Leaky gut and the liver: A role for bacterial translocation in nonalcoholic steatohepatitis

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Abstract

Gut flora and bacterial translocation (BT) play important roles in the pathogenesis of chronic liver disease, including cirrhosis and its complications. Intestinal bacterial overgrowth and increased bacterial translocation of gut flora from the intestinal lumen predispose patients to bacterial infections, major complications and also play a role in the pathogenesis of chronic liver disorders. Levels of bacterial lipopolysaccharide, a component of gram-negative bacteria, are increased in the portal and/or systemic circulation in several types of chronic liver disease. Impaired gut epithelial integrity due to alterations in tight junction proteins may be the pathological mechanism underlying bacterial translocation. Preclinical and clinical studies over the last decade have suggested a role for BT in the pathogenesis of nonalcoholic steatohepatitis (NASH). Bacterial overgrowth, immune dysfunction, alteration of the luminal factors, and altered intestinal permeability are all involved in the pathogenesis of NASH and its complications. A better understanding of the cell-specific recognition and intracellular signaling events involved in sensing gut-derived microbes will help in the development of means to achieve an optimal balance in the gut-liver axis and ameliorate liver diseases. These may suggest new targets for potential therapeutic interventions for the treatment of NASH. Here, we review some of the mechanisms connecting BT and NASH and potential therapeutic developments.

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Key words: Nonalcoholic steatohepatitis; Bacterial translocation; Insulin resistance; Leaky gut; Lipopolysaccharide

INTRODUCTION

Bacterial translocation (BT) and the derangement of gut flora are of substantial clinical relevance to patients with chronic liver disease and cirrhosis. Intestinal bacterial overgrowth and increased bacterial translocation of gut flora from the intestinal lumen predispose patients to bacterial infections and major complications. Furthermore, levels of bacterial lipopolysaccharide (LPS), a component of gram-negative bacteria, are increased in the portal and/or systemic circulation in several types of chronic liver disease. Bauer et al. have demonstrated this phenomenon in cirrhosis. Impaired gut epithelial integrity due to alterations in tight junction proteins may be the pathological mechanism underlying bacterial translocation.
BACTERIAL TRANSLOCATION, THE INNATE IMMUNE SYSTEM AND TOLL-LIKE RECEPTORS PLAY A ROLE IN THE PATHOGENESIS OF LIVER DAMAGE

Inflammation is involved in the pathogenesis of chronic liver diseases and plays a role in the development of progressive hepatic damage and fibrosis[11]. Liver inflammation and chronic damage are mediated by innate immune responses that are regulated by toll-like receptors (TLRs)[12]. Innate immune cells can both initiate and maintain inflammation in the liver. Bacteria translocated from the gut activate lymphocytes immediately interacting at the mesenteric lymph nodes (MLNs)[13].

Innate immune cells, particularly dendritic cells, play a pivotal role in sensing pathogens and initiating adaptive immune responses through the activation and regulation of T lymphocyte responses[11]. The immune system is abnormally activated in patients and experimental models with cirrhosis and ascites[14-16]. In an animal model of cirrhosis, systemic activation of the immune system occurs before ascites develop and is driven by the recirculation of cells activated in hepatic lymph nodes (HLNs)[13]. In compensated cirrhosis, bacterial DNA fragments reach the MLNs, where they elicit a local inflammatory response. Bacterial DNA fragments were present in the MLNs of 54% of rats with cirrhosis, indicating their role in the development of NASH[11].

The liver provides a tolerogenic immune environment for antigen-specific T cells. The liver is a source for activated immune cells present in the blood. The activation of Kupffer cells, recruited macrophages, and inflammatory cells results in the production of cytokines and chemokines that lead to prolonged inflammation and hepatocyte damage[17]. A direct correlation between activated cells in the blood and HLNbs, but not in MLNs, supports this concept, as does the fact that the changes in activated cells in the MLNs, but not in the blood or HLNbs, can be reversed by gut decontamination with antibiotics[19]. TH1 cells and monocytes were expanded and activated to produce intracellular interferon (IFN)-γ and TNF-α in the MLNs of cirrhotic rats[18]. Abrogation of bacterial translocation by bowel decontamination reduced the number of activated TH1 cells and monocytes and normalized IFN-γ and TNF-α production by monocytes in the MLNs and blood[16-19].

TLRs and TLR ligands play roles in the pathophysiology of liver fibrosis[20-23] and cirrhosis, viral hepatitis, ALD[22,23], nonalcoholic fatty liver disease (NAFLD) and hepatocellular carcinoma[24]. TLRs recognize pathogen-associated molecular patterns (PAMPs) to detect the presence of pathogens[24]. TLRs are expressed on immune cells, including Kupffer cells, endothelial cells, dendritic cells, biliary epithelial cells, HSCs, and hepatocytes. TLR signaling induces potent innate immune responses in these cells[25]. The liver is constantly exposed to PAMPs, such as LPS and bacterial DNA, through bacterial translocation via the portal vein system connecting it to the intestine[21].

TLRs also play a role in the regulation of inflammation based on their ability to recognize endogenous TLR ligands, termed damage-associated molecular patterns (DAMPs)[24]. The liver not only represents a major target of bacterial PAMPs in many disease states but also upregulates several DAMPs following injury[11,24]. The activation of inflammatory cells, including Kupffer cells, is a crucial step in the activation of hepatic stellate cells (HSCs)[25]. Intestinal bacterial microflora and functional TLR4, but not TLR2, are required for hepatic fibrogenesis[26]. Crosstalk between TLR4 signaling and transforming growth factor beta (TGF-β) signaling in HSCs has been reported[27]. Quiescent HSCs have been shown to be the target through which TLR4 ligands promote fibrogenesis. TGF-β signaling through the TLR4-MyD88-NFκappaB axis provides a novel link between proinflammatory and profibrogenic signals[28].

The activation of innate immune responses involving TLR4 and complement play important roles in initiating alcoholic steatohepatitis and fibrosis[28]. Activation of the TLR4-mediated myeloid differentiation factor 88 (MyD88)-independent [TRIF/interferon regulatory factor (IRF)-3] signaling pathway in Kupffer cells contributes to alcoholic steatohepatitis, whereas activation of TLR4 signaling in HSCs promotes liver fibrosis[28].

Activation of the innate immune system and increased release of proinflammatory cytokines and other mediators may play an important role in the development of alcoholic liver disease (ALD)[25,26]. Alcohol-induced hepatocellular damage may occur as a result of bacterial endotoxin translocation due to a reduction in reticuloendothelial system function in ALD. The recognition of gut-derived endotoxin by TLR4 contributes to the development of ALD through the activation of TLR-induced intracellular signaling pathways, cytokine production, and ROS[27]. TLR-dependent, ethanol-induced oxidative stress is important for the regulation of NFκappaB activation and
BACTERIAL TRANSLLOCATION IS ASSOCIATED WITH MITOCHONDRIAL DYSFUNCTION

Mitochondrial dysfunction is a pathogenic feature of NASH and there is evidence that mitochondrial damage contributes to apoptotic/necrotic cellular damage in NASH. NASH is associated with an increase in reactive oxygen species (ROS) production in Kupffer cells and hepatocytes. The greater the decrease in cytochrome oxidase activity seen, the more significant is the increase in ROS production. Mitochondrial dysfunction and overproduction of ROS play key roles in the progression of chronic hepatitis C and ethanol-induced liver injury. Ethanol also causes bacterial translocation in the intestine, and the resulting LPS activates Kupffer cells to produce pro-inflammatory cytokines. It has been suggested that NASH may also result from increased ROS production in Kupffer cells and hepatocytes that may be dependent on bacterial translocation. Therefore, in addition to its effects which are directly or indirectly associated with fat delivery, BT may also be associated with mitochondrial dysfunction that further contributes to fat accumulation in NAFLD.

BACTERIAL TRANSLLOCATION IS ASSOCIATED WITH THE DEVELOPMENT OF NONALCOHOLIC STEATOHEPATITIS

Evidence supporting a role for the liver-gut axis in the pathogenesis of NAFLD has been slowly accumulating over the past 7 years. Both preclinical and clinical data suggest an association between BT, small intestinal bacterial overgrowth (SIBO) and NASH. Recently, the presence of SIBO has been associated with the severity of liver steatosis. Exposure to bacterial products of intestinal origin, most notably endotoxin, including LPS, leads to liver inflammation, hepatocyte injury and hepatic fibrosis.

TLR4 and its coreceptor, myeloid differentiation factor-2 (MD-2), recognize LPS and activate proinflammatory signaling pathways. TLR4 can specifically recognize LPS as a danger signal and induce activation of inflammation-associated genes. A 4-wk high-fat diet increased plasma LPS concentration two to three times and the LPS recognition complex (TLR4 and MD-2) activates NADPH in liver steatosis and induces fibrosis in a NASH model in mice. These data support the role of these receptors in the development of steatosis, inflammation and fibrosis in NASH.
The suppression of inflammation and immune tolerance are known to occur in normal livers. Suppressed inflammation has been shown despite bacterial colonization in normal human livers maintaining liver immune homeostasis\[^{[56]}\]. In spite of increased bacterial colonization of liver tissues, lower levels of TLR2/4 mRNA and TLR4 and pIkKalpha, a marker for nuclear factor-kappa B (NFkappaB) activation, proteins were found in liver tissues from healthy subjects compared with samples from patients with primary biliary cirrhosis and NASH. Although these data raise the question of whether BT initiates the inflammatory process in the liver or whether it is instead a result of the inflammatory process, these results further support a role for BT in the pathogenesis of the disease.

SIBO also plays a role in NASH via interactions with TLR-4 and the induction of IL-8\[^{[43]}\]. SIBO has been reported to coexist with NASH\[^{[51-56]}\]. Patients with NASH show higher levels of expression of TLR-4/MD-2 on CD14-positive cells\[^{[43]}\]. Serum levels of the proinflammatory cytokine IL-8 were higher in samples from NASH patients than in those from control subjects and were correlated positively with TLR-4 expression.

Leptin is a proinflammatory cytokine associated with the progression of NASH. Leptin enhanced TNF-α production and caused a dose-dependent increase in MAPK activity in LPS-stimulated KCs\[^{[37]}\]. KCs isolated from the leptin receptor-deficient Zucker rat (fa/fa) showed reduced production of TNF-α upon stimulation with LPS\[^{[37]}\]. Furthermore, treatment of normal rats with leptin increased LPS-induced hepatic TNF-α production in vivo, and leptin receptor-deficient Zucker rats showed reduced hepatic TNF-α production upon addition of LPS in vivo\[^{[37]}\].

Following BT, LPS activates inflammasomes\[^{[38-60]}\]. Inflammasomes respond to endogenous and exogenous danger signals by inducing the processing of pre-IL-1ss into secreted IL-1ss\[^{[56]}\]. In the methionine-choline-deficient (MCD) or high fat diet-induced models, saturated fatty palmitic acid activates the inflammasome and sensitizes hepatocytes to LPS-induced IL-1ss release. Hepatocytes exposed to saturated fatty acid release danger signals that trigger inflammasome activation in immune cells\[^{[38]}\]. LPS treatment significantly increased hepatic TNF-α production in MCD mice. LPS also induced a significant increase in TUNEL-positive cells\[^{[61]}\]. This increase in apoptosis was inhibited by treatment with a neutralizing anti-mouse TNF receptor antibody or pentoxifylline.

In humans, dietary fructose intake has been associated with increased intestinal permeability and translocation of bacterial endotoxin; plasminogen activator inhibitor (PAI-1) may also contribute to the development of NAFLD in humans\[^{[62]}\]. Plasma concentrations of endotoxin and PAI-1 and hepatic mRNA expression levels of TLR4 and PAI-1 were higher in NAFLD patients than in control subjects. Serum levels of LPS-binding protein (LBP) were increased in obese patients with NAFLD\[^{[63]}\]. Plasma levels of LBP were further increased in patients with steatohepatitis when compared with patients with simple steatosis\[^{[58]}\]. TNF-α mRNA expression in liver tissue was significantly higher in patients with NASH than in control subjects and was correlated with the increase in plasma levels of LBP.

BT is also involved in nitric oxide synthase (NOS) upregulation through the activation of both endothelial NOS and inducible NOS\[^{[64-66]}\]. The prevention of intestinal gram-negative bacterial translocation by norfloxacin corrects circulatory changes by decreasing nitric oxide (NO) production in cirrhosis. Norfloxacin administration significantly decreased the incidence of gram-negative bacterial translocation and production of proinflammatory TNF-α, IFN-γ and IL-6\[^{[66,71]}\].

The published data support the hypothesis that BT is associated with the development and maintenance of continued lipid accumulation, inflammation and fibrosis in patients with NASH (Figure 1).

**BACTERIAL TRANSLOCATION IS CLINICALLY RELEVANT IN CHRONIC LIVER DISEASE**

BT was shown to affect the development of chronic liver disease and the associated complications. It is also associated with an impaired prognosis\[^{[58]}\]. Bacterial DNA is a marker of bacterial translocation and can be detected in uninfected patients with cirrhosis and ascites\[^{[69,70]}\]. It is associated with a marked inflammatory response and with the activation of the inducible form of NOS and the release of NO. A similar effect is observed in patients with SBP\[^{[66]}\].

The induction of cirrhosis in rats by CCLs led to prolonged oxidative stress in the intestine, accompanied by increased sugar content in both the intestinal brush border and the surfactant layer\[^{[74]}\]. This was accompanied by changes in bacterial flora in the gut, and these bacteria
showed increased hydrophobicity and adherence to the mucosa. These data support the notion that oxidative stress in the intestine during cirrhosis alters mucosal glycosylation and increases the hydrophobicity of the luminal bacteria, enabling increased bacterial adherence to epithelial cells, facilitating BT\cite{63}.

In a human trial, the presence of bacterial DNA was associated with aggravation of peripheral vasodilatation and with a worsening of intrahepatic endothelial dysfunction\cite{66}. Patients exposed to bacterial DNA had a significantly lower mean arterial pressure and systemic vascular resistance. In response to increased blood flow caused by postprandial hyperemia, these patients had greater increases in hepatic vein pressure gradient and impaired hepatic vasorelaxation\cite{66}. In contrast, a prospective trial of 151 patients with cirrhosis and ascites found no evidence that the detection of bacterial DNA in the ascites of cirrhotics is of clinical or diagnostic relevance to the detection of SBP\cite{73}. This discrepancy in the published data remains to be resolved.

Increased intestinal permeability and abnormal motility were frequently observed in cirrhotics without ascites, even in the absence of evidence of BT. It has been suggested that these factors facilitate BT and thus precede it\cite{73}. Systemic reactivity to microbial components as measured by the development of antibodies was suggested to reflect the compromised mucosal immunity in cirrhotic patients\cite{73}. The presence of bacterial DNA in blood and ascites correlates with BT and is frequent in patients with advanced cirrhosis without overt infection; BT can also precede the occurrence of overt bacterial infection in patients with cirrhosis\cite{73}. Altered permeability of the mucosa and deficiencies in host immune defenses that allow bacterial translocation from the intestine due to intestinal bacterial overgrowth have been implicated in the development of SBP\cite{74}. Altered intestinal permeability was observed in 45% of patients with cirrhosis and was associated with Child-Pugh status, with the presence of ascites, and with a history of SBP\cite{75-77}. SIBO is much more frequent in patients with cirrhosis and was highly correlated with BT, especially in ascitic patients\cite{77}.

Higher levels of *Enterobacteriaceae* were identified in cirrhotic rats than in healthy rats, and *Bifidobacteria* treatment resulted in lower levels of *Enterobacteriaceae*\cite{61}. These results suggest the existence of an imbalance in the gut flora in cirrhotic rats, which may further result in BT and altered liver function\cite{61}.

BT to MLNs in cirrhosis has been linked to impaired host defense in these patients\cite{79}. BT and endotoxemia are contributing factors in the expansion of specific subsets of lymphocyte populations\cite{82}. In a clinical trial of 40 cirrhotics, the percentage of activated monocytes and T lymphocytes was increased in patients, and the proportions of effector cells and of those expressing CD95+ were higher. LBP modulates the biologic activity of circulating endotoxin, and its levels have been shown to rise in response to LPS\cite{80-82}. Patients with elevated levels of LBP showed higher frequencies of regulatory T cells (CD4+CD25+FoxP3+) than those with normal levels of LBP\cite{84}. In a rat model, BT was associated with an increase in the phagocytic capacity of polymorphonuclear leukocytes\cite{83}. Both TNF-α and IL-6 were increased in patients with translocation of bacterial DNA from gram-positive microorganisms regardless of endotoxin and LBP levels\cite{85}.

These data suggest that BT is of clinical relevance in patients with chronic liver disease and may be a contributing factor in the development of liver disease and the degree of severity of the associated complications.

**BACTERIAL TRANSLLOCATION: IMPLICATIONS FOR THERAPY**

Detoxification of gut-derived toxins and microbial products from gut-derived microbes is one function of the liver. Levels of bacterial LPS are increased in the portal and/or systemic circulation in several types of chronic liver diseases. Increased gut permeability and LPS also play roles in several liver disorders. NASH is associated with increased serum LPS levels and the activation of proinflammatory signaling\cite{88,89}, both of which suggest BT as a potential therapeutic target in these disorders.

Probiotics have been suggested as a treatment for different types of chronic liver damage because of their abilities to augment intestinal barrier function and to prevent BT\cite{90,91}. The administration of probiotics reduced BT in a rat model\cite{92}. Both viable and heat-killed yeast cells prevented BT. This effect was suggested to be the result of an immune modulatory effect and the maintenance of gut barrier integrity\cite{90,92}. Oral treatment with viable or heat-killed *Saccharomyces cerevisiae* strain UFMG 905 prevented BT in a murine model of intestinal obstruction. Treatment with either viable or heat-killed yeast reduced intestinal permeability and increased IL-10 levels. Orally administered probiotics, nonpathogenic *Escherichia coli*, and gentamicin decreased BT and attenuated liver damage, decreasing levels of TNF-α, IL-6, IL-10 and IL-12\cite{96}. An enteral diet supplemented with *Chlorella* sp. microalgae had significant protective effects on the intestinal mucosal barrier in a rat model of obstructive jaundice and reduced BT\cite{97}. Oral treatment with resveratrol, curcumin or simvastatin ameliorated small intestinal inflammation by maintaining gut barrier function, preventing BT, and decreasing Th1-type immune responses\cite{98}. Oral administration of these compounds increased regulatory T cell numbers and augmented intestinal epithelial cell regeneration in the ileum. Levels of the anti-inflammatory cytokine IL-10 in the ileum, MLNs and spleen were increased, whereas the proinflammatory cytokines IL-23p19, IFN-γ and TNF-α were decreased\cite{99}. Treated animals displayed fewer proinflammatory enterobacteria and enterococci and higher anti-inflammatory lactobacilli and Bifidobacteria loads.

Glutamine decreased intestinal permeability and BT to physiologic levels in treated animals and preserved intesti-
nal barrier integrity. Arginine supplementation reduced intestinal permeability and BT, leading to mucosal ileum preservation. A specific nutritional combination rich in protein, L-leucine, fish oil and specific oligosaccharides resulted in reduced BT along with reduced production of proinflammatory cytokines, and supplementation with honey in the presence of obstructive jaundice ameliorated BT.

Treatment with an anti-TNF-α mAb in a model of CCl4-induced cirrhosis decreased the incidence of BT in a TNF-α- and TNF-α receptor-independent manner without increasing the risk of systemic infection.

Desferrioxamine attenuated mucosal injury from post-hepatectomy liver dysfunction, and this was associated with decreased BT in the portal circulation, decreased portal endotoxin levels, and decreased systemic endotoxin levels. Low concentrations of histamine inhibited bacteria from entering epithelial cells and inhibited intestinal BT. In histamine-treated rats, the average numbers of bacteria in the liver and lymph nodes were much lower than those in control rats.

The sympathetic nervous system is activated in advanced cirrhosis, particularly in the splanchic circulation, and exerts potent immunosuppressive actions. Splanchnic sympathectomy reduced bacterial translocation to MLNs in cirrhotic rats.

TREATMENT OF BACTERIAL TRANSLOCATION AS A MEANS OF TREATING NONALCOHOLIC STEATOHEPATITIS

In light of the potential role of BT in the development of steatosis, steatohepatitis and fibrosis, several studies have evaluated the potential effects of treatments aimed at BT.

Probiotics exhibit immunoregulatory and anti-inflammatory activity. Administration of the probiotic VSL#3 modulated liver fibrosis via the modulation of collagen expression and impairment of TGF-β signaling in a NASH model, however, this treatment did not prevent inflammation and steatosis.

Oxidative stress contributes to the development of NASH, suggesting that antioxidants, which decrease oxidative stress, may ameliorate the disease. Increasing hepatic α- or γ-tocopherol protected against LPS-induced NASH by decreasing liver damage, lipid peroxidation, and inflammation without affecting body mass or hepatic steatosis. Resveratrol decreased NAFLD severity in rats via TNF-α inhibition and antioxidant activity. Specifically, it decreased fat deposition, increased levels of superoxide dismutase, glutathione peroxidase and catalase and decreased NOS in the liver.

LPS-induced liver injury was prevented by 8A8, a synthetic triglyceride containing an arachidonic acid branch, through the inhibition of TNF-α and NO production by hepatic macrophages. Stimulation of peripheral blood mononuclear cells from NASH patients with LPS resulted in a strong increase in TNF-α production. Pentoxifylline caused a dose-dependent suppression of TNF-α secretion, suggesting that it may be able to serve as a potential treatment for NASH.

In an open-label trial, subjects with biopsy-proven NASH and insulin resistance were orally treated for 30 days with an IgG-enhanced fraction of enterotoxigenic Escherichia coli colostrum (Imm-124, Immuron, Australia). An alleviation of insulin resistance was detected by a decrease in fasting glucose levels, an elevation in the early peak of insulin secretion following glucose administration, improved OGTT, improved insulin secretion during the OGTT, and improvements in the HOMA score and HBA1C levels. Treated patients showed a decrease in serum levels of triglycerides, total cholesterol, and LDL cholesterol. A decrease in liver enzymes was noted in most treated patients. These effects were associated with increased serum levels of GLP-1 and adiponectin. An increase in CD25+ and CD4+CD25+Foxp3+ regulatory T cells was also noted. An anti-LPS effect along with the promotion of regulatory T cells suggests that a combined mechanism is responsible for these effects.

CONCLUSION

Alterations to intestinal microbiota seem to play important roles in the induction and promotion of liver damage progression and in the development and severity of NASH. Bacterial overgrowth, immune dysfunction, alteration of the luminal factors, and altered intestinal permeability are all involved in the pathogenesis of NASH and its complications, including progression to cirrhosis, infections, hepatic encephalopathy, SBP and renal failure.

A better understanding of the cell-specific recognition and intracellular signaling events involved in sensing gut-derived microbes will help in the development of means to achieve an optimal balance in the gut-liver axis and ameliorate liver diseases. The data described here support the notion that BT may serve as a new therapeutic target for NASH.

BT induces an immune imbalance leading to a state of chronic inflammation, fat accumulation in the liver, mitochondrial dysfunction and NASH.

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