Review

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Pathological bacterial translocation in liver cirrhosis

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Introduction

Humans harbor nearly 100 trillion intestinal bacteria, which in terms of numbers, represents around ten times more microbial cells than eukaryotic cells. The gastrointestinal (GI) tract, the largest surface area of the body with an epithelial surface of approximately 400 m^2, is in constant exposure to these live microorganisms. Their peaceful coexistence demonstrated by the lack of pro-inflammatory responses against commensal bacteria implicates the presence of clearly defined lines of communication. In fact, bacterial translocation (BT), being defined as translocation of bacteria and/or bacterial products (lipopolysaccharides, peptidoglycans, muramyl-dipeptides, bacterial DNA, etc.) from the gut to mesenteric lymph nodes (MLN) [1], is a physiological process in healthy conditions and crucial for host immunity. In contrast, in cirrhosis "pathological" BT develops with a sustained increase in quantity (rate and/or degree) of BT. However, at least in humans, lack of access to MLN and/or upstream compartments towards the mucosal barrier until now hamper establishment of "cut-off" levels for physiological levels of BT in individual patients. Nonetheless, there appears to exist a hierarchy of three barriers against pathological BT, each of which encompasses a distinct set of mechanisms (Fig. 1). First, there are mediators that limit direct contact between the intestinal bacteria and the epithelial cell surface. Secondly, a layer of immune protection involves the rapid detection and killing of bacteria that manage to penetrate. Finally, a set of immune responses minimizes exposure of bacteria to the systemic immune system. In advanced liver cirrhosis, at each of these levels marked alterations have been developed throughout the course of the disease.

Background and potential relevance of pathological bacterial translocation

Pathological BT has been termed the "Achilles heel" in liver disease [2] playing an important role in the pathogenesis and complications of cirrhosis. The most evidenced clinical expression of pathological BT is spontaneous bacterial peritonitis (SBP). SBP often originates from bacteria in the gut that belong to the normal intestinal microbiota. It has been shown that green fluorescent protein (GFP) labelled *Escherichia coli* administered orally to cirrhotic rats reveal the presence of these bacteria not only in the intestinal lumen but also in the mesenteric lymph nodes (MLN) and ascites [3] (Fig. 2). However, not only culture-positive SBP and/or bacteremia impact on the cirrhotic host, but also increased inflow of translocating bacterial products into the hepato-splanchnic as well as systemic circulation. Augmented pro-inflammatory response to gut-derived products and failure to control invading bacteria and -products in concert with host susceptibility determine remote organ injury. This may include acute-on-chronic liver failure, hepato-renal-syndrome and hepatic encephalopathy [4]. Therefore, understanding the physiology of gut-bacteria interactions and the pathogenesis of BT can lead to new therapeutic targets in the prevention of infections and other complications of cirrhosis.

Compartments involved in pathological bacterial translocation

Gut associated lymphoid tissue (GALT)

The GALT represents the largest immunological organ in the human body. Despite the vast improvements made in understanding how the microbiota influence host immunity, very little is still known of the intestinal immune system in cirrhosis.

The innate immune system is considered the "first line of defence" against invading bacteria or their associated products. Invading bacteria are detected by the innate immune system through the recognition of highly conserved bacterial motifs that are present in all bacteria (microbial-associated molecular patterns, MAMPs) by germline-coded pattern-recognition receptors (PRR) on intestinal cells [5]. PRR are located on both the cell

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Abbreviations: AMP, antimicrobial peptides; APC, antigen-presenting cell; BDL, bile duct ligation; BT, bacterial translocation; CCl_4, carbon-tetrachloride; DC, dendritic cell; FxR, Farnesoid X receptor; GALT, gut-associated lymphoid tissue; GI, gastrointestinal; GNB, gram-negative bacteria; HE, hepatic encephalopathy; IEL, interepithelial lymphocyte; IFN, Interferon; IgA, Immunoglobulin A; IL, interleukin; LPS, lipopolysaccharide; MCP-1, monocyte chemotatic protein-1; MDP, Muramyl-Dipeptide; MLCK, myosin light chain kinase; MLN, mesenteric lmyph nodes; NO, nitric oxide; NOD, nucleotide binding oligomerisation domain 2; NFkB, nuclear factor kB; PGN, Peptidoglycans; ROS, reactive oxygen species; SBP, spontaneous bacterial peritonitis; SDD, selective gut decontamination; SIBO, small intestinal bacterial overgrowth; TJ, tight junction; TLR, toll-like-receptor; TNF, tumor necrosis factor; ZO, zonula occludens.

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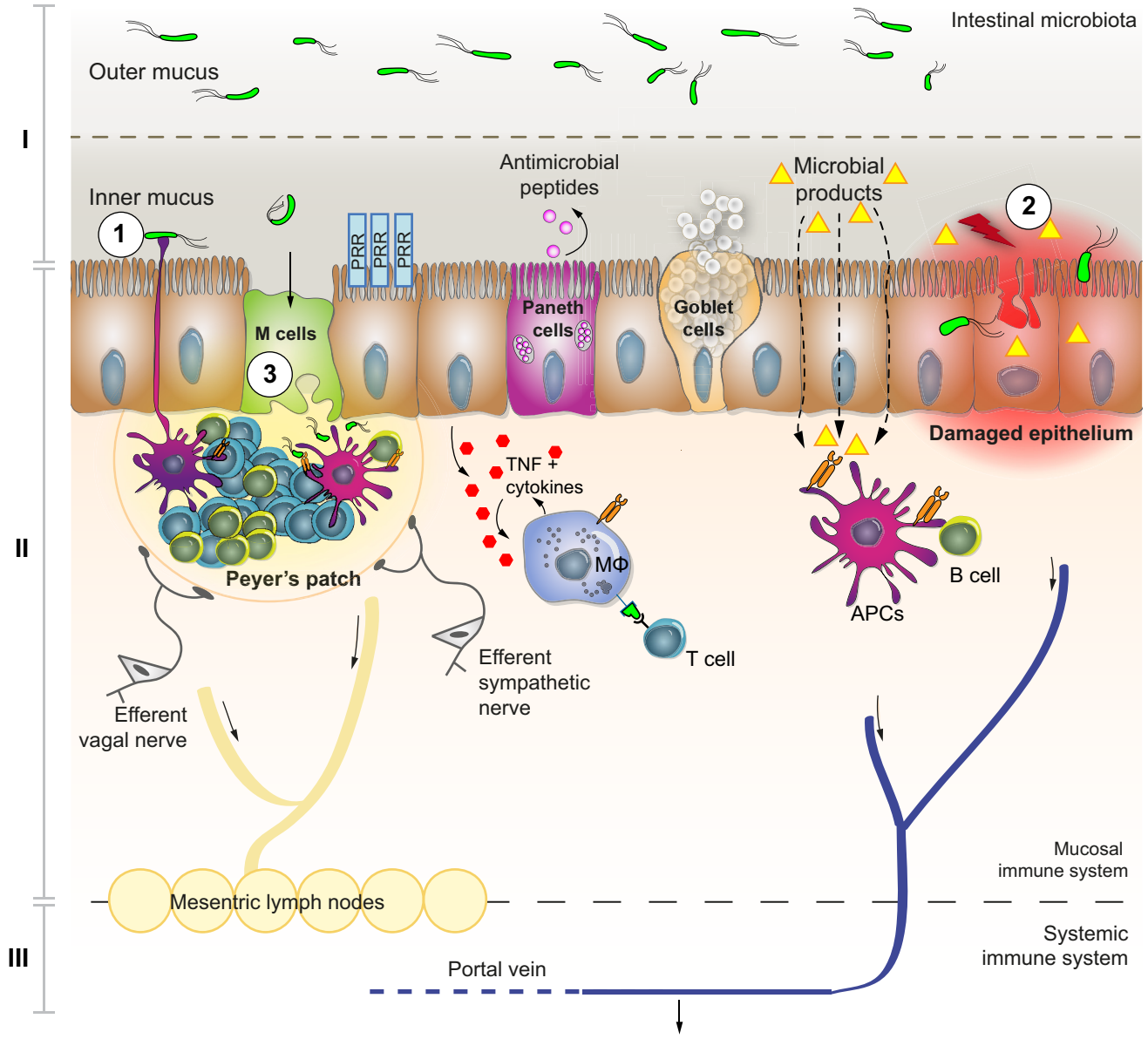

Fig. 1. Compartments and key players involved in mediating pathological BT and the associated host response. Three different routes (1–3) of bacterial translocation can be separated: (1) direct sampling of luminal bacteria (l products) by dendritic cells via processes between epithelial cells, not affecting tight junction function; (2) injured/inflamed epithelium with dysfunctional epithelial barrier and (3) M-cells overlying Peyer Patches as specialized cells providing access of microbial products to antigen-presenting cells. Moreover, three different levels of barriers (I–III) against bacterial translocation are shown: (I) lumen and secretory component (e.g., inner and outer mucus layer, antimicrobial peptides) of gut barrier; (II) mechanical epithelial barrier and the gut-associated lymphatic tissue (GALT) beneath with response elements to BT (e.g., TNF and other pro-inflammatory cytokines) and autonomic nervous system; (III) systemic immune system as third barrier in case of spreading of bacteria(l products) beyond MLN including hematogenous (portal venous) and lymphatic (ductus thoracicus) route of delivery. APC, antigen presenting cell; PRR, pattern recognition receptors; TNF, tumour necrosis factor.

surface and within endosomal compartments and these receptors can be further divided into two subgroups: Toll-like receptors (TLRs) and cytoplasmic NLR (nucleotide binding domain, leucine-rich repeats) proteins.

The mucosal immune system is not ignorant to the commensal bacteria, rather microbial antigens are continuously sampled via various routes (Fig. 1): (1) Dendritic cells (DCs) that underlie the epithelium may open tight junctions (TJ) between epithelial cells, sending processes into the lumen that directly sample microbes [6]; lamina propria DCs compromise two different subsets: *CD103*$^{+}$*CX3CR1*$^{-}$ DCs (inducing development of regulatory T cells) and *CD103*$^{-}$*CX3CR1*$^{+}$ DCs (with features of macrophages, promoting TNF-production and development of *Th1/Th17* T cells) (2) through interaction with antigenic material in underlying tissue that occurs particularly when epithelial integrity is compromised; or (3) through sampling by specialized M cells within villous epithelium or the follicle-associated epithelium overlying Peyer patches [7]. Alterations in these sampling mechanisms in

presence of pathological BT in cirrhosis have yet to be delineated. It has been shown that in CCl_4-induced cirrhotic ascitic rats translocation of bacterial DNA associates with an increase in total number of intestinal *CD103*$^+$ DC's in the lamina propria (as well as MLN) [8]. However, whether in cirrhosis these *CD103*$^+$ DC's or other subsets of DC's (e.g., *CX3CR1*$^+$) or mononuclear cells are actually the "transporter" of living bacteria to MLN with pathological BT remains unanswered.

In response to BT, gut epithelial cells release chemokines that induce the recruitment of DCs to the mucosa. Once activated mature intestinal DCs have the capability to induce and prime mucosal B and T cells ultimately shaping the adaptive mucosal immune system. After maturation, these B and T cells are released into the blood stream and, due to surface expression of the appropriate homing markers, home back to reside within the lamina propria. Microbial antigens presented to B cells induce a commensal-specific IgA response that aids to prevent the commensals from straying beyond the gut mucosa [9]. Interestingly, mice deficient in the TLR-adapter molecule MyD88 on B cells lack commensal-specific immunoglobulin-response with insufficient bacterial killing that leads to lethal dissemination of commensal bacteria during colonic damage [10]. In cirrhotic patients, reductions in memory B cells and hypo-responsiveness to TLR9-stimulation has been reported [11]. However, to what degree this contributes to pathological BT is currently unknown. Likewise, the role of intestinal T cells is ill defined in liver cirrhosis but deserves more attention. T cells are critical in host defense against the translocation of enteric bacteria [12]. In the absence of T cells, there is spontaneous systemic BT of members of the commensal microbiota, such as *E. coli* [13]. Moreover, T cell depletion not only causes accumulation of bacteria in MLN in healthy rats but leads to spreading of bacteria to extraintestinal sites in alcohol- and burn-injured rats [14].

MLN at the centre of BT

In healthy conditions commensal bacteria transported to MLN by DCs induce a local immune response and are killed without inducing systemic immunity. In contrast, if the MLN were surgically removed, bacterial-laden DC carried commensal bacteria to the spleen and ultimately triggering a microbial-specific systemic immune response [15,16]. In humans (or mice) the presence of immunosuppression permits the translocation of intestinal bacteria systemically, which eventually may lead to sepsis and death [17]. Many mechanisms contribute to the spreading of bacterial products and/or living bacteria beyond the MLN in cirrhosis. These include (but are not limited to) relative deficits within both innate and adaptive immunity that result in a reduced chemotactic, opsonic, phagocytic and killing capacity of mononuclear cells [18–23], tuftsin activity [24], and impaired reticuloendothelial system (RES) activity [25].

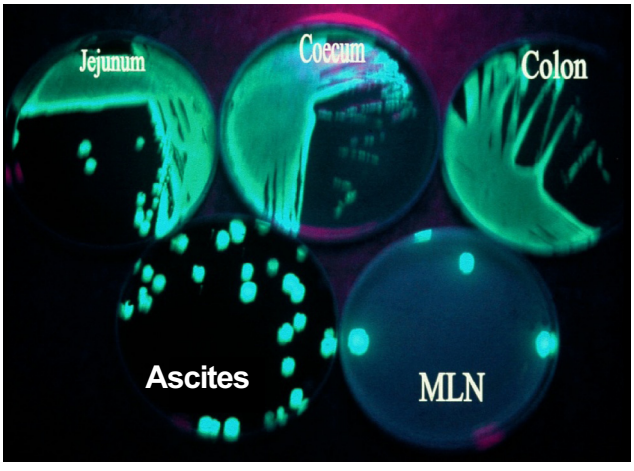

Fig. 2. Visualization of Green fluorescent protein (GFP)-marked *E. coli* in different compartments after oral gavage in an ascitic rat with cirrhosis. 6 h after oral inoculation of 10^8 CFU/ml GFP-labeled *E. coli*, stool along the GI tract, ascites and mesenteric lymph nodes (MLN) were harvested. This clarifies the translocation of those marked bacteria from the gut to MLN as well as into ascites representing the pathophysiological "road" for the development of SBP in advanced cirrhosis. Adapted from Teltschik *et al.* [3].

Intestinal barrier dysfunction: secretory and mechanical components

Only a single layer of epithelial cells separates the sterile host from trillions of live bacteria. This physical barrier functions to deliver critical secretory compounds to the intestinal lumen, such as IgA, mucus proteins and antimicrobial peptides (AMPs) that help to control bacterial attachment and infiltration into the host.

Mechanical component

The mucosal *epithelium per se* closely interacts with antigen-presenting cells beneath and intraepithelial lymphocytes (IEL) within the lamina propria to maintain intestinal integrity. In human cirrhosis, structural changes of the intestinal mucosa including widening of intercellular spaces, vascular congestion, edema, fibromuscular proliferation, decreased villous to crypt ratio, and thickening of the muscularis mucosae have been described [26–28]. It has also been shown by functional studies utilizing dual sugar absorption tests or other test substances that there is an increase in intestinal permeability due to cirrhosis [29–34].

TJs maintain a permeability seal at the apicolateral epithelial surface restricting *paracellular* movement of even very small (2 kDa) molecules and thus, bacteria and macromolecules such as lipopolysaccharides (LPS). More than 50 different TJ-proteins are known and members in the claudin family and the zonula occludens (ZO) proteins [35] are among those most studied. TJ-function is highly dynamic and controlled by signalling molecules including myosin light chain kinases (MLCK). In short-term BDL-mice, increased MLCK activation with concomitant disruption of TJs (diminished expression of occludin and ZO-1) has been reported in colonic epithelium [36]. Also in a descriptive pilot study in human cirrhosis, alterations in TJ-proteins in duodenal biopsies with reduced expression of occludin and claudin-1 that gradually increase from crypt to tip of the villi has been demonstrated [37]. Therefore, in cirrhotic conditions, loosening of TJs may result in an increased accessibility of bacterial products to areas of "free" passage. However, most critical for the translocation of living whole bacteria is the *transcellular* route. In fact, e.g., for *E. coli* C25 transcytosis across *CaCo-2* cells has been evidenced to occur even independent of changes in paracellular permeability [38]. It is epithelial cells under stress that present with decreased transepithelial resistance and increased translocation of commensal bacteria [39]. Corresponding investigations on epithelial transcytosis of commensal bacteria in cirrhosis are lacking.

Epithelial tolerance normally avoids inflammatory changes in response to physiological levels of BT [40]. However, as soon as inflammation occurs or mucosal load of bacteria(l products) becomes overwhelming, DCs and other monocytes and neutro-

phils are recruited, perpetuating the process of BT [41,42]. Indeed, in experimental cirrhosis BT has been found to be associated with mononuclear cell infiltrate in the lamina propria and concomitant submucosal and mesenteric inflammation [28,43,44]. This is in line with increased fecal concentrations of polymorphonuclear elastase [45] and calprotectin [46] being observed in cirrhotic patients. But are those inflammatory changes on a mucosal level normal in relation to the rate and severity of pathological translocation present in advanced cirrhosis? There are no comparative studies but it is tempting to speculate that the intestinal inflammatory mucosal response is reduced and thus, the cirrhotic patient is more tolerant to bacteria reaching the epithelium as compared to the non-cirrhotic healthy individual.

Therefore, at least a state of relative intestinal tolerance to pathological BT can be proposed in cirrhosis. Correspondingly, in cirrhotic rats with culturable pathological BT to MLN there was no significant activation or change in phagocytic and migratory capacity of lamina propria *CD103*$^+$ DC's supporting the yet to be proven hypothesis of relative intestinal tolerance to a vast bacterial challenge [8]. These data are in accordance with the observed "immune paralysis" in patients with sepsis and acute-on-chronic liver failure, attributed to reductions in HLA-DR-expression on circulating monocytes [47,48]. It is tempting to speculate whether such immunosuppression, which has been reliably associated with increased rates of bacterial infections is present in advanced human cirrhosis with pathological BT of living bacteria to MLN.

Secretory component

Much knowledge has been gained recently on this relatively impermeable compartment that may define a confined space, allowing the host to specifically monitor and regulate bacteria that are in close contact with the intestinal surface.

AMPs include defensins, cathelicidines, resistin-like molecules, bactericidial-permeability-inducing proteins and lectins. Among *defensins* only α- and β-defensins have been identified in the intestinal tract. All mature defensins have broad range antimicrobial activity by disrupting the structure and function of microbial membranes. *α-defensin* genes are expressed only in a few cell types, which in humans are predominantly neutrophils and Paneth cells, strategically located at the bottom of each intestinal crypt. The secretion of AMPs by Paneth cells is directly linked to bacteria and LPS exposure [49] and functions to help maintain homeostasis at the intestinal host-microbial interface [50,51]. In contrast, *β-defensins* are expressed constitutively by most epithelial cells in both the small and large intestine [52]. CCl_4-induced ascitic cirrhotic rats with but not without BT to MLN present with a relative deficiency in Paneth cell defensins particularly in the small intestine [3]. In contrast, levels of β-defensins are unchanged or elevated in presence of increased BT, demonstrating a normal ß-defensin response in cirrhotic rats. The observed deficit in α-defensins was accompanied by a diminished *in vitro* antibacterial activity against various *Enterobacteriacea*. The potential mechanisms mediating the impairment in Paneth cell function in cirrhosis are so far unknown but appear not to relate to the level of portal hypertension since pre-hepatic portal hypertensive rats show no alterations in Paneth-cell products [3]. In addition, down-regulation of regenerating islet-derived proteins (RegIIIβ and RegIIIγ) has been demonstrated in the small intestine of mice as well as humans after chronic alcohol intake [53]. These lectins are known to bind cell wall peptidoglycans of gram-positive bacteria and function as bactericidal proteins even at low micromolar concentrations [54,55]. Therefore, deficiency in various AMPs (α-defensins, RegIII proteins) likely leads to decreased mucosal killing activity resulting in a shift of the bacterial composition facilitating bacterial overgrowth and increases in BT in cirrhosis.

Mucus

Mucins create a layer of glycoproteins that prevents direct contact of bacteria with the microvillus [56]. MUC2 is the major secretory mucin being stimulated by a wide array of bioactive factors including microbes/-products, and inflammatory cytokines [57]. The "firm" dense inner mucus layer likely traps immune exclusion molecules [58] rendering it sterile [59]. In contrast, the "loose" outer layer is the habitat for commensal bacteria that consume the mucus proteins as a carbon source [60] and provides specific binding sites for bacterial adhesins [61]. Thus, it is important to differentiate between bacteria that are found within the intestinal lumen and those inhabiting the mucus. In fact, the mucosa-associated microbiome differs from stool flora in cirrhotic patients, particularly in those with hepatic encephalopathy [62]. In addition, recent elegant studies in alcoholic patients indicate increased mucus thickness in the duodenum, suggesting changes induced by cirrhosis and/or alcohol [63]. Surprisingly, MUC2 deficient mice are protected from bacterial overgrowth in response to alcohol most likely due to increases in mucosal antimicrobial peptides (RegIIIβ and RegIIIγ) [63] further emphasizing the proposed role of mucus as an active key player in host-microbial interactions [64].

Bile inhibits small intestinal bacterial overgrowth (SIBO) [65], has a trophic effect on the intestinal mucosa [66], decreases epithelial internalization of enteric bacteria [67], exerts detergent actions with anti-adherence effects and neutralizes endotoxins [68,69]. However, bile also impacts on intestinal immunity by providing retinoids necessary to imprint intestinal *CD103*$^+$ DC with the ability to generate gut-tropic T cells [70]. In cirrhosis, marked decreases in intestinal intraluminal concentrations of bile acids have been ascribed to decreased secretion and increased deconjugation by enteric bacteria. In experimental models the absence of bile in the intestine has been shown to facilitate BT [71,72] and to enhance susceptibility for further translocation in response to endotoxins [73]. Notably these effects are attenuated after oral administration of bile acids [74].

Conjugated bile acids are natural ligands for several nuclear receptors, of which the transcription factor farnesoid X receptor (FxR) has gained much attention [75]. Intestinal FxR limits bacterial overgrowth and BT, which has been demonstrated in BDL mice [76]. A specific FxR agonist (GW4064) repressed bacterial overgrowth, attenuated mucosal injury and reduced bacterial invasion into MLN in wild type but not in mice genetically deficient in *FxR* [76]. Activation of FxR by GW4064 led to the identification of several novel FxR target genes, including those that promote antimicrobial defense. How these FxR target genes function to maintain intestinal homeostasis will surely be an active area of future investigations.

IgA antibodies

On a daily basis 2–5 g of sIgA is secreted into the gut lumen accounting for more than 70% of total body immunoglobulin production. IgA antibodies effectively bind and aggregate bacteria preventing mucosal adherence and colonization (immune

exclusion) [77]. Despite increased BT in IgA-deficient mice [15], commensal-related sepsis is not observed in IgA-deficient animals or humans, which may be due to the overcompensatory function of IgM in the absence of mucosal IgA [78]. In cirrhotic patients, decreased fecal IgA concentrations as well as decreased secretion of mucosal IgA into the jejunum have been reported [45], suggesting a potential relationship between IgA and BT, and the development of infections in cirrhosis, although this hypothesis has yet to be proven.

Intestinal microbiota; qualitative and quantitative changes

The intestinal microflora consists of a dynamic mixture of microbes with considerable quantitative and qualitative differences among individuals and particularly among species. Additionally, only a small proportion of the enteric bacteria can currently be examined by conventional culture techniques [79] limiting diagnostic measures. The proximal small intestine (duodenum, jejunum) is sparsely populated with bacteria; however, from the ileum on there is a sharp increase in microbial density, from 10^5 colony forming units (CFU)/ml in the jejunum to 10^8 in distal ileum and cecum, up to 10^{12} in the colon [80].

Quantitative changes

Small intestinal bacterial overgrowth (SIBO) has arbitrarily been defined as $>10^5$ CFU/ml and/or the presence of colonic bacteria in upper jejunal aspirate [81]. Using this gold standard, the prevalence of SIBO in cirrhotic patients ranges from 48% to 73% [82–86]. SIBO has been shown to be particularly frequent in those with more severe liver disease [87,88] and in those with a prior history of SBP and/or hepatic encephalopathy [89,90]. In advanced liver cirrhosis it has been linked to the development of BT, SBP and endotoxemia [84,86,91]. In fact, in cirrhosis SIBO is one of the main factors that promotes BT and the occurrence of BT to MLN in experimental models routinely associates with SIBO [91,92]. A direct relationship between the density and composition of bacteria populating a segment of the intestine and numbers of viable bacteria of this strain present in MLN has been demonstrated in mouse models [93]. Importantly, in the absence of SIBO in experimental cirrhosis BT occurs rarely (0–11%). However, since BT does not occur in up to half of the cirrhotic animals with SIBO, it appears that SIBO is supportive but not sufficient *per se* for BT to occur. Therefore, other factors, most likely a decrease in local immunity, play the most important role in inducing BT. For instance, in experimental ethanol-induced liver injury, increases in BT do occur prior to changes in intestinal flora [53]. SIBO in cirrhosis has traditionally been attributed, at least partly, to a decrease in small-bowel motility and intestinal transit time [83,94–97]. The proposed contribution of proton pump inhibitors for the development of SIBO [84,98] and SBP [99,100] has recently been questioned in a large cohort of cirrhotic patients [101]. Nonetheless, hypo- and achlorhydria have been observed in cirrhotics even without acid suppressive medication, resulting in higher pH in the small intestine and under these circumstances have been associated with SIBO [102].

Qualitative changes

The full microbial richness in the human population reaches up to 40,000 species and the bacterial metagenome may exceed the human genome by 100 fold [103]. However, only 30–40 species amount to about 98–99% of the microbiota, and Firmicutes and Bacteroidetes are the predominant intestinal phyla across all vertebrates [104]. Using culture-independent techniques such as pyro-sequencing, analyses of fecal contents could demonstrate reductions in microbial diversity and distinct dysbiosis in both animal models as well as human cirrhosis [65,105]. The microbiota of cirrhosis has been associated with the depletion of the beneficial phyla Lachnospiraceae (particularly clostridiae) [105,106] and bacteroidetes (mainly family of Bacteroidaceae) [105] and enrichment in the phyla Proteobacteria (mainly class of Gammaproteobacteria and among those particularly Enterobacteriaceae) [105,106]. Interestingly, particularly the depletion of clostridiae resulted in a pronounced pro-inflammatory profile [106] and correlated negatively with Child-Pugh score [105]. Moreover, the particular relevance of alterations in the mucosa-associated microbiome has been evidenced by distinct differences between cirrhotic patients with and without hepatic encephalopathy being associated with increased levels of inflammation [62]. Finally, similar dysbiosis is observed in inflammatory bowel disease (reviewed in Danese [107]). In conjunction with recent findings that mucosal inflammation *per se* modifies microbial composition inducing the expansion of microorganisms with genotoxic capabilities (such as *E. coli*) [108] it remains to be seen whether inflammation is the cause or the consequence of changes in microbial composition in those entities.

Anaerobic bacteria do not readily translocate whereas aerobic gram negative bacteria translocate easily and even across a histologically intact intestinal epithelium [109,110]. Moreover, anaerobes outnumber aerobes by 100:1 and limit the colonization and overgrowth of other potentially invasive microbes, thereby confining potentially pathogenic bacteria. In fact, selective elimination of anaerobic bacteria facilitates SIBO and translocation of facultative bacteria [111]. Bacteria that translocate most readily are facultative intracellular pathogens (e.g., *Salmonella*, *Listeria*), able to resist phagocytic killing. In contrast, commensal bacteria are easily killed after phagocytosis, surviving only when host defenses are impaired. Gram-negative bacteria (GNB) (specifically *E. coli*, *K. pneumoniae*, *P. aeruginosa* and other Enterobacteriaceae), enterococci and other streptococci, have been found to be

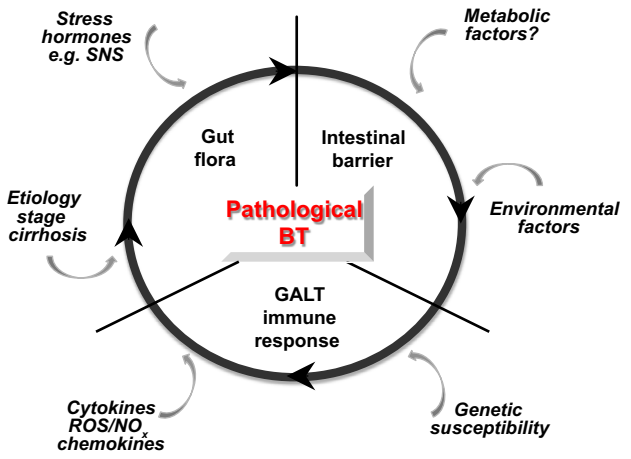

Fig. 3. Compartments and influencing factors promoting pathological BT in cirrhosis. Alterations in the three compartments (= "inner circle") act in concert and most likely synergistically promote pathological bacterial translocation; each of the compartments *per se* influences any of the other (symbolized by arrow heads on the circle) and in end-stage disease may lead to a vicious circle. Influencing factors are multiple and can impact on each of the compartments.

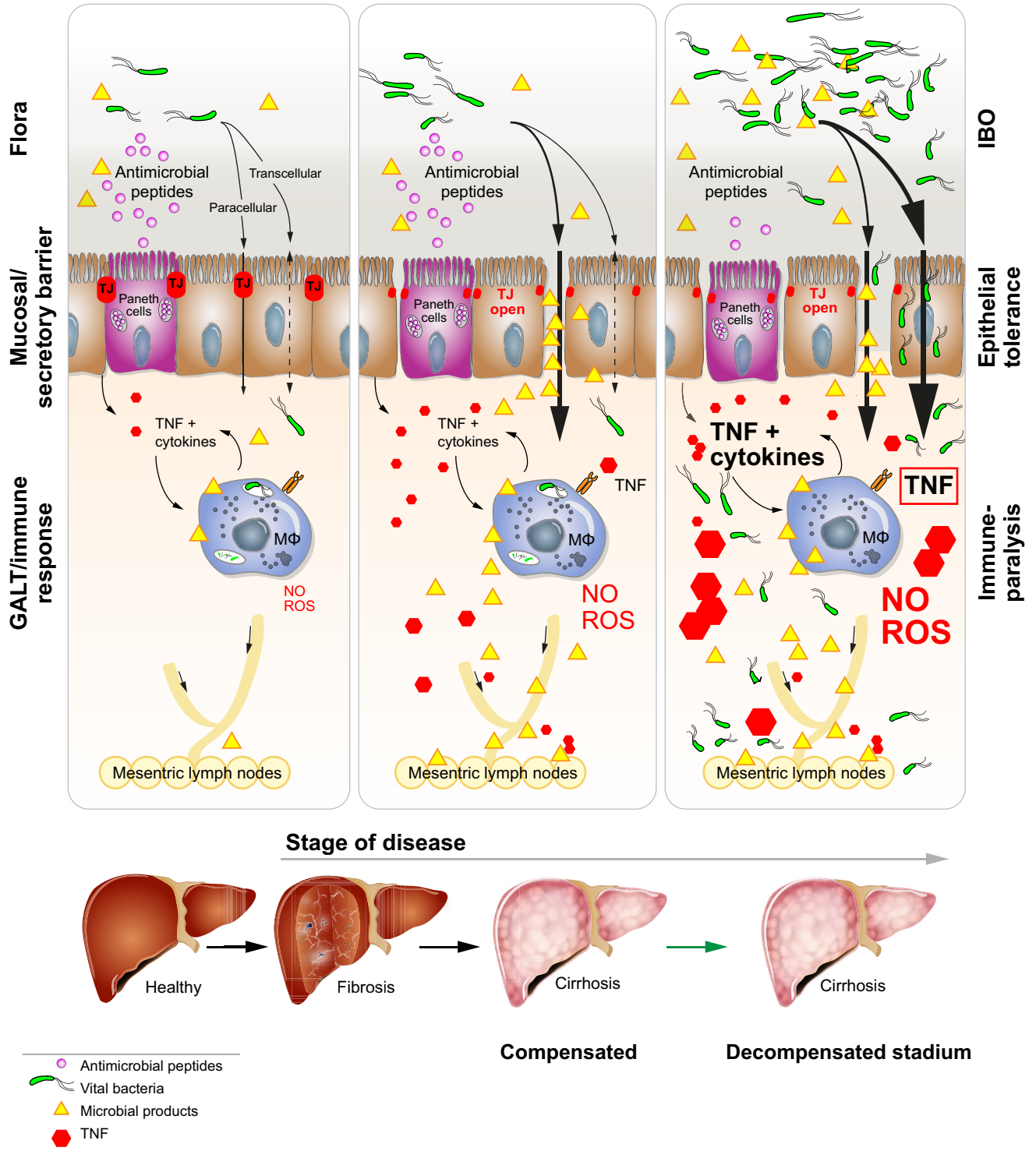

Fig. 4. Stages of liver disease and hypothesis on development of pathological BT. Left: normal healthy conditions with "normal" exclusively low levels of translocation of bacteria(l products); Middle: increases in paracellular translocation of bacterial products stimulate an augmented pro-inflammatory cytokine response and release of ROS and NO_x within the GALT; these mediators impact on the mechanical and secretory barrier as well as most likely on the flora; Right: in ascitic cirrhotic conditions in presence of IBO and a proposed state of epithelial tolerance, enhanced transcytosis of viable bacteria develops ultimately leading to immune paralysis in the GALT (which could lead to a vicious circle perpetuating BT by a relative lack of bacterial killing).

the most adept at translocating to MLN [110]. Interestingly, these species and among those particularly *E. coli* are those that most frequently cause spontaneous bacterial infections in cirrhotic patients [112–116]. As described for other disease patterns which are accompanied by BT, for example intestinal obstruction, burn injury or starvation, the translocation of almost exclusively

coliform bacteria underlines the pronounced preference of these Gram-negative strains to translocate [117,118]. Certain *E. coli* strains (e.g., biochemical phenotype C1–C4 or C25) have been reported to translocate more efficiently than others across intestinal mucosa when it is exposed to metabolic and inflammatory stress [38,117,119]. However, in cirrhosis *E. coli* isolates from SBP cases are genetically diverse [113].

Influencing factors on compartments promoting bacterial translocation (Fig. 3)

Any increase in translocation of bacteria(l) products to the GALT triggers a concert of pro-/anti-inflammatory *cytokine* release. Among those TNF has gained most attention because it increases TJ-permeability in the intestine via decreases in expression of TJ-proteins but also activation of MLCK [120]. In BDL mice, increased numbers of infiltrating monocytes in the lamina propria expressing TNF have been demonstrated to disrupt epithelial TJs resulting in pathological BT [36]. Most importantly, anti-TNF monoclonal antibody as well as pentoxifylline treatment significantly decreases incidences of BT in experimental cirrhosis [121,122]. Interestingly, functional polymorphism of the *MCP-1* gene, known to confer increased *MCP-1* expression and thus increased chemotaxis of monocytes – the major source of TNF – has been shown to be a risk factor for the development of SBP in patients with alcoholic cirrhosis [123]. TNF-secretion is likewise increased in MLN and serum in experimental and human cirrhosis with ascites [124–126] and was found to be predictive for bacterial infections post-transplantation [124]. Therefore, enhanced TNF levels in the GALT appears to play a central role in promoting pathological BT in cirrhosis. Also IL6 and IFN-gamma have been shown to increase intestinal epithelial permeability [127] and induce transcytotic translocation of commensal *E. coli* across epithelial cells [128], respectively. Although increased serum levels of IL6 and IFN-gamma are present in advanced cirrhotic patients [129–131] no data on their role in promoting BT in portal hypertension are available. Important to note is that production of TNF and IL6 stimulated by LPS and/or bacterial DNA is excessively augmented in cirrhosis as compared to healthy controls [8,132–135] setting the stage for a vicious circle to perpetuate pathological BT (see hypothesis).

Reactive oxygen species (ROS) impact on epithelial cells increasing the *in vitro* internalization rate of *E. coli* [136], modulating responses to bacterial stimuli [137] and changing brush border glycosylation increasing bacterial adherence [138]. In experimental cirrhosis, intestinal mucosal oxidative damage has been evidenced by increased lipid peroxidation and altered enterocyte mitochondrial function [139,140]. Besides ROS, *nitric oxide* (*NO*) is important in modulating macrophage function, cytokine release and bactericidal killing capacity [141,142], as well as maintaining gut barrier function [143]. Overproduction of NO, known to be particularly present in the splanchnic circulation [144], has been shown to be deleterious to the integrity of the intestinal epithelium. In fact, NO at high concentrations induces gastric mucosal damage, decreases the viability of rat colonic epithelial cells [145,146], directly dilates TJs in intestinal epithelial monolayers, inhibits ATP-formation and hence, increases intestinal permeability [147,148]. The importance of iNOS-derived NO production in promoting BT has been evidenced experimentally after insults such as endotoxemia, hemorrhagic shock, or thermal injury but also recently in human cirrhosis [149–151]. This has been confirmed in *iNOS* knockout mice exposed to LPS that exhibit a reduced mortality and absent BT [152].

The gut is one of the most intensely innervated organs and the *autonomic nervous system* has recently been realized to influence mucosal barrier function [153]. In cirrhotic ascitic rats splanchnic specific sympathectomy has been shown to prevent translocation and spreading of *E. coli,* being associated with increased chemotaxis and phagocytic capacity of mononuclear cells [154]. Additional proposed beneficial effects of sympathectomy are accelerated intestinal transit time [92], prevention of gram-negative bacterial overgrowth [155] and improvement in gastrointestinal permeability [34]. Propranolol has likewise been used and found to lower rate of BT in experimental cirrhosis [92] as well as incidence of infectious complications in cirrhotic patients [156]. In contrast, parasympathetic input and effects on BT have not been addressed in portal hypertension. However, vagal nerve stimulation attenuates inflammatory response to endotoxin [157] and intestinal inflammation [158], protecting against burn-induced intestinal injury [159]. Finally, neural stimulation of mast cells modulates intestinal barrier function and mast cell stabilization with ketotifen has been reported to reduce splanchnic inflammatory response in portal hypertensive rats [160].

Diet and nutrition are key for host-microbiome interactions. Starvation has deleterious effects on gut mucosal integrity, epithelial cell proliferation and synthesis of mucins and antimicrobial peptides [161]. Moreover, mucosal epithelial cells under metabolic stress perceive commensal bacteria as threat responding with increased endocytotic activity and resulting in increased inflammatory response [162]. Liver cirrhosis in advanced stages is frequently associated with *malnutrition* [163], which has been reported to contribute to enhanced BT [164] and increased permeability [33].

Susceptibility genes for pathological BT have recently been reported mainly influencing innate host defense mechanisms. *NOD2* is highly expressed in monocytes and Paneth cells [165] and recognizes muramyl dipeptide (MDP), a component of the peptidoglycan present in the bacterial wall of gram-positive and -negative bacteria. After ligand recognition, NOD2 switches on the NFkB- and MAPK cascade culminating in the induction of pro-inflammatory cytokines and chemokines. Mutant NOD2 has been implicated in the pathogenesis of mucosal inflammation in Crohn's disease [166,167] and in gastrointestinal graft-versus-host disease [168], conditions also known to associate with increased BT. Three common single nucleotide polymorphisms in *NOD2* (the frame shift mutation *1007fs* (3020insC, SNP 13) the two missense mutations *R702W* (2104C >T, SNP 8) and *G908R* [2722G >C, SNP 12] have been most thoroughly investigated. The presence of any of those mutated *NOD2* alleles has been reported to be an independent risk factor for SBP [169–171]. Mechanisms for increased BT by deficient NOD2 function include (i) promotion of bacterial overgrowth via impaired Paneth cell function and diminished production of subgroups of AMPs [172,173]. Indeed, MDP induces bacterial killing *in vitro* in ileal crypts and intestinal crypts lacking NOD2 are unable to kill bacteria efficiently [174]. (ii) Impaired intracellular bacterial killing after engulfment into mononuclear cells of the GALT via, e.g., failure to recruit autophagy protein ATG16L1 and thus impaired wrapping of invading bacteria by autophagosomes [175].

TLR2 is expressed on the cell surface of macrophages and other immune competent cells and recognizes PAMPs of gram-positive organisms. Among the multiple polymorphisms existing in the *TLR2* gene, increased risk for SBP has been reported for cirrhotic patients with the *TLR2*–16934 TT genotype and carriers with both *TLR2* GT tandem repeat alleles present in frequencies greater than 20 in both alleles [176]. Although not reported for every *TLR2* variant the *TLR2*–16934 TT genotype has been found to associate with increased TLR2 function [177]. This underlines the promotive role of TLR2 in mediating pathological BT in BDL mice since, TLR2 deficient mice present with markedly attenuated BT to MLN and endotoxinemia [36]. In cirrhotic patients *TLR2* and NOD2 variants seem to represent supplementary risk factors since simultaneous presence of both unfavourable polymorphisms markedly increases the risk for SBP in cirrhotic patients [176]. This underlines the known interaction of NOD2 and TLRs in particular the modulation of TLR2-dependent cytokine responses by NOD2 [178]. The latter is particularly relevant for bacterial killing, which appears to be dose dependent [179,180]. Most interestingly to note, benefit of probiotica-pulsed DC treatment in experimental colitis depends on intact functional NOD2- and TLR2-signalling [181]. Therefore, alterations in NOD2- and TLR2-function at various cellular sites appear to play a key role for the susceptibility of pathological BT.

Stage of disease, route and site of pathological bacterial translocation

Severity of liver disease

Rate and degree of pathological BT increases with severity of liver disease (Fig. 4). Pathological translocation of vital bacteria to MLN is a phenomenon of the decompensated stage. In experimental cirrhosis, this occurs only in animals with ascites but not in rats without ascites [43,182]. These data are in accordance with studies in cirrhotic patients demonstrating significant increases in lipopolysaccharide-binding-protein (long-term marker of gram-negative pathological BT) [183] and intestinal permeability [32,184] in ascitic cirrhotics but not in patients without ascites as compared to healthy controls. Correspondingly, modulators of pathological BT such as SNS and malnutrition are typically features of the decompensated stadium [185,186]. In principle however, level of portal hypertension [34] and liver insufficiency [187–189] are the driving forces for BT. The latter appears to be the culprit since, chronic pre-hepatic portal hypertension without liver insufficiency does not lead to pathological BT [190], whereas in galactosamine-induced liver failure, a model that does not develop portal hypertension, BT is observed in all liver failure animals compared to only 0–16% in controls [191]. Also SIBO is observed in increasing frequency with worsening of severity of liver diseases [192], reaching incidences above 80% in advanced cirrhotic patients with ascites [90]. However, today no data on the exact determinant of liver insufficiency mediating the risk of BT and/or utilization of quantitative liver function tests (indocyanine green clearance or methacetin breath test) for prediction of BT are available. Nonetheless, surrogate markers of pathological BT such as systemic endotoxin levels incrementally increase in relation with severity of liver cirrhosis graded by Child-classification [193,194]. Moreover, direct data on culturable BT to MLN revealed a significantly higher rate in Child C cirrhotic patients (30%) as compared to Child B or A (8% and 3%, respectively) patients and Child-Pugh score was the only independent predictor for pathological BT [195]. In contrast, presence of bacterial DNA or LPS in MLN has been evidenced to occur already in pre-ascitic animals [196,197] and the detection of bacterial DNA in the systemic circulation was not associated with differences in severity of liver insufficiency [198]. This hints to different mechanisms responsible for translocation of bacterial DNA as compared to viable bacteria in liver cirrhosis. Finally, gastrointestinal hemorrhage has been shown to increase BT in healthy animals [199] and portal hypertensive rats are particularly susceptible for shock-induced BT to MLN and blood [200]. Pre-existing increases in intestinal permeability in portal hypertension most likely is the underlying mechanism since (i) prior exposure to bacterial DNA has been shown to strongly aggravate systemic inflammation and gut barrier loss in experimental hemorrhagic shock [201] and (ii) higher intestinal permeability in decompensated cirrhotic patients with active GI hemorrhage is an independent predictor for development of bacterial infections [202].

BT-route

Despite obvious differences in size, chemical structure and receptor-ligand interactions between all various types of bacterial products as well as viable bacteria, the question how this impacts on the route of translocation namely para- *vs.* transcellular and lymphatic *vs.* hematogenous is unanswered. This could be of clinical relevance, since the lymphatic route connects the gut with the lung and mesenteric lymph duct ligation has been shown to protect from hemorrhagic shock induced pulmonary injury in rats [203]. In respect to vital culturable bacteria, experimental models of severe inflammatory insults reveal their appearance in the portal circulation earlier and to an excessively higher degree than in the lymphatic system [204]. In experimental cirrhosis positive portal culture has likewise been reported in the majority of cases with BT to MLN [205]. This also points towards the importance of hematogenous spreading of viable bacteria after crossing the epithelial barrier in cirrhosis. Nonetheless, comparative and kinetic studies assessing the lymphatic and portalvenous route in parallel are not available in liver cirrhosis.

Site of bacterial translocation

The colon is used to harbour a vast number of bacteria, and normally is more efficient at eliminating translocating bacteria and presents with higher transepithelial resistance than the small bowel [206]. Experimental studies have shown that after inoculation of equal concentrations of *E. coli* into small or large bowel, BT occurs at higher a rate after small bowel inoculation [207]. In addition, proximal gut colonization has been associated with increased BT and septic morbidity in surgical intensive care patients [208,209], indicating that *small* intestinal bacterial overgrowth has the greatest potential for promoting BT. As for liver cirrhosis, this is supported by a study that showed that lower BT rates in cisapride-treated cirrhotic animals were associated to lower jejunal but not cecal bacterial counts [86]. Histological changes however, have been shown to be most marked in the cecum in experimental cirrhosis [28,43,44]. Moreover, elegant recent loop-experiments, assessing intestinal permeability by local injection of FITC-marked dextran or GFP-marked *E. coli* at different sites in BDL mice, revealed that the cecum and colon

are the sites with the largest rate of BT and increase in intestinal permeability [36,65]. Most interestingly, these changes did precede alterations of the microbiome re-inforcing the primary role of permeability and host response to BT. However, these experiments were performed 1 day after BDL and thus, in non-cirrhotic conditions and it remains to be seen whether this applies also to other models and cirrhotic stages of liver disease.

Hypothesis

The orchestra of players contributing to and/or combating pathological BT in cirrhosis is exclusively complex and likely differs in dependency on type of translocating agents, genetic susceptibility of the host, environmental factors as well as stage and etiology of disease. However, we would like to propose that in early stages (in absence of increased permeation of viable bacteria) slight but constant increases in paracellular translocation of bacterial products trigger an augmented pro-inflammatory cytokine response and release of ROS and NO_x within the GALT aiming to enhance bacterial defence (Fig. 4). This however, further loosens TJ-function perpetuating BT but also shapes the immune system to adapt and tolerize enhanced BT. Ultimately, in decompensated cirrhosis secretion of antimicrobial peptides diminishes, SIBO accelerates and intestinal permeability further increases including enhanced transcellular epithelial crossing of viable bacteria.

Key Points

- Bacterial translocation (BT) is a healthy phenomenon but is pathologically increased in quantity in liver cirrhosis. Whereas rate and degree of translocating bacterial products is increased in early cirrhosis pathological translocation of viable bacteria occurs in the decompensated stage
- Compartments (alterations) involved in promoting pathological BT in cirrhosis include the microbiota (bacterial overgrowth), the intestinal barrier (deficiencies in secretory and mechanical barrier function) and the gut-associated lymphatic tissue (GALT) with an immune response aiming to eradicate invading bacteria and/or bacterial products
- Influencing factors that impact on those compartments driving pathological BT in cirrhosis are multiple and key players are pro-inflammatory cytokines (e.g., TNF), malnutrition, sympathetic hyperactivity (e.g., norepinephrine), genetic susceptibility (NOD2, TLR2) and lack of bile acids
- Hypothesis proposed for pathological BT in cirrhosis includes (1) increased paracellular translocation of bacterial products in early stages, induced by various causes (dependent on etiology) as trigger and priming event and (2) a relative (in proportion to degree of BT) (i) epithelial tolerance avoiding overwhelming mucosal inflammation, (ii) immune deficiency within the GALT, once increased translocation of viable bacteria occurs perpetuating a vicious circle

Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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