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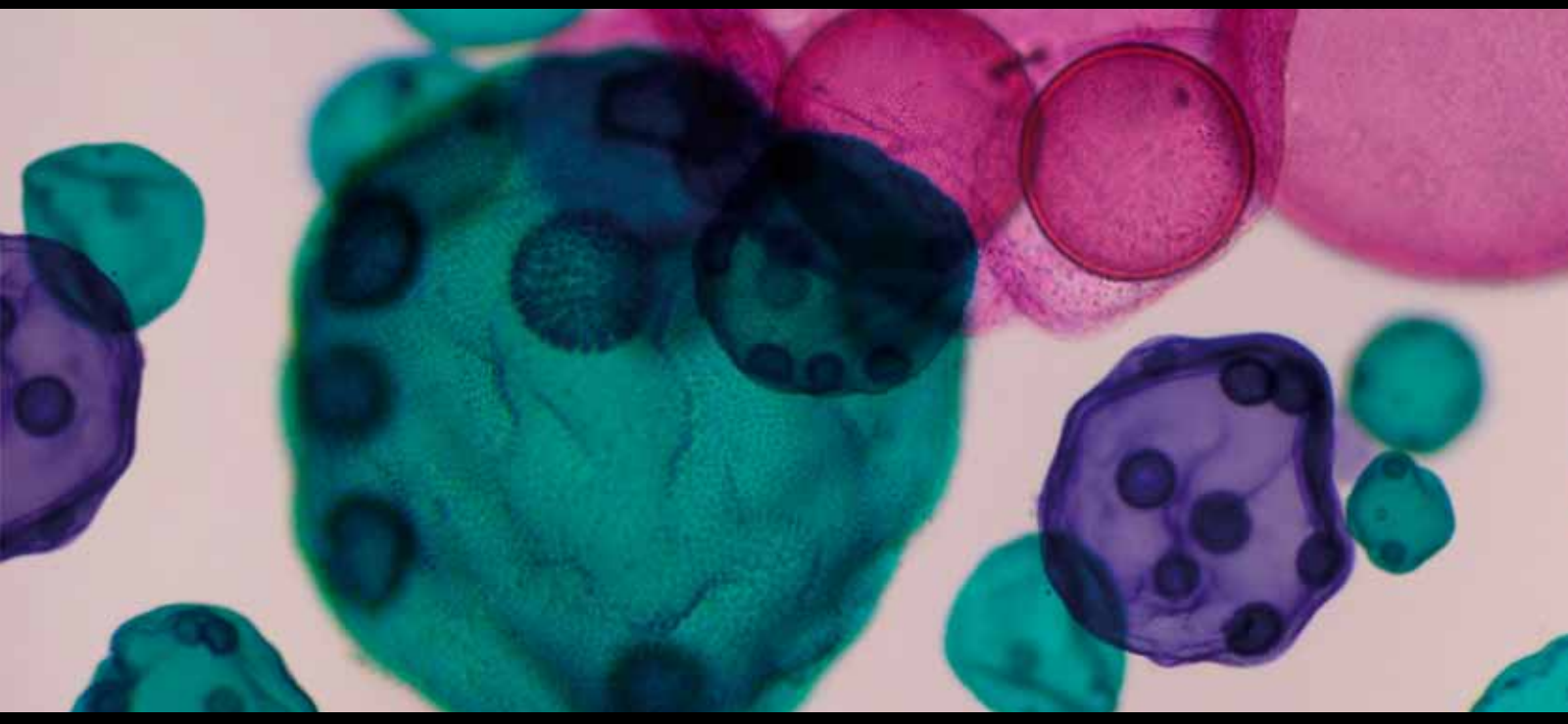
Microbial Translocation and Infectious Diseases: What Is the Link?

Guest Editors: Gabriella D'Ettorre, Daniel Douek, Mirko Paiardini, Giancarlo Ceccarelli, and Vincenzo Vullo



The Role of Microbial Translocation in Infectious Diseases

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Editorial

Microbial Translocation and Infectious Diseases: What Is the Link?

Gabriella D'Ettorre,[1] Daniel Douek,[2] Mirko Paiardini,[3] Giancarlo Ceccarelli,[1] and Vincenzo Vullo[1]

[1] *Department of Public Health and Infectious Diseases, Sapienza University of Rome, 00185 Rome, Italy*
[2] *Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA*
[3] *Department of Pathology & Laboratory Medicine, School of Medicine, Emory University, Atlanta, GA 30322, USA*

Correspondence should be addressed to Gabriella d'Ettorre, gabriella.dettorre@uniroma1.it

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The lumen of the gastrointestinal (GI) tract is a complex ecosystem where an enormous quantity of different bacterial species, termed the microbiota, establishes a generally symbiotic relationship with host immune system and epithelial cells. The correct interaction of the components of the GI tract permits the normal function of this ecosystem, reduces the risk of an excess of microbial translocation (MT) from the lumen of GI tract to the systemic circulation, and prevents its systemic consequences such as immune activation. From the historical observations of Berg and Garlington who defined bacterial translocation as "the passage of viable bacteria through the epithelial mucosa into the lamina propria and then to the mesenteric lymph nodes, and possibly other tissues," this concept has been redefined several times [1, 2]. Currently, this definition has become broader and includes the passage of both viable and nonviable microbes and microbial products such as lipopolysaccharide (LPS) across an anatomically intact intestinal barrier. Recently, the concept has also attracted interest in the study of several infectious diseases such as HIV infection, hepatitis, and leishmaniasis. In the healthy host, several mechanisms are involved to prevent and/or to attenuate MT and generalized immune activation. In contrast, given the several mechanisms responsible for MT, it comes as no surprise that there are multiple infectious diseases which can be associated with MT and the consequent host response. A few years ago, a study was published that suggested a pivotal role for MT in HIV pathogenesis. The authors proposed that this phenomenon contributes to systemic immune activation in people with HIV, and thus plays a causative role in the progression of the disease [3, 4]. From these initial observations many confirmatory studies have subsequently been performed and have led to much scientific debate and reappraisal of the field of HIV disease pathogenesis. An important finding is that MT is not fully controlled by suppressive antiretroviral therapy and is associated with inefficient CD4 T-cell reconstitution. For these reasons, persistent immune activation and inflammation despite sustained antiretroviral therapy- (ART-) mediated viral suppression are able to predict subsequent mortality [5–7]. Moreover, recent studies show that MT may contribute also to the pathogenesis of non-AIDS-related morbidity, including dementia and cardiovascular diseases, and for these reasons it has emerged as a major challenge for the modern HIV treatment era [8, 9].

Notably, hepatitis B and C infections are characterized by increased levels of microbial products in the peripheral circulation. For example, during HCV infection high plasma levels of LPS were observed [10] which decreased after treatment with IFNα. Moreover, LPS-induced inflammation is associated with cirrhosis and predicts progression to end-stage liver disease in patients with HBV or HCV infection [11]. Despite the current data shows that MT may promote liver fibrosis either by direct interaction with Kupffer cells and hepatic stellate cells or indirectly via induction of systemic immune activation and activation-induced apoptotic cell death, further studies are clearly warranted to explore the interplay between MT and liver disease in general [12].

In addition, the HIV/HCV coinfection appears to augment the local and systemic effects of MT. In fact, increased MT in HIV-1/HCV coinfection may play an important role in the more rapid progress to fibrosis observed in coinfected patients compared to their HCV monoinfected counterparts.

In addition to what has been reported with regards to viral hepatitis and HIV infection, recent studies also show that the immunopathogenesis of visceral leishmaniasis is associated with the LPS-mediated cell activation. In fact, GI tract parasitization by Leishmania amastigotes and lymphocyte depletion could also affect the mucosal barrier and gut-associated lymphoid tissue thus predisposing to MT. Moreover, the proinflammatory response described during visceral leishmaniasis is potentiated in HIV coinfected patients and may result in a more aggressive disease progression.

Finally, the concept of MT as a driver of sepsis and multiple organ dysfunction syndrome in surgical and intensive care unit patients has emerged over the last several decades. Although the exact clinical relevance of these phenomena continues to be debated, much evidence support the hypothesis that MT is responsible for increased infectious complications in critically ill patients [13]. Furthermore, MT can be influenced not only by the severity of the patient's illness but also by the use of resuscitation procedures; for example, studies in piglets with experimental pneumonia showed increased bacterial translocation during conventional and high-PEEP ventilation [14].

The continuing debate over the role of MT in infectious diseases continues to garner much interest in the scientific world and is gradually assuming an increasingly multidisciplinary approach. From a therapeutic point of view, different interventions that aim to decrease MT are currently under evaluation. An interesting opportunity under current examination is probiotic administration; in fact, emerging studies support the concept that probiotic bacteria can provide specific benefit in HIV infection [15]. Similarly, there is evidence to support the idea that treatment with pre- and probiotics during critical illness can restore the balance of microbial communities in the gut, with beneficial effects on MT and on clinical outcome of critically ill patients.

On the basis of these findings, it is clear that MT has been recognized as an important mechanism that underlies pathogenesis in a number of different diseases. The link between MT and several infectious diseases seems to be persistent immune activation and the chronic inflammatory state. While the relative contribution of MT to the pathogenesis of different infectious diseases is likely to vary, MT *per se* seems to be a common pathway causing disease progression that is shared by different pathogens.

Gabriella D'Ettorre
Daniel Douek
Mirko Paiardini
Giancarlo Ceccarelli
Vincenzo Vullo

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Review Article

Lipopolysaccharide-Induced Cellular Activation May Participate in the Immunopathogenesis of Visceral Leishmaniasis Alone or in HIV Coinfection

Joanna Reis Santos-Oliveira[1] and Alda Maria Da-Cruz[1,2]

[1] *Laboratório Interdisciplinar de Pesquisas Médicas, Instituto Oswaldo Cruz, FIOCRUZ, 21040-360 Rio de Janeiro, RJ, Brazil*
[2] *Disciplina de Parasitologia, Faculdade de Ciências Médicas, UERJ, 21040-900 Rio de Janeiro, RJ, Brazil*

Correspondence should be addressed to Alda Maria Da-Cruz, alda@ioc.fiocruz.br

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Visceral Leishmaniasis (VL) is an infectious disease which constitutes a serious public health problem, integrating the list of neglected tropical diseases. The disease is characterized by a *Leishmania*-specific immune suppression T-cell depletion and a decrease of other hematopoietic cells. In parallel, an immunostimulatory response also occurs, represented by polyclonal B lymphocytes, T-cell activation, and systemic proinflammatory responses. Parasite antigens were believed to mediate both suppression and activation mechanisms, but these concepts are constantly being revised. Similar to reports on HIV/AIDS, we have proposed that gut parasitation by amastigotes and lymphocyte depletion could also affect gut-associated lymphoid tissue, leading to mucosal barrier breach and predisposing to microbial translocation. An increment of plasmatic lipopolysaccharide (LPS) levels observed in Brazilian VL patients was implicated in the reduced blood $CD4^+$ and $CD8^+$ T cell counts, systemic T-cell activation, pro-inflammatory cytokines and MIF plasma levels, suggesting that a bacterial molecule not associated with *Leishmania* infection can exert deleterious effects on immune system. Recent results also pointed that the proinflammatory response was potentiated in VL/HIV-AIDS coinfected patients. The LPS-mediated cell activation adds another concept to the immunopathogenesis of VL and can bring a rational for new therapeutic interventions that could ameliorate the management of these patients.

Visceral leishmaniasis (VL) is an infectious disease caused by protozoans of *Leishmania* sp. genus. VL is a serious public health problem integrating the list of neglected tropical diseases. In a recent World Health Organization report, 0.2 to 0.4 million cases were globally estimated in the last five years [1]. Ninety percent of them occur in only six countries: India, Bangladesh, Sudan, South Sudan, Brazil, and Ethiopia [1, 2]. The infection is transmitted by sandflies, and nowadays, *L. (Leishmania) donovani* and *L. infantum* (sin. *L. chagasi*) are the main species causing VL [3]. These protozoans are intracellular obligate parasites that infect macrophage cell lineages from lymphoid organs such as bone marrow, spleen, liver, and lymph nodes. VL is classically characterized by fever, hepatosplenomegaly, cachexia, blood cytopenia, and a high parasite burden [4, 5]. It has a high mortality rate, and even in treated patients, the case fatality rates are of 10–20% [1], especially in HIV-coinfected patients [6]. Immunological response is directly involved in the disease's clinical outcome, but the pathogenic mechanisms are still controversial, and the concepts are constantly being revised [7–11].

VL is classically considered an immune suppression-mediated disease characterized by T-cell depletion and decrease of other hematopoietic cells (erythrocytes, platelets, and neutrophils) [4]. Early studies from the 80s showed that active phase of VL evolves with an impairment of the specific effector T-cell response to leishmanial antigens, absence of a delayed-type hypersensitivity reaction to parasite antigens, and an inability of T lymphocytes to proliferate and produce interferon (IFN)-γ and interleukin (IL)-2 cytokine in response to leishmanial antigens [4, 12]. *In vitro* neutralization of IL-10 and IL-4 recovers the type 1 cytokine production in peripheral blood mononuclear cells (PBMC) stimulated with leishmanial antigens [13].

In accordance with this, restoration of the immune response to *L. infantum* is also achieved following specific treatment [13, 14] which reinforced the role of parasite antigens in the suppressive mechanisms [15]. However, other mechanisms have already been implicated in this immunodeficiency such as: circulating molecules acting as soluble receptors for IL-2 [16], immunocomplexes, lipoproteins [17], and deactivating cytokine interactions (IL-4, IL-10, and transforming growth factor [TGF]-β) [15]. These suppressive mechanisms are aggravated by the intense leucopenia especially on T-cell compartment, with a consequent decrease of both $CD4^+$ and $CD8^+$ T lymphocytes [9].

Although immunosuppression is considered a hallmark of acute VL, a remarkable polyclonal B-cell response also occurs. High titers of unspecific and specific immunoglobulins against leishmanial antigens were described [4], suggesting a dual role to parasite antigens, either lymphocytes' activation or inhibition. Therefore, others studies reported an intense release of inflammatory cytokines in the plasma of active VL patients, including tumour necrosis factor (TNF) [18], IL-6, IL-8, and macrophage migration inhibitory factor (MIF) [9, 10]. Besides, elevated levels of plasmatic IL-10 and TGF-β are also detected, showing that these patients present a mixed profile as a result of a cytokine storm [9, 19]. By consequence, VL clinically present as systemic inflammatory response syndrome [5] similar to sepsis, malaria, or dengue fever.

A systemic proinflammatory response is also observed in HIV-1/AIDS patients [20]. As the virus-mediated chronic immune stimulation does not fully explain the intense cellular activation, other mechanisms have been investigated. Of utmost impact, it was demonstrated that $CD4^+$ T cell depletion from gut-associated lymphoid tissue (GALT) [21] leads to mucosal barrier breach and predisposing to microbial translocation (MT). Bacterial products as lipopolysaccharide (LPS) were responsible for an intense cellular activation and proinflammatory cytokine release due to its immunostimulatory activity [22].

Recently, Brenchley and Douek [23] reviewed all the restrictions that humans developed to protect themselves from intestinal microbiota translocation. In this context, any alteration in the gut selective permeability or loss of intestinal immune regulation can facilitate the MT. This phenomenon has been demonstrated in diseases involving the intestinal tract such as inflammatory bowel diseases [24] and graft-versus-host disease [25]. Taking into account that gut parasitation by *Leishmania* amastigotes is known to occur in VL [26, 27], it was also expected that a microbial leakage from gut into the circulation was likely to affect these patients. Moreover, VL shares similar pathogenic features to HIV/AIDS, most notably proinflammatory responses and systemic lymphocyte depletion [4]. Recently, our group demonstrated that the increment of plasmatic LPS levels observed in Brazilian VL patients was implicated in the reduced blood $CD4^+$ and $CD8^+$ T-cell counts, systemic T-cell activation, proinflammatory cytokine plasma levels, and higher MIF levels, suggesting that a bacterial molecule not associated with *Leishmania* infection can exert deleterious effects on immune system [9]. LPS levels are correlated to soluble CD14 (sCD14) and plasma intestinal fatty acid binding protein (IFABP) levels in VL patients. It implies that LPS was bioactive *in vivo*, probably having a luminal origin. An increase in activated status was shaped by increased percentages of activation-associated molecules (HLA-DR, CD38, and CD25) on T lymphocytes and high proinflammatory cytokines response. Consistent with this feature, LPS levels were positively correlated with IL-6, IL-8, and MIF. The hypothesis that bacterial products can impact on chronic immune hyperactivation status in VL prompted us to argue possible benefits of antimicrobial prophylaxis in conjunction with anti-*Leishmania* therapy. Thus, ongoing studies in experimental VL are being performed.

Studies addressing MT in infectious diseases are scarce. To our knowledge, rather than HIV-1 infection [23] and VL [9], a LPS-induced immunostimulatory role was only demonstrated in HBV and HCV virus [28]. A spectrum of changes in the mucosal architecture can be observed in giardiasis, with consequent diarrhea episodes and adherence to the epithelium [29], raising the issue that any condition that causes gastrointestinal barrier damage may allow luminal contents leakage into circulation. In this context, intestinal parasitic disease such as amebiasis, strongyloidiasis, or criptosporidiasis, as well as gastroenteritis due to virus or bacteria could evolve with MT. These possibilities raise such pathogens, as candidates to further studies.

In the last 30 years, the expansion of the HIV/AIDS epidemic over leishmaniasis endemic areas and vice versa has increased the number of coinfected patients [6]. VL is an opportunistic disease in HIV/AIDS patients, although not yet considered an AIDS-defining disease. Both infections with *L. infantum* and HIV-1 share immune-compromising mechanisms that may affect the parasite control in VL coinfected patients [30]. Consequently, coinfected patients present a more severe disease in comparison to patients with VL alone, with increased parasite burden, drug resistance, and frequent relapses [6, 31]. On the other hand, *Leishmania* infection can also contribute to more rapid progression to AIDS, impairing both the lymphocyte depletion and the chronic immune activation, disturbances already observed in HIV-1-infected patients [32, 33].

Considering that MT is involved in activation mechanisms in HIV/AIDS patients and was also detected in VL, it was supposed that this phenomenon could be potentiated in coinfected patients. Recent studies (Santos-Oliveira et al., paper in preparation) showed that *Leishmania*/HIV-coinfected and HIV-1 monoinfected patients presented high LPS and IFABP levels, but the results were not statistically different. However, the plasma proinflammatory cytokines (IL-1β, IL-6, IL-8, IL-17, IFN-γ, and TNF) were much more higher in coinfected group. LPS levels along with immune consequences of *Leishmania* infection were associated with high levels of CD38 in T $CD8^+$ in coinfected patients. These cofactors seem to contribute to the activation status by enhancing the plasma cytokine storm. The parasite influence in this system cannot be ruled out, although patients had experienced a clinical remission of VL symptoms after antileishmanial therapy.

In conclusion, the parasitation of intestinal mucosa and T-cell depletion can lead to GALT compromising, enabling microbial translocation of luminal gram-negative bacteria in VL. The LPS-mediated cell activation adds another concept to the immunopathogenesis of the complex viscerotropic *Leishmania*-host interaction. More importantly, immune activation can profoundly impact the VL clinical course and prognosis, contributing to increase the risk of death even under antileishmanial treatment. This mechanism is aggravated in *Leishmania*/HIV coinfected patients. These findings can bring a rational for new therapeutic interventions that could ameliorate the management of these patients, thus reducing the mortality of VL associated or not with HIV-1 infection.

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Review Article

Gut Microbial Translocation in Critically Ill Children and Effects of Supplementation with Pre- and Pro Biotics

Paola Papoff,[1] Giancarlo Ceccarelli,[2] Gabriella d'Ettorre,[2] Carla Cerasaro,[1] Elena Caresta,[1] Fabio Midulla,[1] and Corrado Moretti[1]

[1] *Pediatric Emergency and Intensive Care Division, Department of Pediatrics, Sapienza University of Rome, Viale Regina Elena 324, 00161 Rome, Italy*
[2] *Department of Public Health and Infectious Diseases, Sapienza University of Rome, 100161 Rome, Italy*

Correspondence should be addressed to Paola Papoff, p.papoff@libero.it

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Bacterial translocation as a direct cause of sepsis is an attractive hypothesis that presupposes that in specific situations bacteria cross the intestinal barrier, enter the systemic circulation, and cause a systemic inflammatory response syndrome. Critically ill children are at increased risk for bacterial translocation, particularly in the early postnatal age. Predisposing factors include intestinal obstruction, obstructive jaundice, intra-abdominal hypertension, intestinal ischemia/reperfusion injury and secondary ileus, and immaturity of the intestinal barrier per se. Despite good evidence from experimental studies to support the theory of bacterial translocation as a cause of sepsis, there is little evidence in human studies to confirm that translocation is directly correlated to bloodstream infections in critically ill children. This paper provides an overview of the gut microflora and its significance, a focus on the mechanisms employed by bacteria to gain access to the systemic circulation, and how critical illness creates a hostile environment in the gut and alters the microflora favoring the growth of pathogens that promote bacterial translocation. It also covers treatment with pre- and pro biotics during critical illness to restore the balance of microbial communities in a beneficial way with positive effects on intestinal permeability and bacterial translocation.

1. Introduction

Despite advances in diagnosis and treatment, bacterial sepsis remains a significant cause of pediatric morbidity and mortality, particularly among critically ill children. Sepsis is the consequence of microbial invasion, or microbial products release, into the bloodstream, which result in systemic inflammatory response syndrome (SIRS). Bloodstream infection may arise through multiple routes, including bacterial translocation across the epithelial-mucosa as in the airways, gastrointestinal tract, kidney or genital tract, and skin breaks as in wounds and during insertion of central venous catheters or other medical devices [1]. Among these different possibilities, bacterial translocation across the gastrointestinal tract has been suggested as one of the principal pathogenetic mechanisms of sepsis and organ dysfunction among critically ill children [2]. There are a number of reasons why bacterial translocation may be relevant to the development of sepsis in children requiring intensive care: the majority of infections diagnosed in children in intensive care unit are due to microorganisms already present in the patients' admission flora of the throat and gut [3]; critical illness, coupled with intensive care treatment, results in a high reduction in microbiota biodiversity with a massive increase of enterococci [4]; gut permeability alterations to large molecules have been documented in critically ill patients [5]; selective gut decontamination seems to reduce infections in a subset of patients admitted to intensive care unit [6]; early enteral feeding is associated with reduced incidence of infections in critically ill patients [7]. Although clinical evidence suggests the importance of the gastrointestinal tract in the development of sepsis syndrome, bacterial translocation itself may not be the primary cause [8].

The purpose of this paper is, therefore, to discuss bacterial translocation, including its definition and role in causing

sepsis syndrome and organ failure in critically ill children. The present paper also includes an analysis of the potential benefits of prebiotic and probiotics supplementation for the prevention of sepsis.

2. Gut Microflora and Its Significance

Human gut contains ~500 different species of microbes as commensals, including obligate anaerobes (about 95%) and facultative anaerobes (1–10%). Obligate anaerobes include *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Fusobacterium*, *Peptococcus*, *Peptostreptococcus,* and *Bacteroides*, and facultative anaerobes *Lactobacillus*, *Bacillus*, *Streptococcus*, *Staphylococcus*, *E. coli*, *Klebsiella*, and *P. aeruginosa*. Bifidobacteria are the predominant microbes representing up to 80% of the cultivable fecal bacteria in infants and 25% in adults [9]. Although commensal bacteria are present in extremely high numbers, they rarely cause local or systemic disease, while have several important physiologic effects in the distal intestinal tract [10]. They directly activate the development and differentiation of the intestinal epithelium and its immune system and contribute to maintain an immunologically balanced inflammatory response. Microflora has nutritive functions, as well. It produces several enzymes for fermentation of nondigestible dietary residue and for secretion of endogenous mucus and helps in recovering lost energy in the form of short-chain fatty acids. It also plays a part in synthesis of vitamins, and in absorption of calcium, magnesium, and iron [11]. Finally, the gut microflora provides a physical barrier against invading pathogens, the so-called "colonization resistance" [12], through a competition for epithelial cell adhesion sites and available nutrients, and by releasing antibacterial substances (e.g., bacteriocins and lactic acid).

Prolonged critical care therapy may disrupt the balance between the host and the gut commensal flora in several ways [13]. Virtually all critically ill patients receive antibiotics which may profoundly affect the intestinal commensal microflora. Using molecular biology techniques, Iapichino et al. evaluated the intestinal microbiota composition of previously healthy patients on admission to intensive care unit [4]. While the first faecal samples showed a banding pattern that was similar to that of healthy subjects, after one week of critical illness and intensive care treatment, including antibiotics, a well definite alteration in the overall microbiota composition was evident, with the presence of a dominant band related to *Enterococcus*. An alteration of intestinal microbial flora has also been observed in premature neonates admitted to intensive care unit. Due to widespread use of broad spectrum antibiotics and late feeding, newborn infants present a delayed colonization with Lactobacillus and Bifidobacteria and a rapid appearance of enterococci, including *Enterococcus faecalis*, *E. coli*, *Enterobacter cloacae*, and the *Klebsiella pneumonia* [14]. Unexpectedly, *C. difficile* colonization does not increase in the early neonatal period despite the use of antibiotics such as cephalosporins [15]. Ferraris et al., who investigated the incidence and perinatal determinants of clostridial colonization in premature neonates, have recently confirmed the absence of effect of either antenatal or postnatal antibiotics on the overall clostridial colonization in neonates admitted to intensive care [16]. In the absence of antibiotics to disrupt the microbiota, it is not clear which event precedes *C. difficile* proliferation. It has been speculated that a decrease in *Bacteroides* and an increase in facultative anaerobes might facilitate colonization by *C. difficile* without a need for the action of antibiotics [17]. The influence of antibiotic use may be more relevant in affecting the severity of *Clostridium difficile* infection as suggested by the results of a recent study by Kim et al. showing that the most significant risk factors for severe *C. difficile* disease in children included young age (adjusted odds ratio [95% confidence interval]: 1.12 [1.02; 1.24]) and receipt of 3 antibiotic classes in the 30 days before infection (3.95 [1.19; 13.11]) [18]. In addition to antibiotic use, several other factors may predispose the gut ecology to alterations during the care of critically ill children. The use of gastroprotectant agents favors proliferation of acid sensitive organisms within the upper intestinal tract. Vasoactive agents that severely limit mesenteric perfusion induce profound luminal hypoxia and hypercarbia that are potent activators of bacterial virulence gene expression [19, 20]. The use of opioids as sedative analgesic agents in mechanically ventilated patients create intestinal atonia and bacterial overgrowth [21]. Finally, nutrients delivered to critically ill patients intravenously or as highly processed foods, whose absorption is nearly complete within the small intestine, create nutrient scarcity within the distal intestinal tract, the area where the highest microbial burden exists. Under such circumstances, the proliferation of highly virulent microorganisms creates a state of perturbed host pathogen balance, and an undesired activation of local inflammation. Systemic inflammation can thus be initiated by mucosally derived cytokines, or when microbial products enter the systemic circulation through the disrupted intestinal epithelial barrier [22].

3. Concepts of Bacterial Translocation

Bacterial translocation has been defined as the process by which live bacteria, their products, or both cross the intestinal barrier where they may either directly cause infection or excite the immune system resulting in a massive inflammatory reaction causing diffuse organ damage and eventually organ failure and death [23]. To some extent, bacterial translocation from the gut occurs regularly even in healthy individuals (5–10%) [24], but bacteremia is generally limited by an intact immune system [8]. This may be a normal physiologic process by which animals and humans sample different luminal antigens in order to produce immunocompetent cells [25]. The first line of defense for preventing bacterial translocation is the mucous coat overlying gut epithelia, produced by goblet cells, which includes degraded mucin and antimicrobial peptides. In the neonatal enterocytes, the ability of mucin to inhibit bacterial translocation may be diminished compared to adults [26]. This might help to explain the propensity of the neonatal rats to spontaneous bacterial translocation in the first two weeks of life when intestinal concentrations of gram-negative bacilli and gram-positive cocci are high, and the concentration of

lactobacilli is low [27]. Translocation occurs in between cells or through cells of the intestinal epithelium after loss of tight junctions between enterocytes. Several mechanisms of bacterial translocation have been identified from studies of enteropathogenic bacteria, such as a zipper mechanism that utilizes transmembrane cell-adhesion proteins as receptors for the bacteria [28]; a bacterial needle-like probe that injects dedicated bacterial effectors into epithelia and the injected molecules modify the cytoskeleton to facilitate bacterial entry [29]; an increased nitric oxide production during inflammatory states that alters expression and localization of the tight junction proteins that surround the upper part and lateral surfaces of enterocytes leading to intestinal hyperpermeability [30]; toll-like receptors that are present on the luminal surface of enterocytes to sense danger and activate host defenses and that can also be harmful by mediating phagocytosis and translocation of bacteria across the intestinal barrier [31]. Once pathogens pass the mucus and epithelial barriers, they are ingested by submucosal macrophages. This process occurs without initiation of an inflammatory response [32]. If intestinal macrophages are dysfunctional as in very low-birth-weight infants [33, 34], this dysfunction may contribute to diffusion of bacteria in the systemic circulation.

Several factors may enhance bacterial translocation in critically ill children. Bacterial overgrowth and breakdown of a tight junction in the setting of intestinal obstruction has been shown to promote bacterial translocation in animal models [35] and in humans [36]. There is evidence in *in vitro* and animal studies that obstructive jaundice impairs reticuloendothelial function [37], interferes with macrophage activation [38], alters Kupffer cell function [39], promotes disruption of desmosomes and formation of lateral spaces between enterocytes [40], and, therefore, alters epithelial barrier permeability. Another possible mechanism for increased translocation associated with obstructive jaundice is considered to be the inhibitory effect of bile on bacterial invasion of enterocytes shown *in vitro* [41]. Intra-abdominal hypertension and abdominal compartment syndrome may also cause gut barrier dysfunction [42]. Another factor that promotes bacterial translocation and predisposes to development of SIRS and organ dysfunction is intestinal ischemia/reperfusion injury. The gut is an organ extremely sensitive to systemic cardiovascular and pulmonary disturbances. The physiological response to hypoperfusion is the shunting of blood away from splanchnic circulation toward more vital organs. The consequence is ischemia/reperfusion injury of villi, release of proinflammatory factors, mucosal disruption, increased intestinal permeability, and bacterial translocation. In addition, secondary ileus seen after ischemia/reperfusion injury seems to promote bacterial overgrowth and proximal gut colonization which are linked with the development of septic complication [43].

Among the factors that influence bacterial translocation, postnatal age and prematurity appear to play a significant predisposing role. Prematurity reduces mucosal barrier function and consequently foster gut permeability [20]. Moy gave definitive experimental evidence that spontaneous bacterial translocation occurs in the neonate by demonstrating that transformed *E. coli* K1 fed to healthy rabbit pups spontaneously translocated from the intestinal lumen and subsequently disseminated to the mesenteric lymph nodes, spleen, and liver [44]. A high proportion of bacterial translocation in neonates results not only from immaturity of host defense functions, but also from the dominant colonization of aerobic bacteria in the intestine. Bacterial colonization develops differently in breast-fed, formula-fed, premature, and full-term infants. In a model of newborn rats, Yajima showed that breastfeeding inhibited systemic bacterial translocation in the suckling period of the rat, even though this phenomenon is not necessarily correlated to modification of the colonizing flora [24].

There are precise criteria to define that bacterial translocation has occurred in a subject including: gut-origin bacteria found in mesenteric lymph nodes (the first organ encountered by the organism undergoing translocation) or portal venous blood; endotoxin found in mesenteric lymph nodes or portal venous blood; bacterial DNA or proteins found in mesenteric lymph nodes, portal venous blood, or the systemic circulation; development of infectious complications with organisms that presumably originated from the gut; increased levels of circulating and tissue cytokines and inflammatory mediators; increased permeability of the gut to large molecules. Increased intestinal permeability as measured by the lactulose-to-mannitol ratio may be a permissive factor for bacterial translocation, but finding an increased lactulose-to-mannitol ratio does not prove that translocation has occurred [8]. In humans in whom direct culture of mesenteric lymph nodes or portal blood is not routinely possible, we often use indirect ways to confirm or monitor bacterial translocation, thus extreme caution should be practiced when drawing conclusions.

4. Bacterial Translocation in Critically Ill Patients

Few studies have investigated the pathogenic potential of bacterial translocation to septic morbidity in critically ill children. Pathan et al. examined the role of intestinal injury and subsequent endotoxemia in the pathogenesis of organ dysfunction after surgery for congenital heart disease [45]. They analyzed blood levels of endotoxin alongside global transcriptomic profiling and monocyte endotoxin receptor expression in children undergoing surgery for congenital heart disease and found that these infants present an increased risk of intestinal mucosal injury and endotoxemia which may contribute to inflammatory activation and organ dysfunction postoperatively. Cicalese et al. evaluated the correlation between bacterial translocation and preservation injury or acute rejection in 50 pediatric small bowel transplant immunosuppressed recipients [46]. Bacterial translocation episodes were considered when microorganisms were found simultaneously in blood or liver biopsy and stool. In this study, a substantial percentage of bacterial translocation was associated with acute rejection, the presence of a colon allograft, and a long cold ischemia time. In a prospective observational cohort study in 94 neonates and

infants who underwent surgical procedures and required parenteral nutrition because of gastrointestinal abnormalities, Pierro et al. explored the relevance of septicemias due to microbial translocation in relation to long-term parenteral nutrition [47]. Microbial translocation was diagnosed when the microorganisms that were isolated from the blood sample were also carried in the throat and/or rectum. Sepsis associated with microbial translocation was found in 15 cases on 6 infants. *Escherichia coli*, *Klebsiella*, *Candida* species, and enterococci were more commonly isolated. The authors concluded that in neonates and infants who are receiving parenteral nutrition, septicemia may be a gut-related phenomenon. From the results of these studies, it appears that bacterial translocation could indeed be a critical component to the development of SIRS; however, the numerous methodological problems that plague these studies make a cause/effect relationship questionable.

5. Prebiotics

The introduction of foods that sustain the growth of intestinal microorganisms might prevent the process by which translocation of potentially pathogen bacteria occurs [48]. In 1995, Gibson and Roberfroid [49, 50] introduced the concept of prebiotic as a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health. Prebiotics, which are not digested in the small intestine, enter the colon as intact large carbohydrates that are then fermented by the resident bacteria to produce short-chain fatty acids. The nature of this fermentation and the resulting pH of the intestinal contents dictate proliferation of specific resident bacteria. For example, infants fed-breast milk containing prebiotics support increased proliferation of bifidobacteria and lactobacilli (probiotic), whereas formula-fed infants produce more enterococci and enterobacteria. Clear criteria have been established for classifying a food ingredient as a prebiotic. These criteria are (1) resistance to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption; (2) fermentation by intestinal microflora; (3) selective stimulation of the growth and/or activity of those intestinal bacteria that contribute to health and well-being. Presently there are only 2 food ingredients that fulfill these criteria, that is, inulin and transgalactooligosaccharides (TOS) [51]. The current most popular targets for prebiotic use are lactobacilli and bifidobacteria. Bifidobacteria are able to break down and utilize inulin-type fructans by the action of the b-fructofuranosidase enzyme. The mechanisms of action of prebiotics are complex and so far not yet well known or understood. These mechanisms are summarized below [52–60].

(1) Sources of carbon and energy for bacteria growing in the large bowel [precursors of short-chain fatty acids (SCFA): acetate, propionate, and butyrate] by saccharolytic fermentation [53, 55, 61]. (2) Immunological effects: (a) activate leukocytes in the gut-associated lymphoid tissue (GALT) system [56–58]; (b) increase cell numbers in Peyer's patches [57]; (c) enhance production of bacteriocins [59]; (d) enhance IgA levels in the small intestine and caecum [60]. (3) Improve gut-barrier function [57, 58]. (4) Acidify intestinal contents. (5) Improve bioavailability of calcium and magnesium [61, 62]. (6) Foster absorption of water and sodium [54]. (7) Promote gut transit.

6. Probiotics

Probiotics are commercially available microorganisms which, when ingested as individual strains or in combinations, offer potential health benefits to the host. These agents are often concurrently administered with substances that promote bacterial colonization and growth (prebiotics): in this instance, they are referred to as synbiotics [63]. The beneficial effects of probiotics in critically ill patients can be summarized as follows: (1) immunological effects, such as activation of leukocytes in the gut-associated lymphoid tissue system, increased cell number in Peyer's patches, enhanced immunoglobulin A levels [64], production of bacteriocins, inhibitory effect on proinflammatory cytokines tumor necrosis factor-alpha, interleukins IL-1 and IL-6, stimulation of anti-inflammatory cytokines (IL-10); (2) improved gut barrier function; (3) acidifying intestinal contents inhibition of pathogenic bacteria; (4) improved bioavailability of calcium and magnesium; (5) facilitated absorption of water and sodium; (6) increased intestinal motility. The effectiveness of probiotics is related to their ability to survive in the acidic and alkaline environment of stomach and duodenum, respectively, as well as their ability to adhere to the colonic mucosa and to colonize the colon [11]. Some probiotics, for example, *Lactobacillus* GG and *L. plantarum* 299v are better able to colonize the colon than others [11]. *Saccharomyces boulardii* are non-LA yeast and secret a protease causing proteolysis of Toxin A and Toxin B of *C. difficile* responsible for antibiotic-associated diarrhea (AAD) and, therefore, should be avoided [11]. Bifidobacteria are gram-positive anaerobic lactic acid bacilli (LAB), colonize the colon within days of birth and its population remains stable until advanced age. Lactobacilli are gram-positive, facultative anaerobic LAB, and are normal inhabitant of human gut. *L. plantarum* 299v adheres to the intestinal mucosa to reinforce its barrier function, thus prevents attachment of pathogens to the intestinal wall [65]. *Lactobacilllus* GG was found to eradicate *C. difficile* in patients with relapsing colitis. *L. plantarum* ST31 produces bacteriocins to limit the growth of potential pathogens. *L. casei* increases the level of circulating IgA. *L. acidophilus* and *B. bifidum* appear to enhance the phagocyte activity of circulating granulocytes. *Bacillus clausii* are gram-positive spore-forming strictly aerobic non-LAB and constitute less than 1% of gut microflora. *B. clausii* stimulates CD4 proliferation and lymphocytic activity in Peyer's patches. It also leads to increase in IgA-positive lymphocytes and HLA-DR positive T lymphocytes [66]. Multistrain probiotics seem to be better than single-strain ones, as individual probiotics have different functions and show synergistic effects when administered together [67]. Various single-strain and multistrain probiotics are commercially available for clinical use, majority being LABs.

7. Pre- and Pro Biotics in Critically Ill Children: Available Evidence

Circumstantial evidence suggests that probiotics alone or in combination with prebiotics are effective in preventing necrotizing enterocolitis (NEC), fungal colonization, and in improving feeding tolerance. They have also proved to be microbiologically safe and clinically well tolerated. To this regard, Manzoni reported a 6-year, two-center experience of routinary *Lactobacillus rhamnosus* GG (LGG) use in very low-birth-weight infants (3×10^9 CFU/day, in single oral dose, since 4th day of life, for 4-to 6-week courses) [68]. No adverse effects or intolerances nor clinical sepsis attributable to LGG occurred. In a randomized controlled trial on 231 infants weighing 750–1499 g at birth, Braga et al. found that oral supplementation of human milk with *Bacillus breve* and *Lactobacillus casei* reduced the occurrence of NEC [69]. It was considered that an improvement in intestinal motility might have contributed to this result. Similarly, Bin-Nun showed in a randomized controlled trial that a probiotic mixture of *Bifidobacteria infantis*, *Streptococcus thermophilus*, and *Bifidobacteria bifidus* reduced the incidence and severity of NEC in very low-birth-weight neonates [70]. In a larger randomized, multicenter controlled trial, Lin et al. showed that *Bifidobacterium bifidum* and *Lactobacillus acidophilus*, added to breast milk or mixed feeding, twice daily for 6 weeks, reduced the incidence of death or NEC [71]. Taken together, these data favor the use of oral probiotics for the prevention of NEC in preterm infants. An improved outcome after treatment with *Lactobacillus reuteri* or *L. rhamnosus* was also observed in a group of 249 preterm infants by Romeo et al. who showed a reduced colonization by *Candida*, protection from late-onset sepsis and reduced abnormal neurological outcome [72]. In contrast with these positive results, Sari and Dani found no benefit in administering *Lactobacillus sporogenes* or LGG in reducing the incidence of NEC [73, 74]. Type of probiotics used, as well as the timing and dosage, may explain such differences. As compared to neonates, fewer studies have been published in older children admitted to intensive care, the majority of which focusing on safety and beneficial changes of intestinal flora. Simakachorn et al. randomized 94 patients between 1 and 3 years old who were requiring mechanical ventilation to receive either a test formula containing a synbiotic blend (composed of 2 probiotic strains (*Lactobacillus paracasei* NCC 2461 and *Bifidobacterium longum* NCC 3001), fructooligosaccharides, inulin, and Acacia gum, or a control formula. Infants in the test group tolerated well pre- and pro biotics [75]. Faecal bifidobacteria and total lactobacilli were higher in the test group, whereas enterobacteria levels diminished. The authors concluded that the enteral formula supplemented with synbiotics was well tolerated by children in intensive care units; it was safe and produced an increase in faecal bacterial groups. Honeycutt et al. evaluated the efficacy of probiotics in reducing the rates of nosocomial infection in 61 patients admitted to a pediatric intensive care unit [76]. Children were randomized to receive either one capsule of LGG or placebo once a day until discharge from the hospital. In this study, LGG was not shown to be effective in reducing the incidence of nosocomial infections. Similarly, a systematic review on 999 critically ill adults revealed no beneficial effect of probiotics/synbiotics in term of clinical outcomes, length of intensive care unit stay, incidence of nosocomial infection, pneumonia and hospital mortality [77]. In conclusion, it appears that beyond the neonatal age no clear evidence supports the use of probiotics in critically ill patients. Well-designed, large-scale, clinical trials are therefore needed to define optimal probiotic species, doses, and whether combination therapy is superior to single-agent therapy.

8. Conclusions

Bacterial translocation as a direct cause of sepsis is an attractive hypothesis that presupposes that in specific situations bacteria cross the intestinal barrier, enter the systemic circulation, and cause SIRS. Critically ill children are at increased risk for bacterial translocation, particularly in the early postnatal age. Predisposing factors include intestinal obstruction, obstructive jaundice, intra-abdominal hypertension, intestinal ischemia/reperfusion injury and secondary ileus, and immaturity of the intestinal barrier per se. Despite good evidence from experimental studies to support the theory of bacterial translocation as a cause of sepsis, there is no definite evidence in human studies to confirm that translocation is directly correlated to bloodstream infections in critically ill children. Besides, attempts at the use of pre- or pro-biotics have not always translated into clinical benefits to patient care, except for prevention of NEC in the neonatal population. Therefore, further research in this field is needed to help clinicians to make correct decisions concerning protection of the gut in the intensive care unit and to decide for possible therapeutic use of pre- and probiotics.

Conflict of Interests

The authors declare that there are no conflicts of interests.

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Review Article

Microbial Translocation in Chronic Liver Diseases

Marilia Rita Pinzone,[1] Benedetto Maurizio Celesia,[1] Michele Di Rosa,[1] Bruno Cacopardo,[1] and Giuseppe Nunnari[1,2]

[1] *Division of Infectious Diseases, Department of Clinical and Molecular Biomedicine, University of Catania, 95125 Catania, Italy*
[2] *Department of Microbiology and Immunology, Jefferson Medical College, Thomas Jefferson University, Philadelphia, PA 19107, USA*

Correspondence should be addressed to Marilia Rita Pinzone, marilia.pinzone@virgilio.it

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The intestinal microflora is not only involved in the digestion of nutrients, but also in local immunity, forming a barrier against pathogenic microorganisms. The derangement of the gut microflora may lead to microbial translocation, defined as the passage of viable microorganisms or bacterial products (i.e., LPS, lipopeptides) from the intestinal lumen to the mesenteric lymph nodes and other extraintestinal sites. The most recent evidence suggests that microbial translocation (MT) may occur not only in cirrhosis, but also in the early stage of several liver diseases, including alcoholic hepatopathy and nonalcoholic fatty liver disease. Different mechanisms, such as small intestinal bacterial overgrowth, increased permeability of intestinal mucosa, and impaired immunity, may favor MT. Furthermore, MT has been implicated in the pathogenesis of the complications of cirrhosis, which are a significant cause of morbidity and mortality in cirrhotic subjects. Therapeutic strategies aiming at modulating the gut microflora and reducing MT have focused on antibiotic-based options, such as selective intestinal decontamination, and nonantibiotic-based options, such as prokinetics and probiotics. In particular, probiotics may represent an attractive strategy, even though the promising results of experimental models and limited clinical studies need to be confirmed in larger randomized trials.

1. Introduction

The intestinal microflora is a complex ecosystem, consisting of more than 500 microbial species, that are involved in the digestion of nutrients and the production of vitamins and short-chain fatty acids; furthermore, the gut microflora plays a role in local immunity, forming a barrier against pathogens, together with the intestinal mucosa [1]. The derangement of the gut microflora and increased microbial translocation (MT) have been widely described in advanced liver disease and associated with the pathogenesis of the complications of cirrhosis [2]; moreover, recent lines of evidence suggest the intestinal microflora to be directly implicated in the induction and progression of liver damage in several chronic liver diseases, including alcoholic and non-alcoholic steatohepatitis, two common causes of cirrhosis [3–5].

Here, we review current concepts regarding the pathogenesis of MT, its role in liver diseases and the potentialities of therapeutic strategies based on the modulation of intestinal flora (i.e. probiotics).

2. Microbial Translocation in Cirrhosis

Microbial translocation (MT) is defined as the migration of viable microorganisms or bacterial endotoxins (i.e., bacterial lipopolysaccharide (LPS), peptidoglycan, and lipopeptides) from the intestinal lumen to the mesenteric lymph nodes (MLN) and other extraintestinal sites [6, 7]. Gram-negative members of the Enterobacteraceae family (such as *Escherichia coli* and *Klebsiella* spp.), enterococci, and other streptococci species are the most effective at bacterial translocation to MLN, across even histologically normal intestinal mucosa [8, 9]. On the contrary, anaerobic species

only rarely translocate and they have been reported to limit the growth of aerobic species with higher translocation potentialities [10].

Increased MT has been described both in experimental animal models of cirrhosis and in cirrhotic patients. In animal studies, the prevalence of MT, defined as a positive bacteriological culture from surgically removed MLN, was around 50% in cirrhotic rats with ascites [11] and up to 80% in cirrhotic rats with spontaneous bacterial peritonitis (SBP) [12–14]. In humans, few studies are available, because of the difficulty in detecting MT to MLN and the lack of widely applicable noninvasive markers of MT. However, MT seems to occur more frequently in Child C cirrhotic patients (about 30% of positive MLN cultures), in comparison with Child B and A cirrhotic patients (8% and 3%, resp.) [15]; in addition, levels of tumour necrosis factor (TNF)-α were found to be higher in MLN of cirrhotic patients than in controls and to correlate with Child-Pugh score and to the risk of developing bacterial infections during the first month after transplant [16].

Detection of bacterial deoxyribonucleic acid (bactDNA) in blood and ascites using the polymerase chain reaction (PCR) has been proposed as a sensitive surrogate marker of MT [17–19]. Such et al. [17] detected the presence of bactDNA (mainly *E. coli*) simultaneously in blood and ascites in as many as 32% of patients with advanced cirrhosis and sterile nonneutrocytic ascitic fluid. Of importance, bactDNA sequence similarity in blood and ascites suggested a shared origin, from a common MT event. Unfortunately, there was no correlation between the severity of liver disease and the detection of bactDNA in body fluids, so that the clinical impact of molecular methods to detect MT remains to be established.

2.1. Pathogenesis of Microbial Translocation. Cirrhosis may lead to MT via different mechanisms, including small intestinal bacterial overgrowth (SIBO), disturbance of luminal factors, increased permeability of intestinal mucosa, and impaired immunity. These factors are summarized in Figure 1.

SIBO. SIBO has been shown to frequently occur in the setting of chronic liver diseases and to be related to the degree of hepatic dysfunction [20]. The diagnosis of SIBO is based on the use of glucose breath hydrogen tests [21, 22] or quantitative culture of jejunal aspirates [20, 23]. The limited sensitivity of breath tests [24] may explain the lower prevalence rates of SIBO (about 30–38% *versus* 48–73%) found when using breath tests [21, 22] rather than jejunal colony counts [23, 24] to detect bacterial overgrowth.

A number of explanations may account for SIBO, including hypochlorhydrosis, malnutrition, and intestinal hypodysmotility [24–26]. The pathogenesis of small intestinal hypomotility in cirrhosis is multifactorial, as a result of increased adrenergic activity, enhanced nitric oxide (NO) production, and structural intestinal damage, due to oxidative stress and portal hypertension [20, 27]. Of note, SIBO itself may further compromise intestinal motility [28, 29], thus creating a vicious cycle that amplifies bacterial overgrowth. In animals, the administration of prokinetics, like cisapride [23, 30], and β-adrenergic blockers, like propanolol [31], has been reported to reduce SIBO and MT; in clinical practice, a six-month trial with cisapride showed decreased orocaecal transit time and SIBO elimination in 80% of patients with SIBO at baseline [23, 32]. Unfortunately, cisapride has been withdrawn from use in some countries because of potential for cardiac arrhythmia.

Disturbance of Luminal Factors. Several luminal factors, such as bile acids, secretory immunoglobulin A, mucins, defensins, lysozyme, and phospholipase A2, physiologically contribute to the intestinal barrier against MT. Bile acids inhibit bacterial overgrowth, especially of anaerobic species; moreover, the absence of bile in the intestine has been shown to promote MT [33]. In cirrhosis, the reduced secretion of bile acids may favor SIBO and MT. Of interest, oral administration of conjugated bile acids, cholylsarcosine, and cholylglycine has been reported to reduce intestinal bacterial content and to decrease the rate of MT in ascitic cirrhotic rats [34]. Further studies should evaluate the potential benefits of bile acids in humans.

Impaired Immunity. Cirrhosis is accompanied by several abnormalities of both systemic and local immune system. The reticulo-endothelial system (RES), whose activities are mainly located in the liver (Kupffer cells), is the main defense against bacteremia; in cirrhosis, phagocytic activity of Kupffer cells may be impaired and the presence of portosystemic shunts bypassing the liver (thereby escaping the action of the RES) may explain the failure to clear not only portal or systemic bacteria, but also endotoxins and cytokines [35]. In cirrhotic subjects, serum complement levels have been reported to be low [36, 37] and to independently predict the risk of infections and mortality [37]. Cirrhosis is also accompanied by a decrease in bactericidal activity by monocytes and neutrophils [38, 39]. An increased number of intraepithelial lymphocytes with markedly impaired proliferative activity and capacity to produce interferon-γ have been reported in a murine model of cirrhosis and correlated with increased MT [40]. Considering the crucial role of intestinal immune cells in regulating the interplay between the host and gut flora, it can be assumed that the derangement of local immunity may allow translocated bacteria to escape from MLN and to reach systemic blood and other extraintestinal sites.

Increased Permeability. Structural and functional changes in intestinal mucosa may increase its permeability, contributing to the development of MT. Structural abnormalities, whose most important determinant appears to be portal hypertension, include vascular congestion, thickened muscularis mucosa, fibromuscular proliferation, and a reduced villus/crypt ratio [41]. In addition, ultrastructural abnormalities in the epithelial layer of small intestine specimens of cirrhotic subjects have been described [42]. In experimental cirrhosis, oxidative damage of the intestinal mucosa has

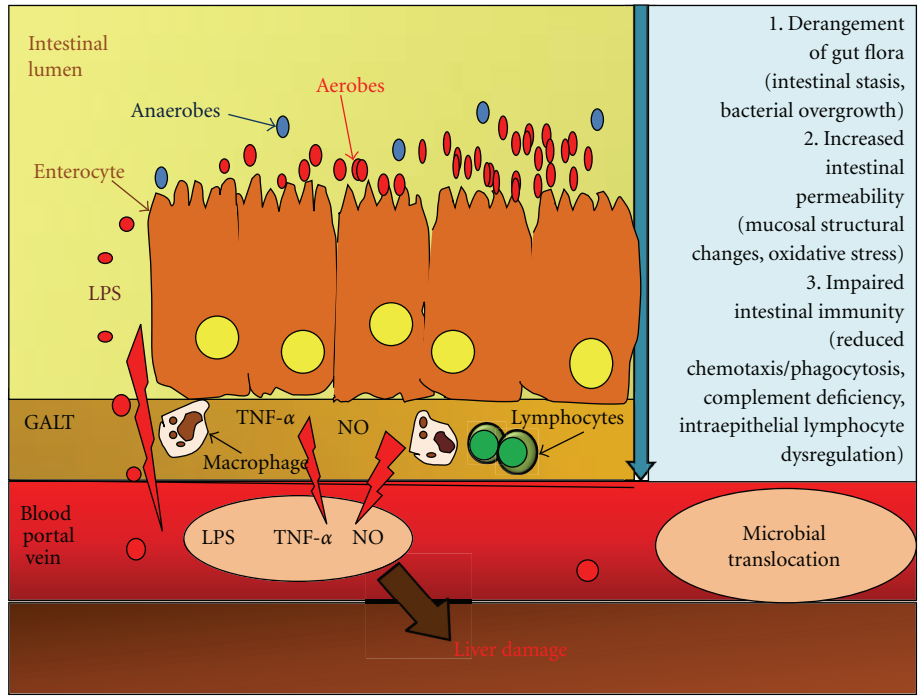

Figure 1: Mechanisms of microbial translocation in chronic liver diseases. LPS: lipopolysaccharide; NO: nitric oxide; GALT: gut-associated lymphatic tissue; TNF-α; tumour necrosis factor.

been shown to cause lipid peroxidation of the brush border membranes and abnormal intestinal transport [43, 44]. Most functional studies, usually dual sugar absorption tests, have found increased intestinal permeability in patients with advanced liver disease [45–48]. Increased permeability is likely to occur via a paracellular route. In humans, morphologically intact tight junctions at the apical pole of enterocytes have been reported in a cohort of clinically stable cirrhotic patients with no prior history of infection with gut-derived bacteria [42], but it remains to be established whether tight junctions are intact in cirrhotic patients with a history of infection with enteric bacteria. Notably, dilatation of the intercellular space below tight junctions has been documented in patients with cirrhosis [32] and, more importantly, NO, whose role in cirrhosis is discussed below, has been shown to reversibly dilate tight junctions, to destroy the cytoskeleton, and to inhibit the formation of adenosine triphosphate in cultured intestinal epithelial cells [49].

2.2. Not Only Microbes: Endotoxins and Cytokines in Cirrhosis. As previously stated, MT does not only refer to the passage of viable bacteria, but also of microbial products, across the gut mucosa, thus causing the production of proinflammatory cytokines and vasoactive mediators. Patients with cirrhosis have been reported to have increased circulating levels of endotoxin (LPS), a cell wall component of Gram-negative bacteria [50, 51]; MT, as well as reduced hepatic clearance, may be responsible for the spill-over of intestinal endotoxin into the systemic circulation. In fact, endotoxemia is significantly more frequent in cirrhotic rats with MT than in cirrhotic animals without MT [52]. Furthermore, MT itself has been reported to make the gut a "cytokine-releasing organ", even in the absence of portal or systemic bacteremia [53, 54]. Endotoxin-primed macrophages release high levels of TNF-α in experimental models of liver damage [55–57]. In rats, TNF-α levels in MLN have been shown to be higher in presence of MT and to correlate with TNF-α plasma levels [58]; in humans, as previously reported, elevated levels of TNF-α in MLN of cirrhotic subjects have been found and significantly correlated with those detected in the blood [16]. The overproduction of proinflammatory cytokines has been associated with elevated plasma levels of LPS-binding protein (LBP), which is synthesized in the liver in response to endotoxin and promotes the binding of endotoxin to the CD14/Toll-like receptor 4 (TLR4) receptor complex [59]. Based on these observations, endotoxemia has been proposed as a major cause of the proinflammatory state in cirrhotic subjects [60–62]. However, other studies have failed to show a significant association between circulating endotoxin and proinflammatory cytokines levels [63–65]. Moreover, contrary to previous assumptions, Riordan et al. suggested to focus more on Gram-positive bacteria rather than Gram-negative ones: in fact, the authors described the upregulation of TLR2, responsible for signaling in response to Gram-positive microbial stimuli, but not of TLR4, on peripheral blood mononuclear cells (PBMCs) of cirrhotic subjects; in addition, TLR2 on PBMCs correlated with circulating levels of both TNF-a and soluble TNF receptors, while TLR4 did not [66].

3. Taking a Step Back: Role of MT in the Induction and Progression of Liver Damage in Chronic Viral Hepatitis, Alcohol-Induced Hepatopathy, and NAFLD

So far, we have focused on MT in the context of cirrhosis pathophysiology, but it must be taken into account that MT may work as a "trigger" in the induction and progression of various kinds of hepatic injury (i.e., inflammation, steatosis, fibrosis, and possibly cirrhosis) in several liver diseases, such as alcoholic, metabolic, and viral hepatitis. In fact, the derangement of the gut microflora and MT has been described even at the early stages of liver disease.

Alcoholic Hepatitis. Even if alcohol metabolism predominantly occurs in the liver, it is known that colonic bacteria are able to produce large amounts of acetaldehyde from ethanol [67–69]; acetaldehyde is responsible, in turn, of increased intestinal permeability and increased translocation of LPS to the portal vein and on to the liver [70]. In fact, plasma endotoxin levels have been shown to be higher than healthy controls not only in subjects with alcoholic cirrhosis, but also in patients with mild forms of alcoholic hepatitis [71, 72]. In the liver, the LPS/TLR4 pathway has been demonstrated to promote fibrogenesis, by sensitizing stellate cells to transforming growth factor-β (TGF-β)-induced signals and promoting TGF-β release by Kupffer cells [73]. Experiments in rats [74] and clinical studies in humans [75–77] have demonstrated both quantitative and qualitative changes in the gut microflora of alcoholic subjects, in comparison with controls. In addition, ethanol-mediated oxidative stress appears to be an important mechanism that leads to gut leakiness and increased MT [78–81]. In particular, the overproduction of NO and its metabolite peroxynitrite (ONOO-) has been associated with the disruption of the intestinal epithelial layer. NO is synthesized from L-arginine by NOS. Three isoforms of NOS have been identified: neuronal (n)NOS, endothelial (e)NOS, and inducible (i)NOS. While nNOS and eNOS are considered protective for the intestinal mucosa, iNOS may be induced by bacterial products and cytokines and it is believed to significantly contribute to local inflammation and hyperpermeability [82], as confirmed by the observation of reduced alcohol-induced gut leakiness when administering iNOS inhibitors [78]. Furthermore, alcohol has been reported to dysregulate immune responses, suppressing natural killer cell activity, antibody-dependent cell-mediated cytotoxicity, and T-cell-dependent antibody responses, thus globally increasing the host susceptibility to pathogenic bacteria of the gut [83, 84].

Viral Hepatitis. Several studies reported elevated LPS levels in the context of chronic viral hepatitis by hepatitis C virus (HCV) and hepatitis B virus (HBV) [85, 86]. In a recent work, Sandler et al. [86] have retrospectively investigated whether the extent and progression of liver disease in patients with chronic HBV or HCV infection were associated with MT and the subsequent local and systemic inflammation. HCV- and HBV-infected individuals had higher plasma levels of LPS, soluble (s)CD14 (produced upon LPS activation of monocytes), and interleukin-6, in comparison with controls. Levels of sCD14 correlated with markers of hepatic inflammation and fibrosis and were significantly higher in subjects with severe fibrosis at presentation, in comparison with those having minimal fibrosis ($P = 0.01$). Importantly, sCD14 was significantly higher in progressors than nonprogressors ($P = 0.003$); moreover, an increase in sCD14 of 1.0×10^6 pg/ml was associated with an odds ratio of disease progression of 3.7 ($P = 0.007$), with no changes after adjustment for other prognostic factors. Of note, elevated sCD14 levels, but not LPS itself, predicted clinical outcome.

Recent lines evidence coming from studies on HIV-HCV coinfected cohorts have reported a similar correlation between sCD14 and the severity of liver disease [87, 88]. In addition, Marchetti et al. [87] have recently investigated whether baseline MT (as assessed by measuring LPS levels) or host response to MT (sCD14) could be predictive of early virological response (EVR: HCV-RNA <50 IU/mL at week 12 of therapy or ≥2 log10 reduction from baseline after 12 weeks of therapy) and sustained virological response (SVR: HCV-RNA <50 IU/mL 24 weeks after end of therapy) to HCV treatment. In the univariate model, the authors found that lower sCD14 levels were predictive of both EVR and SVR, while LPS were not; however, sCD14 lost its independent predictive value in the multivariable model, thus limiting the possibility to use it in clinical practice as measure to *a priori* include/exclude patients from treatment.

Metabolic Hepatitis. Nonalcoholic fatty liver disease (NAFLD) is the most common cause of liver disease in industrialized countries. It usually develops in the setting of obesity and insulin resistance and comprises a continuum of disease ranging from simple steatosis to steatohepatitis (NASH) to cirrhosis. Obesity has been associated with changes in the gut microflora [4] and increased intestinal permeability [89–91]. In subjects with NAFLD, the prevalence of SIBO has also described as higher than controls [91, 92]. A high-fat diet has been reported not only to increase intestinal translocation of endotoxin in mice (the so-called metabolic endotoxemia), but also to reduce enteric *Bifidobacteria* [93, 94], a group of bacteria that have been shown to lower intestinal LPS levels and to improve mucosal barrier function [95]. Furthermore, mice chronically receiving a continuous low rate of LPS, as well as high-fat diet-fed mice, developed insulin resistance, had high circulating levels of proinflammatory cytokines and increased liver triglyceride content. These effects were completely blunted in CD14 mutant mice [94]. Other studies have shown that even the early stages of fructose-induced NAFLD (e.g., steatosis) are associated with increased intestinal translocation of endotoxin and expression of TNF-α in the liver, whereas TNF-α expression and steatosis were markedly lower in the liver of fructose-fed mice treated with antibiotics or TLR-4-mutant mice [96, 97]. In humans with NAFLD, TNF-α expression was reported to be increased too [92, 98]. These data globally support the idea that the LPS/CD14 system has a pivotal role in the induction of the

low tone inflammatory status of metabolic disease, setting the threshold of insulin sensitivity and the onset of diabetes and obesity.

4. Taking a Step Forward: Role of MT in the Pathogenesis of the Complications of Cirrhosis

The gut microflora has been shown to contribute to the pathogenesis of the complications of cirrhosis, including the development of spontaneous bacterial infections and worsening of the hyperdynamic circulatory state (HCS), a hemodynamic perturbation that may lead to ascites, esophageal variceal growth and hepatorenal syndrome.

Spontaneous bacterial infections (i.e., SBP, empyema, and bacteremia) have an incidence rate of 15%–47% in cirrhotic subjects [99–103] and are mainly caused by Gram-negative bacteria, even though the incidence of infections from Gram-positive cocci has increased in recent series, as a consequence of the use of chronic antibiotic prophylaxis [104–106] and more frequent therapeutic invasive procedures [102]. Mortality is significantly higher in cirrhotic patients developing an infection [101, 103]. SBP, an infection of ascitic fluid typically with a single bacterial species, in the absence of any other primary intra-abdominal source, has been shown to be present in up to 23% of cirrhotic patients with ascites undergoing paracentesis [107]. An increased prevalence of SBP has been described in patients with SIBO [108]; conversely, subjects with SBP have a higher prevalence of SIBO than those without SBP [109]. Animal studies have established a causal link between MT and SBP [14]; in humans, this relationship is supported by the observation that selective intestinal decontamination (SID) by the use of oral nonabsorbable antibiotics is effective in decreasing the incidence of spontaneous bacterial infections in cirrhotic subjects [110–113].

MT has been postulated as an important mechanism in the development of the cirrhotic HCS, which is characterized by low vascular resistance, low mean arterial pressure, and by increased heart rate, cardiac output, and regional blood flow. The HCS is mainly due to splanchnic and systemic vasodilatation, which result in "underfilling" of the arterial system and subsequent activation of compensatory mechanisms (i.e., renin-angiotensin aldosterone, sympathetic nervous system, and antidiuretic hormone), which are responsible for increased plasma volume and cardiac output. The HCS contributes to portal hypertension and many of the complications of cirrhosis [2, 114]. NO appears to be the key vasodilator responsible for the haemodynamic abnormalities of cirrhosis [115]. It has been reported that gut-derived endotoxins and the resultant increase in inflammatory cytokines are able to induce the expression of iNOS in vessel walls, causing NO overproduction, vasodilatation, and HCS [116]. Endotoxemia has been shown to correlate with serum NO-metabolite levels [117, 118]; moreover, HCS appears to be more marked in patients with cirrhosis and ascites having high levels of LBP [59]. The role of cytokines, specifically TNF-a, in the development of the HCS is evidenced by studies showing that TNF-a inhibitors ameliorate HCS in cirrhotic rats [119]. The importance of the gut microflora in inducing the production of vasoactive mediators has suggested to use SID as a strategy to decrease bacterial overgrowth and MT, in attempts to ameliorate the HCS. Patients treated with a 4-week course of oral norfloxacin demonstrated improvement in biologic and hemodynamic end points, with trends toward reduction in cardiac output, improvements in mean arterial blood pressure and reduced MT [59, 120].

A growing amount of data suggests that bacterial infections may work as an important trigger for variceal bleeding in patients with cirrhosis [121, 122], possibly as a consequence of enhanced activation of hepatic stellate cells, which may increase intrahepatic vascular resistance and portal hypertension and precipitate the coagulopathy that results from prostacyclin-related inhibition of platelet aggregation, consumption of clotting factors by the extrinsic coagulation pathway and reduction of endogenous heparinoids [122–124]. Conversely, variceal haemorrhage predisposes to bacterial infections with gut-derived microflora, thus creating a vicious cycle between gastrointestinal bleeding and infections, which significantly increases the mortality rates in this group of patients [125–127].

MT may also participate in the pathogenesis of hepatic encephalopathy (HE). HE is associated with the presence of portal and systemic shunts and the failure of the liver to clear ammonia and other toxic products derived from the gut [128, 129]. Ammonia is produced in both the small bowel (from the effects of glutaminase on glutamine) and large intestine (from urease activity of the colonic flora). Colonic bacteria may also produce gamma-amino-butyric acid (GABA) and benzodiazepine (BZD)-like substances [130, 131], which have been implicated in the development of HE [132, 133]. The role of the gut microflora in the pathogenesis of HE is supported by the observation that total colectomy is effective in reducing baseline and protein-induced ammonia production [134], reversing cases of HE which had not been responsive to medical treatment [135]. Nevertheless, HE may recur, probably as a consequence of the colonization of the small bowel [136].

5. Searching for Effective Therapeutic Options: Selective Intestinal Decontamination and Probiotics

Modulation of the intestinal microflora through the use of selective intestinal decontamination (SID) and probiotics has been proposed as an emerging therapeutic strategy in the management of chronic liver diseases.

SID. Long-term use of norfloxacin, a poorly absorbed quinolone, has been reported to markedly reduce the count of intestinal aerobic Gram-negative bacilli, but not Gram-positive cocci or anaerobic bacteria [112]. In cirrhotic animals, some studies have shown that the use of oral norfloxacin or trimethoprim/sulfamethoxazole was associated with a decrease in BT [137, 138], while other studies

have not [139]. In the clinical setting, a double blind, placebo-controlled trial evaluating the long-term efficacy of norfloxacin in cirrhotic patients who had survived a previous episode of SBP, found a significant reduction of the risk of SBP recurrence in the treated group (20% *versus* 68%) at one year of followup [112]. In cirrhotic patients with low ascitic fluid protein concentrations, long-term prophylactic treatment with norfloxacin was effective even in the prevention of the first episode of SBP, but a major concern of long-term antibiotic prophylaxis is the development of quinolone-resistant infections [140]. Of interest, primary prophylaxis with norfloxacin has been associated with higher rates of trimethoprim–sulfamethoxazole resistance and increased incidence of infections with Gram-positive bacteria, including severe hospital-acquired staphylococcal infections [102, 106, 141]. On the basis of these observations, it is crucial to define non-antibiotic strategies to reduce MT and prevent infections.

Probiotics. Probiotics, commonly lactose-fermenting *Lactobacilli* and *Bifidobacteria*, have been reported to stabilize mucosal barrier function and modulate the gut microflora, limiting the growth of pathogenic bacteria, by acidifying the gut lumen, competing for nutrients, and producing antimicrobial substances [95, 142].

In vitro, the probiotic *Lactobacillus casei GG* has been shown to inhibit the translocation of *E. coli* in a dose-dependent manner [143]; however, *in vivo* lines of evidence are controversial. Some studies performed in cirrhotic rats have failed to find significant differences in MT rates between cirrhotic animals receiving *Lactobacilli* and untreated controls [144, 145]. On the other hand, Forsyth et al. described reduced gut leakiness and less severe alcoholic steatohepatitis in alcohol plus *Lactobacillus GG*-fed rats, in comparison with alcohol-fed rats [146]. A combination of *Lactobacillus johnsonii LA1* and antioxidants was effective in reducing MT, oxidative damage, and endotoxemia in rats with CCl_4-induced cirrhosis; unfortunately, the authors did not include a group receiving *Lactobacillus johnsonii LA1* alone [147]. In experimental models of NAFLD, it has been reported that mice treated with VSL#3 (containing *Streptococcus thermophilus, B. breve, B. longum, B. infantis, L. acidophilus, L. plantarum, L. casei,* and *L. bulgaricus*) had lower liver inflammation, serum alanine aminotransferase (ALT), and hepatic total fatty acid content in comparison with controls [148]. Similarly, a recent work of Xu et al. has shown reduced fat accumulation in the liver of high-fat diet-fed rats receiving *Bifidobacterium longum* and in those receiving *Lactobacillus acidophilus*, in comparison with high-fat diet-fed controls. Of interest, intestinal permeability was not affected by probiotics; furthermore, *Bifidobacterium longum* was more effective than *Lactobacillus acidophilus* in attenuating liver fat accumulation [149]. In high-fat diet-fed mice, VSL#3 administration has been associated with decreased hepatic steatosis and insulin resistance, as well as reduced expression of lipid peroxidation markers, TNF-α, iNOS, cyclooxygenase 2, and matrix metalloproteinases (MMPs) [150, 151]; in another study, the use of VSL#3 ameliorated hepatic fibrosis, by decreasing expression of procollagen and MMPs, but not steatosis or inflammation [152].

In humans, VSL#3 administration has been reported to reduce oxidative stress in patients with NAFLD and alcoholic liver cirrhosis, but not chronic hepatitis C [153]. Cirrhotic subjects receiving *Escherichia coli Nissle* for 42 days showed a trend toward lower endotoxin levels and improvement in Child-Pugh score [154]; similar results were obtained by Liu et al. when administering a symbiotic compound (a mixture of lactic acid bacteria and fermentable fiber), Synbiotic 2000, to cirrhotic patients [155]. A significant reduction in Child-Pugh class occurred in 47% of patients receiving Synbiotic 2000 for 30 days, whereas this only occurred in 29% of patients randomized to fiber alone and 8% of patients on placebo. Of importance, stool analysis demonstrated the reduction of Gram-negative fecal flora; furthermore, Synbiotic 2000 was associated with a significant improvement of minimal hepatic encephalopathy (MHE) and decreased endotoxemia. On the basis of these observations, it is possible to hypothesize the beneficial role of symbiotics in modulating the gut flora and reducing MT, as confirmed by the lower circulating endotoxin levels found in symbiotic-treated subjects. Opposing results were recently reported in a study of Pereg et al., who failed to show any beneficial effect of probiotic administration in patients with compensated liver cirrhosis [156]. In this double-blind placebo-controlled study, 36 patients were randomly assigned to receive probiotic capsules containing *Lactobacillus acidophilus, Lactobacillus bulgaricus, Bifidobacterium lactis* and *Streptococcus thermophiles* or placebo for 6 months. No differences in either clinical or laboratory parameters between the two groups were found; the small size of the study population, the reduced number of patients having a Child-Pugh class >A, and the longer period of treatment may globally explain the different conclusions of this trial in comparison with those described above.

As refers to the use of probiotics for the prevention of infections, it has been reported that a symbiotic regimen including *Lactobacillus plantarum* and fermentable fiber was more effective than SID in reducing the incidence of bacterial infections in liver transplant recipients [157]. Analogously, a follow-up randomized double-blind trial in liver transplant recipients has shown the rate of postoperative bacterial infections to be significantly lower in subjects receiving a mixture of lactic acid bacteria and fermentable fiber, in comparison with fermentable fiber alone [158].

The effects of the prebiotic lactitol on the intestinal flora and plasma endotoxin levels have recently been evaluated in patients with chronic viral hepatitis [159]. Lactitol and lactulose are synthetic non-absorbable disaccharides, widely used in the treatment of HE; due to the lack of a suitable galactosidase in the upper part of the gastrointestinal tract, they are able to reach undigested the large bowel, where they are metabolized by colonic bacteria, generating organic acids [160]. The resulting lower pH may inhibit urease-producing intestinal bacteria and promote the growth of non-urease-producing lactobacilli [161]; on the basis of these observations, it is unsurprising the report by

Chen et al. [159] of increased levels of beneficial bacteria, such as *Bifidobacteria* and *Lactobacilli*, in lactitol-receiving patients with HCV, with a consensual decline in endotoxemia, in comparison with untreated controls.

In the setting of HE, current approaches include the use of non-absorbable antibiotics (i.e. neomycin, paromomycin, metronidazole, or rifaximin) and non-absorbable disaccharides [162]. Probiotics appear to be a promising therapeutic option in the management of HE [155, 163–166]. In fact, some studies have shown that probiotics may positively modulate the gut microflora, reducing the amount of bacterial ammonia reaching the portal vein. The long-term oral administration of *Enterococcus faecium SF 68* was at least as effective as lactulose in improving neurocognitive tests and reducing ammonia levels in patients with HE. Of importance, in this trial the improvement in mental status was maintained during the washout periods in probiotic-treated subjects, but not in lactulose-treated ones [163]. Similarly, cirrhotic patients with minimal hepatic encephalopathy receiving *Bifidobacterium longum* plus fructo-oligosaccharides for 3 months had a significant improvement in both biochemical and neuropsychological tests compared to controls [164].

6. Conclusions

The interaction between the gut microflora and the host may play a crucial role in the natural history of chronic liver diseases. In clinical practice, there is a need of surrogate markers of MT and non-antibiotic methods, which may positively modify the gut ecosystem and reduce the passage of bacteria and bacterial products through the gut. Considering the results coming from experimental models and limited clinical studies, probiotics are an attractive strategy, but they need further investigation in larger controlled clinical trials.

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Research Article

Listeria monocytogenes in Ready-to-Eat Seafood and Potential Hazards for the Consumers

Patrizia Gambarin,[1] Cristian Magnabosco,[1] Marina Nadia Losio,[2] Enrico Pavoni,[2] Antonietta Gattuso,[3] Giuseppe Arcangeli,[1] and Michela Favretti[1]

[1] *Istituto Zooprofilattico Sperimentale delle Venezie, Viale dell'Univeristà 10, 35020 Legnaro, Italy*
[2] *Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Via Antonio Bianchi 7/9, 25124 Brescia, Italy*
[3] *Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Roma, Italy*

Correspondence should be addressed to Michela Favretti, mfavretti@izsvenezie.it

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The risk of exposure to *Listeria monocytogenes* (*L. monocytogenes*) when consuming Ready-to-Eat (RTE) seafood was assessed in the Veneto Region (Italy). Thirty-eight samples were analyzed, each sample consisted of three subunits belonging to the same batches. The first of the three units was examined immediately, the second was stored at +4°C (for all of its shelf-life) and the third at +10°C (for the latter third of its *shelf-life*) before the analysis. Chemical-physical and microbiological parameters were tested simultaneously. Culture results showed the presence of viable *L. monocytogenes* in 9 (23,68%) of the 38 samples analysed, 3 (33,33%) of which with a concentration >100 cfu/g. PCR tests yielded 12 *L. monocytogenes* positive samples. Semipreserves with aw (water activity) and pH values that favour *L. monocytogenes* growth were the only ones to result positive to microbiological and PCR tests. Temperature proved to be an important factor as it limits the growth of *L. monocytogenes*, including products with potentially high competitive microbial charges. Four different serotypes were recovered and ribotyping has helped to highlight the genomic variability of *L. monocytogenes* strains in food. This supports the hypothesis that *L. monocytogenes* continues to evolve genetically to the detriment of phenotypic conservation.

1. Introduction

L. monocytogenes is an intracellular pathogen which, especially if foodborne, may induce what is known as listeriosis. Once ingested, *L. monocytogenes* can penetrate the intestinal endothelial barrier, the placental or the hematoencephalic barrier [1, 2]. The groups at higher risk of contracting the disease are young people, the old, (over 65), pregnant women, and immune compromised people, the YOPI, an acronym coined by De Cesare et al. [3, 4]. In healthy subjects, *L. monocytogenes* can lead to episodes of gastroenteritis and fever [5, 6].

Listeriosis is considered a rare disease, its incidence in humans ranges between 0.1 and 11.3 cases/million [7], with a high mortality rate, up to 30% in the categories most at risk (YOPI) [8]. Based on an EFSA 2010 report, the incidence in Europe was of 3 cases/million of inhabitants [9]. Because the incubation period can span from 3 up to 60 days, this disease is often difficult to trace because it is not easy to isolate the food that is responsible for the infection. Europe has some well-documented episodes particularly in France [10], Finland [11], Switzerland [12], the UK [11], Belgium [13], and Ireland [14]. Distinct psychrotolerant characteristics allow *L. monocytogenes* to adapt to acidic conditions and to low water activity environments, making it an insidious threat to some kinds of food as ready-to-eat food (RTE) that is characterized by mild treatments and a medium-to-long *shelf-life*—a highly sought-after quality by today's consumer

[15–22]. The fish products that pose potential risks include mainly cold smoked fish, raw carpaccio, and marinated fish. Smoked fish products in particular were reported to cause human infections [23–25]. In Europe, smoked salmon, more than all the other products, was reported to have surpassed the maximum threshold limits allowed for *L. monocytogenes* contamination [9].

The risk of consuming RTE seafood does not so much entail the contamination of the raw product, which will often have *L. monocytogenes*, but at low concentrations, as much as the product's characteristics which in time encourage its growth [26–28].

Inadequate consumer knowledge on how to store RTE food at home, at the right refrigerated temperature, has led to higher risks of *L. monocytogenes* growth [29, 30].

The goal of this study is to determine the distribution of *L. monocytogenes* in RTE fish semipreserves, as set out by EC Regulations 2073/2005 related to this category which distinguishes the foods that favour *L. monocytogenes* growth from those that do not. Different types of repfed products were examined, these include marinated seafood salads (with cephalopods, surimi, crustaceans, bivalves), marinated shrimps, cephalopods and salmon carpaccio, marinated mackerel, smoked herrings, and cold smoked salmon.

Packaged products marketed in the Veneto Region (Italy) were sampled to determine the levels of *L. monocytogenes*. The products inspected included ones with intrinsically favourable characteristics, thus ideal for *L. monocytogenes* growth, and those with unfavourable characteristics (pH $\leq$ 4.4, $a_w \leq 0.92$; pH $\leq$ 5.0 and $a_w \leq 0.94$). Tests were carried on both these types of products stored, at 4°C and at 10°C, the latter being a more realistic simulation of household conditions which may experience thermal abuse. Each sample unit, aside from examining *L. monocytogenes* (qualitative and quantitative tests) also analysed the following: total aerobic mesophilic count, total psychrophiles count, total psychrophiles H_2S producers, moulds, yeasts, and lactic bacteria to establish whether correlation exists in relation to the presence of *L. monocytogenes* Furthermore, enrichment broths were also tested for *L. monocytogenes* using PCR and serotyping and ribotyping of isolated strains and culture tests.

2. Materials and Methods

The samples, each consisting of 3 sample units coming from a production batch, upon reaching the laboratory, were stored in a refrigerator in their original package at a temperature of 4°C and 10°C until their *shelf-life* expiry (storage at 10°C in the last third of their *shelf-life* expiry). Product *shelf-life* ranged from 8 days (raw salmon carpaccio) to 70 days (seafood salads). During the collection phase at the various traders, only the samples which had not already surpassed half of their expiry period were considered. The products selected were both national and international products taken from 9 different stores. Upon reaching the laboratory, one sample unit was examined immediately, the other two were on the last day of expiry. In total 38 samples were analysed, amounting to 114 sample units.

2.1. Microbial Analysis.

(i) Total aerobic mesophilic count on agar plates with incubation in aerobiosis at 30°C for 72 hours (ISO 4833:2003).

(ii) Total psychrotolerant count on iron agar plates (Lyngby) with incubation in aerobiosis at 15°C for 7 days.

(iii) Total psychrophiles producing hydrogen sulphide count on iron plates (Lyngby) with incubation in aerobiosis at 15°C for 7 days.

(iv) Count of the moulds and yeasts on Rose Bengal Chloramphenicol Agar plates with incubation in aerobiosis at 25°C for 5 days.

(v) Lactic bacteria count on MRS agar plates (final pH 6.4) with incubation in aerobiosis at 30°C for 72 hours.

(vi) *L. monocytogenes* count on ALOA agar at 37∘C for 48 hours (ISO 11290-2:1998/Amd 1 2004).

(vii) Detection of *L. monocytogenes* on ALOA agar and PALCAM agar plates at 37°C for 48 hours following selective enrichment in Half Fraser and Fraser Broth (ISO 11290-1:1996/Amd 1 2004).

The quantitative and qualitative methods were carried out at the same time, the same day.

2.2. Chemical and Physical Analysis.

(i) pH measure using the Mettler Toledo MP 220 instrument, with temperature autocompensation.

(ii) Water activity (a_w) using Rotronic 29539 instrument (ISO 21807:2004).

2.3. Genomic DNA Extraction. Detection of *L. monocytogenes* consisted in taking 1 ml of enrichment broth (Half Fraser), after 24 hours of incubation, to extract DNA and carry out subsequent PCR tests for *Listeriaspp* and *L. monocytogenes*. DNA extraction was carried out on pellet, obtained after centrifugation of the enrichment broth (2,000 g for 2 minutes, followed by 12,000 g for 5 minutes on the surnatant). Once the surnatant was removed, PBS was added and 16,000 g underwent centrifugation for another 2 minutes. GenElute Bacterial Genomic DNA Mini Kit (Sigma) was used, following the manufacturer's instructions, protocol for gram positive bacteria.

2.4. Listerial Genus Detection by PCR. Nested PCR was the method used to target the codifying gene for 16S rRNA in which the amplified product of the first reaction becomes the template for subsequent nested reactions. The primers used in the first reaction were the forward primer LI1 5′-CTC CAT AAA GGT GAC CCT-3′ and the reverse primer U1 5′-CAG CMG CCG CGG TAA TWC-3′ [31]. The reaction took place in a final volume of 25 μL with concentrations of 1X GeneAmp PCR Buffer II (Applied Biosystems), 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of both primers,

1.25 U of Ampli Taq DNA polymerase (Applied Biosystems), and 5 μL of extracted DNA. For amplification, the thermal cyler GeneAmp PCR System 9700 (Applied Biosystem) was used with temperatures set at initial denaturation at 95°C for 3 min, followed by 25 cycles, each with a denaturation phase at 95°C for 90 sec, and an annealing phase at 50°C for 90 sec and an extension phase at 72°C for 2 min, followed by a final extension phase at 72°C for 10 min. The primers used for the nested reactions were forward primer LS1 5′-ACG ACC GCA ADG TTG AAA CT-3′ and reverse primer LS2 5′-GAC GTC ATC CCC ACC TTC CT-3′ manufactured at the Nucleic Acids Technology laboratory applied to foods at the Istituto Zooprofilattico Sperimentale of Brescia. The reaction was prepared in a final volume of 25 μL with concentrations of 1X GeneAmp PCR Buffer II (Applied Biosystems), 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of both primer, with 0.75 U of Ampli Taq DNA polymerase (Applied Biosystems) and 2.5 μL of extracted DNA. For amplification the thermal cycler GeneAmp PCR System 9700 (Applied Biosystem) was used with temperatures for initial denaturation set at 95°C for 3 min, followed by 35 cycles, each comprising a denaturation phase at 95°C for 30 sec, an annealing phase at 59°C for 30 sec, an extension phase at 72°C for 30 sec, followed by a final extension phase at 72°C for 5 min. Analysis of foreseen amplification, of 301 bp, was carried out after electrophoresis in agarose gel at 2.5% stained with ethidium bromide (final concentration on gel: 0.5 μg/mL), by means of exposure to UV radiation.

2.5. L. monocytogenes Detection by PCR. A method to detect the HLY gene target (*haemolytic secreted pathogenic factor or hemolysin*) was used with forward primer LIS1 5′-CGG AGG TTC CGC AAA AGA TG-3′ and reverse primer LIS2 5′-CCT CCA GAG TGA TCG ATG TT-3′ [32]. Reaction was prepared using an end volume of 25 μL with concentrations of 1X GeneAmp PCR Buffer II (Applied Biosystems), 1.5 mM of $MgCl_2$, 0.2 mM for each dNTP, 0.2 μM of both primers, 0.75 U of Ampli Taq DNA polymerase (Applied Biosystems) and 5 μL of extracted DNA. Amplification was carried out in the thermal cycler GeneAmp PCR System 9700 (Applied Biosystem) with a temperature profile for initial denaturation set at 95°C for 3 min, followed by 35 cycles, each comprising a denaturation phase at 95°C for 30 sec, an annealing phase at 58°C for 30 sec and an extension phase at 72°C for 30 sec, followed by a final extension phase at 72°C for 5 min. Analysis of foreseen amplification, of 234 bp, was then conducted after electrophoresis in agarose gel at 2.5% stained with ethidium bromide (final concentration on gel: 0,5 μg/mL), by means of exposure to UV radiation.

2.6. L. monocytogenes Serotyping by Multiplex PCR (M-PCR). Serotyping by M-PCR was performed using primers as described by Doumith et al.[33]. Primers enable the identification of *Listeria* and the subdivision of strains belonging only to the *L. monocytogenes* species into four distinct serogroups.

Serogroup 1 comprises serotype 1/2a and 3a; serogroup 2 of serotypes 1/2c and 3c; serogroup 3 serotypes 1/2b and 3b; and serogroup 4, serotypes 4b, 4d and 4e.

The PCR mix included PCR Master Mix 1X (Qiagen, Milan, Italy), mix of primers (lmo0737, ORF2819, ORF2110, lmo1118 and prs), sterile distilled penta-H_2O and the DNA extracted. PCR reaction conditions included an initial step of denaturation at 94°C for 3 minutes, 35 cycles at 94°C for 0.40 minutes, 53°C for 1.15 minutes, 72°C for 1.15 minutes and a final step at 72°C for 7 minutes.

PCR products underwent 2% agarose gel electrophoretic separation at 90 V for 90 minutes. Then, after being stained with ethidium bromide (10 ng/ml), they were visualised on UV transilluminator.

2.7. L. monocytogenes Serotyping with Seroagglutination. Serotyping with antisera yielded 12 different serotypes of *L. monocytogenes* in terms of cellular surface, and the somatic "O" and flagellar "H" antigens.

Seroagglutination was carried out to confirm the serotypes and serogroups obtained using the molecular method. The "SEIKEN" Listeria Antisera Kit (Denka Seiken co. LTD, Tokyo, Japan) was used according to a modified method outlined by Seeliger and Hohne [34].

2.8. L. monocytogenes Ribotyping. Positive *L. monocytogenes* colonies were detected and characterized using the Du Pont Qualicon Ltd. RiboPrinter Q system [3, 4]. The colonies were taken from agar plates using a plastic stick. They were suspended in a sample buffer. The solution was lysed at 85°C for 20 minutes, and two specific lysing agents were added. Subsequently, a batch containing eight containers to hold eight samples was inserted in the automated ribotyping instrument. In brief, RiboPrinter generates an enzymatic digestion of the solutions using the *Eco*RI restriction enzyme, and electrophoresis of DNA fragments was transferred onto a membrane. The membrane was hybridized using a specific chemiluminescent probe. Finally, the instrument detects the signal emitted by a CCD camera and software converts the images in ribotyping patterns. Ribotyping (of the species) is an automated process if more than 85% of the sample patterns resemble the reference patterns of the instrument's database. The latter were obtained from various international collections (e.g., ATCC, DSMZ, or JMC) and identified with a DUP-ID code (Dupont Identification). Genotypic characterization of bacterial strains consisted of comparing the strains of a batch, assigning to them what is called a ribogroup code (RG). Each ribotyped strain was compared with all the patterns of the profiles contained in the database. In general, if there was a similarity $\geq$93% between the profile of the strain investigated and that of the databank, the strain was assigned to the corresponding ribogroup of that profile. Ribogroup attribution is only possible if strains undergo simultaneous analysis or if analysis is conducted a few days later since the database has to be updated on an ongoing basis, accessing international databanks available on the internet.

To obtain phylogenetic trees the results were extracted from a Pearson correlation (UPGMA method) with BioNumerics software version 6.1.

3. Results

The analyses were carried out on a range of 38 different commercial products 28 (73,68%) of which had a_w and pH values favourable for growth of *L. monocytogenes*, while 10 (26,32%) had unfavourable values instead (Table 1).

Of the 28 samples with favourable characteristics for growth of the pathogen conditions, nine were positive for *L. monocytogenes* (32,14%), according to the standard culture methods (ISO 11290-1:1996/Amd 1 2004); of these, 3 (10,71%) had values exceeding the limit (100 cfu/g) established by EC Reg. no. 2073/2005. No sample with unfavourable characteristics was found positive for viable *L. monocytogenes* in microbiological testing.

Table 2 illustrates the corresponding values of *L. monocytogenes* qualitative test, quantitative test, and PCR results. *Listerial* genus was present in all 38 samples analysed according to the amplification results of the genus specific PCR reaction. Nonetheless, the species specific PCR demonstrated the presence of *L. monocytogenes* after enrichment in 12 samples only, of which 9 were also found to contain the pathogen by classical culture tests. The microbiological parameters are reported in Table 3. In general they rose when thermal abuse at 10°C occurred. In the 3 samples with a charge of >100 cfu/g, *L. monocytogenes* grew despite the presence of competitive flora, both lactic and alternating. Despite the increase of microbial values at the end of *shelf-life*, there were no significant changes in the product pH and a_w values. In evidence that of three samples with a higher than 100 cfu/g value (sample no. 2, 8 and 10), two were analysed after thermal abuse at 10°C (last third of its *shelf-life*), and one proved unfit at the time it was being taken. It is worth noting that the container of sample no. 2, at the end of its *shelf-life*, had bulging but no unpleasant odours. Other samples, when opened, had no significant organoleptic alterations.

Serotyping of 15 isolated *L. monocytogenes* strains coming from RTE fish semipreserves was carried out using Multiplex PCR to separate the 4 main serotypes (1/2a, 1/2b, 1/2c and 4b) into 4 distinct groups. Confirmation of the serotype of single serogroups was achieved with the seroagglutination method (Table 4 and Figure 1).

The 15 strains of *L. monocytogenes* analysed came from 4 different serotypes: the highest percentage was related to serotype 1/2a (73,33%), followed by serotype 4b (13,33%), 1/2b (6,67%) and 4d (6,67%).

Ribotyping of *L.monocytogenes* colonies in microbiological tests (see Table 4) has yielded the broad variation of isolated strains, identifying 15 particular strains on the basis of species. The most diffuse type was DUP-1042, found in 9 samples (60%). DUP-20243, DUP-1043, and -DUP1052 were characterised in the strains examined, in 3 (20%), 2 (13.33%) and 1 (6.66%) sample, respectively.

In terms of ribogroup distribution, RG-568 was the most diffuse, found in 9 of the 15 samples (60%), and the other ribogroups were each found in only one sample, except RG-1296 which was found in 2 samples. Data from phylogenetic analysis on the restriction pattern (Figure 2) yielded a classification of three main strain groups identified with ribogroup 568, being highly phylogenetically related, in turn distant, though still phylogenetically related to the 4 strains grouped in the upper part of the dendrogram. The third and last group comprises of only one sample, no. 3. It was analysed after a *shelf-life* at a temperature of 4°C being very different from rest of the *L. monocytogenes* that were characterized.

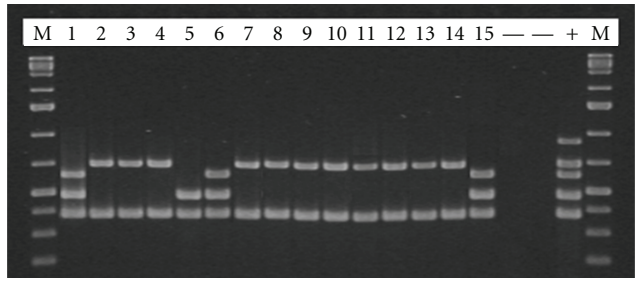

Figure 1: Multiplex PCR on isolated *L. monocytogenes* strains. M: marker; line 1: sample no. 1; lines 2-3-4: sample no. 7; line 5: sample no. 3; line 6: sample no. 5; lines 7-8-9: sample no. 2; lines 10-11: sample no. 8; lines 12-13: sample no. 9; line 14: sample no. 12; line 15: sample no. 10.

4. Discussion

The data demonstrate that there is a rather low probability of *L.monocytogenes* exceeding the 100 cfu/g limit in the RTE seafood distributed in the Veneto Region, which would at any rate be associated with improper storage of the product or with thermal abuse. In fact, of the 3 positive samples out of the 38, 2 had been stored at 10°C in the last third period of their lifecycle and only one was defective at the time it was collected.

Analysis conducted at the end of *shelf-life* provides additional confirmation of the above-mentioned data.

The total charge of psychrophiles and of lactic bacteria found in the three positive samples was high, differently from Jameson's theory which affirms that the lactic charge should develop a sort of microbial competition against *L. monocytogenes* [35].

It is worth noting that 9 semi-preserves resulted positive at the qualitative microbiological test for *L. monocytogenes* (found in 25 g).

If at the end of *shelf-life* the above mentioned 9 samples had a <5 cfu level, the producer would still have to demonstrate, with appropriate studies and challenge tests, that *L. monocytogenes* will not grow and multiply.

Our report reiterates that smoked and fresh salmon are particularly hazardous products.

In case of marinated products, just three samples had viable *L. monocytogenes.* At the end of *shelf-life* however no *L. monocytogenes* growth was detected. Differently from salmon, these products have much lower a_w and pH values, a

Table 1: Types of ready-to-eat seafood, country of origin.

Type of product	No. of samples with characteristics favourable to *L. monocytogenes*	No. of samples with characteristics unfavourable to *L. monocytogenes*	Country of origin
Cold smoked salmon	12	2	Other EU country
Marinated seafood salad	5	3	Italy
Shrimps in brine	3	3	Other EU country
Salmon Carpaccio	4	—	Italy
Octopus Carpaccio	2	—	Italy
Marinated cuttlefish	—	1	Italy
Cooked marinated mackerel	2	—	Italy
Cold smoked herrings	—	1	Other EU country
Total	28	10	—

Table 2: Products resulting positive to *L. monocytogenes*.

Sample no.	Type of product	Temperature of storage	*L. monocytogenes*culture qualitative test	*L. monocytogenes*culture quantitative test (cfu/g)	*L.monocytogenes* PCR
1	Seafood salad	T0	Positive	<5	Positive
		T0	Positive	<5	Positive
2	Salmon carpaccio	4°C	Positive	<5	Positive
		10°C	Positive	**330**	Positive
3	Marinated seafood Cocktail	4°C	Positive	<5	Positive
		10°C	Neg.	<5	Positive
4	Octopus Carpaccio	4°C	Neg.	<5	Positive
5	Cooked and marinated mackerel fillets	T0	Positive	<5	Positive
		10°C	Neg.	<5	Positive
6	smoked salmon	4°C	Neg.	<5	Positive
		10°C	Neg.	<5	Positive
7	Fresh salmon in protective atmosphere	T0	Positive	<5	Positive
		4°C	Positive	<5	Positive
		10°C	Positive	<5	Positive
8	Smoked salmon	T0	Neg.	<5	Positive
		4°C	Positive	<5	Positive
		10°C	Positive	**140,000**	Positive
9	Fresh salmon in protective atmosphere	4°C	Positive	<5	Positive
		10°C	Positive	<5	Positive
10	Smoked salmon	T0	Positive	**6,600**	Positive
11	Salmon Carpaccio	10°C	Neg.	<5	Positive
12	Cooked and marinated mackerel fillets	T0	Positive	<5	Positive

Table 3: Detailed description of microbial charges, a_w and pH values of each *L. monocytogenes*-positive sample (microbiological and PCR tests). Values expressed in CFU/g.

Sample n.	Types of product	Temperature of storage	Mesophiles count	Psicrophiles count	Psicrophiles H2S count	Moulds count	Yeasts count	Lactic count	aw	pH
1	Seafood salad	T0(*)	9×10^8	6.6×10^5	<1	$<1 \times 10^2$	$<1 \times 10^2$	2.4×10^8	0.0680556	5:01
		T0	2.8×10^4	1.4×10^4	6	4×10^2	9×10^2	9.1×10^2	0.0666667	6:01
2	Salmon carpaccio	4°C(**)	6.5×10^5	3.8×10^5	3.5×10^2	2×10^2	2.4×10^3	6.4×10^5	0.0673611	6:01
		10 °C(***)(****)	$\mathbf{1.9 \times 10^8}$	$\mathbf{1.3 \times 10^8}$	$\mathbf{4.7 \times 10^4}$	$\mathbf{9 \times 10^2}$	$\mathbf{1.7 \times 10^4}$	$\mathbf{1.5 \times 10^8}$	**0.0673611**	**6:01**
3	Marinated seafood cocktail	4 °C	7.5×10^4	4.5×10^4	<1	$< 1 \times 10^2$	$<1 \times 10^2$	3.7×10^4	0.0673611	5:01
4	Octopus carpaccio	10°C	5.5×10^7	3×10^6	<1	$<1 \times 10^2$	$<1 \times 10^2$	4.6×10^7	0.0680556	4:09
5	Cooked and marinated Mackerel Fillets(*****)	4°C	4.2×10^6	2.4×10^4	<1	$< 1 \times 10^2$	$<1 \times 10^2$	7.3×10^6	0.0680556	5:03
6	Smoked salmon	T0	7.8×10^4	7.4×10^3	<1	$<1 \times 10^2$	7×10^2	3.6×10^2	0.0638889	6:01
		10°C	7.1×10^7	6.1×10^7	1.1×10^7	$<1 \times 10^2$	1.5×10^5	4.4×10^2	0.0652778	6:03
7	Fresh salmon in protective atmosphere	4°C	1.6×10^5	9.6×10^4	6.9×10^3	4.9×10^3	$<1 \times 10^2$	2×10^3	0.0673611	6:01
		10°C	1.2×10^6	2.2×10^6	8×10^4	6×10^2	5.9×10^3	6.1×10^3	0.0680556	6:00
		T0	2.7×10^6	2.2×10^6	2×10^5	$<1 \times 10^2$	3.6×10^3	9.8×10^3	0.0673611	6:00
8	Smoked salmon	T0	7.6×10^7	4×10^7	1.8×10^5	$<1 \times 10^2$	3×10^2	2.8×10^7	0.0673611	5:08
		4°C	$\mathbf{4.2 \times 10^8}$	$\mathbf{1.5 \times 10^8}$	$\mathbf{1.2 \times 10^7}$	$\mathbf{<1 \times 10^2}$	$\mathbf{4.5 \times 10^4}$	$\mathbf{7.3 \times 10^7}$	**0.0673611**	**6:02**
9	Fresh salmon in protective atmosphere	10°C	3.6×10^6	2.9×10^6	5.2×10^3	$<1 \times 10^2$	3.9×10^3	5.1×10^3	0.0673611	6:02
		4°C	6.7×10^6	1.7×10^5	1.4×10^3	$<1 \times 10^2$	3×10^2	4.1×10^6	0.0673611	6:02
10	Smoked salmon	10°C	$\mathbf{1.4 \times 10^7}$	$\mathbf{1.4 \times 10^7}$	**<1**	$\mathbf{<1 \times 10^2}$	$\mathbf{<1 \times 10^2}$	$\mathbf{4.3 \times 10^6}$	**0.0666667**	**6:02**
11	Salmon carpaccio	T0	7.5×10^6	3.2×10^6	1.3×10^5	1×10^3	3.5×10^3	1.7×10^5	0.0673611	6:00
12	Cooked and marinated mackerel fillets (*****)	10°C	4.1×10^7	1.2×10^5	<1	$<1 \times 10^2$	$<1 \times 10^2$	2.7×10^8	0.0680556	5:03

Note. The lines with bold lettering refer to the 3 samples with a *L. monocytogenes* concentration of >100 cfu/g.
(*): zero time, analysis of one of the three sample units on the day they reached the laboratory.
(**): 4°C, analysis at the end of *shelf-life* at 4°C storage conditions.
(***): 10 °C, analysis at the end of *shelf-life* at 10°C storage conditions in the last third before final expiration.
(****): packaging with bulging at the end of *shelf- life* with no unpleasant odour.
(*****): samples from the same company but from different batches.

Table 4: Serotyping and ribotyping results on isolated *L. monocytogenes* strains.

Sample no.	Types of product	Temperature of storage	Serotype	Ribotype	Ribogroup
1	Seafood salad	T0	4b	DUP 1042	1369
		T0	1/2a	DUP 1043	568
2	Salmon Carpaccio	4°C	1/2a	DUP 20243	568
		10°C	1/2a	DUP 20243	1340
3	Marinated seafood cocktail	4°C	1/2b	DUP 1052	564
		10°C			
4	Octopus carpaccio	4 °C			
5	Cooked and marinated mackerel fillets	T0	4b	DUP 1042	1296
		10°C			
6	Smoked salmon	4°C			
		10°C			
		T0	1/2a	DUP 20243	568
7	Fresh salmon in protective atmosphere	4 °C	1/2a	DUP 1042	568
		10 °C	1/2a	DUP 1042	568
		T0			
8	Smoked salmon	4°C	1/2a	DUP 1042	568
		10°C	1/2a	DUP 1043	568
9	Fresh salmon in protective atmosphere	4°C	1/2a	DUP 1042	568
		10°C	1/2a	DUP 1042	568
10	Smoked salmon	T0	4d	DUP 1042	1533
11	Salmon carpaccio	10°C			
12	Cooked and marinated mackerel fillets	T0	1/2a	DUP 1042	1296

factor that no doubt affects the growth of *L. monocytogenes* [36]. Moreover, smoked salmon production will have *L. monocytogenes* in the raw ingredient which cannot be eliminated in the phases leading to final packaging but can only be contained [37]. Seafood salads instead, since they are made with precooked raw ingredients, do not have *L. monocytogenes*. If there is any *L. monocytogenes* present in the end product, it implies that contamination occurred after the processing phases, after marinating.

The higher number of positive samples in the molecular biology tests and the inability to isolate culture underscore the presence of viable not culturable (VNC) organisms or no longer viable cells [38].

The PCR test compared to the culture is greatly targeted and more sensitive. Used on first enrichment broth it may prove useful in routine laboratory work because at least in PCR-negative cases it does not require microbiological tests.

The samples examined, all being positive to PCR tests for *Listeria*, confirm that these kinds of products are often contaminated with *Listeria*, but that such condition rarely evolves into hazardous *L. monocytogenes* concentrations.

In all the samples with unfavourable a_w and pH values for *L. monocytogenes* growth, the PCR test for *L. monocytogenes* was negative, confirming that even if the product was contaminated in the production phase, *L. monocytogenes* would be overwhelmed by the product's unfavourable environment.

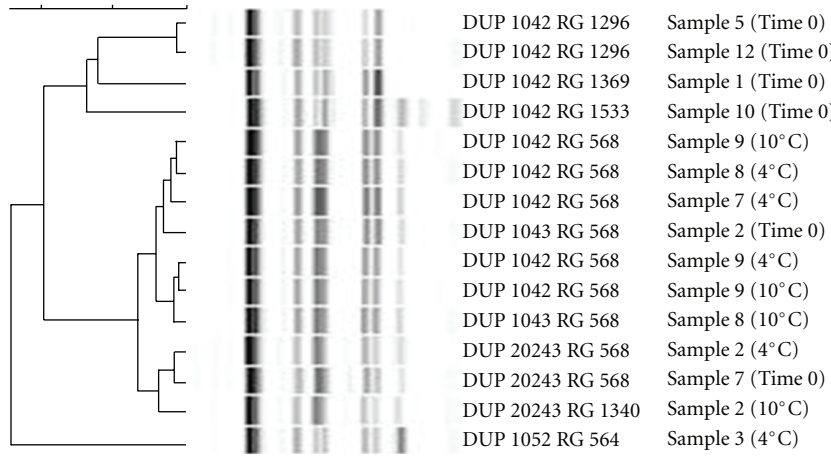

Figure 2: Phylogenetic distances between the various isolated *L. monocytogenes* strains.

The M-PCR method adopted was rapid, reproducible, and cost-effective. It can therefore be used in any equipped laboratory to perform molecular analysis. Its use however is not suggested to identify rare serotypes. For a serotype-specific analysis of isolated strains the traditional sero-agglutination method is recommended. The latter is no doubt less reproducible, more expensive, and requires specially trained staff, but it enables the detection of rare serotypes which in the years to come may be more frequently associated to cases of listeriosis.

It is striking that 10 of the 11 *L. monocytogenes* strains isolated from salmon belonged to serotype 1/2a, as already confirmed by other scientific sources [39–41].

The agglutination method to confirm the serotypes of the single serogroups detected with M-PCR has highlighted a rare strain among the isolates belonging to the 4d serotype.

Rare serotypes have been linked to an epidemic outbreak, as confirmed in a Finnish study by Maijala et al. in 2000 [11]. An epidemic case of listeriosis caused by the strain *L. monocytogenes* serotype 3a, isolated in packaged butter, was described therein. The epidemic outbreak affected 25 people, causing 6 deaths.

Figure 2 shows the variability of strains found in the environment underscoring the need to develop methods that can assess the characterisation of pathogenicity, since same strains may correspond to different levels of pathogenicity.

Literature, in fact, documents *L. monocytogenes* and considers it a ubiquitous microorganism capable of adapting to different environmental conditions [42]. However, despite the various reports on genotyping and characterization [43, 44], there is still very little empirical evidence on the correlation between its presence in food and the ensuing pathologies its consumption generates in humans. This ought to encourage a more ample use of molecular characterization to provide information, even at the epidemiological level, on the distribution of *L. monocytogenes* in the environment, which though it maintains phenotypic homogeneity, undergoes ongoing phenomena of clonal evolution with small, yet significant, genome variations.

In addition, it is interesting to note that the 4 strains grouped in the upper part of the dendrogram identified as RG 1296, RG 1369, and RG 1533 were all isolated in samples analysed at time 0. While ribogroups 564, 568, and 1340 were instead isolated in samples analysed both at the beginning as well as at the end of the *shelf-life* period, this suggests that the latter may be more resistant in time to different storage conditions.

Because of the relatively small number of tested samples, this work should be considered as a preliminary study. Results should be confirmed throught further studies on different kind of products and increasing the number of samples.

5. Conclusions

Temperature plays a key role in preventing the growth of *L. monocytogenes.* In fact, the refrigeration chain is not always respected especially in case of products with a few weeks of *shelf-life*, as the products in this report, which may be subjected to temperature changes which do not bring about any evident organoleptic alterations. Production companies must therefore bear this in mind when certifying their products, using challenge tests to establish how safe a product is in terms of *L. monocytogenes*. They must also foresee temperature tests, in particular for products such as smoked salmon, traditionally from Northern European countries having lower average temperatures than countries like Italy also having less difficulty in managing the cold chain.

Products with a_w and pH characteristics that favour *L. monocytogenes* growth were the only ones to result positive to microbiological and PCR tests in this work, in support of the potential hazard of these products.

The highest percentage related to serotype 1/2a confirm similar investigations in seafood products and the genetic characterization using ribotyping demonstrates the genomic variability of *L. monocytogenes* strains also in this kind of food. This distinctive features could be interesting related to *L. monocytogenes* survival capacity under different storage conditions.

Nearly 30 years have gone by since Canadians Schelch [45] demonstrated that *L. monocytogenes* can infect human

via contaminated food. The significant number of studies conducted since then has provided much data on this pathogen. Currently a lot is known about pathogenesis in humans and about its ability to resist and grow in different types of food.

Rightfully defined as an "*evolving pathogen*" by Bryan [46], it changes its phenotypic and genotypic characteristics under outside-induced stress conditions, with acidic substances, new additives, and with the innovative food technologies applied by food industries. The latter, when producing all the latest food products, have to constantly monitor them for this versatile pathogen. In tandem education campaigns geared at the consumers who may be exposed to *L. monocytogenes* must provide general rules and guidelines for proper food storage and for food preparation in the domestic environment.

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Research Article

Impact of HMGB1/TLR Ligand Complexes on HIV-1 Replication: Possible Role for Flagellin during HIV-1 Infection

Piotr Nowak,[1] Samir Abdurahman,[1] Annica Lindkvist,[2] Marius Troseid,[3] and Anders Sönnerborg[1,2]

[1] *Department of Infectious Diseases, Institution of Medicine, Karolinska University Hospital and Karolinska Institutet, 14186 Stockholm, Sweden*
[2] *Department of Clinical Microbiology, Institution of Laboratory Medicine, Karolinska University Hospital and Karolinska Institutet, 14186 Stockholm, Sweden*
[3] *Department of Infectious Diseases, Oslo University Hospital, Ullevål, 0424 Oslo, Norway*

Correspondence should be addressed to Piotr Nowak, piotr.nowak@ki.se

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Objective. We hypothesized that HMGB1 in complex with bacterial components, such as flagellin, CpG-ODN, and LPS, promotes HIV-1 replication. Furthermore, we studied the levels of antiflagellin antibodies during HIV-1-infection. *Methods*. Chronically HIV-1-infected U1 cells were stimulated with necrotic extract/recombinant HMGB1 in complex with TLR ligands or alone. HIV-1 replication was estimated by p24 antigen in culture supernatants 48–72 hours after stimulation. The presence of systemic anti-flagellin IgG was determined in 51 HIV-1-infected patients and 19 controls by immunoblotting or in-house ELISA. *Results*. Flagellin, LPS, and CpG-ODN induced stronger HIV-1 replication when incubated together with necrotic extract or recombinant HMGB1 than activation by any of the compounds alone. Moreover, the stimulatory effect of necrotic extract was inhibited by depletion of HMGB1. Elevated levels of anti-flagellin antibodies were present in plasma from HIV-1-infected patients and significantly decreased during 2 years of antiretroviral therapy. *Conclusions*. Our findings implicate a possible role of HGMB1-bacterial complexes, as a consequence of microbial translocation and cell necrosis, for immune activation in HIV-1 pathogenesis. We propose that flagellin is an important microbial product, that modulates viral replication and induces adaptive immune responses *in vivo*.

1. Introduction

Antiretroviral therapy (ART) suppresses efficiently the replication of human immunodeficiency virus type 1 (HIV-1) to undetectable levels with standard techniques in most treated patients, but there is still an ongoing low-grade replication in most or all patients [1]. Also, immune activation is a central feature of progressive HIV-1 infection [2–4], and although the degree of immune activation is decreased during ART, it is not normalized [5]. The pathogenic mechanisms for the persistent immune activation remain further to be determined. This is especially important since studies suggest that the remaining immune activation may cause organ damage, for example, an increased risk for cardiovascular diseases and possibly neurocognitive dysfunction [5, 6].

The gastrointestinal (GI) immune system seems to play a central role in the pathogenesis of immune activation [7]. The early dramatic depletion of CD4+ T cells from the gut mucosa may drive immune activation, as this mucosal immune damage impairs the normal barrier function and allows increased translocation of bacterial products from the gut lumen into the circulation [8]. We and others have shown that microbial translocation is present in HIV-1 infection through increased plasma LPS levels in subjects with progressive disease and that the levels are decreased by ART [9–11].

Furthermore, we and others have implied that the alarmin high-mobility group binding 1 protein (HMGB1) modulates HIV-1 replication *in vitro* and contributes to the activation of immune system [12–14]. Thus, plasma HMGB1 levels are elevated in HIV-1-infected patients and reduced with effective ART [11, 15]. HMGB1 is released from damaged or necrotic cells to the extracellular milieu, in which it may act as a potent proinflammatory marker by stimulating cytokine expression in monocytes and endothelial cells [16, 17]. HMGB1 per se does not seem to have a proinflammatory activity [18, 19] but has a high affinity to form complexes with other molecules such as LPS and CpG-DNA [20]. These complexes are likely to bind to various receptors, including TLR4 and TLR9, and promote a large variety of inflammatory and immunological responses [20, 21].

The aim of our study was to explore whether complexes of HMGB1 and TLR ligands, such as flagellin, could synergistically induce HIV-1 replication in a promonocytic cell line.

2. Methods

2.1. Ethic Statement. All research involving human participants has been conducted according to the principles expressed in the Declaration of Helsinki. Patients gave their informed written consent and the study protocol was approved by the Regional Ethics Committee in Stockholm, Sweden (Dnr 2005/3:10).

2.2. Reagents. Lipopolysaccharide (LPS) and (phorbol-12-myristate-13-acetate) PMA were obtained from Sigma (St. Louise, MO, USA), IL-1β from R&D Systems (Minneapolis, MN, USA), and CpG-ODN type B (ODN2006), purified flagellin (*S.typhimurium*), and anti-flagellin (FliC) antibodies from InvivoGen (San Diego, CA, USA and Abcam, Cambridge, UK). Recombinant HMGB1 (HM-116) was purchased from HMGBiotech (Milan, Italy) or from R&D systems (Minneapolis, MN, USA). We also used recombinant HMGB1 [19] that was a kind gift from Professor Helena Erlandsson-Harris CMM/KI, Stockholm.

2.3. Cell Cultures. U1 cells, a subclone derived from U937 cells, were obtained through the AIDS Research and Reference Reagent Program (NIAID, NIH). The U1 cells are chronically infected with HIV-1 and are characterized by low constitutive levels of virus expression that can be upregulated by several cytokines and phorbol esters. The cells were maintained in RPMI medium (Gibco) supplemented with 10% fetal calf serum, glutamine, and antibiotics. Cells were seeded at 200 000 cells/mL in 96-well plates and complexes/TLR-ligands/controls were added and incubated for 48 or 72 hours.

2.4. Patients. Patients ($n = 51$) given ART, followed at the Department of Infectious Diseases, Karolinska University Hospital, Stockholm, and 19 healthy controls were included. Patients' recruitment was based on sample availability as well as virologic response after 2 years of ART. Thirty-three individuals had undetectable viral load and 18 had detectable viraemia (nonresponders) after 2 years of treatment. This cohort (42 of 51 patients) has been described previously [11]. The age and sex distribution of the patients and controls was similar (median age 38 years, 52% women).

2.5. Preparation of Necrotic Cell Extracts. Necrotic extracts were obtained as previously described [22]. Briefly, necrosis was induced in peripheral blood mononuclear cells (PBMCs) from healthy donors (30×10^6 cells/mL) by exposing the cells to six cycles of freezing and thawing. Cell debris was removed by centrifugation and the supernatant was passed through a 0.2 μm membrane and collected. The concentration of HMGB1 in necrotic extracts was 40 μg/mL, as estimated by immunoblot (data not shown). Furthermore, 250 μL of necrotic extract was incubated with polyclonal anti-HMGB-1 antibodies (Abs) from ABCAM (Cambridge, UK). Samples were incubated at 4°C for 16 hours. As control, nonspecific Abs (Rabbit polyclonal IgG) was utilized. Immune complexes were removed by adding 25 μL of Sepharose A/G to the extract, incubated for 1.5 hours at 4°C, and centrifuged. The supernatant was collected and the procedure was repeated again with 25 μL sepharose for 1 hour at 4°C.

2.6. Preparation of HMGB1 Complexes. Necrotic extract or HMGB1was mixed with the TLR-ligands, LPS, CpG-ODN, IL-1β, and flagellin in PBS in different concentrations and incubated for 16 hours at 4°C. Concentrations are given in Figures 1–3. The suboptimal stimulatory concentrations (capable to trigger HIV replication from U1 cells) of necrotic extract as well as LPS, flagellin, CpG-ODN, and Il-1β were estimated in a series of experiments (data not included). Complexes were also mixed and denatured by heating at 95°C for five minutes to verify the stimulatory effect of complex formation on U1 cells.

2.7. Characterization of HMGB1 with Immunoblotting. Equal volumes of necrotic cell extracts, HMGB1-depleted necrotic cell extracts, as well as recombinant HMGB1 proteins were resolved on 10–20% Tris/glycine gel and transferred onto nitrocellulose membrane (Invitrogen, Carlsbad, USA). The membranes were then incubated overnight with anti-HMGB1 Abs at 1 : 2000 dilution. The following day, the membranes were incubated 1 h with horseradish-peroxidase (HRP-) conjugated secondary antibody (GE, Healthcare), raised against rabbit IgG at 1 : 10,000 dilution. The proteins were finally visualized using ECL reagents (GE, Healthcare).

2.8. Immunoblotting of Antiflagellin Antibodies. Approximately 1.88 μg of recombinant flagellin was twofold serially diluted (4 series) and subjected to gel electrophoresis on 10–20% precasted SDS-PAGE gel (Invitrogen) in Tris/glycine/SDS buffer. Similarly, bacterial extracts of flagellated *E. coli* strain O126:H2 and aflagellate *E. coli* O21:H-(CCUG catalog number 11425 and 11326, kind gift from Professor Andrej Weintraub, Clinical Microbiology/KI, Stockholm) that were prepared freshly from an overnight inoculation were also resolved on SDS-PAGE gel as described previously. The proteins were then electroblotted onto iBlot

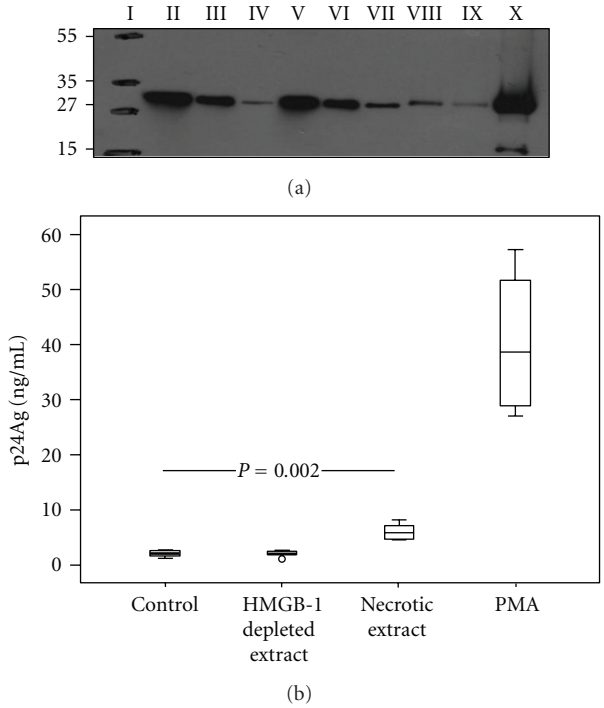

Figure 1: HMGB1 present in necrotic extract induces HIV-1 replication in U1 cells. (a) Western blot of cell supernatants (necrotic extracts) obtained after freeze-thawing cycles of peripheral blood mononuclear cells (PBMC) (30×10^6 cells/mL) from healthy donors: Molecular weight marker (I); supernatants after immune depletion of HMGB1 with nonspecific rabbit polyclonal antibody (II); depletion with anti-HMGB1 antibody $-5\,\mu$g (III) and $10\,\mu$g (IV); necrotic extract loaded $20\,\mu$L (V), $10\,\mu$L (VI), and $5\,\mu$L (VII); 100 ng (VIII) and 75 ng (IX) of recombinant HMGB1; cell debris (X). Numbers to the left depict positions of molecular mass markers (in kDa). (b) Levels of HIV p24 protein in cell culture supernatants after 72 h incubation of U1 cells with necrotic extract (HMGB1 concentration $1\,\mu$g/mL): HMGB1-depleted necrotic extract and mock cells. PMA served as a positive control (20 nM). The levels of viral replication were approximately 2-fold higher after stimulation by necrotic extract compared to the mock cells ($P = 0.002$). Results from three independent experiments in duplicates are presented.

gel transfer stacks nitrocellulose membranes, using the iBlot dry blotting system (Invitrogen), as recommended by the manufacturer. After blocking the nitrocellulose membranes for 1 hour in blocking buffer (PBS supplemented with 0.05% Tween and containing 10% nonfat milk), the blots were probed with primary antibodies overnight at 4°C with a slow agitation. As primary antibody, serum from HIV-1-infected or control subjects diluted 1 : 1000, monoclonal, or polyclonal anti-flagellin Abs was used. The following day, the membranes were washed with PBS containing 0.05% (vol/vol) Tween and bound antibodies were then detected by using HRP-conjugated secondary Abs (Pierce) against human IgG in 1 : 10,000 dilution. Protein bands were visualized by chemiluminescence (Thermo Scientific). To confirm the protein bands, two immunoblotted membranes from HIV-1-infected patients and control subjects were stripped off and reprobed with mouse monoclonal antibody directed against flagellin. Bound antibodies were then detected by using an HRP-conjugated secondary antibody, raised against mouse (DAKO; 1 : 4.000).

2.9. Specific and Total Antibodies Measurement. Antibody titers against flagellin, measles, and total IgG levels were assessed by ELISA. An in-house anti-flagellin-specific IgG ELISA was developed using purified flagellin monomers from *S. typhimurium* (InvivoGen). It has been previously shown that human sera have a similar recognition pattern of flagellin monomers whether isolated from flagellated *E. coli* or *S. typhimurium* [23]. Briefly, microwell plates (MWP) were coated overnight with purified flagellin from *S. typhimurium* (25 ng/well). The following day, plasma samples from HIV-1-infected and control subjects diluted 1 : 1000 were applied to wells coated with flagellin. After incubation and washing, the MWPs were incubated with HRP-conjuggated anti-human IgG. For total IgG ELISA, the manufacturer's procedure was followed (MABTECH, Nacka, Sweden). The Enzygnost Measeles virus IgG ELISA kit (Behring, Germany) was utilized for quantification of antimeasles antibodies.

2.10. Plasma HIV-1 RNA Quantification and CD4+/CD8+ T-Cell Counts. Plasma HIV-1 RNA levels (COBAS Amplicor test Roche Molecular Systems; USA; detection limit 40 copies/mL) and T-cell counts (flow cytometry) were evaluated as part of clinical routine.

2.11. HIV-1 Replication Assay. Supernatants were collected at indicated time points and tested for the presence of HIV p24 antigen with Architect i2000 HIV-1 Ag/Ab combo detection system (Abbott Diagnostics, Abbott Park, IL, USA). The p24 concentration was calculated based on the several standard dilutions of p24 protein included in each run.

2.12. Statistics. Data are presented as median, interquartile range, and total range. Differences between groups were analysed with the Mann-Whitney U-test, and intragroup changes from baseline to the end of the study were evaluated by Wilcoxon test. Jonckheere-Terpstra test was used for trend analyses and correlation analyses were performed using the Spearman method. A two-tailed significance level of 0.05 was used. The statistical analyses were performed with SPSS software, version 15.0 (SPSS Inc, Chicago, USA).

3. Results

3.1. Necrotic Cellular Extract Upregulates HIV-1 Replication in U1 Cells. To determine the impact of an endogenous signal, associated with cell injury, on HIV-1 replication, we generated soluble necrotic extracts from healthy donors PBMCs. Western blot confirmed the presence of large amounts of HMGB1 in the extracts. Additionally HMGB1-depleted extracts were obtained by immune depletion utilizing specific anti-HMGB1 antibodies (Figure 1(a)). In the initial experiment, the U1 cells were exposed to necrotic

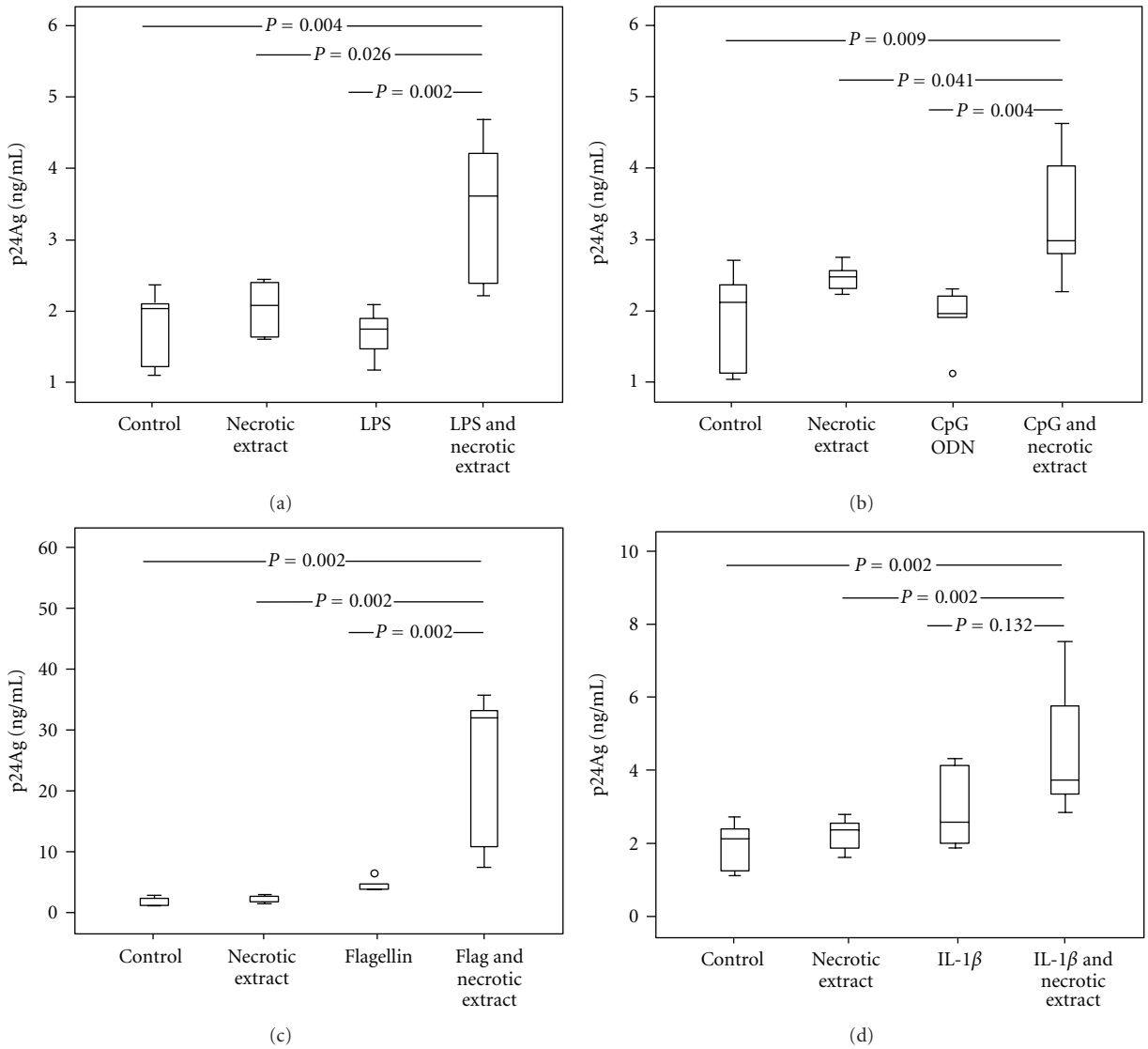

Figure 2: Necrotic extract and TLR-ligands in complexes upregulate viral replication in U1 cells. U1 cell cultures were stimulated with necrotic extract (HMGB1 concentration 1 μg/mL) and Toll-like receptor ligands: LPS 10 ng/mL (a), CpG-ODN 1 μg/mL (b), flagellin 50 ng/mL (c), and IL-1β 0.25 ug/mL (d) alone or in complexes. Supernatants from mock cells served as controls. Supernatants were collected from cell cultures after 72 hours. Results from three independent experiments in duplicates are presented.

extract, HMGB1-depleted extract, and PMA, respectively. The HIV p24 antigen concentration in the cell supernatants was measured after 72 hours (Figure 1(b)). The levels of viral replication were approximately 2-fold higher after stimulation by necrotic extract compared to the mock cells ($P = 0.002$). The stimulation with PMA gave a 10-fold higher viral replication than stimulation with necrotic extract. Notably, addition of necrotic extract depleted of HMGB1 did not result in an increase of viral replication, as compared to the controls, suggesting that HMGB1 crucially contributes to the stimulatory effect of the necrotic extract.

3.2. Interacting Effect of TLR Ligands and Necrotic Extract on HIV-1 Replication in U1 Cells. Thereafter, we stimulated the U1 cells with necrotic extract, TLR ligands (LPS, flagellin, CpG-ODN), and IL-1β alone or with the complexes of necrotic extract and the TLR ligands or IL-1β. Notably, stimulation with all the TLR ligands, in combination with necrotic extract, resulted in a higher viral replication than stimulation with necrotic extract or TLR ligands alone (Figure 2). Hence, stimulation with LPS, CpG-ODN and IL-1β in complexes with necrotic extract resulted in a 1.5–2-fold-increased viral replication compared to each component alone, whereas flagellin in combination with necrotic extract resulted in a 7-fold increased replication compared to flagellin alone and a 13-fold-increased replication compared to necrotic extract alone. The preheating of complexes prior incubation with cells resulted in abrogation of stimulatory signal, implying that the active compound relies on intact protein structure (data not included).

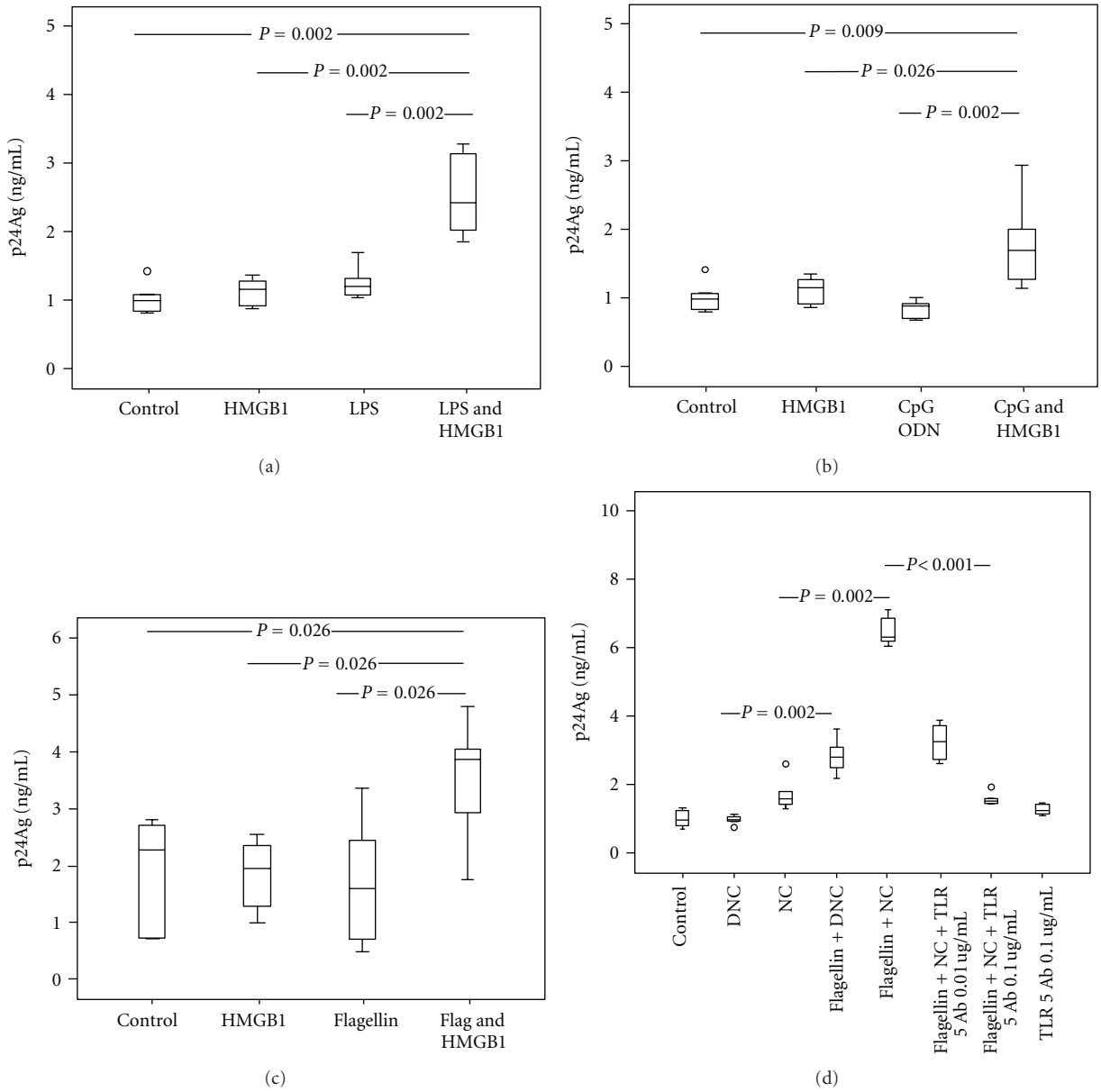

Figure 3: Interacting effect of recombinant HMGB1 and TLR-ligand complexes in U1 cells. Inhibition of flagellin complexes induced HIV-1 replication by anti-TLR5 antibodies. U1 cells were stimulated with recombinant HMGB1 (1 μg/mL) and TLR ligands: LPS 10 ng/mL (a), CpG-ODN 1 μg/mL (b) and flagellin 10 ng/mL (c) alone or in complexes. (d) U1 cells were incubated with 0.1 and 0.01 ug/mL anti-TLR5 antibodies (TLR5 Ab) for 1 hour and then exposed to necrotic extract (NC) and HMGB1-depleted necrotic extract (DNC), alone or in complexes with flagellin (10 ng/mL). HIV-1 replication was estimated after 48 hours of incubation. A dose-dependent inhibition of flagellin-necrotic extract complexes stimulatory effect is present in wells pretreated with anti-TLR5 antibodies (P for trend < 0.001). Results from three independent experiments in duplicates are shown.

3.3. Interacting Effect of TLR Ligands and HMGB1 on HIV-1 Replication in U1 Cells. In order to explore if HMGB1 could mimic the synergistic effects of necrotic extract-TLR ligands, we challenged the U1 cells with complexes consisting of HMGB1 and bacterial substances. Indeed, stimulation with microbial products (LPS, flagellin, or CpG-ODN) in combination with HMGB1 resulted in a higher viral replication than stimulation with HMGB1 or TLR ligands alone (Figures 3(a)–3(c)). Stimulation with LPS, flagellin, and CpG-ODN in combination with HMGB1 resulted in a 1.5–2-fold-increased viral replication compared to each component alone, although the stimulatory effect was not as prominent as with TLR ligands in combination with necrotic extract.

3.4. Dose-Dependent Inhibition of Flagellin by Anti-TLR5. It is known that immune response to flagellin is mediated by TLR5. To investigate whether anti-TLR5 antibodies could block the inducing effects of flagellin, we first preincubated U1 cells with anti-TLR5 antibodies and subsequently added

the necrotic extract complexed with flagellin. Flagellin in combination with HMGB1-depleted extract gave a 3-fold-increased viral replication compared to depleted extract alone, whereas flagellin in combination with necrotic extract gave a 4-fold increase compared to necrotic extract alone (Figure 3(d)). Preincubation of U1 cells with anti-TLR-5 antibodies before addition of necrotic extract-flagellin complexes resulted in a dose-dependent inhibition of this stimulatory effect (P for trend < 0.001). Addition of anti-TLR5 antibodies alone did not affect viral replication.

3.5. Detection of Antiflagellin Antibodies in HIV-1-Infected Patients. Encouraged by the *in vitro* data we aimed to evaluate whether flagellin is a potentially important antigen *in vivo* during HIV-1 infection. Therefore, serum samples from HIV-1-infected patients and control subjects were used to measure the level of flagellin-specific antibodies by Western blot analysis. When diluted 1 : 1000, all of the sera samples from the HIV-1-infected patients analyzed exhibited easily detectable bands that recognized the first two dilutions of flagellin derived from *S. typhimurium* (Figure 4(a), upper panels). A relative increase in flagellin-specific IgG in HIV patients was observed if serum samples were diluted 1 : 500. In contrast, in only one control subject (CS#2) flagellin was detected faintly at the highest dilution (Figure 4(a), lower panels). Although semiquantitative analysis of detected bands was not performed, the levels of flagellin-specific IgG observed were in all cases strikingly elevated in HIV-1-infected patients. Similar pattern of anti-flagellin IgG was observed when plasma instead of sera was used. In order to address the specificity of the flagellin IgG, we subjected the bacterial lysates from flagellated and aflagellate *E. coli* to protein separation on the SDS-PAGE gel. The Western blotting with HIV-1 serum (as a primary antibody) showed similar pattern as when the polyclonal anti-flagellin antibody was used confirming that the specificity of the antibodies was not limited to the recombinant protein (Figure 4(b)).

Furthermore, we used the anti-flagellin ELISA to evaluate the levels of flagellin IgG in plasma of HIV-1-infected patients before and after two years of ART. At baseline significantly elevated levels of flagellin antibodies were found in HIV-1-infected patients as compared to controls ($P < 0.001$) (Figure 5(a)). This difference persisted ($P < 0.001$) when the flagellin antibodies were adjusted to the total IgG (Figure 5(b)), suggesting that the elevation of flagellin antibodies was not due to hypergammaglobulinemia. Moreover, analysis of antimeasles antibodies in 10 patients with severe immune deficiency (CD4+ T-cell counts ≤ 200) supported that the elevation of the flagellin antibodies was not caused by polyclonal activation Supplementary Table 1 (see supplementary material available online at doi:10.1155/2012/263836). The levels of flagellin IgG, total IgG, and the ratio flagellin IgG/total IgG were significantly reduced after two years of ART for the whole group ($P < 0.001$, $P = 0.03$, and $P < 0.001$, resp. Figures 5(a)–5(c)). Additionally a significant reduction of flagellin IgG levels was observed also when the patients were subdivided into those with successful ART and the nonresponders who had remaining low levels of viral replication two years after initiating the ART ($P = 0.009$; $P = 0.001$, resp.). The total IgG levels after ART did not decrease in nonresponders as they did in successfully treated patients ($P < 0.001$) (data not shown).

We found no correlation between the levels of flagellin IgG and the viral load nor the CD4/CD8 T-cell counts. In contrast, among the subgroup of 42 patients in whom we had earlier analysed HGMB1 and LPS in plasma, significant correlations were found between the levels of flagellin IgG and LPS ($r = 0.32$; $P = 0.02$) as well as between the flagellin IgG/total IgG ratio and LPS ($r = 0.25$; $P = 0.007$) (data not shown).

4. Discussion

Microbial translocation has been described in different conditions like inflammatory bowel disease, neutropenia, and chronic viral infections [6, 24]. In HIV-1 infection, the proof for the translocation of bacterial products is based mainly on LPS data [9, 11, 25, 26]. However, the original observation that increased LPS levels were associated with both activated memory CD8+ T cells and enhanced IFN-α levels implies the involvement of other factors [9].

We therefore hypothesized that HMGB1 could be such a link between the microbial products and hyperinflammation [27]. Mounting evidence shows that HMGB1 does not act alone but forms stable potent proinflammatory complexes with other molecules, such as bacterial products or single stranded DNA [18–20, 28]. Since we have earlier shown that HMGB1 alone activates latent HIV-1 replication *in vitro* [12], we decided to expand our analysis to the effect of HGMB1 in complexes with bacterial products. Here, we present that HMGB1 in complex with the TLR ligands (LPS, CpG-ODN, flagellin) and IL-1β induce viral replication in a promonocytic cell line, U1 cells. The data obtained with both the HMGB1 derived from necrotic extract as well as recombinant protein yielded similar results, although the stimulatory signals associated with necrotic HMGB1 were more potent. This is not surprising as other endogenous danger signals should be anticipated in this process [20]. The reduction of the stimulatory effect by depletion of HGMB1 also supports our hypothesis that HMGB1 is an important component of these complexes.

These *in vitro* findings brought our attention to flagellin as a potent activator of HIV-1 replication alone or in complexes with HMGB1. Bacterial flagellins are present in all motile bacteria and play an important role in mediating gut inflammation associated with infection by enteric pathogens or in inflammatory bowel diseases [29]. Their proinflammatory activity is exerted mainly through TLR5 [30, 31]. It has been recently demonstrated that flagellin is the major antigen activating innate and adaptive immune response in intestinal inflammation observed in Crohn's disease [32, 33]. Disruption of the intestinal barrier promotes translocation of flagellated commensal bacteria across the epithelium driving the activation of innate immune cells residing in the lamina propria. This phenomenon results also in abnormal exposure

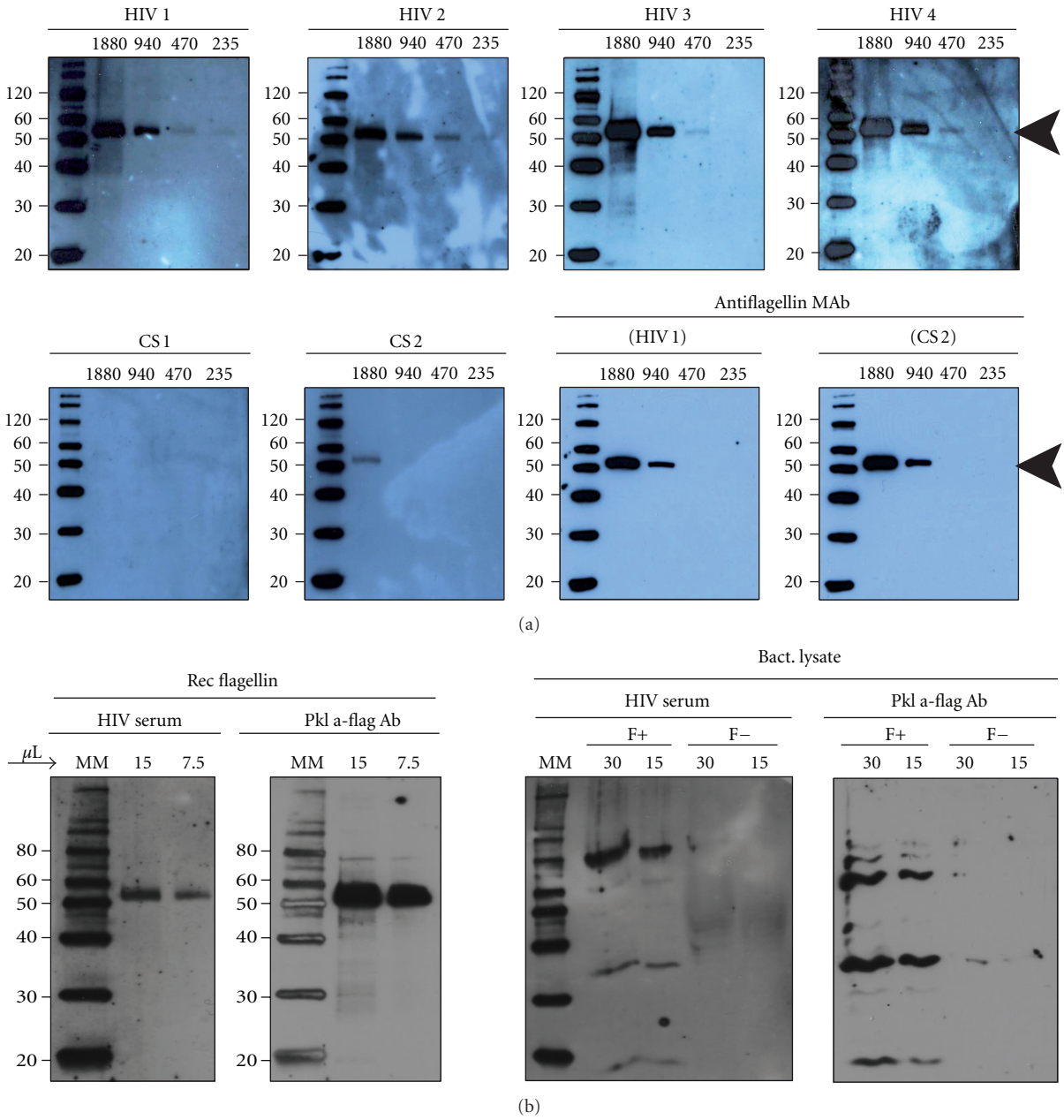

Figure 4: HIV-1-infected patients exhibit elevated levels of flagellin-specific antibodies. (a) Approximately 1.8 μg of recombinant flagellin was twofold serially diluted (4 series) and resolved on 10–20% SDS-PAGE gel, transferred onto a nitrocellulose membrane, and detected with immunoblot assay. Serum from HIV-1-infected or control subjects diluted 1 : 1000 was used as a primary antibody. Each panel is a separate immunoblot of the same recombinant flagellin detected with serum from HIV-1 patients (HIV 1, HIV 2, HIV 3, HIV 4) or control subjects (CS 1, CS 2). To confirm equal sample loading and protein transfer, immunoblotted membranes from HIV 1 and CS 2 were stripped off and reprobed with monoclonal antibody directed against flagellin (blots in the lower panel). The experiments were performed using sera from ten HIV-1-infected patients and four control subjects. These presented data are representative for all immunoblots. The position of recombinant flagellin protein is indicated with an arrow to the right. Numbers to the left depict positions of molecular mass markers (in kDa). (b) Recombinant flagellin (column 1) and lysates of flagellated or aflagellate *E. coli* (columns 3 and 4) were subjected to SDS-PAGE and immunoblotted using serum from HIV infected patient (column 1 and 3, diluted 1 : 1000) as primary antibody or polyclonal anti-flagellin antibody (columns 2 and 4, diluted 1 : 1000). The membrane in column 1 was stripped off and reprobed with polyclonal anti-flagellin antibody (column 2). The numbers above the figures indicate the amount of sample loaded in each well (in μL). MM, magic marker (Invitrogen); F+: flagellated; F−: aflagellate whole bacterial lysate; Pkl: polyclonal; Ab: antibody. Numbers to the left depict positions of molecular mass markers (in kDa).

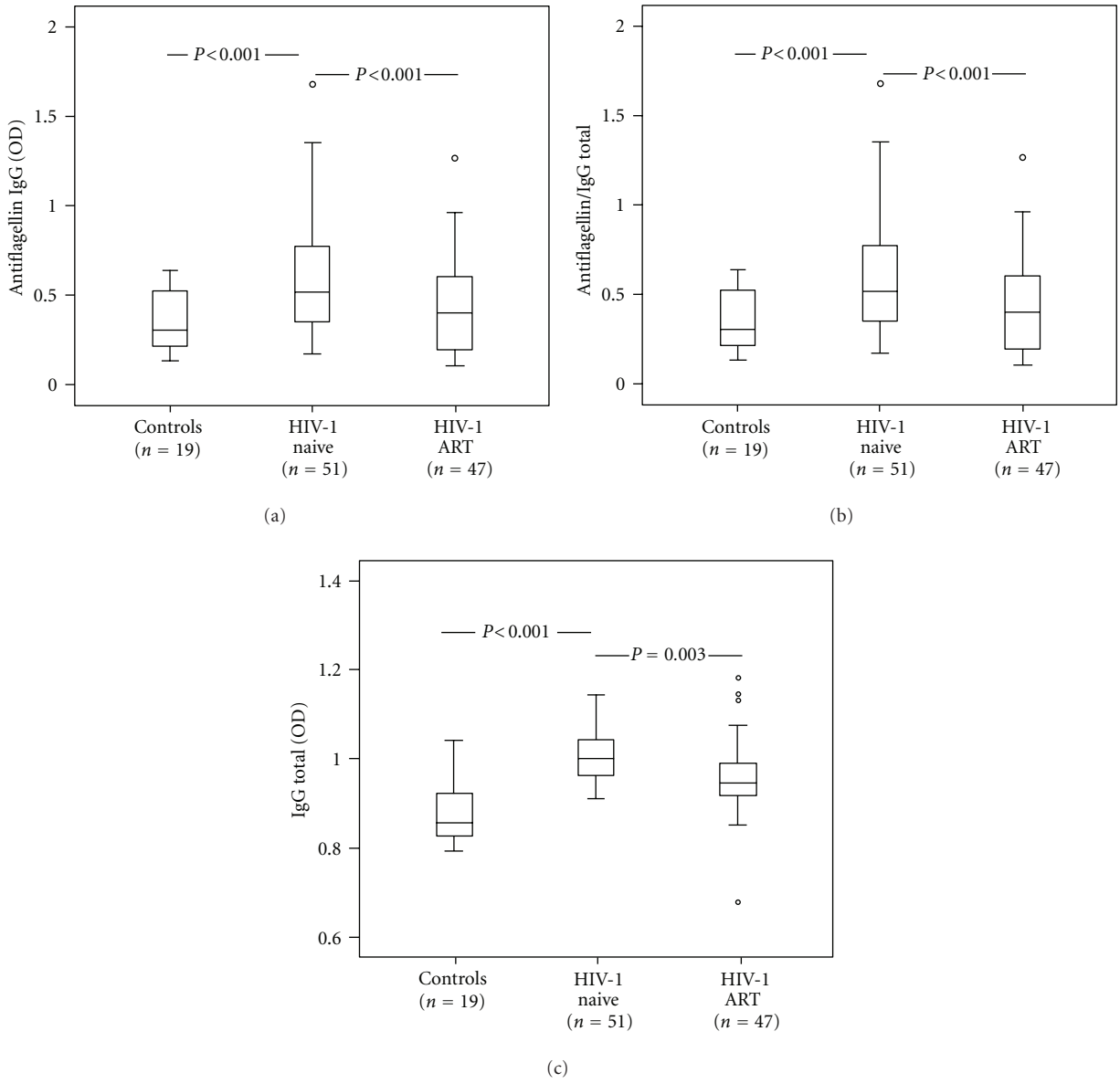

Figure 5: Elevated levels of anti-flagellin IgG are reduced during ART. Plasma levels (OD) of anti-flagellin IgG (a), ratio anti-flagellin IgG/total IgG (b), and total IgG (c) in healthy controls, HIV-1-infected individuals before (= naïve) and after two years of ART (antiretroviral therapy). The plasma samples were diluted 1:1000. The data are presented as median 25–75 interquartile range and total range. *P* values refer to intergroup differences.

of immune cells to flagellin, a process that may influence the balance and function of the different T-cell subsets present in the gut-associated immune system (GALT) and promote inflammation [29, 34].

The contribution of flagellin to immune activation during HIV-1 infection has been anticipated [7], but the data are scarce. Thus, exposure of PBMCs to flagellin resulted in activation of T cells predominantly of central memory and effector memory phenotype [35]. Moreover, flagellin is able to induce HIV-1 gene expression in resting memory CD4+ T cells that are considered as a key cellular HIV-1 reservoir in infected individuals [36].

Our *in vitro* and *in vivo* findings are clearly in line with the hypothesis of an important role of flagellin in HIV-1 pathogenesis. Thus, we demonstrated that flagellin complexes are able to significantly stimulate the HIV-1 replication, at least from cells of monocytic origin. Furthermore, our finding that elevated levels of anti-flagellin IgG are present in HIV-1-infected individuals anticipates that this observation has *in vivo* implications. Hence, we show not only presence of elevated levels of flagellin antibodies before the initiation of ART but also a reduction after two years of ART suggesting decreased exposure to the antigen probably due to partial restoration of gut-blood barrier [25, 37].

The hypergammaglobulinemia present during the HIV-1 infection cannot be solely responsible for the elevation of flagellin IgG levels as the normalisation to the IgG did not influence the results.

An elevated adaptive immune response to flagellin has been previously observed in conditions associated with gut barrier dysfunction such as Crohn's disease and short bowel syndrome [23, 24] and relates to the severity of Crohn's disease [38]. Also, Kamat et al. [39] reported recently the presence of a subgroup of anti-flagellin antibodies (anti-CBir1) in 4/26 HIV-1-infected patients with CD4+ T-cells counts <300 cells/ul and high LPS levels. The CBir1 flagellin has been identified as an immune dominant flagellin in Crohn's disease and linked to Clostridia species. Interestingly a recently presented work has shown alterations in bacterial composition of microbiota during HIV-1 infection with significantly lower ratio of Clostridia taxa in faeces obtained from HIV-1-infected patients as compared to controls [40]. Although our assay is based on the recognition of *Salmonella typhimurium* flagellin which spans the two well conserved N- and C-terminal domains of flagellin [41] enabling us the detection of broader range of flagellin antibodies, additional studies are needed to determine the antibody specificity. Furthermore, our results deserve future studies of adaptive flagellin immune response in a larger cohort of HIV-1-infected individuals.

In summary, the novelty of our findings is that the bacterial products and HMGB1 form active complexes which can efficiently not only create a proinflammatory milieu but also directly trigger viral replication in infected cells. This synergistic effect may require lower levels of the interacting substances when present in complexes, as suggested by others [19]. We also report that flagellin has to be considered as a microbial product that can contribute to the immune activation during the HIV-1 infection. The formation of HMGB1/TLR ligand complexes has direct implications on immune activation, particularly in late stage of disease, where cell destruction and necrosis are dominant phenomena due to CD4+ T-cell loss, opportunistic infections, and other pathological conditions [27].

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