Gut-Liver Axis and Sensing Microbes

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Abstract
‘Detoxification’ of gut-derived toxins and microbial products from gut-derived microbes is a major role of the liver. While the full repertoire of gut-derived microbial products that reach the liver in health and disease is yet to be explored, the levels of bacterial lipopolysaccharide (LPS), a component of Gram-negative bacteria, is increased in the portal and/or systemic circulation in several types of chronic liver diseases. Increased gut permeability and LPS play a role in alcoholic liver disease where alcohol impairs the gut epithelial integrity through alterations in tight junction proteins. In addition, non-alcoholic fatty liver disease is also associated with increased serum LPS levels and activation of the pro-inflammatory cascade plays a central role in disease progression. Microbial danger signals are recognized by pattern recognition receptors such as the Toll-like receptor 4 (TLR4). Increasing evidence suggests that TLR4-mediated signaling via the MyD88-dependent or MyD88-independent pathways may play different roles in liver diseases associated with increased LPS exposure of the liver as a result of gut permeability. For example, we showed that in alcoholic liver disease, the MyD88-independent, IRF3-dependent TLR4 cascade plays a role in steatosis and inflammation. Our recent data demonstrate that chronic alcohol exposure in the liver leads to sensitization of Kupffer cells to LPS via a mechanism involving upregulation of microRNA-155 in Kupffer cells. Thus, understanding the cell-specific recognition and intracellular signaling events in sensing gut-derived microbes will help to achieve an optimal balance in the gut-liver axis and ameliorate liver diseases.

Introduction

Increasing evidence suggests that in healthy individuals, maintenance of homeostasis between the gut microbiome and the host plays an important role in health while disruption of such homeostasis occurs in disease [1–3]. It is estimated that there are multiple times more microbial cells in the gut than the total number of cells in the human body [4]. Microbes in the gut play important roles in digestion of food, vitamins and shaping immunity [4, 5]. Changes in the composition of the microbiome or alterations in gut permeability, however, can promote translocation of microbes into the portal circulation that delivers blood directly to the liver. There is increasing evidence that gut-derived microbial components represent danger signals for the host in the liver, induce inflammatory cascade activation in immune cells and modulate functions and responses of liver parenchymal cells [2]. The important role of the liver, particularly of residing innate immune cells, is emerging in different types of chronic liver diseases and it will be discussed in this review article.
Gut-Liver Axis in Homeostasis and Disease

Inflammatory responses in the body to gut-derived and blood-borne pathogens occur in the liver and spleen, the major organs that remove bacteria and their lipopolysaccharide (LPS) from the bloodstream. Several mechanisms have been identified and proposed in this process that relies on a balance between the barrier functions of the gut and the ‘detoxifying’ capacity of the liver [1, 2]. The role of gut-derived LPS has been identified in various liver diseases [6, 7]. The liver has different mechanisms to ‘detoxify’ gut-derived LPS including host lipases [8] and the lack of this protective mechanism was shown to increase inflammatory responses [9]. There is a positive correlation between liver dysfunction and bacterial translocation [10, 11]. Increased gut permeability and/or LPS levels were also noted in liver disease associated with HIV and HCV infection, cirrhosis and cholestasis of pregnancy [6, 12, 13].

The Microbiome

The gut microbiome has been a focus of recent investigations [3–5]. The composition and the frequency of the microbiome changes with the different segments of the elementary tract and its composition is influenced by the environment, consumed diet and host factors. Studies are controversial regarding the definition of the ‘normal’ microbiome, but reports are consistent on association between certain diseases and changes in the gut flora. Most reports agree that the number of microbiota increases from the mouth to the rectum within the gastrointestinal tract. Changes in the composition of the intestinal bacterial content have been shown in different diseases associated with liver abnormalities. For example, high-fat diet-induced obesity is associated with changes in the gut flora in rats [14] and in humans, gut microbiota changes in obesity and after gastric bypass [15]. In a recent experimental model, alcohol feeding of mice resulted in an increase in the total number of bacteria as well as the composition of the bacteria in the gut compared to mice fed an isocaloric diet [16].

Gut Permeability

The gut epithelium plays a central role in demarcating microbes in the gut from the host immune system. The simple columnar epithelial cells exhibit physical adaptations to maintain barrier integrity including microvilli, tight junctions, production of antimicrobial peptides and specialized intestinal epithelial cell (M) cells that overlie Payer’s patches and lymphoid follicles [3, 17, 18]. Gut epithelial cells have tight junctions consisting of zona occludens, occludin, etc. [3, 17, 18]. The integrity of tight junctions and gut barrier can be affected by several factors.

For example, in alcoholic liver disease, alcohol was found to disrupt the gut barrier function (fig. 1). Suggested mechanisms include direct effects of alcohol as well as acetaldehyde-mediated inhibition of tight junction protein expression [19, 20]. Alcohol also acts via nitric oxide induction to impair microtubule cytoskeleton in intestinal epithelial cells [21]. We also found that alcohol exposure of Caco-2 colon epithelial cells results in reduced transepithelial electrical resistance (fig. 2) and disruption of the pattern of zona occludens protein (ZO-1) expression compared to control cells (fig. 3) suggesting impaired barrier function. It was reported that alcohol induces microRNA-212 that in turn inhibits ZO-1 protein levels [22]. Further investigation of the integrity of the gut mucosa will likely provide novel insights into liver diseases other than alcohol-induced liver injury.
Sensing Microbes

The host immune system recognizes pathogen-associated molecular patterns as danger signals via pattern recognition receptors including families of the Toll-like receptors (TLRs), NOD-like receptors and helicase receptors [23–26]. These are evolutionarily conserved and expressed in multiple cell types in non-immune cells including hepatocytes, sinusoidal endothelial cells, biliary epithelia and stellate cells [23–26]. Of the TLRs, some are expressed on the cell surface (TLR4, TLR1, TLR2, TLR6, TLR5) that recognize mostly bacterial-derived molecular signals. Intracellularly localized TLRs recognize nucleic acid sequences from pathogens including bacterial DNA (TLR9), single-stranded RNA (TLR7/8), or double-stranded RNA (TLR3) [23–26]. Nucleic sequences from viruses (dsRNA) are also sensed by the helicase receptors, RIG-I and MDA5 [23–26]. NOD-like receptors are expressed intracellularly and sense intracellular pathogens as well as damaged cells in sterile inflammation [27]. Ligand engagement of these receptors induces activation of distinct intracellular signaling pathways to induce production of pro-inflammatory cytokines and/or type-I interferons (IFNs) [23–27].

LPS and TLR4 Signaling

While increased gut permeability is likely to permit translocation of components from various microbial sources from the gut into the portal circulation, our un-
derstanding is rather limited on the specifics regarding the type and nature of translocated microbial components. LPS, a component of the cell wall of Gram-negative bacteria, is the most studied PAMP in relation to gut-derived pathogenic signals. LPS is sensed by TLR4 through participation of the co-receptors CD14 or MD-2 that bind LPS [26, 28–30]. Ligand engagement of TLR4 induces downstream signaling via recruitment of two adapter molecules [26]. The MyD88-dependent TLR4 signaling pathway results in NF-κB activation, phosphorylation, nuclear translocation and activation of pro-inflammatory cytokine genes. Recruitment of the adapter TRIF to TLR4 triggers downstream signaling through TBK/IKKe and IRF3 phosphorylation, nuclear translocation and induction of type-I IFNs [31]. Activation of both of these pathways after LPS-TLR4 interaction is unique because all other TLRs activate only one of these two pathways.

**Gut-Derived LPS in Alcoholic Liver Disease and in Other Chronic Liver Diseases**

The role for gut-derived LPS in alcohol-induced liver pathology has been suggested by Bode and Bode [32] followed by studies in the late Thurman’s laboratory where gut sterilization with antibiotics prevented alcohol-induced liver damage, steatosis and inflammation [33]. Early studies by Thurman’s group also suggested a role for LPS and Kupffer cell activation in ALD as mice with mutant TLR4 or elimination of Kupffer cells with gadolinium chloride in rats’ attenuated features of alcohol-induced liver disease [34]. Our studies followed up on the role of TLR4/LPS-induced signaling in ALD. In testing the hypothesis that TLR4-induced two downstream signaling pathways may be differently affected in ALD, we found that the lack of MyD88-dependent TLR signaling in MyD88-deficient mice could not prevent ALD [35]. Importantly, we also determined that blocking the MyD88-independent TLR4/LPS signaling pathway at the level of IRF3 prevented alcohol-induced liver damage, steatosis and inflammation [36]. Data in figure 4 show that unlike in wild-type, C57BL/6 mice, liver steatosis was not present in alcohol-fed IRF3-deficient mice on hematoxylin-eosin or Oil-red-O staining (fig. 4a). Mice with IRF3 deficiency were protected from alcohol-induced liver damage indicated by no increase in serum ALT (fig. 4b) or the pro-inflammatory cytokine gene, TNF-α or IL-1β induction in the liver compared to paired IRF3-deficient mice (fig. 4c, d). Alcohol-induced activation of the MyD88-independent TLR4 signaling pathway in wild-type mice was indicated by the increased expression of the IFN-inducible gene, ISG-56 which was absent in the IRF3-deficient mice (fig. 4e). These results confirmed that TLR4-mediated and IRF3-dependent signaling pathways are critical in development of alcoholic liver injury, steatosis and inflammation (fig. 5). Because TLR4 and its downstream signaling pathways are active not only in Kupffer cells and immune cells but also in parenchymal cells in the liver, we recently extended studies to delineate a cell-specific role for IRF3 in alcoholic liver damage and showed that IRF3 in hepatocytes has protective effects, while in myeloid cells it mediates liver damage [36].

The importance of gut microbiota and microbe-derived danger signals is also being explored in obesity [37, 38]. Consistent with this notion, impaired gut permeability was reported in non-alcoholic fatty liver disease [39] where activation of Kupffer cells via TLR4/LPS has been identified as a major pathogenic component in disease development [40].

**Molecular Mechanisms of LPS Sensitization: New Insights**

Exposure of immune cells, particularly macrophages, to LPS elicits an immediate robust pro-inflammatory cytokine response characterized by NF-κB activation and induction of TNF-α, IL-1β, IL-6, IL-12 [41, 42]. In the later stage of LPS response, IL-10 and TGF-β are induced that have autocrine negative regulatory effects to terminate the initial pro-inflammatory cytokine activation [41, 42]. It has also been noted that an initial LPS stimulation results in ‘tolerance’ to a subsequent LPS stimulation, a phenomenon referred to as LPS or TLR tolerance. Recent studies explored molecular mechanisms underlying LPS tolerance within the TLR4 signaling pathway and also identified that the absence of upregulation of negative regulators of TLR signaling that mediate TLR4 tolerance permit overactivation of macrophages [41, 42]. For example, in monocytes/macrophages, IRAK-M induced by an initial LPS stimulation acts as a negative regulator of TLR4 signaling and loss of IRAK-M is associated with increased LPS-induced TNF-α production. Our group reported that chronic alcohol exposure in monocytes/macrophages results in a loss of IRAK-M expression which is associated with increased LPS-induced cytokine production [43].
**Fig. 4.** IRF3 deficiency protects against alcohol-induced liver damage. Wild-type (WT) and IRF3-deficient (IRF3-KO) mice were fed Lieber-DeCarli ethanol or control (pair-fed) diet and sacrificed after 4 weeks. Livers were fixed in formalin and stained with HE or with Oil-red-O. 200× (a). Serum ALT levels (b) were analyzed. Messenger RNA levels of liver, TNF-α (c), pro-IL-1β (d) and IFN-stimulated gene ISG-56 (e) were analyzed by real-time PCR and normalized to 18 s. Values are shown as mean ± SEM fold increase over WT pair-fed control group (9–12 mice/group). Numbers in graphs denote p values: * p < 0.05 vs. pair-fed WT mice; # p < 0.05 vs. ethanol-fed WT mice.

**Fig. 5.** IRF3 is a key regulator of alcoholic liver disease. LPS is sensed by TLR4, which induces the MyD88-dependent and MyD88-independent downstream signaling pathways. Deficiency in IRF3 in mice protects from alcoholic liver disease, while MyD88 deficiency has no protective effects.

**Model of TLR4 signaling in ALD**

LPS

MyD88-dependent

TLR4

MyD88-independent

Pro-inflammatory cytokines

TNF-α, IL-1, IL-6, chemokines

Type I IFNs

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Kupffer cells to LPS in alcoholic liver disease. We found increased miR-155 expression in Kupffer cells isolated from livers of alcohol-fed mice [45]. The data in figure 7 show that alcohol feeding results in increased serum endotoxin levels in mice (fig. 7a) and this correlates with alcohol-induced liver injury indicated by elevated serum ALT levels (fig. 7b). We also found that in the total liver of chronic alcohol-fed mice miR-155 expression was significantly increased (fig. 7c), suggesting a role for miR-155 in alcoholic liver disease. Further exploration of the role of microRNAs in liver inflammation and different types of chronic liver diseases will likely provide further insights into the role of LPS in the gut-liver axis.

The newly discovered microRNAs (miRNAs) also play an important role in regulation of inflammation. miR-155 is a master regulator of inflammation as it has a positive regulatory effect on TNF-α production [44]. We found that chronic alcohol treatment increases miR-155 levels in RAW 264.7 macrophages in vitro (fig. 6a) and this correlated with an increased sensitivity of macrophages to LPS stimulation and LPS-induced TNF-α production (fig. 6b). In a recent work, we investigated the mechanisms by which alcohol increases miR-155 and showed that miR-155 inhibition can prevent alcohol-induced sensitization to LPS [45]. Upregulation of miR-155 is also likely to contribute to the increased sensitivity of Kupffer cells to LPS in alcoholic liver disease. We found increased miR-155 expression in Kupffer cells isolated from livers of alcohol-fed mice [45]. The data in figure 7 show that alcohol feeding results in increased serum endotoxin levels in mice (fig. 7a) and this correlates with alcohol-induced liver injury indicated by elevated serum ALT levels (fig. 7b). We also found that in the total liver of chronic alcohol-fed mice miR-155 expression was significantly increased (fig. 7c), suggesting a role for miR-155 in alcoholic liver disease. Further exploration of the role of microRNAs in liver inflammation and different types of chronic liver diseases will likely provide further insights into the role of LPS in the gut-liver axis.

**Fig. 6.** Enhanced miR-155 expression and TNF-α production in RAW 264.7 macrophages after prolonged alcohol treatment. a RAW 264.7 macrophages were treated or not with 50 mM alcohol for 1 or 2 days and total RNA was extracted with miRNeasy kit and used to analyze miR-155 expression by TaqMan PCR primers. The values were normalized to Sno202 and are shown as the fold change over the unstimulated cells. b RAW macrophages were treated with 50 mM alcohol for 2 days or 100 ng/ml LPS for 6 h or pretreated with alcohol for 2 days and challenged with LPS for 6 h. TNF-α levels were measured in cell-free supernatants by ELISA. Data represent the average of three experiments as a mean ± SD (*p < 0.05 compared to unstimulated cells).

**Fig. 7.** Increased serum endotoxin, alanine aminotransferase, and liver miR-155 in chronic alcohol-fed mice. Mice (6–8/group) received the Lieber-DeCarli diet with 5% (v/v) of ethanol or isocaloric liquid control diet for 4 weeks. After 4 weeks of feeding, serum was separated from blood and analyzed for endotoxin level (a) and alanine aminotransferase (ALT) (b). c Total RNA was isolated from livers and used to quantify miR-155 expression by real-time PCR. The values were normalized to Sno202 (endogenous control) and are shown as the fold increase over the pair-fed control group. Data represent mean values ± SD (*p < 0.05 compared to pair-fed mice). EU = Endotoxin units.
Conclusions and Remaining Questions

Strong evidence suggests that pathogen-derived compounds from the gut have a major role and/or modulating effect on liver diseases. The integrity of the gut epithelium, immune defense in the gut and in the liver and the composition of the microbiome all appear to play an integrated role in maintenance of health and the balance in the gut-liver axis. Further studies are needed to better understand the cooperation between these components, the molecular mechanisms of 'detoxification' mechanisms in the liver and the importance of the microbiome in specific liver diseases. Future studies should concentrate on targets for potential therapeutic interventions to improve liver health.

References


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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of the article.