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General issues on microbial translocation in HIV-infected patients

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Abstract. **The lumen of the gastrointestinal tract is home to an enormous quantity of different bacterial species that thrive in an often symbiotic relationship with the host. It is the principal source of microbial products because of its massive bacterial load.**

Injury to the immune component of the gastrointestinal mucosal surface, along with damage to the intestinal epithelial microenvironment with its antimicrobial functions, may affect systemic immune activation during the chronic phase of HIV infection through the increased translocation of luminal microbial products. Moreover, microbial translocation, which is defined as "the passage of both viable and nonviable microbes and microbial products such as endotoxin across anatomically intact intestinal barrier", may be a fundamental mechanism through which HIV accelerates progression of chronic viral hepatitis. Improvements in the tools available to microbiota research, and especially advancement of our knowledge in this area may help us in controlling the evolution of HIV disease, although population complexity and diversity between individuals make this challenging.

Key words:
Gut microbiota, Bacterial traslocation, HIV infection, HIV immunity, Lipopolisaccaride (LPS), HIV-HCV coinfection, Liver fibrosis.

Introduction

The role of gastrointestinal tract with its complex polymicrobial ecology and the interactions with internal and external environment in causing human diseases is currently considered of great interest and not fully understood. In HIV infection, where the mucosal associated lymphoid tissue damage is recognized as a key factor in the pathogenesis of disease, the alterations of epithelial intestinal barrier resulting in the translocation of microbial products to body compartments should have a role on the course of the infection. Anyway, the mechanisms by which these components interplay are not yet elucidated. The aim of this review is to summarize whether and how microbial translocation influences the natural history of HIV disease. As the issue is complex we tried to discuss this topic by considering several items. After having reviewed the framework of the normal gut with its intestinal barrier function and the immunological events during acute and chronic HIV infection, we further will focus to the different conditions involved in mucosal dysfunction in chronically HIV infected patients. Since circulating microbial products derived from the gastrointestinal tract are among the supposed causes of HIV-related systemic immune activation, we described the microbial translocation process, (the passage of luminal antigens to the inner environment) and the principal methods used to measure the different markers as indexes of microbial translocation. Then, we discussed the results of published studies on the putative role of MT markers in HIV patients at the different stages and treatment conditions and in different settings with the aim to find an existing correlation between plasma levels of MT markers and immune activation parameters. Existing data on MT and specific population such as non HIV immuno-compromised subjects, and HIV/HCV coinfected patients are reviewed in the conclusive paragraph.

Intestinal Barrier

Intestinal barrier is a function of the intestinal wall, whose task is to separate potentially harmful, luminal contents, from the strictly controlled inner environment of the human body. This ability is though to be placed mainly into two different

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structures: the immunological barrier and the anatomical barrier[1]. The latter is located in the intestinal epithelium, which is a single layer of cells covered by mucous, immunoglobulin A and glycocalyx. To go through to it, two different routes can be followed: the transcellular with specific membrane pumps and channels and the paracellular route controlled by tight junctions. The latter, consisting of a complex of many different proteins, seal each other to intestinal epithelial cells tight adjusting themselves according to physiological needs. The anatomical barrier separates luminal antigens from the lymphoid tissue lying in the lamina propria (immunological barrier). The immunological barrier consists of the complex network of lymphoid and non-lymphoid cells and humoral factors associated with the gut where the vast majority of mucosa-associated lymphoid tissue is located. Dendridic cells, macrophages, granulocytes, mast cells, B and T cells and most bodily CD4+CD25+ cells reside in these tissues. The small intestine also hosts clusters of lymphoid tissue (Peyer's patches) covered by highly specialized epithelial cells (M cells) through which antigens reach lymphocytes. From the functional point of view, these are considered, the inductive sites of intestinal immune system, i.e. where mucosal antigens are collected and immune response induced[2]. On the other hand, epithelial cells and lamina propria associated lymphoid cells (intraepithelial lymphocytes mostly CD8+ T cells and B-cells and plasma cells) are the effector site that is where adaptive immune response is exerted. Beyond the barrier function, this extremely complex network of cells is also actively involved in the generation, on the host side, of a phenomenon called "oral tolerance". This can be described as the ability of the intestinal tissue to maintain a low level of immune activation in the presence of overwhelming exposure to environmental antigens. The other side of the homeostasis of this mechanism is represented by luminal microorganism. Actually, the gut contains a large community of microorganisms most of which come from the domain bacteria, but there are some eukaryotes, viruses and bacteriophages too[3]. Among bacteria some species permanently inhabit the gut and are acquired primarily during the first years of life. Other species temporarily colonize the gastrointestinal tract and are continuously ingested from the external environment. This bulky mass of bacteria, called microbiota, encompasses more than 500 different species, with 30-40 species representing 99% of the total[4]. Microbiota is in a symbiotic relationship with its host and to some extent specific to every single host[5]. Among the many different interactions between microbiota and its host, intestinal bacteria play an essential role in the development of the immune system[6]. Exposure of intestinal epithelia to symbiotic bacteria elicits immunoregulatory pathways and down regulation of inflammatory response[7]. Besides these two barriers, a third one (biological barrier) constituted by a broad spectrum of antimicrobial molecule (such as alfa and beta defensin) is produced in the mucosal environment and plays a primary role against pathogens[8].

Up to the beginning of the nineteen, the scientific community defined bacterial translocation as "the passage of viable bacteria through the epithelia mucosa into the lamina propria and to the mesenteric lymph nodes, and possibly other tissues" (Figure 1)[9]. In a seminal paper, published in October 1990 on Annals of Surgery, Ash et al[10] showed that in burned guinea pigs not only can the whole bacteria/fungi passage through the intestinal barrier can be highly increased, but also bacterial products can reach lymphoid tissue through morphologically intact enterocytes with a mechanism which is different from classical phagocytosis and exocytosis. This process was called "microbial translocation" and is defined as "the passage of both viable and nonviable microbes and microbial products such as endotoxins across the anatomically intact intestinal barrier". While bacterial translocation was a well known phenomenon increased by systemic pathologic conditions such as hemorrhagic shock[11], antibiotic therapy[12], burns[13] and intestinal obstruction[14], causes and mechanisms of bacterial products whose passage through the intestinal barrier are still under debate.

HIV Immunity

HIV infection is marked by an acute mononucleosis-like initial phase with high levels of viremia and low CD4+ T cells, followed by an apparently asymptomatic period of variable length. Eventually a clinically evident immunodeficiency occurs. At the onset of HIV pandemic, HIV related immune-deficiency was considered to be caused by chronic uncontrolled replication of the virus, which was supposed to be highly cytopatic. Subsequently, studies on SIV infected primates showed that lentivirus was not always pathogenic even in the presence of high levels of viremia[15]. Furthermore, on elite controllers (chronically HIV-infected individuals which are

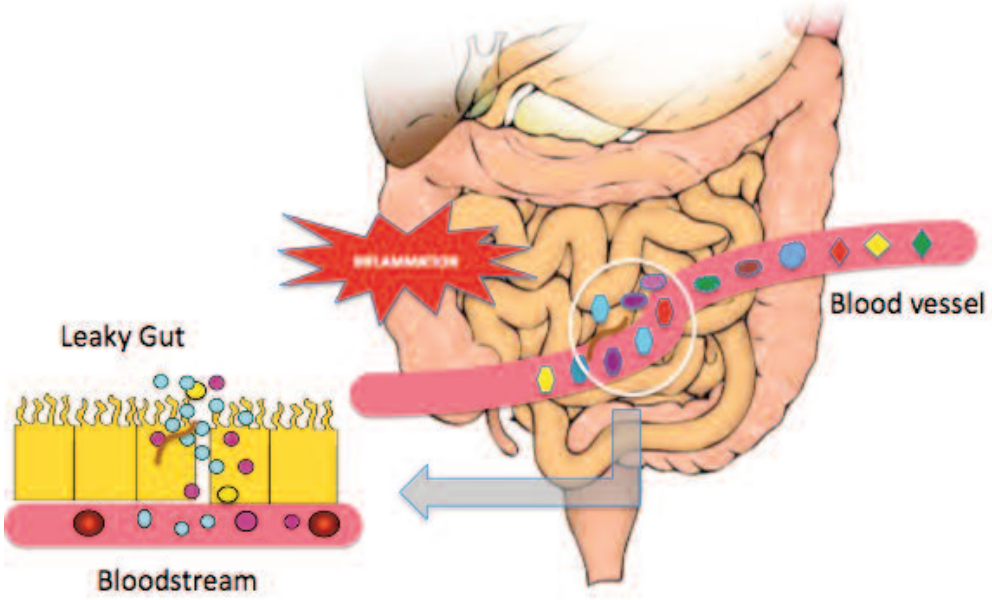

Figure 1. Microbiota translocation occurs as the result of an internal cause, such as impaired microvilli function. Damaged microvilli limit intestinal motility in the gut, promoting the translocation of bacteria from the colon to the small intestine, causing small intestine bacterial overgrowth.

capable of maintaining clinically undetectable plasma HIV RNA levels (< 75 copies/mL) in the absence of antiretroviral medications)[16] it was established that, even in subjects with an undetectable plasma viral load there is a progressive loss of CD4+ T cells. At the moment the cellular and molecular basis of HIV related immune-deficiency still remain unclear and only recently more light has been shed on the problem of HIV disease pathogenesis. First it has been cleared that the highest rate of CD4+ T cell destruction by the virus occurs during acute infection[17] and takes place mainly on CD4+ T activated cell belonging to gut associated lymphoid tissues[18] (GALT). In physiological conditions, in fact, constant exposure to luminal antigens, induces on a large proportion of GALT associated CD4+ T cells the expression of chemokine receptor 5 (CCR5). CCR5 is a principal co-receptor that enables HIV entry as the first step for viral replication. Accordingly, during acute HIV infection, the primary target of HIV replication are those cells endowed in the gut mucosa. As a consequence a massive depletion of mucosal CD4+ T cells occurs which is much deeper than in lymphoid tissue and peripheral blood[19] and is probably due not only to direct viral infection, but also to host derived cytotoxic responses and activation-induced apoptosis[20].

Recent findings suggest that in addition to viral replication other factors could drive immune activation, in fact which is probably the leading cause of the immune impairment[21]. Immune activation in HIV infection is characterized by the increased turnover of T cells, monocytes and natural killer (NK) cells which express markers of activation, proliferation and apoptosis[22]. Furthermore, a high serum level of proinflammatory cytokines and chemokines is registered[23]. Several different factors influence immune activation in HIV. The virus itself elicits both adaptive T and B cells mediated immune response and innate immune system reaction through receptors such as Toll like receptors. But this is not the only factor, because complete viral suppression through HAART does not normalize T cell activation[24]. Even elite controllers show some evidence of immune activation[16]. Opportunistic infections and occasional pathogens can contribute to immune activation. Herpes zoster and genitalis reactivation, both common in HIV infected patients, elicit a strong T cell proliferation at mucosal level[25]. Moreover, many literature reports have suggested an association between immune activation and microbial translocation secondary to mucosal dysfunction due to CD4+ T cells depletion in acute and chronic infection. Immune activation correlates steadily with disease progression in

chronically HIV-infected patients[26]. Several studies have identified that the association between immune activation, metabolic disorders, traditional risk factors (such as obesity, tobacco use,and genetic predisposition) and HIV and ART-specific contributors may drive to an increased risk of comorbidities, such as ischemic heart disease, bone frailty and chronic kidney disease[27].

Mucosal Dysfunction and Microbial Translocation in HIV Infected Patients

Several different conditions are involved in mucosal dysfunction in chronical HIV infected patients. First of all, the loss of CD4+ T cells in GALT cannot be completely restored even after a suppressive highly active antiretroviral therapy (HAART) during acute infection[28]. Depletion persists through the whole course of HIV infection without antiretroviral treatment, but even on HAART Dandekar et al[29] showed a marked delay in CD4+ T cells recovery in GALT compared to peripheral blood. This can be a consequence of the role played by intestinal mucosa as a key viral reservoir even in patients on HAART with undetectable peripheral viral load for long time. Indeed in their GALT the frequency of cells carrying HIV proviral DNA is higher than in PBMC, suggesting that even low numbers of CD4 T cells can support HIV replication in the intestinal compartment[30,31]. Secondly, among different CD4+ T cell subsets involved in GALT depletion, Th17 are the most one. Opposite to classical Th-1 IFNα producing and Th-2 interleukin-4 (IL-4) producing cells, the recently discovered new lineage Th-17, a subset of T cells separate from Th-1 and Th-2, produce IL-17, IL-22, IL-21, GM-CSF and potentially TNF-α and IL-6. IL-17 and IL-22 promote recruitment of neutrophils and proliferation of enterocytes, thus, playing a critical role in immunity against extracellular bacteria and fungi and in enterocytes homeostasis[32]. Brenchley et al[33] reported that Th17 cells are preferentially diminished compared with Th1 cells in the GI tracts of HIV-infected patients. During HAART, an effective CD4+ T cell restoration (> 50% of normal burden in gut) can be achieved only when enhanced Th17 CD4 + T-cell accumulation occurs[34]. In a group of long term treated patients (median no detectable HIV-RNA 6.7 years range 48-129 months) the size of the sigmoid provirus reservoir was inversely correlated with the sigmoid Th17 frequencies[35]. More recently, the problem of GALT restoration has been focused on by Ciccone et al. They found that after prolonged HAART in terminal ileum and colon only the absolute number of CD4+ T cell can normalize even in patients with CD4+ nadir < 250/mmc, but as stated also by Mehandru et al[28], never normalize the percentage of CD4+ in the intestinal compartment. On the other hand, due to persistence of elevated levels of CD8+ T cell, percentage of CD4+ in this group of patients remained consistently lower than in HIV-uninfected controls[36]. Thirdly, HIV infection induces enterocytes apoptosis and impairment of mucosal repair mechanisms[37]. Finally, biopsies of patients with HIV infections demonstrate crypt hyperplasia, blunted villi and inflammatory infiltrates[38]. According to some authors the final results of this complex array of injuries could be a leaky gut which allows passage of luminal antigens to the inner environment. This process, called microbial translocation, can have some correlation with pathogenic mechanism of AIDS.

In 2006, Brenchley et al[39] published the seminal paper on microbial translocation in HIV/SIV infection. In their study the authors highlighted how microbial products derived from the gastrointestinal tract are among the causes of immune activation documented in HIV infected patients. Among microbial products they measured lipoplysaccaride (LPS) in the blood of chronically HIV infected subjects and SIV infected rhesus-macaques. In both groups of samples, LPS plasma levels were increased and significantly related to immune activation. Enteric pathology has been a defeating problem in AIDS patients since the discovery of HIV as the causative agent of AIDS. In that very year (1984) Kolter et al[40] published data concerning histologic abnormalities in the gut mucosa characterized by lymphocyte depletion. Subsequently, the hypothesis of a relation between the well-known gastrointestinal damage, microbial products passage to blood and HIV progression, had been proposed only by Stein et al[41] in a paper published in 1997 on Cytokine. In their paper the authors hypothesized for the first time that microbial translocation due to altered integrity of the gut wall can occur in AIDS patients. They measured it using butyric acid in urine as a marker; furthermore they hypothesized that microbial translocation could induce a stress/inflammatory response which was measured through Il-6 urinary levels and that this response would play a pivotal role in weight loss during the wasting syndrome. Among 35 patients

in different HIV staging, they found the highest level of butyrate and IL-6, in AIDS subjects with lowest CD4 and significant weight loss, concluding that in this group of patients there could be a low but chronic rate of bacterial products seeping across the colonic wall.

Microbial Translocation Assays

Subsequently, other methods had been proposed to demonstrate bacterial products leakage through the gut wall and up to recent times the vast majority of studies were based on bacterial LPS measurement on plasma. Alternative methods support semi-quantitative real time PCR amplification of bacterial ribosomal DNA[42], ELISA for antiendotoxin core antigen immunoglobulin M (IgM) antibodies (EndoCab) and soluble CD14 (sCD14). All these methods show advantages but suffer from drawbacks too. The LPS measurement is based on the limulus amoebocyte lysate (LAL) assay which has been in use for more than 15 years and has a high sensitivity rate but it shows inter-assay variability[43,44]. Furthermore, as LPS is specific of gram negative bacteria, LAL do not detect gram positive bacterial products. Plasma endotoxin core antibody is consumed when endotoxin derived from Gram-negative bacteria are present in the blood, so it has the same intrinsic limits as LPS in measuring microbial translocation; moreover, EndoCab standard median-units (MU) are arbitrary and based on medians of ranges for 1000 healthy adults in a particular locality and study results should always be matched with selected healthy controls[45]. CD14 is a receptor shown to be expressed mainly on a myriad of cells as well as on monocytes and B-cells; it is a membrane-bound GPI-anchored glycosylated protein, but also exists in a soluble form in the circulation. Soluble CD14 (sCD14), is secreted by monocytes, dendridic cells and by hepatocytes too; it binds both LPS and peptoglycan of Gram positive bacteria, but its level increases in inflammatory conditions and in response to pro-inflammatory cytokines such as α-IFN and β-IFN[46]. Considered as related to CD14, is LPS-binding protein (LPB); LBP is a soluble acute-phase protein that binds to bacterial LPS to elicit immune responses by presenting LPS to CD14 and TLR4. It is involved in the acute-phase immunologic response to gram-negative bacterial infections and its plasma level has been used as an indirect index of LPS presence in blood[47] even if an accurate comparison between the two indexes has never been done. Bacterial DNA is recognised as a potent inducer of innate immune response through interaction with TLR9 and TLR[48] and it has been previously demonstrated to circulate in the blood of healthy subjects[49]. Bacterial 16 s rDNA is an ideal target for PCR amplification as it encompasses highly conserved regions among different bacterial taxa suitable to synthesize universal primers. Different methods of detection 16 s rDNA have been published in scientific literature, but the interpretation of the results and their significance into a clinical context is made difficult by the high percentage of false positive probably due to possible reagent or sample contamination.

Anti-a-galactosyl immunoglobulin G (anti-Gal) is a natural antibody present in unusually high amounts in human sera. It was found to interact with a variety of Gram negative bacterial strains, some of which isolated from normal stool too[50], and it is increased in inflammatory bowel disease[51] when an intestinal barrier damage is present.

Microbial Translocation, HIV Patients Immuno/Virological/Therapeutic Status and Immune/Response Activation

The levels of plasma LPS in acute/early HIV infected patients were found by Brenchley et al[39] comparable to uninfcted controls; on the other hand AIDS patients showed the highest with chronically infected intermediate levels of LPS. Moreover, they found a decrease in plasma LPS after HAART treatment and an association between CD4+ T recovery and LPS reduction. Similar data were produced by Marchetti et al[52] who demonstrated significantly lower levels of LPS in treated patients compared to advanced naïve ones; among those treated with comparable HIV-viremia suppression immunologically non responders showed higher level of plasma LPS compared to full responders. Among these patients 14 randomly selected samples were tested using 16s rDNA broad range primers: five out of seven immunologically non responders and seven out of seven full responders gave negative PCR products. These results are consistent with low sensitivity of the PCR method used. Jiang et al[53] showed that blood level of bacterial DNA, as determined by quantitative PCR on 16S rDNA fragments, was significantly higher in HIV-infected patients than in uninfected controls. Moreover, bacterial DNA in blood was shown to decrease after antiretroviral treatment. But they found no 16S rDNA in the blood from healthy subjects

which was the opposite to international studies on this topics[49,54] and probably their method suffered from low PCR sensitivity. In a study conducted by our group using both relative and absolute quantification, we determined rDNA levels in plasma of 20 HIV subjects using eubacterial broad range PCR primers widely used for this purpose[55]. We found no correlation between rDNA plasma levels and HIV staging. Subsequently, PCR products were sequenced through pyrosequencing, determining that part of the amplified rDNA was of human origin due to lack of specificity of the primers (unpublished data). Recently, Kamski et al[56] proposed a novel real-time PCR method to quantify 16s rDNA using a shrimp nuclease to purify samples from exogenous DNA; in their work they found a strong correlation between LPS and 16s rDNA levels. Among patients enrolled in the SMART study[57], those who died, whose samples were available were analysed for microbial translocation markers in comparison both with matched HIV seropositive controls. LPS, 16rDNA, sCD14 and EndoCAb merely measured in all enrolled subjects[58]. Baseline sCD14 resulted significantly higher in subjects who died compared to matched controls ($p < 0.001$). In comparison with unmatched healthy subject LPS resulted higher in SMART patients, EndoCAb lower, while the concentration 16s rDNA was equal. If any of these values reached the statistical significance was not reported. Even if the authors use the same method of Jiang et al[53] to quantitate 16s rDNA they state they found "no differences in 16s rDNA between subjects and healthy volunteers". In a study by Chege et al[35], LPS, EndoCab and sCD14 were determined among three different group of patients: HIV positive antiretroviral naïve subjects (n=16), HIV positive on long term HAART (n=15) end healthy controls (n=10). Compared to healthy controls LPS remained elevated and blood Th17 count reduced despite HAART; EndoCAb IgM titres resulted lower in treated patients than both untreated HIV subjects and healthy controls and sCD4 was higher in untreated vs treated HIV subjects, remaining anyway steadily higher than healthy subjects even after a median of undetectability of viral load of 6.7 years. In a very interesting study microbial translocation indexes (LPS, sCD14, EndoCAb and antiGAL IgM/IgG[50]) were studied in relation to innate and adaptative immunity[59] in 96 (20 HAART naïve and 76 treated patients) and 50 healthy controls. Patients were selected among a group of otherwise healthy HIV subjects vaccinated with penumococcal vaccines. sCD14 was the only MT markers significantly higher in HIV subjects compared to healthy controls; LPS showed only a trend towards higher concentration, while EndoCAb and anti-GAL IgM/IgG levels were comparable among the two groups.

Among 107 Uganda patients with known HIV seroconversion, Redd et al[60] did not find increased levels of LPS in their plasma; moreover, they were unable to confirm any correlation between HIV disease outcome and LPS, EndoCAb and sCD14. Only LPB plasma levels showed a correlation with the speed of progression of HIV infection, with long term non progressor displaying the lowest level of LPB. HAART did not influence markers of microbial translocation among 80 South African infected patients too; but in this group Cassol et al[61] found high levels of plasma LPS in strict connection with sCD14 and tumor necrosis factor levels. Similarly, 57 female sex workers from the Nairobi cohort showed higher LPS level than HIV negative matched controls, independently from antiretroviral treatment e/o plasma viral load[62]. No difference in LPS were detected between 44 HIV suppressed (HIV-RNA < 50 copies/ml) and 10 uninfected controls, but when HIV pts were stratified according to residual viremia, only fully suppressed patients (less than 2.5 copie/ml of HIV), had LPS values comparable with HIV sero-negative. The same patients were longitudinally monitored under structured treatment interruptions, but no significant impact on LPS plasma levels, checked after 15 days of interruption, were detected[63]. Twelve weeks of treatment interruptions are needed to 41 HIV infected chronically suppressed subjects, to exhibit significantly increased LPS levels compared to the baseline. In the same study[64] a negative association between plasma HIV-RNA and LPS levels and EndoCAb changes was observed.

In sharp contrast with a previous study published by the same group[52], in which, among HIV patients in different staging, marked differences in LPS plasma levels were detected, is the paper of Merlini et al[65]. As previously, they investigated either LPS, sCD14 and rDNA levels in plasma of two groups of patients with low CD4+ T cell nadir divided into two groups according to their immunological response after 12 months on HAART. Compared to HIV sero-negative controls, they found a steady state in the LPS level both at the baseline and after 12 months of anti-

retroviral therapy. On the other hand, immunologically non responders presented increased circulating 16s rDNA levels; in this case too, in 30/44 at the baseline and in 37/44 at T12 no 16s rDNA was detected in the blood, even if a shorter amplicon (300 bp) compared to the previous study, had been considered. Furthermore, in a study conducted among patients of the ICONA cohort LPS was found as the only biomarker associated with the disease progression[66]. Finally, in a recently published paper by Marchetti et al[67] the authors concluded that microbial translocation has been shown to be a clinically significant event, as it is associated with HIV clinical progression, suboptimal CD4 T-cell recovery on ART, and the early onset of non-AIDS comorbidities such as liver disease progression, atherosclerosis and cardiovascular disease, and neurocognitive impairment. Recently, some papers have tried to define the effects on microbial translocation (MT) and enterocyte damage of different antiretroviral therapy (ART) regimens and their capacity of promotes HIV replication. As a marker of enterocyte damage, the authors tested the usefulness of flagellin and the kinetics of antiflagellin antibodies levels during the chronic phase of infection. They found that markers of MT and enterocyte damage are elevated in untreated HIV-1 infected patients and long-term ART reduces their levels and that flagellin is an important microbial product, modulating viral replication and induces adaptive immune responses *in vivo*[68,69].

As previously reported in this paper, a HIV subject normally presents a pattern of immune activation (Figure 2). Trying to demonstrate that this pattern is at least partly related to microbial translocation has not always been possible. Brenchley et al[39] succeeded in proving that there is a correlation between LPS plasma levels and IFN-a and activated CD8+ with CD38+HLA-DR+ phenotype. Ki67 is a nuclear marker of proliferating cells; a positive correlation between both peripheral blood CD4+ and CD8+ T cells expressing the Ki67 pattern with LPS was proven by Marchetti et al[52] but confirmed only for Ki67+CD4+ T cells in blood and gut tissues by Ciccone et al. On the other hand, the percentage of cycling Ki67+CD8+ T cells was unrelated to LPS plasma concentration[36]. Among patients enrolled in the SMART study[58], the highest quartile of sCD14 plasma concentration resulted as the only predictor of mortality among studied patients. Subjects whose sCD14 plasma level resulted in this quartile, have 6-fold increased rate of death compared to the other ones. In the previously quoted papers by Chege et al[35], LPS, EndoCab and sCD14 were also studied in comparison either with kinetics of both peripheral blood and tissue Th17 and with immune activation. As markers of immune activation, expression of CD3, CD8, CD4, CD69, HLADR, FoxP3, IFNγ and IL-17A was studied on blood and sigmoid mononuclear cells. All the three indexes of microbial translocation did not result in a statistically significant correlation with any immune activation markers. High-mobility group protein 1 (HMGB-1) is a protein secreted by activated macrophages and monocytes as a mediator of in-

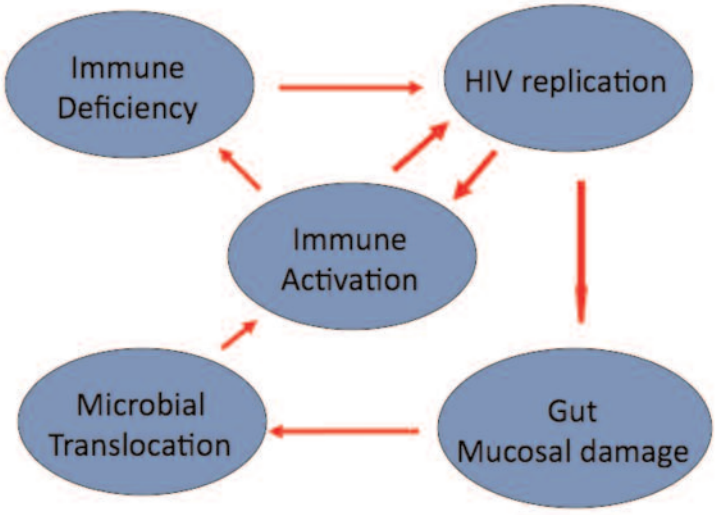

Figure 2. Supposed mechanism leading to immune-activation linking gut damage and HIV infection.

flammation through interaction with TLR4[70,71]. This protein and LPS were found higher in 42 naïve HIV subjects compared to unmatched 19 healthy controls. After two years of successful HAART, plasma levels of both HGMB1 and LBS resulted decreased by 35% and 36% respectively although not yet normalized. Both markers were correlated neither to each other nor to CD4+ T, CD8+ T cell count or to a viral load[73]. In a very interesting study microbial translocation indexes (LPS, sCD14, EndoCAb and antiGAL IgM/IgG) were studied in relation to innate and adaptive immunity[60] in 96 (20 HAART naïve and 76 treated patients) and 50 healthy controls. Patients were selected among a group of otherwise healthy HIV subjects vaccinated with penumococcal vaccines. sCD14 was the only MT markers significantly higher in HIV patients compared to healthy controls; LPS showed only a trend towards higher concentration, while EndoCAb and anti-GAL IgM/IgG levels were comparable among the two groups. No association was detected among HIV-RNA, current or nadir CD4+ T cell count, treatment status, studied pro-inflammatory cytokines and all the MT markers. On the other hand, LPS concentration resulted in an inversed independent predictor IgG antibody response to a scheduled vaccination.

Immune tolerance to overwhelming inflammatory stimuli is a very well known phenomenon during sepsis[72]. As TLR-4 is a major receptor of LPS to induce a pro-inflammatory response, becausethe highest levels of LPS are related to a reduced expression of TLR4 mRNA in HIV infected Nairobi sex workers[62], to some extent a chronic tolerance can be hypothesized in this setting. On the contrary, to this tolerance, HIV is a strong inducer of pro-inflammatory signaling of TLR4; the consequences of this double effect on the TLR4 expression are unknown, but at least an altered innate immune response to the HIV infection can be expected. Decreasing levels of circulating innate cytokine have been reported in African patients during disease progression and these levels do not exhibit correlation with markers of microbial translocation. In particular TNF-α, lower than in Western controls at the baseline, significantly increased in standard and rapid progressors compared to LTNP[60]. TNF-α, remained persistently elevated even after 12 months of successful treatment in eighty African HIV-1 infected patients; in this group no correlation was detected between LPS and TNF-α, at the baseline, but only in patients receiving HAART[61]. Impaired innate immunity was also detected by Nowroozalizadeh et al[73] in a group of both HIV-1 and HIV-2 infected patients from Guinea Bissau. Compared to HIV negative controls from the same area, impaired expression of IL-12 after stimulation of TLR7/8 and TLR9 was revealed in whole blood; the impairment was in inverse correlation with the LPS concentration. It would be interesting to check responsiveness of TLR4 too, as it is directly stimulated by the complex LPS/LPB/sCD14[48]. In the same paper, compared to negative controls, significantly higher levels of LPS, inversely correlated with CD4+ T cell count and directly with plasma viral load, were noted both in HIV-1 and HIV-2 infected patients.

Microbial Translocation in Special Population

Idiopathic CD4 lymphocytopenia (ICL) is a rare syndrome defined as a clinical condition characterized by a repeated control of peripheral CD4 T lymphocyte counts < 300 cells/μL or < 20% of total lymphocytes in the absence HIV-1 or HIV-2 infections or other known causes of immunodeficiency[74]. Among 11 ICL subject, 10 HIV-1 naïve patients and 8 healthy controls LPS and sCD14 were measured. ICL and HIV-1 positive subjects showed increased levels of both microbial translocation markers, even if only LPS were statistically significant[75]. Moreover, LPS plasma concentration were inversely related with cell proliferation, measured as naïve CD4+CD45RO-CD27+ peripheral blood count. On the other hand, no relation between Th17CD4+ T cell functionality and any of the MT indexes studied was detected. In two groups of HIV/HCV co-infected patients and HCV mono-infected patients whose history and outcome is well known, LPS, LPB, sCD14 and EndoCAb were studied. In mono-infected patients, LPS resulted associated with older age, female sex but not with ethnicity or alcohol use or with HCV sero-conversion. HIV patients tested had higher levels of LPS plasma levels compared to HIV uninfected subjects and there was an inverse correlation with CD4+ T cell count[76]. Furthermore, liver disease progression was found related to lymphocytes count and LPS, showing patients in the upper quartile of LPS blood levels having liver disease progression 19-fold more often (95% CI: 2.98-120.79, $p = 0.0018$).

In a cohort of AIDS patients with high frequencies of intra venous drug abuse and HCV co-infection, high levels of LPS and LPB were

found to be associated with HIV related dementia and with minor cognitive motor disorders independently of HIV-RNA and CD4+ T cell count[77].

"Elite controllers" are HIV infected patient with undetectable viral load without having any antiretroviral treatment. The existing data on this group of patients are obviously limited by the small number of the case series. In a study of Hunt et al[16], published in 2008, elite controllers have higher blood LPS and CD4+ and CD8+ T cell activation levels, when compared to healthy subjects and LPS concentration was directly related to CD8+ activation levels. Furthermore a higher CD8+ activation level was inversely related with CD4+ absolute count suggesting that immune activation plays a critical role in promoting HIV disease progression independently of HIV replication. More recently, Moreno et al[78] reported the results obtained on 126 plasma samples from 18 elite controllers patients showing that there is a good correlation in the quantification of LPS, sCD14, and LBP levels, but not with bacterial 16S rDNA, and did not exists any significant association between these markers of microbial translocation and immune activation.

The mechanisms of accelerated liver fibrosis in HIV-1/HCV coinfection are complex and their relationship with MT are not yet well understood. Products of microbial translocation may promote liver fibrosis either by direct interaction with Kupffer cells and hepatic stellate cells (HSCs) or indirectly via induction of systemic immune activation and activation-induced apoptotic cell death. According to this model, Sandler et al. described high density of CD14+CD68+ in the liver correlating with hepatic disease progression in HIV-1/HCV coinfection, strongly suggestive for LPS-driven activation of hepatic Kupffer cells[58] although it is possible that microbial translocation is a result of liver disease progression and not a cause. Kupffer cells bind LPS and other gut-derived microbial products and shunting of portal blood past the liver is a well known consequence of cirrhosis and could explain the elevated levels of plasma LPS[76] HIV-1 enteropathy is associated with increased microbial translocation and systemic immune activation. Mechanisms that underlie increased microbial translocation include direct effects of HIV-1 infection on the epithelial barrier function and alteration in intestinal permeability secondary to inflammatory cytokines and CD4 T cell depletion. Risk of liver fibrosis is increased in HIV-1/HCV coinfection and associated with reduced CD4 T cell counts and raised lipopolysaccharide levels and/or depletion of hepatic Kupffer cells (Figure 3)[79].

Conclusions

To understand the significance of the data discussed above we have to consider that there are some doubts about the method used to determine LPS concentration. All these kits are not intended for the detection of endotoxin in clinical samples or as an aid in the diagnosis of human disease[80]. Also the results of home made PCR amplifica-

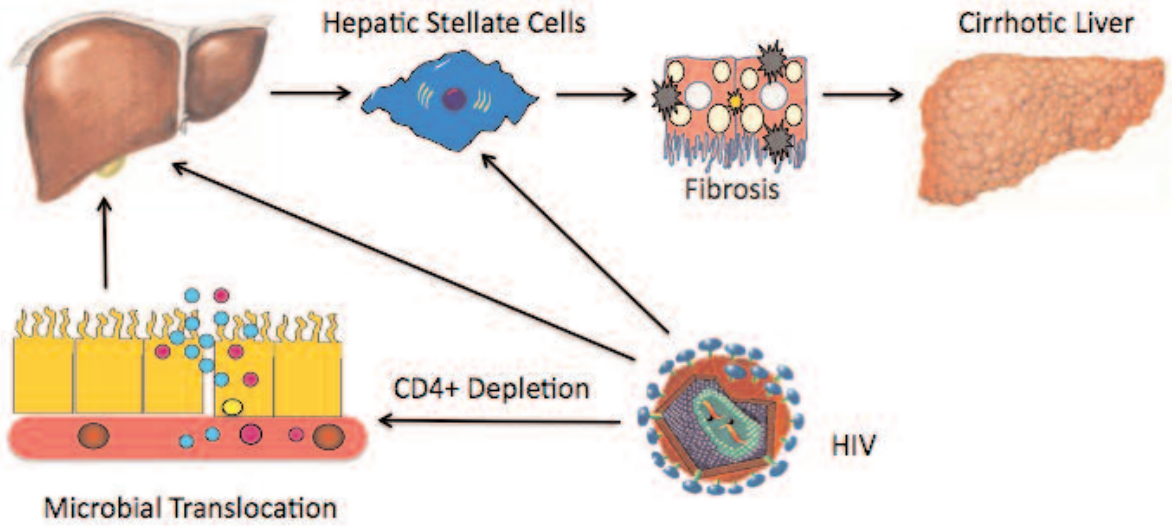

Figure 3. Products of microbial translocation may promote liver fibrosis either by direct interaction with Kupffer cells and hepatic stellate cells (HSCs) or indirectly via induction of systemic immune activation and activation-induced apoptotic cell death.

tion based methods are to be considered erratic. The intrinsic variability of LPS tests can partly explain discrepancy among results obtained in patients in apparently matching HIV conditions. LPS and 16s rDNA are the only two MT related indexes of direct microbial provenance; the others MT indexes as sCD14, EndoCAb and LPB are of bodily origin; that is they are produced in response to exogenous stimulation of both innate and adaptive immunity. Soluble CD14, a specific co-receptor of LPS when tested through a more consistent kit, is probably a better index for microbial translocation. On the other hand, sCD14 plasma levels could be increased by exogenous interferons[46] but also a direct *in vitro* stimulation of monocyte by GP120 can increase its release[81]. EndoCab test was originally devised to screen blood donor plasma for high-titer antibodies to endotoxin core. Healthy subjects show a steady state of these antibodies but with extremely high inter-subject variability. It is only during acute events (sepsis, surgical interventions) that their level can be perturbed: initially significantly reduced and afterward increased. So far, we don't have a reliable method to measure MT products.

Anyway, the most of published data suggest that microbial products derived from the gastrointestinal tract are among the causes of immune activation documented in HIV infected patients. Among microbial products LPS plasma levels were increased and significantly related to immune activation in patients with HIV disease and decreased after HAART treatment. Also sCD14 was a MT marker significantly higher in HIV subjects compared to healthy controls.

Significantly lower levels of LPS have been found in treated patients compared to advanced naïve ones; among those treated with comparable HIV-viremia suppression immunologically non responders showed a higher level of plasma LPS compared to full responders.

The same conclusion has been suggested by the systematic review of Vassallo et al[82] which found that exisisting published data confirms that LPS is a marker of microbial translocation, responsible for chronic immune activation in HIV-infected patients. Even in successfully treated patients, LPS values are rarely normal. Several studies suggest a role for LPS as a negative predictive marker of immune restoration in patients with blunted CD4 T cell gain.

In patients with HIV/HCV coinfection, liver disease progression was found related to lymphocytes counts and LPS, showing that patients in the upper quartile of LPS blood levels have liver disease progression 19-fold more often.

The methods to quantify LPS have not been sufficiently standardized to overcome the interpersonal variability, so further research is needed to assess the role of MT in the course of HIV disease.

Conflict of interest

The Authors declare that there are no conflicts of interest.

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