease in animal models, but more studies are required to clarify the role of TLR3 in human autoimmune diseases.

Abbreviations

D-GalN: D-galactosamine
HSC: Hepatic stellate cells
IFN: Interferon
MAPK: Mitogen activated protein kinase
MyD-88: Myeloid differentiation factor-88
Poly I:C: Polyriboinosinic:polyribocytidylic acid (poly I:C)
TLR3: Toll-like receptor 3
TRIF: TIR domain-containing adaptor inducing IFN-β (TRIF).

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Review Article

NF-κB, JNK, and TLR Signaling Pathways in Hepatocarcinogenesis

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Hepatocellular carcinoma (HCC) is the third largest cause of cancer deaths worldwide. The role of molecular changes in HCC have been used to identify prognostic markers and chemopreventive or therapeutic targets. It seems that toll-like receptors (TLRs) as well as the nuclear factor (NF)-κB, and JNK pathways are critical regulators for the production of the cytokines associated with tumor promotion. The cross-talk between an inflammatory cell and a neoplastic cell, which is instigated by the activation of NF-κB and JNKs, is critical for tumor organization. JNKs also regulate cell proliferation and act as oncogenes, making them the main tumor-promoting protein kinases. TLRs play roles in cytokine and hepatomitogen expression mainly in myeloid cells and may promote liver tumorigenesis. A better understanding of these signaling pathways in the liver will help us understand the mechanism of hepatocarcinogenesis and provide a new therapeutic target for HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is the third largest cause of cancer deaths worldwide, particularly in Africa and Eastern Asia. Major HCC risk factors include infection with hepatitis B (HBV) or C viruses (HCV) and cirrhosis associated with chronic inflammation. The fungal contaminant aflatoxin B_1 and environmental pollutants, such as aromatic amines, vinyl chloride, polycyclic aromatic hydrocarbons, and nitrosamines are also risk factors [1].

The role of molecular changes in the acquisition of resistance or susceptibility to HCC and the importance of genetically susceptible and resistant murine models have been used to identify prognostic markers and chemopreventive or therapeutic targets. Studies with genetically modified mice have revealed many genes associated with HCC. Among them, we focus on toll-like receptors (TLRs) as well as the nuclear factor (NF)-κB and the JNK pathways in HCC development in this paper.

2. Putative Mechanisms of HCC Development

Although many putative causal factors are involved in the development of HCC, carcinogenic mechanisms have been difficult to elucidate. Among them, particular HBV or HCV proteins induce HCC without other oncogenic alterations in mouse models [2]. It is thought that chronic liver injury resulting in compensatory proliferation of differentiated hepatocytes is one of the major pathogenic mechanisms underlying HCC development [3, 4]. Several signal transduction pathways such as NF-κB and JNK are involved in the pathogenesis of these viruses. For example, the HCV core and HBx proteins are the most potent signal inducers for NF-κB and AP-1 [5].

Another important etiology of HCC development is obesity-induced hepatosteatosis, which includes more severe complications such as nonalcoholic steatohepatitis (NASH), classified as nonalcoholic fatty liver disease (NAFLD) [6]. Several mechanisms have been proposed to explain how NASH increases HCC risk, including the prevalence of insulin resistance among obese individuals, which results in elevated circulating concentrations of insulin and insulin-like growth factor 1 (IGF-1) [7] and a low-grade inflammatory response with elevated production of cytokines, such as TNF and IL-6 [8]. In these cases, it seems that the NF-κB and JNK pathways are critical regulators for the production of the cytokines associated with tumor promotion.

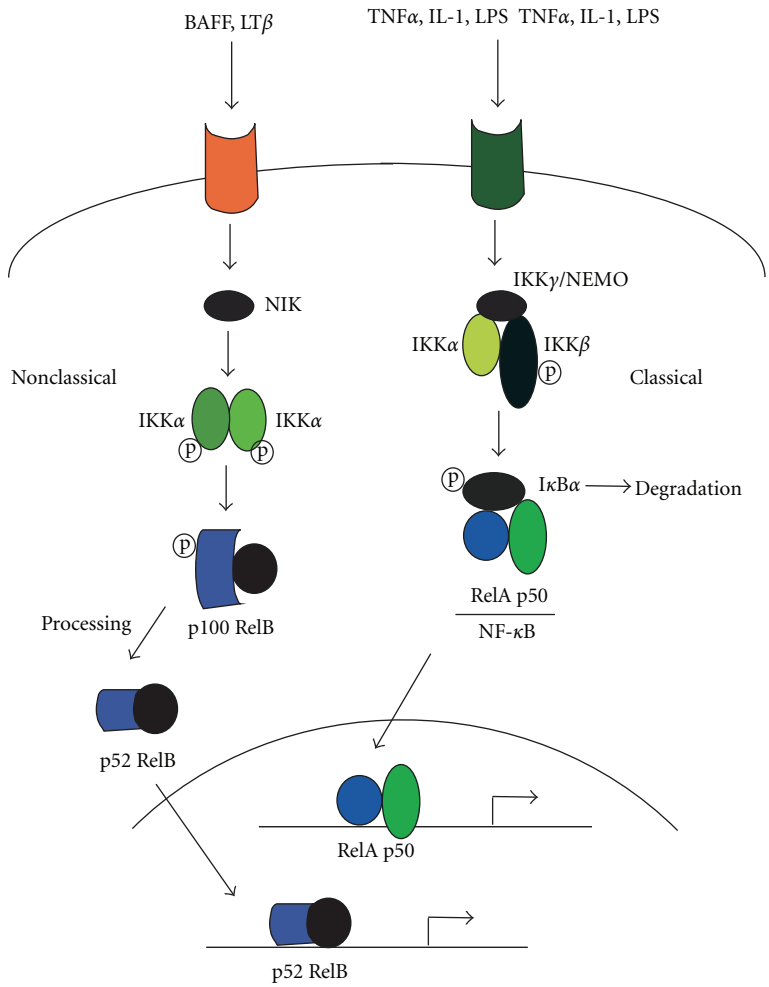

Figure 1

3. NF-κB and HCC

3.1. NF-κB Signaling Pathway. NF-κB transcription factors are key regulators of innate and adaptive immune responses, inflammation, and cell survival [9]. Many proinflammatory stimuli activate NF-κB, mainly via IκB kinase-(IKK) dependent phosphorylation and degradation of the κB inhibitor (IκB) proteins. Five members, p65 (RelA), p50/NF-κB1, c-Rel, RelB, and p52/NF-κB2, belong to the mammalian NF-κB family and are assembled by dimerization. Once activated, NF-κB dimers stimulate transcription of genes encoding cytokines and antiapoptotic factors [10]. IKK consists of two catalytic subunits, IKKα and IKKβ, and a regulatory component, NEMO/IKKγ. IKK activation occurs primarily through IKKβ [10], whose absence increases susceptibility to tumor necrosis factor-α-(TNFα-) induced apoptosis [11] (Figure 1).

In the nonclassical pathway, NF-κB activation is triggered by members of the TNF family, including B-cell-activating factor, which belongs to the TNF family (BAFF), the CD40 ligand, and lymphotoxin β/LTβ. Dimerized IKKα is activated by NF-κB-inducing kinase (NIK), after which the p100 proteins are processed. After processing p100, p52/NF-κB2 complexes move into the nucleus, resulting in the expression of genes that activate the adaptive immune response and the development of secondary lymphoid organs [12] (Figure 1).

3.2. NF-κB Activation and Carcinogenesis. Tumor initiation means cellular immortality, which happens through DNA mutation, but the relationship with NF-κB activation has not been considered in detail for this process. However, the first clue linking NF-κB to cancer was recognizing that c-*rel*, which is a v-*rel* oncogene cellular homologue, encodes an NF-κB subunit and that all of these proteins share the Rel homology DNA-binding domain [13]. Not surprisingly, overexpression of normal Rel proteins promotes oncogenic transformation.

Participation of NF-κB activation in the carcinogenic promotion and progression stages has become clear in recent years. This promotion stage is the cell proliferative stage, and proliferation, antiapoptosis, angiogenesis, invasion, and metastasis become important [14].

It is unclear whether NF-κB activation is directly associated with tumor cell proliferation. There are a few reports of a strong participation in transcriptional regulation of

the expression of growth factors through NF-κB activation. However, for the activation and infiltration of inflammatory cells, inhibition of NF-κB activation may reduce expression of these growth factors. TNFα, which is a strong NF-κB-activating factor whose expression is regulated by NF-κB, is produced by macrophages due to inflammation and plays a central role in inflammation but has also been suggested as an accelerator factor of cell proliferation [15]. In addition, the possibility that NF-κB activation operates on cell growth through cyclin expression is indicated in certain cell types [16]. In contrast, *Ikkβ* deletion accelerates hepatocyte growth. *Ikkβ*-deficient hepatocyte proliferative responses show heightened sensitivity to TGF-α [17], suggesting that involvement of NF-κB activation in proliferation differs among cell types.

Antiapoptosis is also important for maintaining cancer cells. A large number of antiapoptotic factors, such as cIAPs, c-FLIP, and BclX, are controlled by NF-κB activation [18]. Tumor cells with constitutive NF-κB activation are highly resistant to anticancer drugs, and the inhibition of NF-κB activity in those cells increases tumor sensitivity to these therapies [19–22].

Invasion and metastasis are pivotal processes for prognosis. Matrix metalloproteinases (MMPs) are produced by inflammatory cells and tumor cells and are key players in the degradation of the extracellular matrix and basement membranes; thus, they are important in tumor invasion. Gelatinases (MMP-2 and MMP-9), in particular, are prognostic factors in many solid tumors, and their expression is regulated by NF-κB activation. The clinical application of an MMP inhibitor aimed at antimetastasis is expected [23]. Recent observations suggest that IKKβ/NF-κB activation controls the development of liver metastasis through IL-6 expression, which is associated with tumor cell proliferation and angiogenesis [24].

It is likely that NF-κB activation is most critical in cancers in which inflammation acts as a tumor promoter, but a more general role for NF-κB remains to be established for the inflammation mediated by its activation in cancers that are not associated with inflammation.

3.3. NF-κB Activation and HCC. In clinical cases, constitutive NF-κB activation has been frequently observed in tumor tissues rather than in nontumor tissues [25]. High expression of IKKα and IKKβ, which are critical kinases for NF-κB activation, is necessary to produce the malignant properties of liver cancer [26].

Although IKKβ-deficient mice die at mid-gestation from uncontrolled liver apoptosis [27], the IKKβ deficiency in the hepatocytes does not influence their function and growth [28]. However, following an injection of concanavalin A (ConA), liver damage becomes worse in IKKβ hepatocyte-specific IKKβ knockout mice, and TNFα, which causes ConA expression on the T cell surface, is responsible for the apoptotic action. This result suggests that IKKβ/NF-κB activation of hepatocytes is important for the survival of TNFα-mediated acute hepatitis and that inactivating NF-κB may injure hepatocytes [28].

The roles of IKKβ in carcinogenesis have been examined using diethylnitrosamine (DEN) as a chemical carcinogen. DEN does not require any assistance from inflammation-inducing tumor promoters if it is given to 2-week-old male mice. An increase in hepatocyte death coincident with an increase in reactive oxygen species (ROS) was observed in knockout mice when the acute reaction was analyzed following chemical carcinogen administration. Furthermore, cell death was accompanied by inflammation, and elevated hepatocyte death enhanced compensatory proliferation due to the strong regenerative capacity of the liver [29].

Decreased NF-κB activity and elevated JNK activity promote TNF-α-induced cell death [30]. Accordingly, hepatocyte IKKβ ablation results in higher DEN-induced JNK activity and increased cell death. Enhanced JNK activation in the absence of IKKβ is dependent on ROS accumulation. Interestingly, a reduction in ROS production by the antioxidant BHA controls liver cell death and proliferation and reduces carcinogenesis [29].

Compensatory proliferation depends on the production of factors such as TNF-α, IL-6, and hepatocyte growth factor by nonparenchymal cells [31], and NF-κB activation is important for producing these cytokines. Accordingly, hepatocytes and blood cells, including Kupffer cells, which are liver macrophages, were used in a carcinogenesis model of IKKβ knockout mice, and the occurrence of HCC was reduced. These results suggest that IKK/NF-κB activation in myeloid cells is important for producing liver growth factors [29].

In fact, DEN-induced hepatocarcinogenesis has been examined in IL-6 knockout mice and wild-type controls, and a marked reduction in HCC incidence was seen in IL-6 knockout mice. Interestingly, gender disparity is observed in mice given DEN, and ablation of IL-6 abolishes this gender difference [32].

Recently, IKKβ deletion was observed to accelerate HCC development and enhance tumor cell proliferation. These effects of IKKβ were correlated with increased ROS accumulation, which led to JNK and STAT3 activation. Accordingly, hepatocyte-specific STAT3 ablation prevents HCC development. These results suggest that the negative crosstalk between IKKβ and STAT3 is a critical regulator in HCC development [33].

The IKK subunit NEMO/IKKγ is essential for activating the NF-κB transcription factor, which regulates cellular responses to inflammation. Hepatocyte-specific ablation of NEMO causes the spontaneous development of HCC in mice. Tumor development is preceded by chronic liver disease such as NASH. These results reveal that NEMO-mediated NF-κB activation in hepatocytes plays an essential physiological role to prevent the spontaneous development of NASH and HCC [34]. However, viral-induced activation of IRF3 and IRF7 depends on NEMO, suggesting that NEMO acts as an adaptor protein allowing RIG-I to activate not only NF-κB, but also IRF signaling pathways. Ablation of NEMO in the liver may influence the IRF pathway [35].

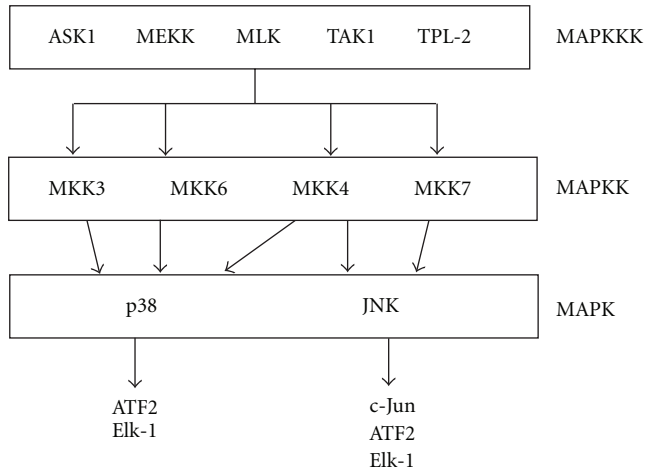

Figure 2

3.4. NF-κB and Therapeutic Target for HCC. Applying NF-κB inhibitors for use as anticancer agents is expected. Through an antiapoptosis effect of NF-κB, the inhibition leads cells to apoptosis. Furthermore, constitutive NF-κB activation is observed in many cancer cells, and connection with a malignancy of the cancer is assumed. IKKs or IκBs are now the main targets of NF-κB inhibitors. Many researchers have discovered various inhibitors that attenuate IKKα, IKKβ, IKKγ, or IκBα, and some are effective in animal models. In our group, NEMO-binding domain peptide (NBD), one of the inhibitors of IKKβ, has shown chemopreventive effects in a mouse colitis model [36]. Discovering drugs that target these molecules appears to be attracting greater attention, and the development of specific IKKβ inhibitors has progressed rapidly. Several novel, small-molecule IKKβ inhibitors have demonstrated anti-inflammatory activity, and the advancement of IKKβ inhibitors into clinical development is anticipated in the near future [37].

4. JNK and HCC

4.1. JNK Signaling Pathway. The c-Jun NH_2-terminal kinase (JNK) belongs to a family of mitogen-activated kinases (MAPKs), together with extracellular regulated kinases (ERKs) and p38. The JNK subgroup of MAPKs is encoded by three loci; *Jnk1* and *Jnk2* are ubiquitously expressed, and *Jnk3* is expressed primarily in heart, testis, and brain [38–40]. JNKs are activated by stress signals and proinflammatory stimuli, and their activity increases following phosphorylation by the MAPK kinases, MKK4, and MKK7 [41] (Figure 2).

Immunoblot analyses suggest that a 46-kDa molecule represents JNK1 and a 54-kDa molecule represents JNK2. However, a recent analysis revealed that the 46- and 54-kDa molecules represent two isoforms of JNK1 and JNK2, whereas the 46-kDa JNK1 and 54-kDa JNK2 are dominantly expressed in most cell and tissue types [42]. Activated JNKs phosphorylate c-Jun, JunD, ATF, and other transcriptional factors, which are involved in the formation and activation of the AP-1 complex [43]. In addition, JNKs also phosphorylate other proteins that induce apoptosis, cell proliferation, or transformation, depending on the cell type and stimuli [44].

Using gene knockout mice, it has been shown that JNKs are involved in many processes, including liver inflammation and proliferation, neuronal apoptosis, T-cell activation, and insulin resistance [45–49].

4.2. JNK Activation and Liver Disease. In the liver, activating JNK was thought to be important for proliferation and apoptosis. In a knockout-mice analysis, JNK1 was associated with increased apoptosis in ConA-induced acute hepatitis [28]. Acetaminophen-induced liver injury is also JNK dependent [50, 51]. Furthermore, mice lacking both JNK1 and 2 expression in hepatocytes exhibit the same degree of injury in the development of hepatitis as control mice do, whereas mice without JNK1/2 in the hematopoietic compartment exhibit a profound defect in hepatitis that is associated with a markedly reduced expression of TNF-α. It is suggested that a role for JNK in the development of hepatitis, but identified hematopoietic cells as the site of the essential function of JNK [52]. JNK plays a pivotal role in the development of metabolic syndrome including NAFLD [48, 53]. Hepatic steatosis, inflammation, and fibrosis have been examined in mice fed a choline-deficient L-amino-acid-defined diet. The results showed less hepatic inflammation and less liver fibrosis despite a similar level of hepatic steatosis in JNK1-deficient mice compared with wild type, suggesting that JNK1 may be associated with the induction of diet-induced steatohepatitis and liver fibrosis [54].

4.3. JNK and Carcinogenesis. Tong et al. reported that JNK1 knockout mice spontaneously develop intestinal tumors, suggesting a role for JNK1 in suppressing intestinal tumor formation [55]. In contrast, mice lacking JNK1 were much less susceptible to N-methyl-N-nitrosourea-induced gastric carcinogenesis, which was correlated with decreased tumor initiation and diminished cell proliferation [56]. These findings suggest that the JNKs have tumor-promoting or

tumor-suppressing functions, depending on the cell type or organ.

Tumor initiation indicates cellular immortality, which occurs due to a DNA mutation, and the relationship with the JNK activation is considered important. The presence of mutated ras genes in 30% of all human cancers suggests an important contribution to the development of human cancers. Overexpression of mutated ras genes causes transformation of a variety of rodent fibroblast and epithelial cell lines. Ras causes activation of a Raf-independent MAPK cascade, which leads to JNK activation [57], and inhibition of JNK activation also inhibits Ras transformation in NIH3T3 cells [58]. JNK1-dependent c-Myc and p21 are responsible for the diminished checkpoint function in tumorigenic hepatocytes [59].

JNK function is critical in the carcinogenic promotion and progression stages, as JNK phosphorylates a variety of genes associated with carcinogenesis. The promotion stage is the proliferative stage of the cell, which becomes immortal, and proliferation, anti-apoptosis, angiogenesis, invasion, and metastasis are important [14].

Growth factors activate receptor tyrosine kinases, and phosphorylated receptors transmit the signals through JNKs [60]. There is also participation in the transcriptional regulation of growth factors such as EGF through JNK activation [61]. Numerous studies have considered the proliferative effect following JNK activation. For example, in a liver regeneration mouse model, the number of Ki67-positive proliferating hepatocytes in *Jnk1*$^{-/-}$ mice was reduced by 80% compared with that in controls at 48 hours after a partial hepatectomy [59]. In an HCC model induced by DEN, several important genes associated with proliferation such as PCNA, cyclin D, and CDKs increased [4].

The expression of several angiogenic factors is also regulated by JNK. Vascular endothelial growth factor (VEGF) promotes proliferation and migration of endothelium cells. VEGF expression is also controlled by JNK activation [62]. Chemokines such as interleukin (IL)-8 are factors controlling leukocytes at the time of inflammation, but they function as blood vessel growth factors in tumor tissue [63, 64].

4.4. JNK Activation and HCC. JNKs are protein kinases, and many molecules are phosphorylated by JNKs. Approximately, 70% of HCC tissues, but not background tissues, show positive immunostaining for phosphorylated JNK, suggesting that JNK is frequently active in human HCC [65]. *In vitro* studies suggest that several HBV and HCV proteins are common etiologic pathogens for HCC-activated JNK in cancer cells [66].

In addition to the clinical samples and *in vitro* studies, JNK activation has been critically important in *in vivo* mouse models. In hepatocyte-specific *Ikkβ*-deficient mice, DEN-induced liver cancer increased and JNK activation was elevated simultaneously [29]. To determine the role of JNK in these models, JNK1-deficient mice were interbred with *Ikkβ*-deficient mice, and the results showed that JNK1 is a critical factor for the increased incidence of HCC in *Ikkβ*-deficient mice [4]. Additionally, to determine the role of JNK1 in DEN-induced hepatocarcinogenesis, wild-type and *Jnk1*-deficient mice were injected with DEN. All mice given DEN developed typical HCCs, but the number of detectable HCCs was reduced fivefold by the JNK1 deficiency. Thus, JNK1 is required for efficient HCC induction in response to DEN administration [4].

The downstream molecules phosphorylated by JNK have been extensively investigated. Originally, JNKs were identified as protein kinases that phosphorylated c-Jun on serine residues [67]. c-Jun is a well-characterized oncogene, especially in liver [68], and its phosphorylation by JNK may be relevant in HCC development. The AP-1 transcription factors, c-Jun and c-Fos, are composed of homo- and heterodimers with basic region-leucine zipper proteins belonging to the Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra-1, and Fra-2) subfamilies, all of which recognize the AP-1 binding site or TPA-response element in the regulatory region of AP-1 target genes [69]. Liver carcinogenesis induced by DEN is dramatically reduced in mice lacking c-Jun in hepatocytes. A putative mechanism accounting for the reduced tumorigenic effect was prevention of apoptosis by c-Jun, and the prevention of apoptosis was associated with p53 [68].

The tumorigenic effect of JNK1 in the liver is mediated through positive gene regulation or by cell proliferation molecules such as cyclins and CDKs and metastatic factors such as MMPs, VEGF, and others [4].

Based on an NF-*κ*B and JNK study in an animal hepatocarcinogenesis model, regulation of ROS-mediated JNK activation is critical for developing cancer. Recent studies have revealed that ROS production accompanies many signaling events, including receptor signaling, and that ROS play critical roles in determining cell fate as second messengers and modifying various signaling molecules. Sustained JNK activation is induced by ROS by activating upstream MEKKs or inhibiting MKPs [70–72]. In fact, enhanced JNK activation in IKK*β*-deficient mice has been assessed. Hepatocyte-specific *Ikkβ*-knockout mice and *Jnk1*-knockout mice were inter-bred and treated with DEN at 15 days of age. Double-knockout mice developed three-fold fewer tumors compared with hepatocyte-specific *Ikkβ*-knockout mice, and a similar decrease was found in maximal tumor diameters. These results suggest that increased ROS and ROS-mediated JNK activation is a critical regulator for HCC development (Figure 3). Viral proteins including the HBV X and HCV core proteins are capable of inducing ROS accumulation in hepatocytes [73].

TAK1 is one of the MEKKs that activate both the NF-*κ*B and JNK pathways. Hepatocyte-specific Tak1-deficient mice display spontaneous hepatocyte death, compensatory proliferation, inflammatory cell infiltration, and perisinusoidal fibrosis at the age of 1 month. Older mice develop multiple cancer nodules characterized by increased expression of fetal liver genes including alpha-fetoprotein. Cultures of primary hepatocytes deficient in Tak1 exhibit spontaneous cell death, which is further increased in response to TNF-*α*. These results indicate that TAK1 is an essential component of cellular homeostasis in the liver [74].

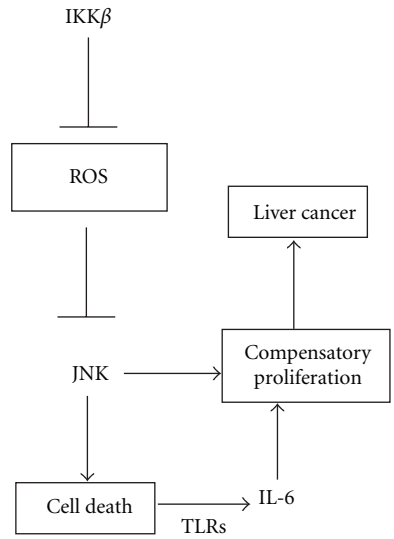

Figure 3

4.5. JNK1 and Therapeutic Target for HCC. The findings implicating a pivotal contribution of JNK in HCC development suggest the use of a JNK inhibitor for treating HCC. The most commonly used JNK inhibitor is SP600125, which shows high efficacy in blocking JNK kinase activity. In animal models, this compound is effective against acute hepatitis induced by acetaminophen or LPS/GalN [51, 75]. In a mouse model, the cell-permeable JIP peptide, which interferes with the interaction between JIP and JNK to inhibit JNK activity, suppresses chemically induced HCC [59].

5. HCC and TLRs

5.1. TLR Signaling. Innate immunity represents the first line of protection against microbial pathogens and is mediated by macrophages and dendritic cells. Although it was initially suggested to be a nonspecific response, innate immunity discriminates a variety of pathogens through the function of pattern-recognition receptors (PRRs) such as TLRs. These receptors recognize microbial components known as pathogen-associated molecular patterns [76]. Thirteen mammalian TLRs have been described; 10 are expressed in humans, and each is responsible for recognizing distinct bacteria, virus, and fungi microbial structures. The two most eagerly studied are TLR2 and TLR4, the PRRs for gram-negative and gram-positive bacterial products, respectively. TLR4 is also the major receptor recognizing endogenous ligands released from damaged or dying cells. TLRs are characterized by two conserved regions: the extracellular leucine-rich region and the cytoplasmic Toll/IL-1 receptor (TIR) domain. A detailed characterization of the TLRs is described in other chapters.

TLRs share the initial common activation pathway mediated by the TIR domain. After receptor activation, two signaling pathways mainly exist; one is through the adapter protein myeloid differentiation factor 88 (MyD88), and the other is not. All superfamily receptors, with the exception of TLR3, use MyD88 to initiate signaling. In some cases, MyD88 acts in concert with other adaptors, such as MAL/TIRAP, in the response triggered by stimulating TLR4, TLR1/2, and TLR2/6. In contrast, TLR3-mediated signaling requires only the TRIF adaptor molecule, which is also recruited by TLR4 in association with the other adaptor, TRAM [77]. In the MyD88-dependent pathway, MyD88 is associated with IRAK4, IRAK1, and/or IRAK2. IRAK4 in turn phosphorylates IRAK1, and IRAK2 promotes their association with TRAF6, which serves as a platform to recruit TAK1 kinase. Once activated, TAK1 activates the IKK complex and eventually activates NF-κB and JNK. The IRF7 transcription factor is also activated downstream of TLRs 7, 8, and 9, leading to its translocation into the nucleus and to activation of IFN and IFN-inducible genes. TLR3 and TLR4 both signal through the TRIF adaptor, which interacts with TRAF3 to activate the nonclassical IKKs and the IKK, resulting in the dimerization and activation of IRF3, which then translocates to the nucleus activating the transcription of IFN and IFN-induced genes [78].

5.2. TLR Signal and Liver Disease. The liver may be exposed to bacteria from the intestine via the portal vein, leading to an uncontrolled innate immune system that may result in inflammatory liver disorders [79]. Many factors are capable of activating TLRs in the liver. Among them, HBV, HCV, alcoholic liver disease, and NASH are important etiologies for HCC.

The TLR ligands TLR4 and 9 inhibit viral replication in HBV-transgenic mice [80]. In the absence of HBeAg, HBV replication is associated with upregulation of the TLR2 pathway, leading to increased TNFα production, demonstrating a potentially important interaction between HBV and the innate immune response [81].

HCV can activate innate immune systems to produce inflammation. The HCV core and NS3 proteins activate TLR2 on monocytes to induce cytokines in a NF-κB- and

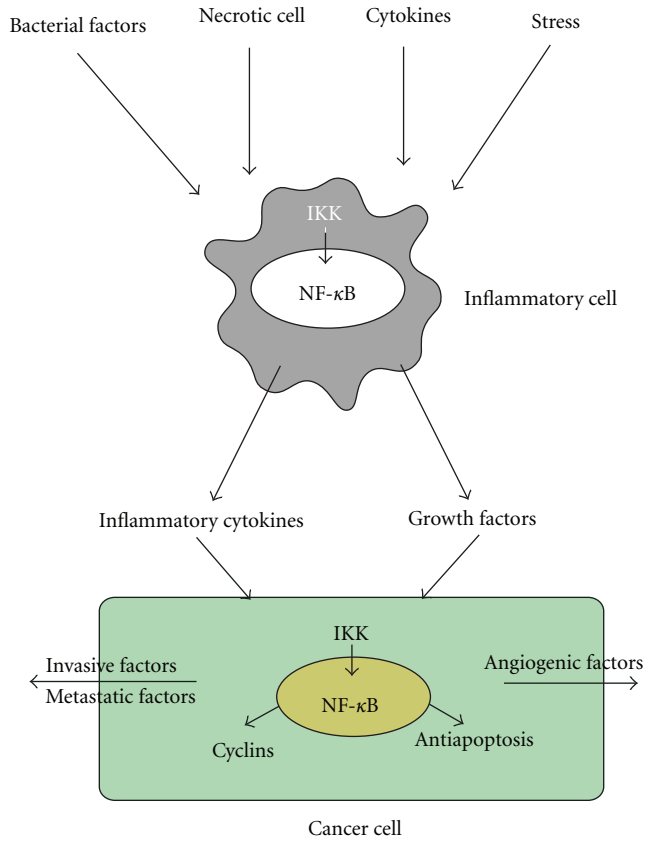

Figure 4

JNK-dependent manner [82]. The NS3 protein interacts directly with TBK1, resulting in decreased TBK1-IRF3 interaction and inhibition of IRF3 and IFN transcription. The NS3 protein also impedes both IRF3 and NF-κB activation by reducing functional TRIF abundance [83]. Many other *in vitro* studies have been reported, but the *in vivo* condition is still unclear.

Excessive alcohol intake is associated with increased intestinal permeability and elevated endotoxin levels [84]. LPS activates TLR4 on Kupffer cells and increases proinflammatory cytokine production. Antibiotic treatment reduces the sensitivity of alcoholic liver disease [85, 86].

Intestinal bacteria seem to be important in NASH pathogenesis. In NASH, ob/ob mice exhibit increased hepatic sensitivity to LPS and developed steatohepatitis [87]. In a methionine/choline-deficient NASH model, TLR4-deficient, but not TLR2-deficient mice, exhibited less intrahepatic lipid accumulation [88].

5.3. TLR Signal and HCC. All of the diseases described above are associated with the development of HCC. Therefore, it seems clear that TLRs are involved in the development of HCC. It is very important to analyze which cell types are involved in the process to understand liver tumorigenesis. Kupffer cells may be the major cells expressing TLRs in liver. Kupffer cells are resident liver macrophages and express most major TLRs [89]. In contrast, hepatocytes, the liver parenchymal cells, show weak TLR 2 and TLR4 expression and less response against their ligands [90, 91]. TLR2 expression in hepatocytes is upregulated by LPS, TNF, and others, suggesting that hepatocytes become more sensitive in the inflammatory condition [92].

Mice deficient in TLR4 and MyD88, but not TLR2, have a marked decrease in the incidence, size, and number of chemically induced (DEN) liver cancer tumors, indicating a strong contribution of TLR signaling to hepatocarcinogenesis [79]. It is assumed that dying hepatocytes following DEN may activate myeloid cells such as Kupffer cells via TLRs and induce proinflammatory cytokines and hepatomitogens, which enhance the development of HCC [93].

MyD88 is an adapter molecule for TLRs necessary for NF-κB, and MyD88 ablation strongly suppresses DEN-induced hepatocarcinogenesis [32]. IL-6 induction and liver injury are dependent on signaling via MyD88. Collectively, TLR4-MyD88 signaling appears to be essential for hepatocarcinogenesis.

6. Conclusion

Participation of the IKKβ/NF-κB and JNK signaling pathways in carcinogenesis differs among organs, cells, and models. The crosstalk between an inflammatory cell and a neoplastic cell, which is instigated by the activation of NF-κB, is critical for tumor organization (Figure 4). JNKs

also regulate cell proliferation and apoptosis, making them the main tumor-promoting protein kinases. TLRs play roles in cytokine and hepatomitogen expression mainly in myeloid cells and may promote liver tumorigenesis. These observations suggest that a better understanding of these signaling pathways in the liver will help us understand the mechanism of hepatocarcinogenesis and provide a new therapeutic target for HCC.

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Review Article

Contribution of Gut Bacteria to Liver Pathobiology

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Emerging evidence suggests a strong interaction between the gut microbiota and health and disease. The interactions of the gut microbiota and the liver have only recently been investigated in detail. Receiving approximately 70% of its blood supply from the intestinal venous outflow, the liver represents the first line of defense against gut-derived antigens and is equipped with a broad array of immune cells (i.e., macrophages, lymphocytes, natural killer cells, and dendritic cells) to accomplish this function. In the setting of tissue injury, whereby the liver is otherwise damaged (e.g., viral infection, toxin exposure, ischemic tissue damage, etc.), these same immune cell populations and their interactions with the infiltrating gut bacteria likely contribute to and promote these pathologies. The following paper will highlight recent studies investigating the relationship between the gut microbiota, liver biology, and pathobiology. Defining these connections will likely provide new targets for therapy or prevention of a wide variety of acute and chronic liver pathologies.

1. Introduction

Receiving approximately 70% of its blood supply from the portal vein which is the direct venous outflow of the intestine, the liver is continually exposed to gut-derived factors including bacteria and bacterial components. To combat this influx, the liver contains a large number of resident immune cells including macrophages (i.e., the Kupffer cell), lymphocytes, natural killer cells, dendritic cells, and B cells. Together, these immune cell populations in conjunction with other nonparenchymal cells including endothelial cells and stellate cells orchestrate a controlled and organized response to these potentially highly inflammatory factors. However, when normal liver physiology is disrupted and inflammatory cells are activated, gut-derived factors likely augment or exacerbate certain liver diseases leading to enhanced tissue damage and propagation of inflammation. Thus, understanding the mechanisms both of control and of activation by gut-derived factors as well as the functionality of the gut barrier are critical to the development of new therapeutic modalities to treat or prevent acute and chronic liver diseases such as viral hepatitis, alcoholic liver disease, and/or liver cancer. The current paper will provide an overview of gut bacterial populations, gut barrier function, and the potential interactions of gut bacteria with acute and chronic liver disease.

2. The Gut Microbiome

The human intestine provides residence to 1×10^{13} bacteria, a number which dwarfs the total number of cells in the human body (1×10^{12}) [1, 2]. Referred to as commensal bacteria, these microorganisms play a crucial role in human physiology and metabolism, providing key metabolic functions during absorption and waste breakdown [3]. Moreover, they also contribute to gut epithelial cell responses including proliferation and differentiation and play a key role in barrier development and function [4]. It is clear, however, that these same luminal contents may also contribute to intestinal pathology in the setting of colitis where immune cell dysfunction, barrier disruption, and/or

overgrowth of pathogenic bacterial species have been shown to be involved [5–8]. The following sections will provide a brief overview of the populations present and their proposed functions.

3. Populations of Microflora

A number of studies have sought to identify the populations present with estimates of approximately 800 different species [2]. The reasons for these differences in numbers are likely the result of mechanism(s) of detection as early studies used both microscopy and culture where as more recent studies utilize highly sensitive sequencing approaches of 16 S rRNA for identification including 454 pyrosequencing [1]. The predominant bacteria present within the gut are from the genera *bacteroides*, *clostridium*, *bifidobacterium*, *peptostreptococcus*, and *ruminococcus* with *escherichia*, *lactobacillus*, *enterobacter*, and *enterococcus* constituting a minor but significant proportion [1, 9]. Interestingly, the types and proportions of bacteria within the intestine from lower small intestine to distal colon are different, likely regulated both by the microenvironment (i.e., pH and nutrient availability) and the intestinal motility itself [10, 11]. For example, *Escherichia coli*, *enterococci*, and *lactobacilli* account for greater than 50% of cecal bacteria whereas their numbers dwindle to less than 10% in the distal colon. The specific populations present also varies both between individuals and in the same individual during periods of illness or alterations in food intake [9]. Indeed, treatment with antibiotics or acute diarrheal illness have been shown to alter gut microbe densities and populations while alterations in diet (i.e., high fiber) may also cause a minor shift in these populations [12]. Alternatively, specific physiological abnormalities may also contribute to gut bacteria content. For example, pancreatic exocrine insufficiency or vagus nerve defects can reduce antibiotic factors (i.e., pancreatic enzymes) and peristalisis, respectively, leading to increased bacterial growth in the upper gastrointestinal (GI) tract while strictures and adhesions can themselves limit gut content mobility, promoting bacterial overgrowth [13]. Moreover, in animal models, significant differences in gut microbiota have been shown between vendors and as well as in genetically deficient mice. For example, significant differences in both total bacterial load and specific bacterial populations (i.e., segmented filamentous bacteria) were shown between mice purchased from Jackson Laboratories and Taconic Farms [14]. In addition, mice deficient in MyD88, an adaptor molecule associated with numerous innate immune receptors, showed altered gut microbiota in the distal intestine suggesting that immune cell function can interact with and alter luminal bacterial populations [15]. Thus, physiological and pathological changes in intestinal function including alterations in immune cell responses can significantly influence the proportions, quantity, and spatial distribution of bacteria present within the intestine.

The reason for and importance of interindividual differences in gut microbial content in otherwise normal individuals is less clear [16]. The human microbiome project has been initiated by the National Institutes of Health in the United States to characterize the bacterial populations present both within healthy and diseased individuals from all over the world. Early results demonstrate what appear to be a core group of bacteria which is shared by most individuals though the vast majority of bacteria are still heterogeneous among different individuals [16]. Together, it is clear that significant diversity exists among intestinal bacterial species between individuals and that certain environmental, physiological, pathological, or therapeutic interventions can modulate these populations.

4. Function of the Gut Microflora

As noted above, intestinal microbiota are referred to as commensal as they coexist without initiating inflammatory or infectious responses. It is becoming clear that these same bacteria provide at least three key functions to the mammalian intestine including epithelial cell health, nutrient metabolism and breakdown, and indirect mucosal defense against pathogenic bacterial strains. Perhaps the most easily understood function of these bacteria is their contribution to metabolism and nutrient breakdown. Commonly represented genera of bacteria within the human intestine are known to express key polysaccharide metabolizing enzymes capable of breakdown of routinely consumed sugars including cellulose, pectins, and gums [17]. These same bacteria also affect gut barrier function. Indeed, these nonpathogenic bacteria compete for nutrients and adherence with other pathogenic bacteria. A good example of this effect is seen in experimental animals as well as patients receiving high-dose antibiotic treatment where reductions in normal gut flora allow for overgrowth of pathogenic bacteria including *Clostridium difficile* [18, 19]. The generation of germ-free mice has further confirmed the importance of commensal bacteria in health and barrier function. Intragastric infection of germ-free mice with *E. coli* O157:H7 resulted in rapid intestinal colonization and morbidity associated with glomerular toxicity, a response not observed in conventionalized mice [20]. Again, overgrowth of pathogenic bacteria in a germ-free host is the likely cause for these findings though alterations in barrier integrity or immune cell development, localization, or responsiveness may also contribute.

Finally, intestinal microbiota also influence epithelial cell health and function. When biota are absence as in the germ-free mice, intestinal epithelial cells are underdeveloped [21, 22]. As discussed above, gut flora play a key role in metabolism of complex sugars. During this process, fermentation of sugars forms a number of short chain fatty acids including proprionate, acetate, and butyrate among others [1]. Interestingly, intestinal epithelial cells derive a large percentage of their metabolic fuel from these products, specifically butyrate. *In vitro* butyrate administration to cultured intestinal epithelial cells promotes their survival, differentiation, and proliferation thereby supporting barrier integrity [23]. Together, these studies and numerous others support a specific and tightly regulated role for enteric bacteria in metabolism, defense, and barrier integrity within the intestine.

5. Gut Barrier Function and Dysfunction

In as much as gut bacteria contribute to normal gut physiology, their presence poses a continuous risk for systemic infection [6]. The direct physical barrier against bacterial translocation is complex. Mucous producing goblet cells secrete a thick layer of polysaccharide called mucin which coats the intestinal epithelial surface and provides a physical barrier suppressing epithelial-bacteria contact. This layer constitutes in part the unstirred layer covering the intestinal epithelium, slowing the movement of solutes and bulk fluid through the barrier [24]. The specific importance of mucins in protection against intestinal inflammation can be seen Muc2-deficient mice [25]. Mice lacking Muc2 show increased susceptibility to dextran sodium sulfate-mediated colitis. Moreover, in humans, polymorphisms in Muc3A correlate with increased frequency of ulcerative colitis [26]. Thus, the mucous layer is a critical component of the intestinal barrier limiting direct access to the intestinal epithelium.

Below the mucous layer resides the intestinal epithelial layer. Organized in a crypt and villus arrangement to increase surface area, intestinal epithelial cells (IECs) are held together by a series of cell-cell protein interactions which tightly regulate paracellular solute movement [27]. Nearest the surface of the epithelial cell, claudins interact with intracellular support provided by zonula occluding (ZO-1) and F-actin forming the tight junction. Secondary to the tight junction exists the adherens junction. E-cadherins attach cell membranes, supported by intracellular catenins α and β. Near the basolateral surface, the desmosome exists consisting of desmogleins and desmocollins anchored to intracellular keratin by desmoplakin [6]. Together, these three structures support strong epithelial cell contacts and prevent paracellular movement of large molecules and bacteria to the underlying tissue.

Despite this tight and redundant barrier, gut bacteria are continuously sampled by the underlying lamina propria immune cells. Indeed, this underlying layer contains a large population of lymphocytes, dendritic (DC) cells, and neutrophils which serve to intercept invading pathogens and modify the underlying immune response to commensal bacteria populations. DCs extend projections through the epithelial layer, sample enteric bacterial antigens, and present them to underlying lymphocytes, thereby priming the immune system in case of barrier dysfunction [28, 29]. T cell development is then regulated by the production of key cytokines produced by myeloid cells where IL10 principally contributes to T regulatory cell development and maintenance and suppression of inflammation [7]. However, other very recent studies indicate that direct interactions of gut-derived antigens signaling through Toll-like receptor (TLR) 4 on $CD4^+$ T cells contributes to their regulatory development and function [30]. In summary, the epithelial barrier and underlying immune cells work together to protect against translocation, inflammation, and systemic infection.

When this complex barrier and/or underlying immune cell network is damaged or disrupted, intestinal inflammation, tissue damage, and absorptive dysfunction result. Key mechanisms in this disruption have been elucidated and involve immune cell dysregulation, pathogenic bacterial overgrowth, and/or primary barrier dysfunction. Absence of interleukin (IL) 10, a key regulatory cytokine, is known to lead to spontaneous intestinal inflammation in a gut bacteria-dependent manner [31]. Likewise, reconstitution of severe combined immunodeficient mice or recombinase activating gene 1 deficient mice with naïve $CD4^+CD45RB^{Hi}$ positive T cells results in significant intestinal inflammation and barrier disruption again due to dysregulation of lymphocyte responses [32]. Infection of germ-free mice with certain pathogenic strains of *Campylobacter* can also lead to mild to moderate intestinal inflammation [33]. Similar correlations have been established in human inflammatory bowel disease (IBD) where increased proportions of *E. coli* are noted and can be correlated with the severity of disease [34]. Finally, and as discussed above, alterations in mucin production predispose the intestine to inflammation and bacterial translocation. It is likely, however, that in mice and humans, a combination of factors exist which alter the intestinal barrier, enhance bacterial translocation, and promote intestinal inflammation. Nevertheless, key participants in the regulation of gut barrier function have been established and serve as targets for therapeutic intervention.

6. Gut-Liver Interactions in Liver Disease

As the preceding discussion has indicated, gut barrier function is critical to prevent inflammation of the underlying mucosa and submucosa. Receiving ~70% of its blood supply from the intestine through the portal circulation, the liver, much like the intestine, is also exposed to gut-derived factors including bacteria and bacterial products and thus must be prepared to handle these potential systemic pathogens. To accomplish this task, the liver contains a large number of immune cells, of both the innate and adaptive immune systems which participate both in tolerance and inflammation within the liver. The following section will provide a brief overview of these immune cell populations, including their locations, proportions, and general functions.

7. Hepatic Immunology: An Overview

Perhaps the most characterized of these immune cell populations is the Kupffer cell (KC), the resident hepatic macrophage. Making up approximately 4% of the total hepatic cell population and 80–90% of all tissue macrophages, KCs are well known for the ability to engulf bacteria and respond to bacterial antigens including lipopolysaccharide (LPS) derived from gram negative bacteria such as E. coli [35]. Through the expression of TLR4 and CD14, KCs are able to efficiently take up endotoxin and phagocytose portally delivered bacteria while also contributing significantly to inflammation and tissue damage through the production of tumor necrosis factor alpha (TNFα) and reactive oxygen intermediates in a wide variety of acute and chronic liver disease [36, 37]. Alternatively, KCs may serve to tolerize the immune response through antigen presentation and concomitant nitric oxide and prostaglandin production [38]. It

is clear given their sheer numbers that KCs are an important component of the innate immune response of the liver.

Working in concert with these resident macrophages are DCs. DCs are also capable of engulfing particles including bacteria but play a key role in antigen presentation, cytokine production (i.e., IL4 and 12 production), and T and B cell development and reactivity [39]. DCs may also promote natural killer (NK) and natural killer T (NKT) cell activation via IL12 production and accelerate tumor cell clearance and their reduced numbers in the hepatitis C virus (HCV) infected liver may enhance HCV infectivity and carcinogenicity [40, 41].

Complimenting the functions of KCs and DCs are those of natural killer (NK) cells. NK cells express specific receptors (NK1.1, NKG2D in mice; NKp46, CD56, and CD57 in humans) and produce large amounts of perforin and granzyme B in addition to immunomodulatory factors such as interferon gamma (IFNγ) and TNFα upon activation [42, 43]. NK cells are particularly responsive to malignant or infected cells while also potentially contributing to transplant rejection and autoimmunity [44, 45]. Indeed, depletion of NK cells promotes graft survival while their activation suppresses cancer cell survival and proliferation. Moreover, NK cells suppress fibrogenesis through direct killing of hepatic stellate cells (HSCs) in an NKG2D and IFNγ dependent manner [46].

Bridging the gap between innate and adaptive immunity is the natural killer T (NKT) cell. Expressing receptors for both innate (NK1.1, CD49b, CD56, and CD57) and adaptive (T cell receptor) immune cells, NKT cells represent an important source of IFNγ and IL4 within the liver [47]. A large proportion of hepatic NKT cells recognize antigens presented through the MHC Class I-like receptor CD1d, rely heavily on IL12 and IL15 for survival and activation, and contribute both to the regulation of T helper (T_h) cytokine production and to acute and chronic liver injury through cytokine production and Fas expression [48–50]. Much like NK cells, activation of NKT cells results in tumor cell clearance while also contributing to early alcohol-induced liver injury [50, 51].

Fulfilling the adaptive immune functions within the liver are a large population of traditional $CD4^+$ and $CD8^+$ lymphocytes. Constituting approximately 35% of the hepatic lymphocyte population, these cells play a key role both in antigen recognition and in tolerance [52]. Accumulation and/or survival of hepatic T cells is associated with worse fibrogenesis while their early accumulation in the ischemic liver is a known trigger for neutrophil infiltration and tissue damage [53, 54]. $CD8^+$ T cells contribute to stellate cell activation during carbon tetrachloride induced fibrosis and directly damage hepatocytes in the HCV-infected liver in an antigen specific manner [55, 56].

In summary, and as is shown in Figure 1, the liver provides residence to a large and heterogeneous population of immune cells, each with specific functions of protection, tolerance, and/or inflammation. It is this third aspect, during inflammatory responses or chronic injury, where the function of hepatic immune cells is perhaps most interesting and extensively studied. And of even greater interest is the potential impact which gut-derived factors may have on this process. As noted earlier, the liver is a unique position where its normal function to sample, metabolize, synthesize, and/or degrade both absorbed and circulating products also places it in potential direct contact gut-derived bacteria and bacterial antigens. And previous studies would suggest that a connection exists as either small intestinal bacterial overgrowth or infection with helicobacter alone contributed to hepatic pathology including increased serum alanine aminotransferase release and inflammatory cell recruitment [57–59]. Likewise, experimental colitis models in rodents and inflammatory bowel disease in patients were associated with periportal inflammation similar to that seen in primary biliary cirrhosis and primary sclerosing cholangitis, respectively, suggesting that gut-derived factors likely activate inflammatory processes within the liver [60, 61]. The following sections will highlight the current knowledge regarding the influence of gut-derived factors on hepatic biology and pathobiology, focusing on several important mechanisms of liver injury.

8. Gut Bacteria and the Undamaged Liver

The contribution of gut bacteria to the formation of the hepatic immune system has not been intensively investigated. It is clear from the previous discussion that the liver contains a large number of immune cells though the specific mechanisms governing their localization is not well understood. Crispe and colleagues identified TLR4 as a potential indirect regulator of activated $CD8^+$ T cell trapping within the murine liver suggesting a potential interrelation between the gut antigens, specifically endotoxin, and liver lymphocyte populations [62]. Recent studies from our laboratory were directed at better understanding the connection between gut bacteria and resident hepatic immune cells. Using germ-free C57Bl/6 wild type mice or specific pathogen free (SPF) mice, we demonstrated that gut bacteria have little effect on the proportions of or total numbers of lymphocytes (CD4, CD8, NK, or NKT cells) or macrophages present within the murine liver. Moreover, examination of serum alanine aminotransferase levels and basal expression of key inflammatory cytokines including TNFα and antiinflammatory cytokines (i.e., IL10) were not different between germ-free and SPF mice (Son and Hines, unpublished observation). Finally, analysis of basal hepatocyte proliferation revealed no substantial differences between these groups. Together, these data demonstrate that resident hepatic immune cells and hepatocytes themselves are not overtly affected by normal gut-derived antigen exposure.

While gut bacteria do not significantly affect liver physiology or immune cell populations, its potential to initiate and/or propagate liver injury has been investigated. For example, experimental damage of the intestine with dextran sodium sulfate (DSS) leads to periportal liver inflammation likely the result of increased gut bacterial delivery to the liver [63]. Similarly in patients with ulcerative colitis there is often evidence of primary sclerosing cholangitis including significant periportal inflammation [64]. Thus, it is clear that a relationship exists between gut barrier

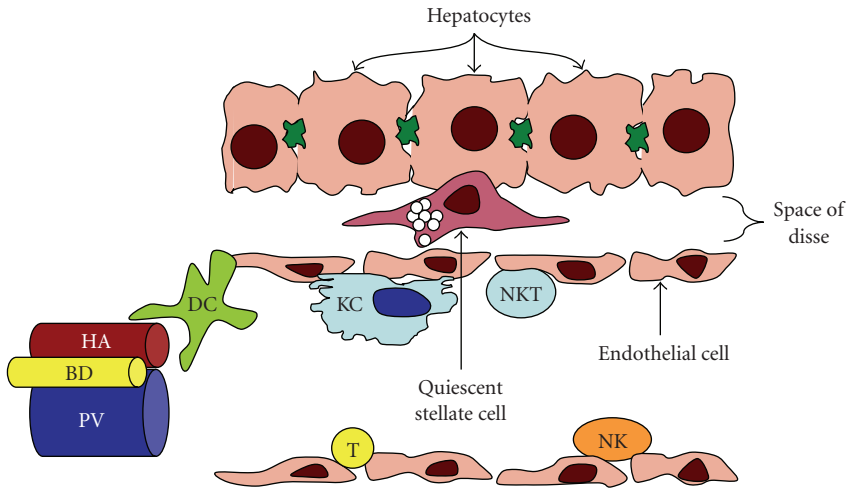

Figure 1: Diagramatic representation of the liver sinusoid. HA; hepatic artery. BD; bile duct. PV; portal vein. DC; dendritic cell. KC; Kupffer cell. NKT; natural killer T cell. T; T cell. NK; natural killer cell.

function and secondary liver inflammation. The following sections will provide a review of the current understanding of gut-derived factors in a number of primary liver pathologies.

9. Alcoholic Liver Disease (ALD)

Perhaps the best characterized model of liver disease which is nearly completely dependent on gut-derived factors for its pathogenesis, chronic alcohol consumption remains an important clinical problem alone and in combination with other liver diseases [65]. Early clinical studies revealed increased plasma endotoxin levels following acute ethanol exposure in patients with and without chronic liver disease suggesting that ethanol could potentially alter gut barrier function [66]. Experimental studies confirmed these findings identifying the ability of alcohol to injure the rodent liver through augmentation of gut-derived bacterial translocation (specifically increased periportal levels of LPS) and specific activation of Toll-like receptor 4 on KCs [67–70]. Indeed, sterilization of the gut, depletion of KCs, or mutation in TLR4 caused a near complete inhibition of ethanol-induced liver injury as characterized by serum alanine aminotransferase release, inflammatory cell infiltration, and hepatocellular lipid accumulation. This central role for endotoxin in the pathogenesis of early ALD could not be argued though the mechanism by which ethanol altered gut permeability was less clear. Consumed ethanol is rapidly absorbed by the upper GI tract with near complete absorption occurring by the mid-jejunum. However, the majority of bacteria are held, as described earlier, within the cecum and upper large intestine. Careful studies have demonstrated the ability of ethanol to suppress endotoxin uptake by KCs and the function of acetaldehyde, the principle by-product of ethanol metabolism, to directly interfere with tight junction and adherens junction support [71, 72]. Indeed, absorbed circulating ethanol directly inhibits phagocytosis of macrophages including KCs thereby limiting LPS clearance while acetaldehyde promotes ZO-1 dissociation from occludin and E-cadherin adherence to β-catenin [73, 74]. Further compounding these effects is the ability of gut bacteria to metabolize ethanol and thus increase the luminal concentration of acetaldehyde and the potential for bacterial overgrowth to occur in ethanol consuming individuals [71, 75]. Thus, gut bacteria play a key role in early alcohol-induced liver injury both through the metabolism of ethanol and through the activation of key hepatic innate immune cell populations. Key questions remain, however, including the net effect of ethanol on the gut microbiota and the influence of gut-derived antigens on the progression of ALD, specifically fibrogenesis.

10. Nonalcoholic Fatty Liver Disease (NAFLD)

NAFLD continues to increase in westernized countries. Once considered a benign event, accumulation of lipid within the liver is a known risk factor for the development of inflammation and fibrosis and a component of metabolic syndrome, insulin resistance, and obesity [35]. The mechanism(s) underlying the development of NAFLD are not well understood, though obesity is a key risk factor. Numerous studies have implicated liver TNFα as a potential regulator of its development and TNFα levels are elevated in several models of NAFLD in rodents and in patients with NAFLD [76, 77]. Likewise, deficiency in leptin is known to result in hepatic lipid accumulation in conjunction with peripheral obesity [48, 78]. The potential influence of gut bacteria on the development of hepatocellular steatosis has been postulated. Models of bacterial overgrowth have shown promise as an initiator of fatty liver and patients with NAFLD present with upper intestinal bacterial overgrowth and enhanced intestinal permeability [79, 80]. Consistent with gut-derived endotoxin mediating these effects, TLR4-mutant mice showed reduced lipid accumulation following feeding

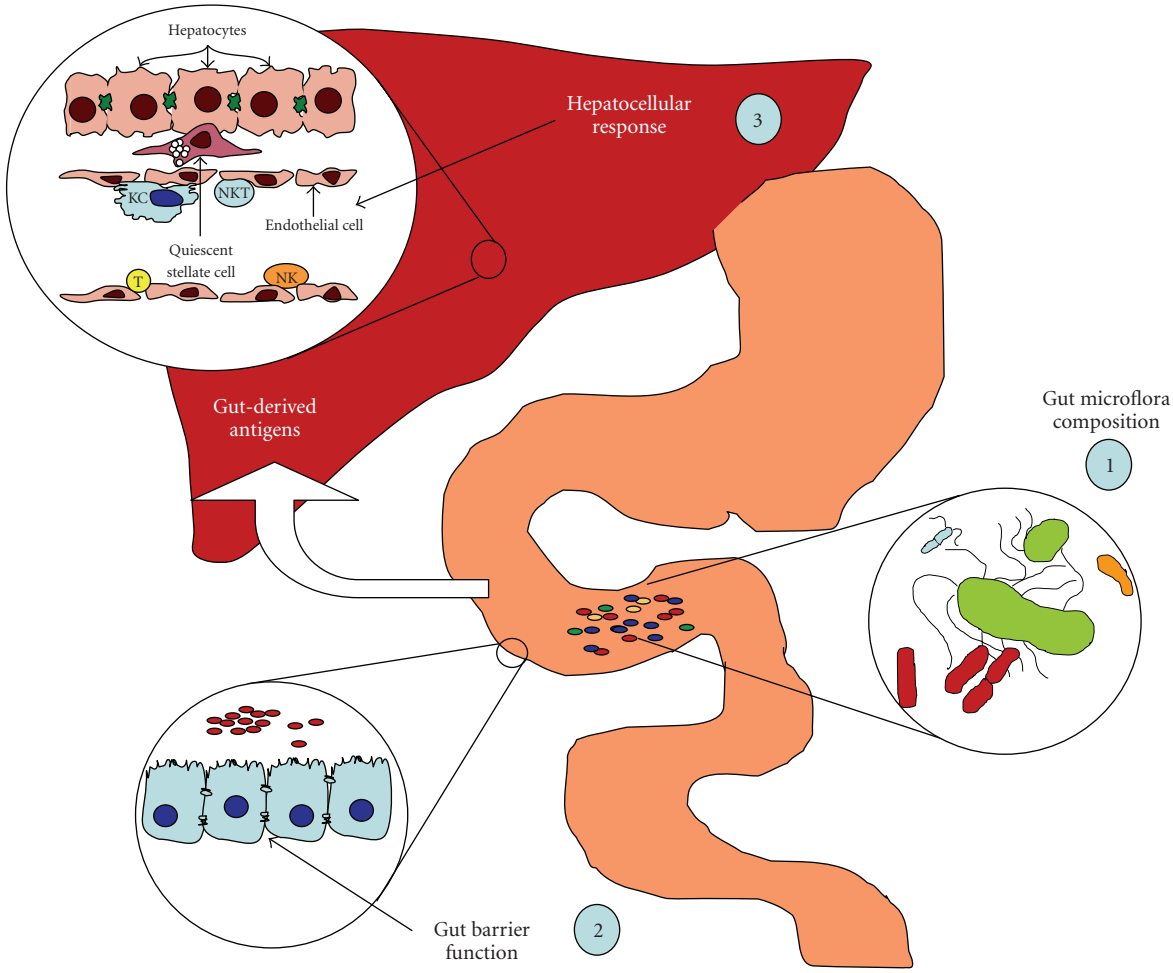

Figure 2: Diagram of the points of future study/intervention to treat or prevent various liver diseases. Discussed in summary section of the text.

a high-fructose diet or methionine-choline deficient diet when compared to their TLR4-wild type controls suggesting that LPS may contribute to disease progression [81, 82]. Studies from our laboratory and others have confirmed enhanced sensitivity of the liver to endotoxin treatment suggesting that hepatic immune cells, likely KCs, are primed to respond with increased production of TNFα and IL12 [49]. Similarly, TLR9 signaling may also promote NAFLD. Miura and colleagues demonstrate a significant reduction in hepatic lipid accumulation in choline deficient, amino acid defined diet in TLR9-deficient mice when compared to their wild type controls, a process which appears to involve TLR9-dependent Kupffer cell activation and subsequent production of IL1β [83]. Thus, both cell wall components and DNA derived from bacteria could be involved in the disruption of normal hepatocyte function in the setting of NAFLD.

While the above mentioned studies have focused on the direct interactions of gut-derived bacteria and bacterial products, the potential influence of certain metabolites of gut bacteria including short-chain fatty acids on hepatic energy homeostasis has not been thoroughly explored. It is clear that gut bacteria provide important metabolic functions, metabolizing complex sugars into short-chain fatty acids including proprionate and acetate, two molecules which are key sources of energy for the liver and muscle, respectively [1]. This important function is reinforced by findings in germ-free mice which are substantially leaner than their colonized counterparts. The specific mechanism by which SCFAs affect energy balance is not entirely clear though increased delivery of short chain fatty acids to the liver and the periphery could disrupt normal metabolic processes, specifically reducing glucose utilization and promotion lipid storage. However, high levels of propionate derived from fiber metabolism inhibit cholesterol and fatty acid biosynthesis in rodent livers [84]. Moreover, high-fat diet feeding supplemented with fermentable dietary fiber reduces plasma endotoxin levels when compared to high-fat diet feeding alone suggesting that fiber may affect the gut microbiota and potential influence intestinal barrier function [85–87]. Thus, the net effect of the gut microbiota on nutrient and energy balance is complex and warrants further investigation as it is likely to contribute, in addition to the direct effects on hepatic cells, to hepatic lipogenesis, endotoxemia, and NAFLD.

11. Ischemic Liver Injury

Liver transplantation remains the primary treatment for a number of chronic liver diseases including cirrhosis. Organ preservation and associated ischemic tissue damage represents an important determinant of graft survival. Critical mechanism(s) in reperfusion injury have been delineated and include early $CD4^+$ lymphocyte recruitment, KC activation and subsequent TNFα production, and later neutrophil recruitment and hepatocellular damage [54, 88, 89]. The contribution of gut bacteria to these processes has not been thoroughly investigated though it is clear that bone marrow derived cells and Kupffer cells and TLR4 are critical for early tissue damage following warm ischemia and reperfusion injury [88, 90–92]. It is also well established both experimentally and perhaps more so clinically that intestinal injury occurs during liver surgery and transplantation. Congestion of the portal vein due to clamping, even intermittently during transplantation or resectional surgery, reduces barrier integrity and promotes bacterial translocation [93]. Supporting the impact of intestinal microbiota directly, gut sterilization suppresses transplant-induced liver injury in rodents and reduces the incidence of sepsis early following transplantation in patients suggesting that intestinal microbiota are involved in these processes [94, 95].

12. Hepatocellular Carcinoma

The occurrence of hepatocellular carcinoma (HCC) continues to rise clinically. Both environmental and genetic factors have been implicated in its initiation. While susceptibility genes have been identified and range from immune cell markers to traditional tumor suppressor genes, the potential environmental cues are less well established [96]. It is clear that certain hepatotoxins including nitrites, organochlorine compounds, and aflatoxin contribute significantly to its development as do preneoplastic injuries associated with HCV infection [97–99]. The particular contribution of gut bacteria to the development and progression of HCC remains somewhat unclear. Gut bacteria convert intestinal nitrates to nitrites and nitrosoamines which are linked to colorectal cancer [1]. Moreover, recent studies have highlighted the ability of helicobacter hepaticus to promote aflatoxin-induced HCC in mice, a process involving inflammatory and proproliferative cytokine production [100]. These studies correlate well with findings in humans where *helicobacter sp.* can be both cultured from the liver and shown to be in greater quantities in the intestine [58, 101, 102]. Thus, bacterial metabolism of ingested materials and byproducts as well as inflammatory responses to the bacteria themselves likely play critical roles in liver cancer development and/or progression.

13. Liver Fibrosis

Chronic liver injury arising from a number of etiologies ranging from chronic ethanol consumption to viral infection is associated with increased risk for the development of fibrosis, cirrhosis, and liver failure [103]. It is clear from a large body of experimental work that repeated and continuous hepatocellular damage leads to the activation of HSCs and their production of key extracellular matrix proteins, specifically Type I fibrillar collagens. A number of studies have implicated immune cell activation, specifically macrophages and lymphocytes, in the initiation and propagation of disease [55, 104, 105]. Indeed, depletion of T and B lymphocytes or macrophages significantly reduced carbon tetrachloride-induced liver injury [104, 105]. The specific role which gut-derived antigens play in the setting of fibrotic liver disease has recently been examined [104]. Sterilization of the gut prevented both toxin-induced and cholestasis-induced hepatic fibrosis [104]. Further characterization revealed a critical role for gut-derived endotoxin as mice deficient in either CD14 or Toll-like receptor 4 were protected from cholestasis-induced fibrogenesis [104, 106]. Indeed, it appears that toxin-induced tissue injury, either by carbon tetrachloride or cholestasis, leads to increased portal delivery of endotoxin, activation of hepatic macrophages, induction of growth factor production, specifically transforming growth factor beta, and subsequent HSC activation [104, 106]. Moreover, recent studies also highlight the ability of endotoxin to directly activate HSCs further amplifying the fibrogenic response in these models [107, 108]. Together, it is clear that gut microflora, and specifically gram negative bacteria, contribute to fibrosis induction and progression experimentally.

The process of fibrogenesis may itself promote bacterial overgrowth and barrier dysfunction. Cirrhotic patients are at increased risk for spontaneous bacterial peritonitis in conjunction with reduced blood flow through the portal vein, intestinal vascular congestion, and barrier leakiness [109, 110]. Additionally, fibrosis and associated defective liver function itself may promote changes in bacterial populations, intestinal motility, and nutrient absorption and availability. For example, decreased bile acid production by the cirrhotic liver is associated with bacterial overgrowth [111]. Indeed, bile acids play a critical role in regulation of bacterial survival within the intestine [112]. Bile acids are directly, though weakly, bacteriocidal but are capable of activating specific bile acid receptors including farnesoid X receptor which regulates the expression of key bacteriocidal genes including inducible nitric oxide synthase and IL18 within the intestine [113]. Cirrhosis also limits small intestine motility which has been associated with bacterial overgrowth [110]. Finally, decreased absorption increases nutrient availability throughout the small and large intestine further enhancing bacterial growth [114]. Together, it is clear that hepatic fibrosis is critically regulated by gut-derived antigens and that cirrhosis itself may influence the populations of bacteria present within the intestine promoting a positive feedback loop perpetuating tissue injury and fibrogenesis.

14. Autoimmune Liver Diseases

Autoimmunity is associated with several forms of chronic liver damage including autoimmune hepatitis, primary

biliary cirrhosis, and primary sclerosing cholangitis. The initiating events in these diseases are not well understood though it is clear that antibody formation to self antigens is key to the development. The influence of the gut microbiota on these disease processes again has not been thoroughly investigated though some connections have been suggested.

Autoimmune hepatitis (AIH) accounts for approximately 20% of chronic hepatitis in Caucasians and is characterized by hypergammaglobulinemia and liver-directed autoantibodies resulting in large hepatic lymphocytic infiltrates [115]. Activation of hepatic T lymphocytes with the plant lectin Concanavalin A leads to the expression of key T helper cytokines including IL4 and IFNγ, macrophage activation, neutrophil recruitment, and hepatocellular injury similar to that observed in autoimmune hepatitis [116]. It is clear from this model system that interruption in the early expression of either IL4 or IFNγ or disruption in Fas-FasL signaling protect the liver from this T cell-mediated tissue injury [117–121]. The contribution of gut bacteria to this response has not been thoroughly investigated. Previous studies have demonstrated the contribution of TLR4 signaling to the trapping of $CD8^+$ T cells within the murine liver [62]. More recently, TLR9 was shown to contribute to the homing and activation of hepatic NKT cells, a process dependent on KCs and IL12 [51]. Similarly, TLR4 expression on intestinal $CD4^+$ T cells contributed to the induction of T regulatory cells and suppression of colitis resulting from absence of IL10 [30]. Thus, T cells appear to be capable of responding to conserved antigens such as endotoxin directly and this cascade likely contributes to their responsiveness within the liver. Consistent with this notion, recent studies in our laboratory demonstrate the necessity of gut-derived bacteria during ConA-induced T cell-mediated hepatitis (Son and Hines, unpublished observation). Absence of gut bacteria significantly reduced ConA-induced liver injury in the absence of major alterations in resident T cell number or activation. Indeed, absence of gut bacteria-reduced early IFNγ and IL4 production, and later eosinophil recruitment and hepatocellular apoptosis. Together, these studies suggest that gut-derived products regulate, either directly or indirectly, T cell function within the liver. Further study will be required both in animal models and in patients with AIH to more specifically delineate the mechanism governing these responses.

Similar to AIH, primary biliary cirrhosis affects approximately 40 per 100,000 people in the United States and is a consequence of immune cell activation and directed damage to cholangiocytes, specifically intrahepatic bile ducts with nearly 95% of patients presenting positive for antimitochondrial antibodies [115]. Progressive intrahepatic biliary tract damage promotes bile acid buildup, stellate cell activation, and hepatic fibrogenesis with failure occurring in 26% of patients within 10 years of diagnosis with liver transplantation constituting the primary treatment. Thus, understanding the factors which may promote or exaggerate this process are needed. Studies by Hopf and others detailed an association of *E. coli* rough form and the presence of PBC in patients as healthy individuals rarely show measurable levels of this bacterial subspecies. Moreover, they demonstrated the presence of lipid A within the liver of PBC but not healthy control patients further demonstrating the presence of bacteria within the liver [122]. Given this association, further study is warranted to determine if modulation of gut microbiota, particularly E. coli subpopulations might aid in the treatment of this complex disease.

Very similar to PBC, primary sclerosing cholangitis can be described as a progressive autoimmune disease process leading to destruction of intrahepatic and extrahepatic bile ducts, inhibition of bile acid secretion, toxin buildup, and chronic hepatocellular injury [123]. Interestingly, as mentioned previously [64, 115], a large number of patients (~75%) show signs of inflammatory bowel disease suggesting potential interactions of the gut and liver and/or common pathological causes (i.e., autoimmune disorders, defective immune cell regulation). Experimental models of inflammatory bowel disease have been associated with periportal inflammation suggesting potentially that gut factors may initiate the response in the absence of underlying immune cell dysfunction [124]. Further examination of the gut microbiota in conjunction with PSC may unlock new information into the mechanisms of PSC and aid in therapeutics development.

15. Viral Hepatitis

Hepatitis arising primarily from HCV infection represents the leading cause of liver disease in the world [125]. Indeed, hepatitis B and C viral infections account for 75% of the cases of liver disease worldwide [126]. The pathogenesis, particularly of HCV infection, is complicated and involves primary hepatocyte infection, disruption of immune cell responses including inhibition of endogenous antiviral responses and activation of adaptive immunity including antigen specific $CD8^+$ T cell recruitment [126]. The contribution of gut-derived antigens to the pathogenesis of viral hepatitis has not been explored. HCV infection is associated with a number of hepatic diseases from hepatocellular lipid accumulation to stellate cell activation, immune cell recruitment, and cancer development. To this third end, very recent studies by Machida and colleagues identify an important connection between ethanol consumption, viral infection, TLR4 signaling, and carcinogenesis within the murine liver. Indeed, TLR4 signaling promotes Nanog/CD133 production and promotes ethanol/HCV-induced hepatic tumor formation [127]. Future study in this complex system using rodent models is warranted to better understand the overall impact of gut bacteria to the multiple pathologies present. Indeed, it could be that gut-derived antigens serve to prime hepatic innate immune cells to produce important antiviral cytokines including IFNγ while also promoting hepatic T cell function and responsiveness. Further study is warranted to dissect out the potential multiple pathways of involvement of gut-derived antigens in this complex injury scenario.

16. Concluding Remarks

From the above discussion, it is clear that gut bacteria contribute to normal intestinal epithelial cell biology and

function while also contributing substantially to the breakdown of complex sugars in the diet. It is also evident that these same bacteria, in the absence of appropriate immune cell regulation or when gut barrier function is impaired, contribute significantly to intestinal inflammation and damage. Likewise, these same antigens, when delivered to the liver, contribute significantly to various acute and chronic liver diseases through activation of both innate and adaptive immune responses and wound healing processes. Thus, modulation of the gut microbiota may represent a new avenue for therapeutic intervention to treat or prevent a variety of liver diseases. As detailed in Figure 2, key questions remain, however, including (1) what are the specific populations of bacteria present within the intestine and can these be correlated with or used as a screening tool for the progression of liver disease, (2) how do different microbiota populations influence gut barrier integrity, and (3) what are the cell-specific effects of gut-derived antigens within the injured liver (i.e., KC, stellate cell, T cell, endothelial cell, etc.) and does the type of injury influence their effects (i.e., ischemic damage versus viral infection). Future studies directed at these questions will provide important new information into the connection between the gut microbiota and liver disease and likely contribute to new therapies for or predictors of liver pathobiology.

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Review Article

LPS-Toll-Like Receptor-Mediated Signaling on Expression of Protein S and C4b-Binding Protein in the Liver

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Protein S (PS), mainly synthesized in hepatocytes and endothelial cells, plays a critical role as a cofactor of anticoagulant activated protein C (APC). PS activity is regulated by C4b-binding protein (C4BP), structurally composed of seven α-chains (C4BPα) and a β-chain (C4BPβ). In this paper, based primarily on our previous studies, we review the lipopolysaccharide (LPS)-induced signaling which affects expression of PS and C4BP in the liver. Our *in vivo* studies in rats showed that after LPS injection, plasma PS levels are significantly decreased, whereas plasma C4BP levels first are transiently decreased after 2 to 12 hours and then significantly increased after 24 hours. LPS decreases PS antigen and mRNA levels in both hepatocytes and sinusoidal endothelial cells (SECs), and decreases C4BP antigen and both C4BPα and C4BPβ mRNA levels in hepatocytes. Antirat CD14 and antirat Toll-like receptor (TLR)-4 antibodies inhibited LPS-induced NFκB activation in both hepatocytes and SECs. Furthermore, inhibitors of NFκB and MEK recovered the LPS-induced decreased expression of PS in both cell types and the LPS-induced decreased expression of C4BP in hepatocytes. These data suggest that the LPS-induced decrease in PS expression in hepatocytes and SECs and LPS-induced decrease in C4BP expression in hepatocytes are mediated by MEK/ERK signaling and NFκB activation and that membrane-bound CD14 and TLR-4 are involved in this mechanism.

1. Introduction

Protein S (PS), a vitamin K-dependent plasma glycoprotein (*M*r 75,000), is a physiologically important regulator of blood coagulation, as patients with hereditary PS deficiency have severe thrombotic diseases [1–3]. In the blood coagulation system, PS is a cofactor of activated protein C (APC), which inactivates the blood coagulation factor Va and factor VIIIa [4–6] (Figure 1). PS may also directly inhibit the prothrombinase complex by binding to factor Va and factor Xa [7, 8]. In human plasma, PS circulates in free form and in complex with C4b-binding protein (C4BP), a protein of the classical complement pathway [9, 10]. C4BP consists of seven α chains (*M*r 70,000) and a β chain (*M*r 45,000), with the β chain being important in complex formation with PS [11–13]. Both forms of PS are capable of binding to APC, but only the free form acts as a cofactor [14, 15] (Figure 2). Additionally, PS may promote phagocytosis of apoptotic cells by macrophages [16] and mediate neuroprotection via APC [17]. These observations suggest that PS has important functions in both blood coagulation and inflammation.

Human PS is mainly synthesized in hepatocytes, endothelial cells and megakaryocytes [18–20], and C4BP in hepatocytes [21]. Acquired decreased plasma PS levels have been associated with liver diseases [22], pregnancy [23], oral contraceptive use [24], sepsis-associated disseminated intravascular coagulation (DIC) [25], and systemic lupus erythematosus [26], and patients with these conditions have an increased incidence of thrombotic events. Decreased levels of free PS may also lead to thrombotic tendency; therefore, it is believed that increased levels of plasma C4BP, as found in type III PS deficiency, are a risk factor of thrombosis [27].

Previously, we demonstrated that PS acts as a cofactor for APC in rats, and that it forms a complex with rat C4BP, as in humans [28]. Studies in various species demonstrated that both humans and rats have the PS-C4BP complex in

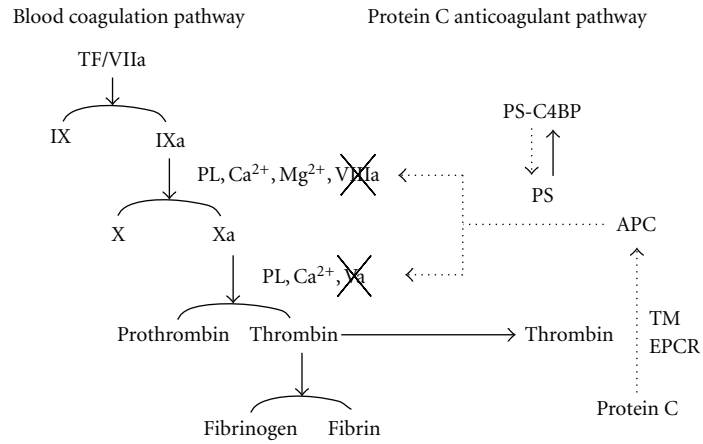

Figure 1: *Blood coagulation pathway and protein C anticoagulant pathway*. Thrombin, bound to thrombomodulin (TM), activates protein C which binds to endothelial protein C receptor (EPCR). Activated protein C (APC) inactivates coagulation factor Va (Va) and factor VIIIa (VIIIa) in the presence of protein S (PS). PS circulates in free form and in complex with C4b-binding protein (C4BP) in plasma, and the free form of PS plays a role as a cofactor of APC. TF: Tissue factor, PL: Phospholipids.

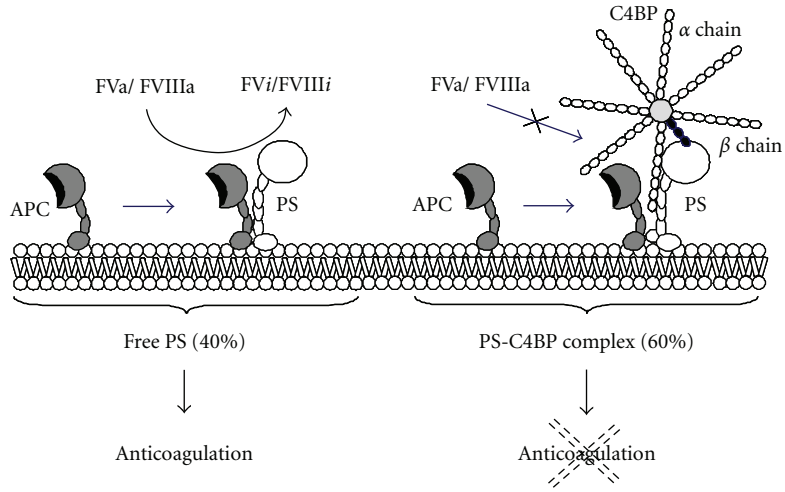

Figure 2: *Functions of PS and PS-C4BP complex*. PS circulates in free form (about 40%) and in complex form with C4BP (about 60%) in plasma. C4BP is comprised of seven α chains and a β chain, and PS binds to the β chain. APC proteolytically converts factors Va and VIIIa (Va/VIIIa) into inactivated factors Va and VIIIa (V*i*/VIII*i*) in the presence of free form PS, but not the complex form PS with C4BP.

plasma [10, 14, 28, 29]; therefore, rats are considered to be the most appropriate animal to study the pathophysiological role of PS. In this paper, we describe changes in the plasma levels of PS and C4BP. We also evaluate the *in vitro* effect of LPS on PS and C4BP expression in hepatocytes and/or sinusoidal endothelial cells (SECs) isolated from rats and the LPS-mediated signaling that affects PS and C4BP expression in these cells.

2. Effect of LPS on PS Expression In Vivo and In Vitro

We showed that in a rat endotoxemia model [30, 31], prepared by intraperitoneal injection of LPS, the total PS level in plasma was significantly decreased while the free PS level was markedly decreased in plasma after LPS injection (Figures 3(a) and 3(b)). APC cofactor activity of plasma isolated from rats 24 hours after LPS injection was also evaluated by activated partial thromboplastin time (APTT), suggesting that plasma obtained from rats 24 hours after LPS injection prolonged the APTT significantly less than plasma from nontreated rats (data not shown). These results suggest that LPS-induced reduction of plasma PS is a cause of thrombotic tendency in patients with sepsis. Thses results were consistent with the report by Hesselvik et al. that patients with sepsis have decreased plasma levels of PS, and that this is associated with thrombotic events [32]. In the liver, PS mRNA transiently decreased from 4 hours to 8 hours after LPS treatment and then returned to baseline levels; however, the plasma antigen level of PS did not recover concomitantly with the PS mRNA expression [30]. The detailed mechanism of this phenomenon is unclear, thus

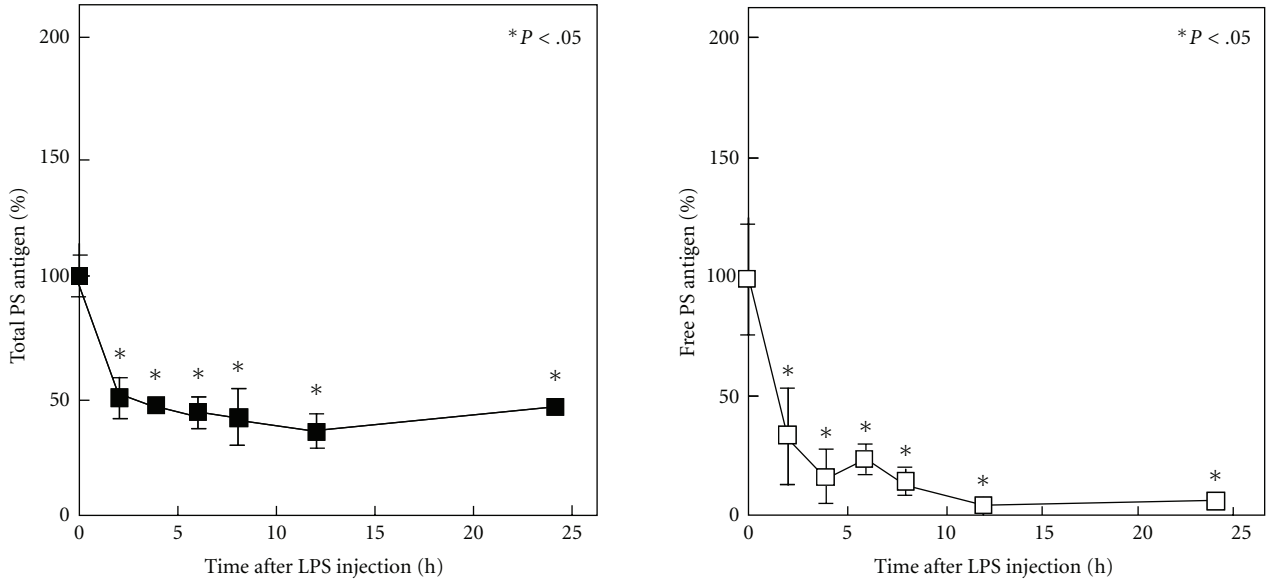

Figure 3: *Changes in plasma levels of total PS and free PS in LPS-treated rats*. Citrated plasma was obtained from three rats treated with LPS intraperitoneally ($2\,\text{mg} \cdot \text{kg}^{-1}$) at each time point to determine (a) plasma total PS antigen levels and (b) plasma free PS antigen levels, as described previously in [31]. Data are expressed as the mean ± S.D. ($n = 3$). $^*P < .05$ versus time 0.

future investigations are needed. The *in vitro* studies using hepatocytes and SECs isolated from normal rats indicated that LPS dose-dependently decreased mRNA expression of PS in both cells, and these decreases occurred at the transcriptional level [30]. These data suggest that decreased plasma level of PS in LPS-treated rats is mainly due to reduced PS mRNA expression in both hepatocytes and SECs.

3. Effect of LPS on C4BP Expression In Vivo and In Vitro

It is reported that plasma C4BP levels are significantly increased in patients with severe infection and septic shock [32], but it is unknown whether C4BP expression in the liver is directly affected by LPS. We examined the effect of LPS on C4BP expression *in vivo* in the liver of rats and *in vitro* in isolated rat hepatocytes. We observed that LPS transiently decreased the plasma level of C4BP antigen with a maximum decrease between 4 hours and 6 hours, followed by a significant increase by 24 hours after LPS injection (Figure 4(a)) [31]. This result was consistent with the data that free PS was significantly decreased for 24 hours after LPS injection (Figure 3(b)). However, the PS-C4BP complex level was not significantly changed within 24 hours after LPS injection (Figure 4(b)). The *in vitro* studies using hepatocytes isolated from normal rats indicated that LPS directly decreased both C4BPα and C4BPβ mRNA expression in hepatocytes [31]. These data suggest that the early decrease of plasma C4BP is caused by a direct effect of LPS. It is also reported that interleukin (IL)-6 increased C4BP expression in HepG2 cells [33], suggesting that in LPS-treated rats the relatively late increase in the plasma level of C4BP is caused by IL-6. It is reported that IL-6 also increased PS expression in HepG2 cells [34] and in rat hepatocytes [30]. However, it is unclear whether IL-6 is one of the causes of thrombotic tendency. To clarify this point we prepared IL-6-injected rats, and *in vivo* effect of IL-6 on plasma PS, C4BP and PS-C4BP complex level was examined. Our data indicated that both C4BP and PS-C4BP complex levels in plasma are increased until 8 hours after IL-6 injection, and then gradually decreased, and free PS level is decreased 24 hours after IL-6 injection, so that APC cofactor activity of plasma 24 hours after IL-6 injection is decreased as compared with nontreated rats (data not shown). These results suggest that IL-6 causes thrombotic tendency by increasing C4BP expression in hepatocytes followed by increasing plasma PS-C4BP complex and decreasing plasma free PS level. These results also suggest that IL-6-induced reduction of free plasma PS is also the cause of thrombotic tendency in endotoxemia rats, and the major effect of IL-6 is increasing of C4BP expression in hepatocyte rather than increasing of PS expression in hepatocytes and SECs.

4. Signal Transduction Pathway Involved in LPS-Induced Expression of PS in Hepatocytes and SECs and C4BP in Hepatocytes

CD14 and TLR-4 are necessary for signal transduction induced by LPS in which LPS, bound to CD14, can interact with TLR-4 in the presence of myeloid differentiation protein-2 (MD-2) [35, 36]. It is known that induction by LPS on NFκB activation occurs via CD14 and TLR-4 in human endothelial cells [37], and mouse hepatocytes [38]. Recently, we showed that rat hepatocytes and SECs also

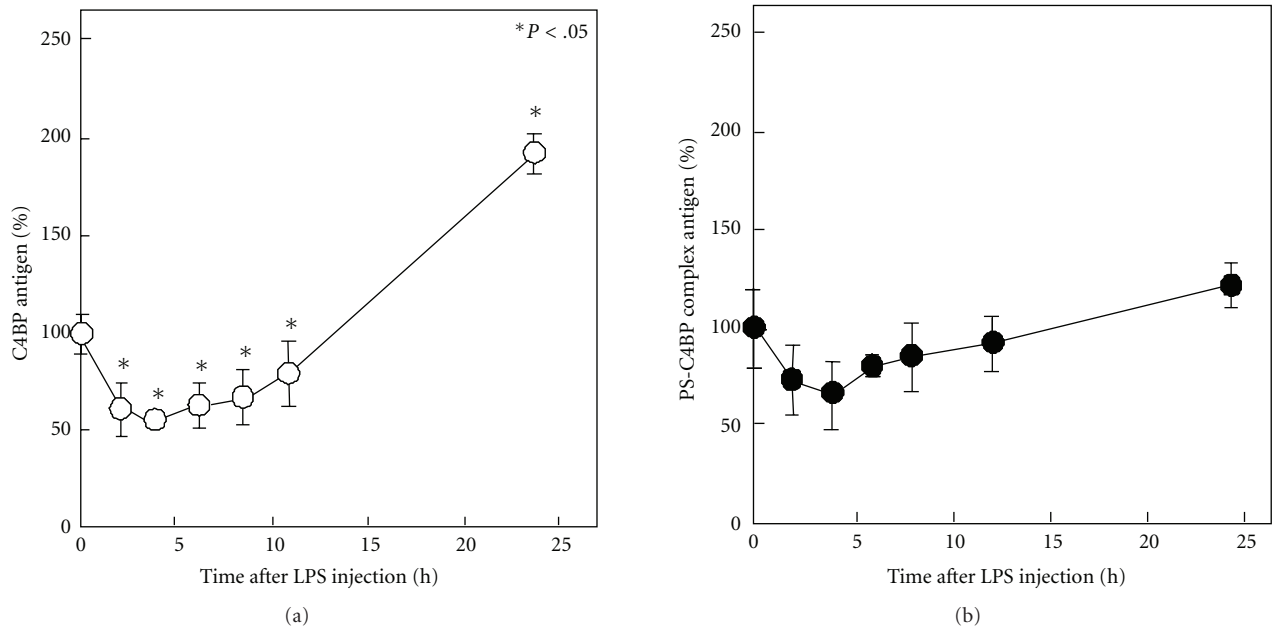

Figure 4: *Changes in plasma levels of C4BP and PS-C4BP complex in LPS-treated rats.* Citrated plasma was obtained from three rats treated with LPS intraperitoneally ($2\,\text{mg} \cdot \text{kg}^{-1}$) at each time point to determine (a) plasma C4BP antigen levels and (b) plasma PS-C4BP complex antigen levels, as described previously in [31]. Data are expressed as the mean ± S.D. ($n = 3$). $^*P < .05$ versus time 0.

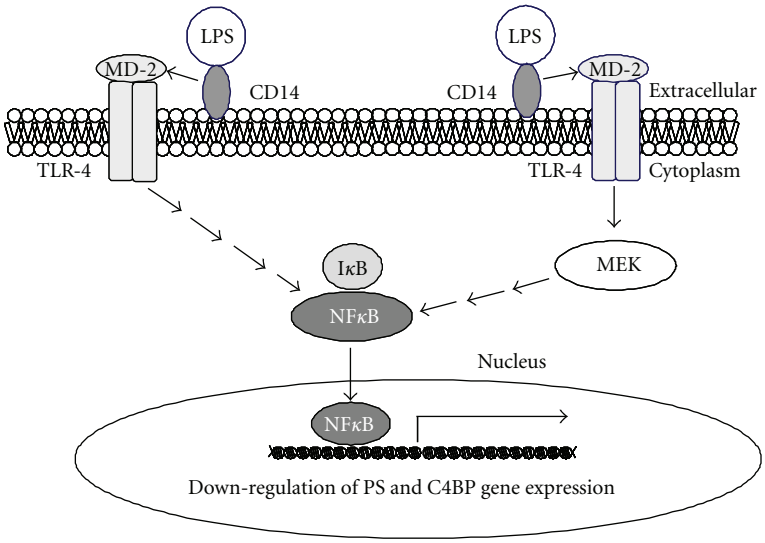

Figure 5: *Mechanism of LPS-induced decreased expression of PS and C4BP in hepatocytes.* LPS binds to membrane-bound CD14 and TLR-4, followed by activation of MEK/ERK and NFκB. Activated NFκB interacts with the promoter regions of the PS and C4BPα and C4BPβ genes, leading to decreased PS expression in hepatocytes and SECs, and decreased C4BP expression in hepatocytes.

express CD14 and TLR-4 [30]. Further, gel mobility-shift assay indicated that anti-CD14 and anti-TLR-4 antibodies inhibit LPS-induced NFκB activation in both hepatocytes and SECs. These results suggest that LPS also induced NFκB activation via CD14 and TLR-4 in both rat hepatocytes and SECs [30].

Furthermore, we found that a NFκB inhibitor blocked LPS-induced decreased expression of PS in both hepatocytes and SECs and the LPS-induced decreased C4BPβ expression in hepatocytes [30, 31], suggesting that NFκB activation is involved in the expression of PS and C4BP in hepatocytes and/or endothelial cells. These data also suggest that the rat PS, C4BPα and C4BPβ gene promoters contain a NFκB consensus sequence. In addition, we found that a MEK inhibitor blocks LPS-induced reduction of PS expression in hepatocytes and SECs, and LPS-induced reduction of C4BP in hepatocytes [30, 31]. These data are consistent with previous studies [36, 37], that LPS induces activation

of the MEK/ERK pathway and NFκB nuclear translocation in hepatocytes and endothelial cells. These findings suggest that NFκB activation and MEK/ERK pathway, but not the protein kinase C, JNK and p38 MAPK pathways, are linked to LPS-induced decreased PS expression in rat hepatocytes and SECs, and also linked to LPS-induced decreased C4BP expression in rat hepatocytes (Figure 5).

5. Effect of Inflammatory Cytokines on PS and C4BP Expression in Hepatocytes and SECs

LPS stimulates monocytes and endothelial cells to express various inflammatory cytokines [39, 40]. Among these inflammatory cytokines, tumor necrosis factor-alpha (TNF-α) decreased PS expression in rat SECs [30], and IL-6 increased PS expression and C4BPβ expression, but not C4BPα expression, in rat hepatocytes [30, 31]. On the other hand, LPS directly decreased PS expression in human umbilical vein endothelial cells (HUVECs) (our unpublished observations). These findings are consistent with previous reports showing that TNF-α decreases PS expression in HUVECs [41] and that IL-6 increases PS expression in human hepatoma cell lines [34]. As described above plasma PS antigen and activity are decreased in LPS-treated rats, and this suggests that both LPS and TNF-α induce decreased plasma PS levels, which causes thrombotic tendency in patients with sepsis. On the other hand IL-6 increased PS expression in hepatocytes, but this IL-6-induced increase of PS expression is not enough to compensate for LPS- and TNF-α-induced decreased expression of PS in hepatocytes and/or endothelial cells. Furthermore, IL-6 itself increased C4BP expression in rat hepatocytes, and the resulting decrease of free PS in plasma is thought to be the cause of thrombotic tendency. Overall, these results suggest that various cytokines which are induced by LPS in monocytes and endothelial cells induced thrombotic tendency in patients with sepsis. Recently, it was reported that IL-6-induced increased PS expression in HepG2 cells is regulated through the signal transducer and activator of transcription (STAT3) binding site, which is located in the 5′-flanking region of the human PS gene [42]. The mechanisms by which TNF-α specifically decreases PS expression in SECs and IL-6 specifically increases C4BPβ expression in hepatocytes are unknown. Further investigations are needed to elucidate the signal transduction pathway for IL-6 and TNF-α action in hepatocytes and SECs.

6. Conclusion

LPS directly decreases PS expression in hepatocytes and SECs and decreases C4BP expression in hepatocytes. Membrane-bound CD14 and TLR-4 mediate the LPS-induced activation of MEK/ERK and NFκB and the activated NFκB interacts with promoter regions of the PS and C4BPα and C4BPβ genes. Moreover, LPS activates TLR-4 in monocytes to express IL-6 which stimulates expression of PS and C4BP in hepatocytes and/or endothelial cells. Our findings may be useful for the development of anticoagulation therapy involving PS and C4BP to regulate LPS-TLR-4-mediated activation of the NFκB and MEK system.

Abbreviations

LPS: Lipopolysaccharide
TLR: Toll-like receptor
PS: Protein S
C4BP: C4b-binding protein
IL-6: Interleukin-6
PKC: Protein kinase C
APC: Activated protein C
JNK: Jun kinase.

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Review Article

TLR4 and Insulin Resistance

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Chronic inflammation is a key feature of insulin resistance and obesity. Toll-Like Receptor 4 (TLR4), involved in modulating innate immunity, is an important mediator of insulin resistance and its comorbidities. TLR4 contributes to the development of insulin resistance and inflammation through its activation by elevated exogenous ligands (e.g., dietary fatty acids and enteric lipopolysaccharide) and endogenous ligands (e.g., free fatty acids) which are elevated in obese states. TLR4, expressed in insulin target tissues, activates proinflammatory kinases JNK, IKK, and p38 that impair insulin signal transduction directly through inhibitory phosphorylation of insulin receptor substrate (IRS) on serine residues. TLR4 activation also leads to increased transcription of pro-inflammatory genes, resulting in elevation of cytokine, chemokine, reactive oxygen species, and eicosanoid levels that promote further insulin-desensitization within the target cell itself and in other cells via paracrine and systemic effects. Increased understanding of cell type-specific TLR4-mediated effects on insulin action present the opportunity and challenge of developing related therapeutic approaches for improving insulin sensitivity while preserving innate immunity.

1. Introduction

1.1. Insulin Resistance. Insulin resistance is a primary defect leading to and a characteristic feature of type 2 diabetes [1, 2]. The state of insulin resistance leads to increased insulin secretion by pancreatic β-cells and compensatory hyperinsulinemia. As long as compensatory hyperinsulinemia is sufficient to overcome the insulin resistance, fasting glycemia and glucose tolerance remain relatively normal. In patients destined to develop type 2 diabetes, β-cell compensation efficiency declines and relative insulin insufficiency develops leading to impaired glucose tolerance and eventually frank type 2 diabetes. Although there is still some debate as to whether the insulin resistance or the β-cell defect comes first, most epidemiologic studies indicate that in the early, prediabetic state, insulin resistance is the initiating abnormality.

Type 2 diabetes only develops in insulin resistant patients with a concomitant β-cell defect. As such, many subject groups with insulin resistance who do not have diabetes. These include patients with simple obesity, polycystic ovarian syndrome, and advanced age. There are a number of other abnormalities associated with insulin resistance that are included in the state of metabolic syndrome. Patients with metabolic syndrome are insulin resistant, hyperinsulinemic, and dyslipidemic (usually elevated triglyceride and decreased HDL levels) and frequently also have hypertension, nonalcoholic fatty liver disease, albuminuria, and increased plasminogen activator inhibitor 1 (PAI-1) levels. Epidemiologic evidence demonstrates that patients with metabolic syndrome have a high likelihood of developing type 2 diabetes and cardiovascular disease. Thus, while it is well established that treatment of insulin resistance has beneficial effects in patients with type 2 diabetes, it is becoming increasingly clear that enhanced insulin sensitivity is also therapeutically important in nondiabetic individuals with insulin resistance.

1.2. TLR4 Activity and Insulin Resistance-Associated Inflammation. Activation of the pro-inflammatory pathway has been described in a variety of insulin resistant states [3–6]. Chronic inflammation inhibits insulin sensitivity through

the activation of signaling pathways that directly interfere with the normal function of key components of the insulin signaling pathway [5, 6]. Inflammation impairs insulin sensitivity in part via the activation of the Toll-Like Receptor (TLR) family of pattern recognition receptors, specifically TLR2 and TLR4. For the purposes of this review, we will focus on the role of TLR4 in insulin resistance. TLR4 is a cell surface receptor that generates innate immune responses to pathogens by inducing signaling cascades of kinase and transcription factor activation (Figure 1). These cascades lead to the generation of pro-inflammatory cytokines, chemokines, eicosanoids, and reactive oxygen species (ROS), all effectors of innate immunity. Notably, TLR4 is expressed in many cell components of insulin target tissues, including liver, adipose tissue, skeletal muscle, vasculature, pancreatic β cells, and brain (Figure 2). Thus, activation of TLR4 can dampen insulin action directly, through activation of pro-inflammatory kinases and ROS, and indirectly, via activation of cytokine signaling cascades and systemic release of pro-inflammatory, insulin-desensitizing factors (Figure 1).

Lipopolysaccharide (LPS) and its endotoxic moiety Lipid A are potent agonists of TLR4. LPS is an outer membrane component of gram-negative bacteria and is composed of oligosaccharides and acylated saturated fatty acids (SFA). Free SFA are also reported to bind and activate TLR4. However, there are conflicting interpretations of these data which are discussed in more detail below. Endogenous activators of TLR4 include S100A8/S100A9 (calprotectin) [7], high-mobility group 1 (HMBG1) [8], fibronectin [9], and minimally modified low-density lipoprotein (mmLDL) [10]. LPS binding protein (LBP), CD14 and MD-2 serve as TLR4 accessory proteins that facilitate ligand delivery in the circulation and receptor binding. Two signaling pathways are initiated by TLR4 activation (Figure 1). One, modulated by MyD88 and TIRAP, activates IKK, p38, JNK, CREB, AP2, and NFκB and leads to the induction of pro-inflammatory genes. The other pathway, modulated by TRAM and TRIF, requires internalization of TLR4 (not depicted in Figure 1), activates IKK, NFκB, and IRF3, and leads to induction of type 1 interferon genes. Transcriptional activation by these pathways induces robust expression of thousands of genes, depending on the cell type, that propagate the defense mechanisms of innate immunity. These signaling cascades induce feed forward signaling cascades (e.g., via IL-6 and TNFα receptor activation) and negative feedback loops (e.g., via transcriptional activation of the IκB gene) [3]. In addition, TLR4-activated pathways inhibit the components of other signaling systems, for example, insulin signaling via IRS1 serine phosphorylation (Figure 1).

1.3. Regulation of TLR4. TLR4 expression and signaling are regulated by a variety of inputs, explained briefly here. Adiponectin impairs LPS activation of TLR4 signaling pathways in hepatic Kupffer cells and macrophages through mechanisms involving AMPK, IL-10, and heme oxygenase-1 [11–15]. AMPK also impairs LPS-induced IκB degradation and CREB activation in macrophages [16]. Activated nuclear receptor transcription factors glucocorticoid receptor (GR), peroxisome proliferators-activated receptor gamma (PPARγ), and liver X receptor alpha (LXRα) each transrepress TLR4-activated gene transcription and impair TLR4-mediated inflammation [17]. PPARγ activation also inhibits expression of TLR4 [18, 19] and, conversely, TLR4 activation inhibits expression of PPARγ [20]. Sex hormones can also affect TLR4 expression. Progesterone impairs LPS/TLR4 signaling efficacy via GR and progesterone receptor [21, 22]. Estrogen treatment of ovariectomized mice increases cell surface localization of TLR4 but does not change total cellular protein levels [23]. Testosterone downregulates TLR4 expression in macrophages both *in vitro* and *in vivo* [24]. Long-chain polyunsaturated omega-3 fatty acids (ω-3 FA) including DHA and EPA are antagonists of TLR4 activation by LPS and SFA in humans and mice [25–27]. One mechanism by which ω-3 FA interfere with TLR4 signaling is by altering plasma membrane lipid raft composition and function. For example, ω-3 FA block the ability of LPS and SFA treatments to stimulate assembly of TLR4 homodimers and signaling component complexes within lipid rafts, preventing subsequent signal transduction [28]. Stearoyl CoA desaturase 1 (SCD1) deficient mice, although protected from high-fat diet-induced insulin resistance, accumulate macrophage plasma membrane SFA, exhibit greater susceptibility to atherosclerosis, and are hypersensitive to inflammatory stimuli including those signaling through TLR4 [29, 30]. Supplementation with ω-3 FA completely protects SCD1 deficient mice from diet-induced atherosclerosis and metabolic syndrome [31]. These SCD1 deficient phenotypes may, in part, relate to lipid raft compositional changes that alter TLR4 signaling efficiency. SFA are conducive to formation and function of lipid rafts, essential sites of particular signal transduction pathways including TLR4, but lipid raft function and signaling component assembly are disrupted by ω-3 FA [32–34].

1.4. Role of Saturated Fatty Acids and Gut-Generated LPS as TLR4 Ligands. Are saturated fatty acids actual ligands of TLR4? SFA activation of TLR4 is an attractive link between obesity, insulin resistance, and inflammation, as cellular exposure to SFA greatly increases in the obese state. SFA are acyl components of LPS, activate TLR4 *in vitro*, and bind directly to TLR4/MD2/LPS crystal structures, although in an orientation that would probably rely on their presentation in an acylated form [35, 36]. Recent studies document endotoxin contamination of experimental reagents (such as bovine serum albumin, BSA) which would generate false-positive experimental results regarding the TLR4 agonistic effects of SFA [37–39]. LPS contamination is pervasive and LPS levels can only be assayed indirectly [31, 40]. Nonetheless, many publications document activation of TLR4 via SFA and many of these include samples that control for possible endotoxin contamination, for example, studies wherein BSA-complexed SFA treatments activate TLR4 effects but BSA alone and BSA-complexed monounsaturated fatty acid treatments do not. Extensive literature suggests that high-fat diet-augmented postprandial endotoxemia is a possible mode by which dietary SFAs induce inflammation through TLR4 in diet-induced obesity (DIO) models.

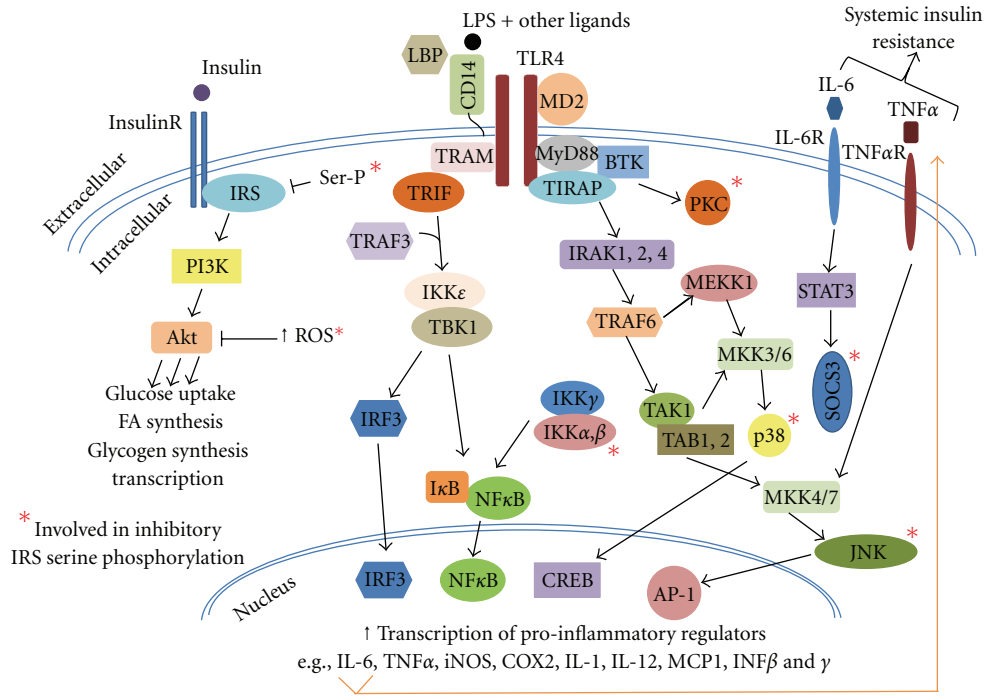

Figure 1: Schematic of TLR4 signaling cascades. Activation of TLR4 signal transduction through MyD88/TIRAP and TRAM/TRIF pathways leads to activation of innate immune responses and inhibition of insulin signal transduction, primarily through IRS serine phosphorylation. Additional cellular responses to TLR4 activation not shown include activation of NADPH oxidase, cytoskeletal rearrangement, and internalization of TLR4 complexes to endosomal compartments.

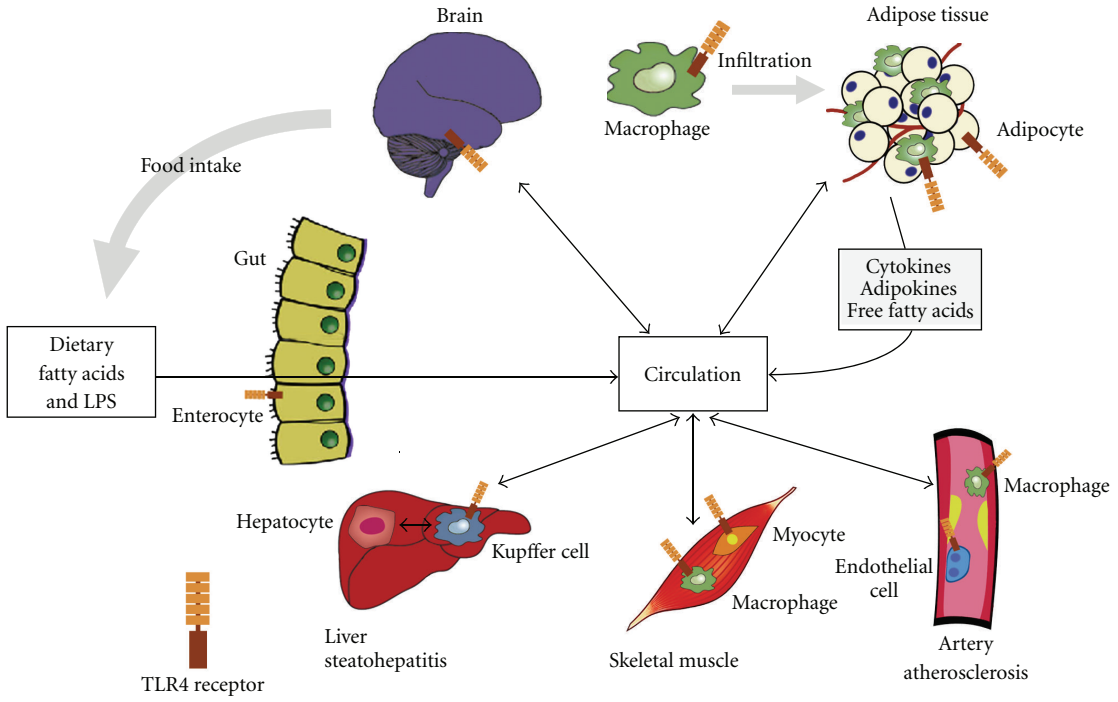

Figure 2: Distribution of TLR4 expression in the integrated organ/tissue systems that modulate energy homeostasis and insulin sensitivity. Schematic representation of the cross-talk between tissues. Adipose tissue shown in the macrophage-enriched, inflamed state.

Both high-fat diets and high-fructose diets influence enterobacterial bacterial production and circulating levels of LPS/endotoxin by altering gut flora growth and composition and gut permeability [41–44]. Dietary lipids facilitate LPS incorporation into chylomicrons [45] and TLR4 is responsible for phagocytosis of gram-negative bacteria by gut enterocytes [46], each contributing to postprandial endotoxemia. Insulin resistant DIO and genetically obese mice and type 2 diabetic humans [41, 46–48] all exhibit elevated plasma LPS levels and endotoxemia is correlated with insulin resistance and atherogenic markers. Thus, chronic elevation of circulating gut-generated LPS or "metabolic endotoxemia" [49] would result in sustained, systemic pro-inflammatory stimulation of TLR4. Interestingly, germ-free mice or mice treated with antibiotics specific for gram-negative bacteria do not acquire high-fat diet-induced insulin resistance or other associated metabolic abnormalities [50–52]. Genetically obese *ob/ob* mice treated with an LPS inhibitor or in a CD14 KO background have reduced inflammation and metabolic abnormalities compared to normal *ob/ob* mice [51, 52] which suggests that these *ob/ob* phenotypes are partly mediated by gut LPS and TLR4 signaling.

1.5. Mouse Model Overview. Several mouse model studies have demonstrated the importance of TLR4 and its signaling components in diet-induced insulin resistance, inflammation, and atherosclerosis. These studies include those conducted in whole body TLR4 knockout (KO) or loss-of-function mutations [26, 53–55], hematopoetic TLR4 KO [56], and whole body KOs of MyD88 [57] or CD14 [58, 59]. Some discrepancies in phenotypic reports with regard to food intake, weight gain, and adipose tissue macrophage accumulation will be discussed in the sections below. There are two nonsynonymous polymorphisms (SNPs) in the human TLR4 gene that result in changes in the TLR4 extracellular domain. These polymorphisms are reported to alter responsiveness to TLR4 activation and correlate with protection from atherosclerosis, CVD and the metabolic syndrome in some populations [60–62].

In the next sections, we will highlight tissue-specific effects of TLR4 activation and its role in insulin resistance. As many of the mouse models are whole body KOs of TLR4 signaling, it is unclear which exact cell type(s) mediate the phenotypes observed in these models. TLR4 expression in tissues, the mediate glucose homeostasis, and insulin sensitivity is shown in Figure 2 along with a schematic representation of the intra- and intertissue crosstalk that exists *in vivo*.

1.6. Adipose Tissue. Adipose tissue acts not only as a storage depot for excess calories, but also secretes large numbers of hormones, cytokines, and chemokines that influence energy homeostasis and metabolism (Figure 2). Adipose tissue consists of a variety of cell types, including adipocytes, immune cells (macrophages and lymphocytes), preadipocytes, and endothelial cells. Among these cell types, adipocytes and macrophages release cytokines and chemokines such as MCP-1, IL-1β, IL-6, and TNF-α [5] that promote inflammation. In addition, adipocytes are the unique source of hormones termed "adipokines" such as leptin [63, 64] and adiponectin [65], which can promote insulin sensitivity as well as resistin and retinol-binding protein 4 (RBP4), which can impair insulin sensitivity [6].

In states of chronic nutrient excess and obesity, insulin resistance manifests through several mechanisms such as increased free fatty acid (FFA) flux, ER stress, and microhypoxia in adipocytes, all of which are associated with increased inflammation [6]. In recent years, much interest has been focused on this association between obesity, chronic inflammation and insulin resistance. In 2003, two landmark studies showed bone-marrow derived macrophages invade adipose tissue in obese states [66, 67]. Recruited macrophages may initially remove dying cells and contribute to adipose tissue vascularization, but their activation towards an inflammatory phenotype soon results in cytokine production [68]. These macrophages contribute to the development of inflammation in adipose tissue and are believed to be a key contributor to insulin resistance [69]. This relationship between increased macrophage infiltrates and obesity has since been verified in human studies [70]. A growing body of literature is focused on the role of these adipose tissue macrophages (ATMs) in insulin resistance, characterizing their activation, recruitment, and function.

Attenuating the inflammatory signaling pathway by gene knockout experiments has been shown to reduce obesity-related insulin resistance in mice [69, 71–74]. For example, mice with myeloid cell-specific deletion of IKKβ or JNK1 have significantly improved glucose tolerance and insulin sensitivity despite high-fat diet-induced obesity. Several lines of evidence point to the involvement of TLR signaling in this paradigm. For example, TLR4 expression is highly abundant in pro-inflammatory macrophages [75, 76] and differentiated adipocytes [77, 78]. Moreover, TLR4 expression in adipocytes increases with obesity [26] and it can be activated by LPS to induce NFκB activation and cytokine production in both rodent and human tissues [79–81]. SFA acylated in the lipid A moiety of LPS is essential for the biological activity of LPS [82]. LPS and SFA have also been shown to attenuate insulin signaling in adipocytes with decreased phosphorylation of Akt and GSK3β [78].

Most intriguingly, TLRs in adipocytes [26, 83] and pro-inflammatory macrophages [76] may be directly activated by nutrients, particularly SFA. Although it is not yet clear if LPS contamination may contribute to any of these findings, dietary SFA appear to differentially modify the risk of developing many chronic inflammatory diseases in both human and animal studies [84]. In contrast, fish oil-derived ω-3 FA can mitigate the effect of LPS and free SFA-induced inflammation in macrophages [25] and adipocytes [26] and prevent high-fat diet-induced insulin resistance in rodents [85]. Taken together, this evidence suggests that elevated FFAs in obesity activate TLR signaling and impair insulin action, providing yet another link between obesity, inflammation, and insulin resistance.

Toll-like receptors in adipose tissue play a key role in initiating the inflammatory response, thereby promoting insulin resistance. Several studies to date have shown that disruption of the TLR4 gene in mice confers protection

from obesity-induced inflammation and insulin resistance [26, 54, 86–88]. Several models of TLR4 deficiency have been studied in this context. Two LPS-resistant naturally occurring mouse strains have been identified with loss-of-function (C3H/HeJ mice) or deletion (C57BL/10ScN mice) mutations in the TLR4 gene [55, 81]. TLR4 null mice have also been generated using homologous recombination [80]. Body composition differs between mouse strains following high-fat feeding, with unaltered [88] or decreased [55, 86, 87] weight in C3H/HeJ and C57BL/10ScN mice, and unaltered or increased weight in *TLR4*$^{-/-}$ mice [26]. It is unknown whether this divergence results from differences in TLR4 mutations, mouse strains, or other factors. However, in both models, TLR4 signaling in macrophages and adipose tissue appears to regulate whole body glucose homeostasis via effects on adipose, muscle, and liver tissues.

Since body composition strongly influences insulin sensitivity, it is challenging to ascertain whether improved glucose metabolism results from altered TLR4 expression or decreased body weight. Therefore, models in which body weight is unaltered or increased are advantageous. There are two papers published to date showing increased adiposity following high-fat feeding in mice with disrupted TLR4 gene expression [26, 88]. In these studies, adipose inflammation was greatly attenuated with reduced expression of pro-inflammatory genes, despite their greater adiposity. TLR4 deletion also improved insulin sensitivity, with higher rates of glucose disposal into skeletal muscle and adipose tissue, and reduced inflammation and insulin resistance induced by either LPS or FFAs in isolated primary adipocytes [26, 88]. Moreover, while *in vivo* lipid infusion promotes insulin resistance and NFκB activation in adipose tissue of control mice, similar effects were not observed in TLR4 knockout mice [26].

Additional work indicates that TLR4-mediated alterations in macrophage activity underlie the adipose-specific improvements in inflammation and insulin. Saturated fatty acids such as palmitate, lauric acid, and oleate fail to elicit TNFα production or IKKβ degradation in elicited peritoneal macrophages from TLR4 mutant mice [26, 89]. Furthermore, mice with myeloid-specific ablation of TLR4, generated by transplanting bone marrow from *TLR4*$^{-/-}$ animals into wild-type mice, demonstrate significantly improved insulin sensitivity in adipose tissue with reduced ATMs and adipose pro-inflammatory gene expression [54]. Because these chimeric mice possess TLR4 deficiency only in bone marrow-derived cells, it is possible to determine the contribution of hematopoietic TLR4 to insulin sensitivity. In addition, body weight and adipose depot sizes are equal between chimeric and control mice, such that differences in adiposity are not a confounding factor in the comparison between groups. Similar conclusions were drawn from studies in mice lacking the TLR4/TLR2 coreceptor CD14. Adipose tissue from obese CD14 null mice had fewer macrophages, with reduced glucose intolerance despite increased body weight [59]. Interestingly, TLR4 expression in peripheral blood mononuclear cells is reduced in overweight individuals with metabolic syndrome that undergo weight loss [90]. Although our understanding of macrophage function is incomplete, it appears that their absence in adipose tissue confers protection against insulin resistance during nutrient overload.

1.7. Liver. The liver is comprised of heterogeneous cell types including hepatocytes, stellate cells, endothelial cells, and immune cells. Liver-resident macrophages called Kupffer cells make up 10% of cells in the liver and 80%–90% of all tissue macrophages in the body [91]. They are localized at sinusoids where they are in close contact with circulating factors (hormones, cytokines, lipids, danger signals, post-prandial LPS, etc.). Kupffer cells, able to migrate and cross-talk with other cell types within the liver, are important mediators of liver inflammation and non-alcoholic fatty liver disease (NAFLD), reviewed in more detail within this volume [92]. TLR4 is expressed on Kupffer cells and other liver cell components and modulates liver pro-inflammatory activity induced by high-fat diet- and fructose-induced hepatic steatosis and insulin resistance in mouse models [26, 44, 54, 93], although a direct role for liver tissue TLR4 in these processes is unclear. In obese patients with nonalcoholic steatohepatitis (NASH), liver tissue levels of activated pro-inflammatory NFκB and AP-1 were correlated with oxidative stress and insulin resistance [94]. Both whole body and myeloid-specific TLR4 signaling deficiency result in reduced lipid accumulation, inflammation, JNK and IRS1 serine phosphorylation, and insulin resistance in liver [26, 54, 59, 86–88]. Interestingly, acute LPS treatment inhibits hepatic glucose production *in vivo* and *in vitro* via a TLR4 signaling pathway and induces insulin resistance 48 hours post-treatment *in vivo*, suggesting possible cross-talk between TLR4 and insulin receptor signaling pathways in this system [95]. The hypoglycemic effects of acute LPS are PI3K- and TNFα-independent and additive when combined with insulin. There are several models of how steatosis could facilitate activation of TLR4 in Kupffer cells [93]. Increased lipid content and exposure can affect TLR4 signaling complex assembly, endosomal sorting, and signaling cascade flow by altering lipid raft composition and membrane fluidity. Liver hypertrophy and elevated lipid content can impair sinusoidal perfusion efficacy and constrict circulatory flow. Circulating leukocytes, stuck within the sinusoids, would be more likely to activate liver resident cells, including Kupffer cells. Liver tissue infiltration of circulating monocytes might also be facilitated in the inflamed, hypertrophic, and steatotic state. In the insulin resistant state, liver-expressed TLR4 would be exposed to elevated circulating ligands including adipose tissue FFA and, in obese/high-fat diet states, LPS and dietary SFA.

1.8. Muscle. Skeletal muscle is the primary site for insulin-stimulated glucose utilization, accounting for over 75% of this process under normal physiological conditions [96, 97]. Glucose disposal into muscle is markedly reduced in obese, hyperinsulinemic subjects [98], underscoring the importance of skeletal muscle in normal glucose homeostasis and in the development of insulin resistance. Putative mechanisms of insulin resistance in skeletal muscle include direct effects of intramyocellular FFA metabolites, paracrine effects

of adipocytes, and macrophages (interspersed between muscle fiber bundles) in muscle tissue as well as endocrine effects from adipocytes and macrophages present in adipose tissue. As an example of the latter, the production of the pro-inflammatory cytokine TNFα from adipose tissue can impair insulin signaling in muscle through inhibitory serine phosphorylation of IRS-1 [99].

TLR4 has been shown to regulate substrate metabolism in muscle, favoring glucose oxidation rather than fatty acid oxidation in the absence of insulin [100]. Emerging data indicates that TLR signaling may also underlie the development of chronic inflammation and insulin resistance in skeletal muscle. Skeletal muscle cells and intact whole muscle express multiple TLRs, including TLR2 and TLR4 [101], that are responsive to LPS [102]. Moreover, skeletal muscle TLR4 gene and protein expression are significantly elevated in muscle from obese subjects with type 2 diabetes [103]. In these individuals, TLR4 protein expression in muscle correlates with the severity of insulin resistance. TLR4 contributes to skeletal muscle metabolism. Activation of TLR4 has also been shown to regulate substrate utilization in muscle, favoring glucose oxidation rather than fatty acid oxidation in the basal state.

Much of the current data implicates saturated FFAs as ligands for TLRs in muscle tissue. For example, in human myotubes and in muscle from lean human subjects, acute palmitate treatment induces robust NFκB-activation via TLR4 [103]. In C2C12 mouse myotubes, palmitate has been also shown to activate NFκB, as well as JNK1/2 and novel PKC pro-inflammatory pathways via TLR2 [104]. Rodent studies also show that disrupted expression of TLR4 protects against saturated fatty acid-induced insulin resistance in muscle resulting in improved insulin-stimulated glucose uptake, improved IRS1 tyrosine phosphorylation, reduced IRS1 serine phosphorylation, and decreased JNK1 phosphorylation in TLR mutant mice [26, 87]. More recent work indicates that conditioned media from macrophages treated with palmitate, but not LPS, can impair glucose uptake in muscle cells, suggesting that saturated fatty acids mediate their effects on muscle via macrophage cells [68].

1.9. Other Tissues

1.9.1. Brain. In recent years, much interest has been focused on the role of the brain in regulating glucose metabolism. Several investigators have identified the hypothalamus and mesolimbic area as important sites in the regulation of food intake, energy expenditure, peripheral insulin resistance, and pancreatic β-cell function. For example, neural influences on the liver and muscle directly influence glucose output and uptake in these tissues [105]. Inflammatory signaling pathways in adipose tissue have been shown to regulate energy balance by increasing thermogenesis [106]. Studies in TLR4 mutant mice suggest that TLR signaling in the CNS may also contribute to obesity phenotypes by affecting nutrient intake. For example, TLR4 null females demonstrate increased adiposity secondary to increased food intake on both normal chow and high-fat diets [26]. In addition, a study of C3H/HeJ mice showed decreased food intake when fed ad libitum, and developed obesity when pair-fed with control mice [88]. It is difficult to ascertain direct effects of TLR signaling in the brain since TLR4 expression is disrupted in the whole body in both models. However, high-fat diet has been shown to increase JNK1 and NFκB activation in the hypothalamus with impaired insulin signaling and apoptosis that may result in dysregulated feeding control [107]. TLR4 in the hypothalamus may activate pro-inflammatory pathways that contribute to the development of insulin and leptin resistance. For example, mice deficient for the TLR adaptor molecule MyD88 in the CNS are protected from hypothalamic inflammation and leptin resistance induced by acute central application of palmitate as well as from impairment of peripheral glucose metabolism induced by either centrally administered palmitate or by HFD [108]. However, the role of TLR4 is still unclear as some work suggests that TLR4 may in fact play dual roles in the hypothalamus by both activating pro-inflammatory pathways and restraining apoptosis [109].

1.9.2. Pancreatic Islet. Several TLRs (TLR2, 3 and 4) are highly expressed in human and rodent islets [110]. Although types 1 and 2 diabetes differ in etiology, β-cell destruction eventually occurs in both cases, leading to clinical manifestations of absolute or relative insulin deficiency. Islet inflammation is a well-established observation in autoimmune-mediated type 1 diabetes. However, it has been more recently described in type 2 diabetes and is thought to be secondary to toxicity induced by high glucose, FFAs, cytokine signaling, or ER stress. TLR signaling in β-cells has been primarily implicated in autoimmune type 1 diabetes [111–114]. Nevertheless, recent studies also implicate TLR2/4 and MyD88 in insufficient β-cell compensation in type 2 diabetes. For example, the chemokine CXCL10 is highly expressed in islets isolated from individuals with type 2 diabetes and has been shown to impair insulin secretion and promote β-cell apoptosis via TLR4 [115]. In addition, TLR activation by FFAs can induce the expression of proinflammatory cytokines in purified mouse and human islets [116], suggesting that the dyslipidemia associated with insulin resistance promotes islet inflammation.

1.9.3. Artery and Endothelial Cells. TLR4 activity impairs endothelial cell function and contributes to atherosclerosis. TLR4 is required for LPS-stimulated NFκB activation in endothelial cells [117]. Kim et al. report that mice with whole body deletion of TLR4 were protected against high-fat diet-induced vascular inflammation (aorta) *in vivo* [53]. The mice were also protected from high-fat diet-induced insulin resistance while exhibiting the same body weight, adiposity, and plasma insulin and FFA levels as wild-type controls. These authors also demonstrated that the SFA palmitate stimulated TLR4-dependent IKK and NFκB activation and impairment of insulin signaling and NO production in wild type aortic explants and cultured human endothelial cells. Whole body disruption of TLR4 signaling prevents atherosclerosis in proatherogenic genetic mouse models [57, 58]. Interestingly, in hematopoetic cell TLR4 KO/whole body LDLR KO agouti mice, only females fed normal chow

plus cholesterol diet exhibited less ATM, inflammation, and atherosclerotic lesion size than TLR4 wild-type controls [56]. In these studies, metabolic and atherogenic sequelae were the same in hematopoetic cell TLR4 KO versus wild type females fed three different high-fat diet plus cholesterol formulae. The authors state that no significant phenotypic differences were observed between the male genotypes. Direct action of TLR4 signaling in vascular inflammation and atherosclerosis in vivo is unclear as the phenotypes described above could be mediated by TLR4 indirectly via reduced inflammation elsewhere (e.g., adipose tissue).

1.9.4. Sex-Specific Dimorphism. Numerous observations in the literature reveal sexual dimorphism in the regulation of TLR4 and TLR4 signaling. Sex hormones differentially regulate TLR4 localization, gene expression, and sensitivity, described above. Males and females exhibit dramatic differences in their immune responses and susceptibility to sepsis [118]. Innate immunity modulation and insulin resistance are observed in pregnancy during which estrogen and progesterone levels are dramatically elevated [119–121]. Two of the TLR4 knockout mouse studies described above report TLR4-dependent phenotypic effects in females only [26, 56]. Sex differences in acute lipid infusion-induced insulin resistance have been reported in both human [122] and rat [123] studies where only males become insulin resistant during lipid infusion. Future studies should provide more clarity about the mechanisms by which TLR4 signaling is modulated by sex hormones.

2. Conclusion

TLR4 activation promotes insulin resistance. Given the wide-spread expression pattern of TLR4 in tissues and cell types that modulate energy homeostasis and insulin action (Figure 2), the direct, cell type-specific relationship between TLR4 activation and insulin resistance is unclear. TLR4 plays an important insulin-desensitizing role in myeloid-derived cells and future studies of nonmyeloid, cell type-specific transgenic and knockout models are of great interest. Our increasing understanding of TLR4 and insulin resistance will facilitate the design of novel therapeutic approaches that can derail the negative metabolic effects of TLR4 activation from the important functions of innate immunity.

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