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Is intestinal inflammation linking dysbiosis to gut barrier dysfunction during liver disease?

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Abstract

Changes in the intestinal microbiota composition contribute to the pathogenesis of many disorders including gastrointestinal and liver diseases. Recent studies have broadened our understanding of the “gut-liver” axis. Dietary changes, other environmental and genetic factors can lead to alterations in the microbiota. Dysbiosis can further disrupt the integrity of the intestinal barrier leading to pathological bacterial translocation and the initiation of an inflammatory response in the liver. In this article, the authors dissect the different steps involved in disease pathogenesis to further refine approaches for the medical management of liver diseases. The authors will specifically discuss the role of dysbiosis in inducing intestinal inflammation and increasing intestinal permeability.

Keywords

gut-liver axis; microbiome; intestinal inflammation; intestinal barrier; microbiome; metagenome; metabolome; bacterial translocation

Introduction

The human intestine contains trillions of microbes that live in a homeostatic state with the host [1]. Microbes are important for the digestion of food, absorption of nutrients, synthesis of vitamins and for the development of lymphoid structures in the gastrointestinal tract. The intestinal microbiota prevents colonization of pathogens by competing for nutrients and space [2]. On the other hand, the host supplies nutrients for the microbiota and provides a protected space to flourish [3]. It is therefore not surprising that loss of this homeostasis can result in disease. And in fact, several diseases are associated with changes in composition or function of the intestinal microbiota (dysbiosis). For example, patients with inflammatory

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bowel disease (IBD) have a reduced diversity of the microbial community compared with healthy subjects [4]. Changes in the microbial composition can contribute to intestinal inflammation and are causatively linked to the onset of IBD [1]. Tumor necrosis factor (TNF) is a pleiotropic cytokine and plays a central role in IBD. TNF is produced by invading immune cells, stromal cells and epithelial cells during intestinal inflammation [5]. TNF binds to TNF-receptor 2 (TNFR2), which activates myosin light chain kinase (MLCK) in intestinal epithelial cells [6]. MLCK causes rearrangement of tight junctions and a reduction in the intestinal barrier function [6]. MLCK levels are also increased in patients with ulcerative colitis and Crohn's disease [7]. TNF has not only been implicated in the disruption of intestinal tight junctions, but also in epithelial cell death and perpetuation of the intestinal immune response [5]. Chronic intestinal inflammation leads to defects in the epithelial barrier.

Although IBD is characterized by chronic intestinal inflammation and is associated with increased intestinal permeability, only a minority of patients with IBD develops extraintestinal liver manifestations. Primary sclerosing cholangitis (PSC) is one well-documented example of chronic liver disease associated with IBD. PSC occurs more often in patients with ulcerative colitis (UC) than in patients with Crohn's disease (CD), although the exact reason for this is not known. The low incidence of PSC in UC patients (approximately 5 percent) suggests that intestinal inflammation alone is not sufficient to drive liver disease [8]. On the other hand, sixty to eighty percent of patients with PSC have concurrent IBD [9]. One could speculate that a "second hit" is necessary that accounts for liver disease development. PSC runs in families and several genetic risk loci have been identified [10]. Another important pathogenic factor in the development of PSC might be an altered bile composition. Genetically or chemically altered bile formation leads to the development of PSC in several animal models [11].

HIV enteropathy is a second example of changes in the intestinal immune system and gut barrier dysfunction. CD4⁺ T-cells and Th17 cells are reduced in the gut-associated lymphoid tissue following HIV infection [12]. This leads to an intestinal barrier dysfunction that exposes the lamina propria immune cells to microbial antigens [12]. Microbial translocation is associated with systemic immune activation in HIV patients, but not necessarily with liver disease [13].

We therefore conclude that intestinal inflammation and a disrupted gut barrier alone are not sufficient to cause liver disease.

Is dysbiosis able to induce intestinal inflammation?

Different factors change the composition of the microbiota, including genetic factors, diet and other environmental factors [14]. Inflammasome or NOD-like receptor family pyrin domain containing (NLRP) deficient mice provide a genetic model of dysbiosis [15]. Inflammasomes are cytosolic multiprotein complexes that respond to microbial products or a disruption of cellular physiology. Inflammasomes activate caspase-1, which cleaves proinflammatory cytokines pro-interleukin (IL)-1 β and pro-IL-18 into their active forms [16,17] Deficiency of the major inflammasome component NLRP6 leads to intestinal

dysbiosis, particularly evidenced by the expansion of *Prevotellaceae* and *Porphyromonadaceae* families [18,19]. The effector cytokine IL-18 (but not IL-1 β) was responsible for intestinal dysbiosis. The aberrant microbiota in NLRP3 and NLRP6 deficient mice induces colonic inflammation via the induction of chemokine (C-C motif) ligand (Ccl5) from epithelial cells [15]. Ccl5 recruits a variety of innate and adaptive immune cells further promoting inflammation [15]. As a consequence of colonic inflammation, Toll-like receptor (TLR) agonists including lipopolysaccharide (LPS) and bacterial DNA translocate to the portal vein and liver [18]. These microbial products bind to TLR4 and TLR9 in the liver and induce downstream signaling that enhances the progression of non-alcoholic fatty liver disease (NAFLD) to non-alcoholic steatohepatitis (NASH) [15]. Increased innate immune signaling in the liver via TLRs has also been associated with progression of other liver diseases including alcoholic liver disease, liver fibrosis and chronic viral hepatitis [20]. Taken together, dysbiosis induces intestinal inflammation and a subsequent translocation of microbial products to the liver enhances the progression of liver disease.

Quantitative changes of the microbiota alone can trigger liver disease. Using jejunal self-filling blind-loops as a model, small-bowel bacterial overgrowth was sufficient to induce hepatobiliary injury in rats [21]. The underlying mechanism might involve damage of the bacteria to the intestinal mucosa, the formation of a disrupted gut barrier and pathological translocation of bacterial products to the liver.

Other factors that cause changes in the composition of microbiota involve dietary factors. Chronic alcohol consumption results in qualitative and quantitative changes of the microbiota [22,23]. Qualitative changes include a decrease in Firmicutes (e.g. *Lactobacillus*) and an increase in Bacteroidetes following alcohol exposure in mice [23]. Another study found a decrease in *Bifidobacterium* and *Lactobacillus* in the stool of alcohol-dependent individuals [24]. In line with these results, probiotic *Lactobacillus* ameliorates alcohol-induced liver disease in animal models and in human subjects [23,25,26]. Interestingly, during alcohol abstinence suppressed *Lactobacillus* ssp. and *Bifidobacterium* ssp. are restored. This suggests that bacteria, known to have beneficial effects, could play a role in the recovery process of the intestinal tract [27].

Our own recent data provides mechanistic insight on how alcohol administration causes intestinal bacterial overgrowth and dysbiosis [28]. Alcohol feeding to mice leads to a reduced capacity of the intestinal bacteria to synthesize saturated long-chain fatty acids (LCFA). LCFA are important for maintaining eubiosis and for preventing overgrowth of intestinal bacteria. The presence of LCFA correlates with intestinal levels of beneficial lactobacilli in alcoholics, which are important for maintaining the integrity of the intestinal barrier. Accordingly, feeding mice saturated fatty acids prevents dysbiosis, leads to reduced intestinal inflammation and leakiness, and ameliorates alcohol-induced liver damage. This study also supports a concept on how a dietary intervention can prevent the development of alcoholic liver disease [28].

Feeding mice high fat diet is also associated with intestinal inflammation; specifically the interaction between high fat western diet and gut microbiota can promote intestinal inflammation. When conventionally raised mice were placed on high fat diet, increased

inflammation was detected as measured by TNF gene expression and NF κ B activation [29]. The presence of microbiota seems indispensable, as high fat diet did not cause an upregulation of those markers in germ-free mice. As a consequence of intestinal inflammation, conventional mice developed obesity, weight gain and adiposity in contrast to germ-free mice which were devoid of these symptoms. An interaction between the microbiota and the dietary change is therefore necessary to cause intestinal inflammation [29].

Taken together, dysbiosis induced by environmental factors, dietary changes or genetic components can lead to intestinal inflammation. Such inflammation in combination with a liver insult can result in progression of liver disease.

How is intestinal inflammation characterized?

Intestinal inflammation is a complex process including the response of several immune cells to tissue damage and bacterial products. One of the primary goals of the initial inflammatory response is to contain bacterial invasion and to repair tissue defects. Persistent failure in repairing tissue damage and containing bacterial invasion results in chronic inflammation [30]. Several proinflammatory mediators are involved in the onset of intestinal inflammation and liver disease development. As discussed above, chronic TNF stimulation plays a major role in the pathogenesis of inflammatory bowel disease as evidenced by the successful treatment of IBD patients with approved anti-TNF drugs [31]. Studies have also shown a role for proinflammatory mediators TNF [23,32] and Ccl5 in the progression of liver disease [15]. Intestinal TNF gene expression is higher in patients with NAFLD patients as compared with healthy subjects [32]. In addition, chronic alcohol administration in mice and alcohol abuse in humans increased intestinal TNF levels. Depletion of the microbiota in mice reduced intestinal TNF expression after chronic alcohol feeding suggesting that alcohol-associated enteric dysbiosis induces intestinal inflammation. However, the exact mechanism of how dysbiosis induces intestinal TNF is not known [33].

Intestinal inflammation also stimulates regenerative processes. For example, binding of IL-6 to its receptor gp130 triggers transcriptional regulators such as Yes-associated protein (YAP) and Notch, which are involved in tissue growth and regeneration [30]. As expected, mice deficient in IL-6 are more susceptible in a model of experimentally induced colitis [34]. In addition, transgenic mice that express activated gp130 in intestinal epithelial cells show less severe colitis compared with wild-type mice [30]. Another protective cytokine is IL-22. IL-22 is highly expressed in the inflamed colon, and mice receiving IL-22 deficient T cells lost significantly more weight in a T cell transfer model of colitis. This suggests that IL-22 can ameliorate colitis and in fact IL-22 expression correlates with protection from colitis [35]. In line with these results, IL-22 has also been shown to be hepatoprotective. Administration of an IL-22 expressing adenovirus or recombinant IL-22 ameliorates liver damage in several models of liver injury including ethanol-induced liver injury using a chronic binge model [36] and high fat diet-induced hepatic steatosis [37]. Whether IL-22-mediated stabilization of the gut barrier is involved in the hepatoprotective effect, deserves future studies.

In addition to protective cytokines, inflammatory cells produce antiinflammatory cytokines. IL-10 deficient mice develop enterocolitis by the age of 4–8 weeks when raised under conventional conditions [38]. IL-10 ameliorates liver disease in various models. IL-10 deficient mice show larger hepatic inflammatory infiltrates in an animal model of liver fibrosis [39–41]. Additional investigations need to address whether IL-10 plays a protective role in the intestine in animal models of liver disease.

What are the consequences of chronic intestinal inflammation?

Once intestinal inflammation becomes chronic, a major consequence is a change in the integrity of the epithelial barrier, often referred as a “leaky gut”. Studies in mice have suggested that intestinal inflammation (e.g. experimentally induced by dextran sulfate sodium (DSS) can lead to a disrupted mucosal barrier. A disrupted intestinal barrier allows bacteria and their products to translocate to the portal circulation and reach the liver. This translocation process induces an inflammatory response in the liver and aggravates liver disease and NASH [42]. It is important to remember that translocation of microbial products occurs even in healthy individuals. However, this process is increased following a breach of the intestinal barrier [43]. The integrity of the intestinal barrier is of specific importance to limit bacteria and bacterial products from translocating and reaching extraintestinal sites. The intestinal epithelium has the capacity to segregate the microorganism from the host [44].

Which factors control the exposure of microbes to the systemic circulation?

Epithelial cells together with tight junctions serve as physical barrier that minimize the contact between bacteria and the systemic circulation. Goblet cells secrete glycoproteins forming a mucus layer that covers the epithelium [45]. This mucus layer is composed out of an inner and an outer layer [46]. The outer layer is a loose mucus layer and contains a high number of bacteria yielding an ideal place for the commensal bacteria, since the O-glycans of mucin (Muc)-2 serve as important energy sources [45]. The inner layer is devoid of any bacteria and is a firmly attached, dense mucus layer, preventing direct contact of the bacteria with epithelial cells. The importance of this first-line defense is demonstrated by the development of colorectal cancer in Muc-2 deficient mice [47,48].

Muc-2 deficient mice were also subjected to an animal model of alcoholic liver disease [49]. Against our expectation, Muc-2 deficient mice showed decreased susceptibility to alcoholic steatohepatitis. Our data suggest that Muc-2 deficiency leads to an increase in the expression of antimicrobial molecules (specifically regenerating islet derived (Reg)-3b and Reg3g) and an altered microbiome composition. This increase in antimicrobial molecules enhances the killing of bacteria in the intestinal lumen. As a consequence intestinal bacterial growth is reduced in Muc-2 deficient mice after alcohol feeding compared with wild-type mice. Therefore, due to a lower amount of luminal bacterial products, less translocation of bacterial products to the systemic circulation was detected causing less alcoholic liver injury and steatosis. This study supports not only an important role for the mucus barrier, but it also underlines the importance of the composition of the intestinal flora to start with. The presence of a mucus barrier has obvious advantages e.g. for preventing the colonization and

invasion of intestinal pathogens [50]. On the other hand, a failure of a physical barrier turns on an alert state of the mucosal innate immune system, which triggers a beneficial intestinal inflammatory response and controls the luminal bacterial burden.

Commensal bacteria can have a beneficial effect for the integrity of the intestinal barrier. Some of these beneficial bacteria adhere to enterocytes and maintain a proper barrier function. This has recently been suggested for the commensal bacteria *Akkermansia muciniphila* [51].

Antimicrobial molecules including defensins, cathelicidins, lysozyme and C-type lectins, are other important molecules that prevent the interaction of bacteria with the epithelial layer [52]. Antimicrobial proteins can kill bacteria either through enzymatic attack (e.g. lysozyme) or through non-enzymatic mechanisms that include the direct disruption of bacterial membranes through electrostatic interactions (defensins, cathelicidins and C-type lectins). Reg3g is a C-type lectin that is expressed in epithelial cells in a TLR-dependent fashion, and both TLR4 and TLR5 ligands induce Reg3g expression [53–57]. In addition, Reg3g expression is dependent on IL-22, which is secreted by intestinal innate lymphoid cells [58]. Interestingly, Reg3g deficient mice showed increased bacterial colonization of the intestinal epithelial cells and in particular of the inner mucus layer, suggesting that Reg3g has a major role in the segregation of the epithelium and luminal bacteria [57]. Therefore, Reg3g is an important contributor to maintain spatial homeostasis in the intestinal tract [52].

The functional role of Reg3 molecules in liver disease has not completely been elucidated. Alcohol feeding in mice was associated with a decrease in intestinal Reg3b and Reg3g expression. Furthermore, when prebiotics were administered to alcohol-fed mice, Reg3g levels were partially restored, and alcoholic steatohepatitis was decreased [23]. Future studies need to address whether suppressed Reg3b and/or Reg3g functionally contribute to microbiome changes, pathological bacterial translocation and liver disease.

Finally, epithelial cells actively transport and secrete IgA into the intestinal lumen. Among all the immunoglobulins secreted into the lamina propria, IgA is the most abundant [59]. Microbe specific IgA within the intestinal lumen is made with the help of intestinal dendritic cells (DCs) that sample luminal bacteria with their transepithelial DC extensions. DCs then interact with B and T cells in Peyer's patches inducing B cells to produce IgA. Microbe specific B cells home to the lamina propria and secrete IgA. IgA binds to the polymeric immunoglobulin receptor (pIgR) on the basolateral surface of mucosal intestinal epithelial cells, and is then shuttled across the epithelium. IgA in the intestinal lumen can bind to bacteria and prevent translocation [59–61]. To what extent IgA functionally contributes to liver disease, has not yet been addressed.

Although the intestinal tract has developed these multiple layers of protection to minimize the contact of the bacteria with the epithelial barrier, there is an occasional breach of the barrier and bacteria can enter the lamina propria. Macrophages in the lamina propria can phagocytose and kill bacteria. As they are located directly under the epithelial layer they are ideally positioned to catch and destroy bacterial products that have passed the epithelium [62,63]. However, intestinal macrophages produce less proinflammatory cytokines than

macrophages elsewhere in the body [64]. This allows intestinal macrophages to eliminate bacteria without initiating an inflammatory response. This is most likely due to a decreased expression of intracellular response genes (like MyD88 and TRAF6), which are important to propagate TLR signaling [64].

Which molecular mechanisms are involved in the dysfunction of the intestinal barrier?

Following a dysfunction of the intestinal barrier, bacterial products are able to translocate from the intestine to the blood stream. The transition of bacteria and bacterial products from the luminal site to the lamina propria can be achieved by several distinct mechanisms depending on the specific bacteria/ pathogen. Many bacteria translocate through M-cells. This requires a specific interaction of bacterial proteins with M-cells followed by internalization, transcellular transport and the extrusion of the bacteria at the other site of the cell. Although bacteria need specific equipment for the initial contact with M cells, commensal bacteria can also translocate together with pathogens [65]. Although rare, another possibility is paracellular transport. Epithelial cells are joined together by junctional complexes including tight junctions, and the size of the tight junction pores determines which molecules can be transported paracellularly [65]. Functional tight junctions allow the transport of water, solutes and ions but not intact bacteria. However, some pathogens can alter tight junction molecules and therefore change the paracellular permeability. Some bacteria are activating MLCK which leads to tight junction disruption [65]. To what extent translocated viable bacteria contribute to progression of pre-cirrhotic liver disease is currently not known.

Although intestinal inflammation has been associated with “leakiness” of the epithelial barrier [42,66], a defined mechanism has not been identified until recently. Our own studies demonstrated that intestinal production of TNF is a major contributor to intestinal barrier dysfunction during alcoholic liver disease. In mouse models and humans, chronic alcohol use leads to the activation of monocytes and macrophages in the lamina propria and to the production of TNF. Signaling via TNFR-1 and downstream events via activation of MLCK in enterocytes contributes to the epithelial barrier dysfunction. The mechanism most likely involves removal of occludin, a major component of tight junctions. Therefore, TNF production by monocytes and macrophages and signaling via TNFR-1 contributes to a loss in tight junction proteins and a reduced barrier function. Increased translocation of microbial products to the liver augments alcoholic liver disease. Most importantly, intestinal inflammation, gut leakiness, pathological bacterial translocation and alcoholic liver disease are dependent on intestinal dysbiosis, because non-absorbable antibiotics prevent all of these changes. In addition, intestinal inflammation precedes the onset of increased intestinal permeability [33].

Reactive oxygen species have also been implicated in causing intestinal inflammation, followed by a disruption of the gut barrier and liver damage [67]. Cyp2E1 enzyme metabolizes ethanol and is expressed in the liver and the intestine [66]. Cyp2E1 activity contributes to oxidative stress. Cyp2E1 null mice that were fed with high fat diet, showed significantly less NASH compared with wild-type mice [67]. Hepatic production of reactive

oxygen species might contribute to the expression of proinflammatory cytokines. Cyp2E1 deficient mice also showed decreased endotoxin levels and enterobacterial contents in the liver after binge alcohol, suggesting a role for Cyp2E1-mediated induction of reactive oxygen species in promoting gut leakiness [66].

What are consequences of a disrupted gut barrier for liver disease?

The liver has a unique dual blood supply. While it receives arterial blood from the hepatic artery, venous blood coming from the portal vein mixes with the arterial blood in the hepatic sinusoids. Venous blood from almost the entire small and large intestine drains into the portal vein. Portal venous blood carries not only nutrients, but also translocated microbial products and viable bacteria. The liver is therefore the body's "first pass" organ that encounters all of these molecules. In the liver, bacterial products can bind to their specific pathogen recognition receptor (e.g. TLR4, TLR9) on parenchymal and non-parenchymal cells triggering an inflammatory response and further enhancing disease progression.

Several studies demonstrated that patients with alcoholic liver disease have increased intestinal permeability as evidenced by higher plasma endotoxin levels [68–70]. Increased levels of LPS and other bacterial products were also detected in mouse models of alcoholic liver disease [71]. Studies in rodents provide some insight into the underlying molecular mechanism responsible for liver disease progression. Once translocated, bacterial products bind to their respective pathogen recognition receptor initiating intracellular signaling and triggering an inflammatory cascade. For example, LPS binds via MD-2 to TLR4, using CD14 as a co-receptor; CpGs bind to TLR9 [72]. Mice deficient in CD14, TLR4 or TLR9 are resistant to experimentally induced NASH [73,74]. TLR9 signaling is associated with inflammasome expression in hepatic macrophages and in the production of IL-1 β , leading to steatosis, inflammation and NASH fibrosis [20]. This data suggest that bacterial products, once translocated, signal via their respective receptors in the liver and trigger hepatocyte damage, inflammation and fibrosis. Hepatocytes can be a direct target of microbial products. This is evidenced by the requirement for IRF3 signaling triggering hepatocyte death in alcoholic liver disease [75]. Alternatively, PAMPs can activate Kupffer cells to initiate hepatocyte damage following alcohol administration [76]. A role for bacterial products like LPS is further evidenced by the fact that antibiotic treatment reduces plasma endotoxin levels and prevents alcohol-induced liver injury in rats [77].

However, it has been shown that the translocation of bacteria and bacterial products is not exclusively detrimental. Our own data shows that the commensal microbiota is protective in a setting of experimentally induced liver fibrosis. Administration of thioacetamide or carbon tetrachloride induced significantly more liver injury and fibrosis in germ-free mice as compared with wild-type mice. In line with those results, mice deficient in the major innate immunity signaling molecules MyD88 and Trif also show increased liver injury and fibrosis [78]. These results suggest that microbes and their products are required for liver homeostasis during an injury process and have a beneficial role in liver disease. Further attempts to identify beneficial bacterial species or microbial metabolites offer an opportunity for therapeutic disease intervention.

Expert commentary

Changes in the microbiome have been associated with the progression of liver disease. Dysbiosis can contribute to a subtle intestinal inflammation adding to a disruption of the intestinal barrier. Once the mucosal barrier becomes dysfunctional, bacterial products are able to translocate and trigger an inflammatory response in the liver. However, translocated microbial products without an additional liver insult will lead to liver disease in only rare instances. Thus, increased intestinal permeability and pathological bacterial translocation can be considered as strong enhancer of a primary liver disease.

Five-year view

One possible target within this sequence of events is the manipulation of the microbiome. Restoring eubiosis could be one goal in the treatment of liver disease. This might also help turning a proinflammatory intestinal inflammation into a “good” inflammation that restores the integrity of the gut barrier and promotes healing. Whether this could be accomplished by microbiome-independent interventions remains to be determined in future studies. Recent advances in microbiomics, metagenomics, metatranscriptomics and metabolomics will help not only to elucidate the pathogenesis of liver disease but also to reveal new treatment options for patients with liver disease.

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Key issues

- Changes in the microbiome are associated with liver disease
- Several factors can contribute to changes in the microbiome including genetic factors, diet and other environmental factors
- Change in the microbiome can lead to intestinal inflammation and contribute to gut barrier dysfunction
- Once the intestinal barrier is disrupted, bacterial products can translocate and can enhance liver disease
- The understanding of the microbiome-host interactions will help to develop patient specific therapies for liver disease

Figure 1. Host-microbial interactions that contribute to liver disease

Diet, other environmental factors and genetics can trigger dysbiosis. In addition, bacterial synthesis of long-chain fatty acids (LCFA) maintains eubiosis as LCFA can be utilized by beneficial bacteria such as *Lactobacillus*. Dysbiosis triggers intestinal inflammation which is characterized by the production of proinflammatory cytokines (right crypt) and antiinflammatory cytokines (left crypt). Stimulation of macrophages with bacterial products or metabolites can lead to the production of proinflammatory cytokines (e.g. TNF), which can disrupt tight junctions. A subsequent translocation of bacterial products into the portal vein results in accumulation of these molecules in the liver, which then induce hepatic inflammation and cause disease progression.

Stimulation of dendritic cells (DCs) with bacterial products can lead to the production of IL-23 that stimulates IL-22 production from innate lymphoid cells (ILC). IL-22 triggers the release of Reg3g from epithelial cells, which is an important antimicrobial molecule preventing bacterial overgrowth. IL-18 is another beneficial cytokine that is produced in a NLRP3 dependent manner. The presence of IL-18 further contributes to eubiosis.