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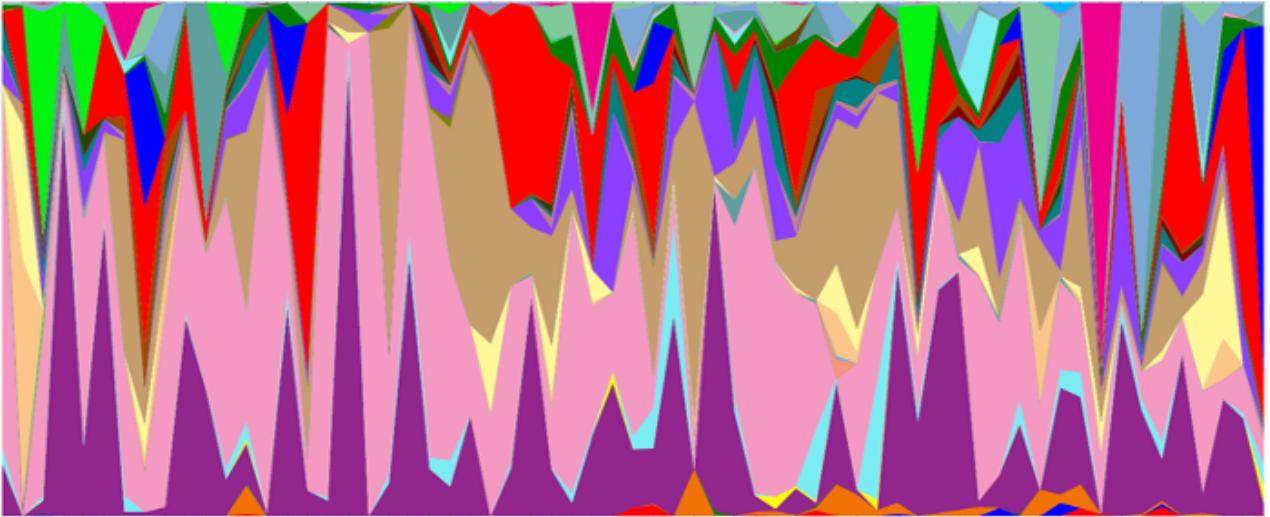
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The Gut Microbiome, Mucosal Immunity and Disease Pathogenesis in Humans with HIV or Idiopathic CD4 Lymphocytopenia and in non-Human Primates with SIV



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Chapter 1

General introduction

The acquired immune deficiency syndrome: an overview

In 1981, the medical community in the United States observed an increasing number of men who have sex with men developing unusual opportunistic infections [1]. In 1982 the Centers for Disease Control and Prevention (CDC) used the term acquired immune deficiency syndrome (AIDS) to describe the disease for the first time, and increasing numbers of AIDS cases were also reported from other countries [2]. In 1983, a retrovirus was isolated and identified as the primary cause of AIDS which subsequently received the name human immunodeficiency virus type 1 (HIV-1) [3]. Since the start of the epidemic, 75.7 million people have become infected with HIV and 32.7 million people have died worldwide [4].

HIV spreads by sexual, percutaneous and perinatal routes. However, the majority of adults acquires HIV following exposure at mucosal surfaces, as a primarily sexually transmitted infection [5, 6]. HIV preferentially infects CD4⁺ T-lymphocytes. Specifically, the envelop protein of the virus binds to the CD4 receptor, which induces a conformation change supporting the interaction with either the chemokine receptor type 5-(CCR5) or C-X-C chemokine receptor type 4 (CXCR4) co-receptor, and finally the virion fuses with the CD4 cell membrane allowing the virus to enter the cell. Once inside the cell, viral RNA is released, reverse transcribed through the action of the virally encoded reverse transcriptase into HIV-DNA, and subsequently integrated into the host cell DNA by way of the virally-encoded integrase enzyme. Once encoded into the host cell's DNA, de novo HIV RNA and HIV proteins can be generated, which assemble into novel immature viral particles, which when exiting the CD4⁺ T cell through the action of the virally-encoded protease mature into infectious virions. One of the major reservoirs of CD4⁺ T cells in the body is the

gastrointestinal tract, which represents the primary target for HIV infection [7, 8]. The following paragraphs describe the pathological changes triggered by HIV in the GI tract in more detail.

Impact of HIV on mucosal immune cells and epithelial integrity in the gut

The GI tract is a large interface that is in contact with the external environment, aiding the function of food processing and providing selective mechanical and immune barriers that prevent translocation of harmful substances, as well as of pathogens and their products, from the lumen into the blood stream [9, 10]. The mechanical barrier is comprised of a continuous single layer of intestinal epithelial cells (IECs), sealed together by tight junction (TJ) proteins including e-cadherin and claudins [11]. Moreover, the mucus layer of the gut separates the host from luminal bacteria through the action of antimicrobial peptides such as defensins, while also providing a nutrient-rich source to promote commensal bacteria colonization [12]. On the other hand, secreted immunoglobulin A (IgA) by plasma cells in the lamina propria, macrophages, neutrophils, natural killer cells (NK), T-cells, Peyer's patches, and mesenteric lymph nodes create an immune barrier with specialized lymphocyte subsets residing within GI tract tissues that are further contributing to maintain gut barrier integrity [9, 13]. Epithelial cells within the GI tract express receptors for the cytokines IL-17 and IL-22. CD4⁺ T cells, CD8⁺ T cells, and innate lymphoid subsets residing within the lamina propria of the gut are important sources of these cytokines, and they promote gut integrity through induction of epithelial cell proliferation and inducing expression of claudins, defensins, and mucin [9, 12, 14].

During the natural history of HIV infection and SIV infection the dysfunction of the GI tract is associated with alterations in number and function of resident leukocytes. In line with this, starting from the acute phase of the infection, high levels of viral replication occur in the gut associated lymphoid tissue (GALT), where the majority of gut-resident CD4⁺ T cells possess an effector memory phenotype and express high levels of the HIV co-receptor CCR5 [15]. These gut-resident T cells are lost disproportionately when compared to CD4⁺ T cells in peripheral blood and lymphoid tissues of humans with acute HIV infection and rhesus macaques with acute SIV infection [16, 17]. Despite the profound loss of CD4⁺ T cells in gut tissues being a salient feature of the HIV disease course, these events alone do not fully explain severe dysfunction of the GI tract in PWH and ultimately disease progression due to sustained systemic inflammation. Indeed, during non-pathogenic SIV infection of natural primates, such as African green monkeys (AGMs) and sooty mangabeys (SMs), moderate CD4⁺ T-cell depletion occurs, but this does not result in disease progression [17]. Similarly to HIV-infection, people with idiopathic CD4⁺ lymphopenia (ICL), a syndrome defined by low CD4⁺ T-cell count (<300/ μ L) and an increased susceptibility to opportunistic infections, including with *Cryptococcus neoformans* and non-tuberculous mycobacteria, also exhibit very low numbers of CD4⁺ T-cells in the GI tract, but without evidence of systemic inflammation [18].

Taken together, these data suggest that there are additional pathophysiological phenomena that must contribute to HIV pathogenesis, and alterations in the epithelial integrity have been proposed to play a crucial role. Specifically, T-helper type 17 (Th17) and T-helper type 22 (Th22) cells, a predominant source of the cytokines IL-17 and IL-22 which promote gut integrity, are preferentially lost from the overall CD4⁺ T-cell pool of the GI tract and mesenteric lymph nodes within weeks after infection, and this remain the case throughout the disease course [19, 20].

Similarly, other groups of leukocytes capable of producing IL-17 and IL-22 in the gut show functional defects during HIV and progressive SIV infection. Innate lymphoid cells (ILCs), particularly ILC3s, a subset that provides an innate source of IL-17, are depleted in gut tissues of SIV-infected rhesus macaques very early in the disease course and remain so throughout its chronic phase [21]. IL-17-expressing CD8⁺ T cells, referred to as Tc17 cells, are also depleted in SIV-infected rhesus macaques in end-stage disease. IL-17-producing CD8⁺ mucosal-associated invariant T (MAIT) cells are irreversibly lost in SIV/HIV-infected subjects [22]. MAIT cells are a subset of unconventional, innate-like T cells, expressing a semi-invariant T cell receptor (TCR) and exert important innate-like antimicrobial activity through the recognition of the antigens in complex with the MHC-Ib-related protein (MR1) [23]. In parallel to a reduction in cell populations secreting enterocyte p-promoting factors, IL-17 and IL-22, increased concentrations of proinflammatory cytokines such as TNF- α , β -chemokines, IL-6, IL-10, and IFN- γ enhance enterocyte apoptosis in PWH and pathogenic SIV infection of rhesus macaques [24]. As a result, the altered intestinal microenvironment leads to decreased gastrointestinal epithelial barrier integrity in HIV infection and progressive SIV infection [25, 26]. The specific functional consequences of this phenomenon are further elucidated in the next paragraph.

The loss of mucosal integrity as a mechanism of HIV-associated systemic inflammation

As described above, the immunological abnormalities that occur in the gut during the HIV or SIV disease course are linked to structural damage of the GI tract in the sense of massive enterocyte apoptosis, decreased expression of tight junction proteins, and increased intestinal permeability [24]. These abnormalities result in the breakdown of mucosal integrity, thus allowing microbial

products, like lipopolysaccharide (LPS), to translocate from the lumen of the GI tract into the peripheral circulation [25]. As a consequence, the increased systemic exposure to microbial products exacerbates immune activation through monocyte subsets, which directly respond to microbial products by means of Toll-like receptors (TLRs), thus sustaining systemic inflammation [10]. In line with this, although the best characterized biomarkers of immune dysfunction in untreated HIV infection are the absolute CD4⁺ T cell count and the frequency of activated T cells, in the context of treated HIV infection, monocyte and macrophage-related inflammation seem to better predict, and potentially cause, disease progression [26].

Studies performed using animal models of disease also support a role for microbial translocation in HIV-associated inflammation. Unlike SIV-infected rhesus macaques, SIV infection of natural hosts is characterized by the absence of chronic immune activation and microbial translocation, as shown by the lack of LPS or sCD14 in the plasma of SIV-infected AGM [25, 27]. SIV-infected natural hosts generally do not progress to AIDS but live healthy life spans despite continual viral replication and high levels of plasma SIV viremia [28, 29]. In this context, the absence of microbial translocation is interestingly associated with preservation of cell subsets integral to mucosal health, including $\gamma\delta^+$ T-cells, fundamental for mucosal immunity and response to bacterial antigens, and Th17 cells, which are preferentially depleted from mucosal tissues during pathogenic HIV/SIV infections [30]. Of note, experimentally induced immune activation with LPS in natural hosts leads to increased viral replication and CD4⁺ T-cell depletion [31]. These data together support the hypothesis that lack of systemic immune activation, and of microbial translocation, might be linked to the absence of disease progression as observed in natural hosts.

In conclusion, microbial translocation due to loss of GI epithelial integrity, and consequent systemic activation of monocytes and macrophages, represents a crucial mechanism underlying residual inflammation, in the context of HIV. During physiological conditions, gut immunological homeostasis is maintained by the interaction between host immunity and intestinal microbiota [32]. Therefore, it is appealing to hypothesize that alteration in the gut microbiome and dysfunctional interactions with immune cells might be another contributing factor upstream to the local and systemic inflammation. The next paragraph elucidates this possibility in greater detail.

Gut microbiota and mucosal immunity in physiological conditions and disease

The collection of microbes colonizing the GI tract, termed the ‘gut microbiota’, is a tightly regulated community of microorganisms, mostly bacteria [33, 34]. In healthy adults, gram-negative Bacteroidetes and gram-positive Firmicutes constitute a large majority of the colonic microbiota, with Proteobacteria, Tenericutes, and Fusobacteria represented with lower abundance [35]. The gut microbiota is affected by innumerable factors including host genetics, age, gender, sexual practices, diet, ethnicity, which renders it unique to each individual [36-38]. Despite the extensive interpersonal variability in bacterial community composition, there is a shared core of functionalities for the microbiome namely supporting host homeostasis and immune processes. The immune system, and especially the mucosal immune system, has indeed developed a complex connection with its associated microbiota, and it seems that both the innate and adaptive immune systems have evolved to require microbial interactions during their development. Studies of germ-free mice have shown that the gut microbiota is required for normal generation and maturation of GALT [39, 40]. In particular, the resident microbiota regulates the development of T_H17 cells,

which preferentially accumulate in the intestine and are crucial for host defense. The absence of a Clostridia class commensal bacteria in the murine gut results in a decrease in IL-17-secreting CD4⁺ T cell subpopulations [41]. The microbiota is also required for the differentiation of ILCs and for their production of IL-22, which, in turn, promotes antimicrobial peptide production by intestinal epithelial cells [42, 43]. Microbial communities enriched in riboflavin-synthesizing bacteria regulate the development of MAIT cells and their long-term frequencies within tissues [44]. Further, commensals can promote gut homeostasis through the release of microbial products, including metabolites such as short-chain fatty acids (SCFA), which are a source of energy for the enterocytes and have important immunomodulatory effects [45].

Alteration in the abundances of these microbes has been linked to a number of inflammatory and metabolic disorders including inflammatory bowel disease (IBD), cardiovascular disease, cancer, obesity, diabetes and HIV infection [46-48]. Specific to HIV, it has been proposed that alterations in immune and epithelial cells, including Paneth cells, macrophages, epithelial cells, and T_H17 cells, due to the infection might impact on microbiota homeostasis [24, 49]. In turn, given the above-mentioned role of the gut microbiome in supporting tissue integrity and immune homeostasis, it is not unlikely that unbalanced gut bacterial communities may play a critical role in sustaining the disruption in gut homeostasis, thus creating a vicious cycle which further contributes to HIV-associated immune activation and systemic inflammation. Our understanding of which specific gut bacteria are responsible for these processes, and whether gut homeostasis-promoting or pro-inflammatory gut bacteria might be differentially abundant in PWH, has been the focus of intense research. The most relevant results thus far are summarized in the next paragraph.

The role of gut dysbiosis in HIV-associated inflammation

Over the course of the years, evidence has shown gut dysbiosis in PWH. Although the majority of studies exhibit great heterogeneity with regards to the characteristics of subjects investigated, cohort sizes, type of sampling method used, and level of bacterial taxonomic classifications reported, some common features in microbiota composition of PWH have been reported. Specifically, an overall enrichment of members of the phylum Proteobacteria, which includes potentially pathogenic bacteria (e.g. Salmonella, Yersinia, Escherichia), and pathobionts (e.g. Acinetobacter), was observed in the mucosal tissue of both untreated and treated PWH [50-52]. Increased levels of Proteobacteria members were also reported in colonic mucosa and stool of untreated and treated PWH and linked to key markers of inflammation and immune activation, including the kynurenine pathway of tryptophan metabolism, plasma concentrations of the inflammatory cytokines IL-6, sCD14, IFN- γ and TNF- α [53-55]. Concomitantly with the increased abundance in Proteobacteria, numerous studies have described a decrease in the abundance of members of the phylum Firmicutes, which comprises Gram-positive species (Staphylococci, Streptococci, Lactobacilli, Clostridia), including many with immune-regulatory properties and even some used as probiotics [56]. Decreased abundance in Firmicutes was shown to be mucosa-specific in some studies while also observed in stool samples in others, in both untreated and treated PWH [50-52, 55-58]. For instance, Lactobacillus abundance was detected in stool samples of PWH with lower peripheral blood CD4⁺ T cell count and higher viral loads compared to those individuals with higher CD4⁺ T cell counts and lower viral load [56, 59]. Members of the Firmicutes phylum are involved in the production of the SCFA butyrate as a byproduct of fiber fermentation [60]. Butyrate is a metabolite involved in the regulation of gut homeostasis as well as a source of energy for epithelial cells and a signaling molecule that modulates intestinal immune

cell responses [60]. However, not all Firmicutes members were found to be depleted in PWH, the family Erysipelotrichaceae, reported to be linked to other inflammatory disorders, was instead found to be increased in the mucosa and stool of untreated and treated PWH in several studies [61]. Studies have also described a decrease in Bacteroides, most of which are inducers of regulatory T cell function, and a concomitant increase in Prevotella sp. abundance in PWH compared to HIV-uninfected controls [50, 54, 57, 62]. A recent study suggested that the Prevotella-rich/Bacteroides-poor community structure was more prevalent among men who have sex with men (MSM), rather than being associated with HIV infection status per se [36], thus highlighting the importance of appropriately accounting for the myriad of possible factors that might confound HIV microbiome studies [56].

In line with the human data, studies on SIV-infected nonhuman primates, which can better control for confounding variables, have shown that wild chimpanzees, which develop progressive immunodeficiency with SIV infection, also develop changes in their gut microbiota following infection [63, 64]. This is in contrast to findings in wild gorillas, which maintain a stable gut microbiome following SIV infection, although it is unknown whether gorillas develop progressive CD4 depletion from SIV [64, 65]. Similarly, rhesus macaques have repeatedly been shown to have an enteric microbiome that is not substantially altered by SIV infection, except temporarily during acute infection [20, 64]. Despite long-term stability of the gastrointestinal microbiome in macaques, microbial translocation in advanced disease occurs with the phylum Proteobacteria, which has been found in lymph nodes and liver and was overrepresented in the stool in the acute phase of SIV infection [64, 66].

To conclude, increasing knowledge suggests HIV infection and the resultant immune compromise might alter the gut microbiome. Yet, the extent of this phenomenon remains controversial mainly due to limitations in the experimental design. Similarly, it is known that the gut microbiota is influenced by several factors both biological, e.g. age, gender, ethnicity [36-38] among others [67, 68]. Controlling for these confounders is quite difficult given the relatively small sample sizes generally available for study of the human microbiome especially in light of several confounding variables (diet, genetics, smoking, etc) and the sampling method.

Despite the fact that more research is clearly required to reach strong conclusions regarding the HIV-associated microbiota signature and its role in disease progression, therapeutic strategies aiming to modulate immunological dysfunction and restore the gut microbiota have been tested. Such approaches will be discussed in more detail below following an overview of the mainstream strategy for treating HIV infection with the use of antiretroviral therapy (ART).

Development of effective ART: from a deadly epidemic to a chronic condition

The development of ART started in the mid-80s following the identification of potential drug targets to inhibit key steps in the HIV replicative process. In 1987, zidovudine, a nucleoside reverse transcriptase inhibitor (NRTI), was the first approved antiretroviral agent for clinical use [69]. Although NRTI monotherapy showed a reduction in viral load, the use of a single agent, and subsequently combinations of two of these agents, did not provide sustained viral suppression. The approval of HIV protease inhibitors (PI) and non-nucleoside reverse transcriptase inhibitors

(NNRTI) in the mid-1990s marked the beginning of what was then called highly active antiretroviral therapy (HAART), and this dramatically changed the course of the HIV epidemic.

The use of combinations of three drugs, consisting of a PI or NNRTI together with 2-NRTI, resulted in rapid and sustained reduction of HIV RNA, improved immune function and reduced mortality [70, 71]. Since those early days, therapies have further evolved. New drugs and drug classes with novel mechanisms of action have been introduced, exhibiting greater potency, better tolerability and less toxicity, as well as a reduced likelihood of selecting for drug resistance. Alongside the development of better drugs and treatment regimens, insight into the optimal time to initiate treatment has also developed over time. In 2010, treatment guidelines in the United States and the European Union recommended the initiation of ART with three fully active antiretroviral agents once CD4⁺ cells in the peripheral blood had declined to 350 cells/ μ l or less [72]. In 2015, the Strategic Timing of Antiretroviral Treatment (START) trial showed a lower risk of both AIDS-related and non-AIDS-related events and mortality PWH with more than 500 CD4⁺ T cells/ μ l who were randomized to receive immediate ART compared to those receiving deferred ART once CD4-cells had declined to 350 cells/ μ l or less [73]. Therefore, as of 2015, global guidelines were adopted to recommend universal start of ART in all PWH, regardless of CD4⁺ T cell count [74].

AIDS as a manageable chronic disease: unmet clinical challenges

The implementation of effective combination antiretroviral therapy (cART) has led to a markedly increased life expectancy, and thereby to a growing population of middle-aged and elderly PWH. As the cohort of PWH has aged, and the incidence of AIDS-related conditions has reduced with

effective cART, the spectrum and burden of age-associated non communicable comorbidities (AANCC) including liver, cardiovascular and kidney disease as well as cancer, has increased.

This phenomenon appears to be accentuated by a state of systemic residual inflammation in PWH [75, 76]. Indeed, levels of markers of inflammation and coagulopathy such as C-reactive protein, interleukin 6 (IL-6), a cytokine released by monocytes and macrophages, and D-dimer generally decline in PWH upon initiation of cART but remain higher than in HIV-uninfected individuals. The underlying causes of this residual inflammation are still far from being fully elucidated and remain the focus of intense research. Residual viral replication, incomplete immune restoration, microbial translocation, co-infections, antiretroviral drug toxicity and behavioral habits are among the culprits of this phenomenon identified so far [77, 78].

As described above, important effects of HIV-infection include breakdown of mucosal integrity, allowing microbial products, like LPS, to translocate from the lumen of the GI tract into the peripheral circulation. The increased systemic exposure to microbial products in turn can cause monocyte and macrophage activation, thereby driving innate immune and coagulation activation.

Given the importance of monocyte and macrophage-driven inflammation in cardiovascular disease (CVD) and fibrosis, a role for microbial translocation in the development HIV-related comorbidities has also been suggested [79]. In particular, circulating LPS has been proposed to be an early trigger of inflammation, insulin resistance, and subsequent cardiovascular risk. Plasma levels of both LPS and sCD14 have been found elevated in PWH compared to HIV-uninfected controls, predicted sustained hypertension, and were intercorrelated in subjects who developed hypertension several years later. [80]- Moreover, circulating bacterial products, pro-atherogenic lipids and altered blood flow dynamics are shown to damage the endothelium and upregulate

adhesion factors, ultimately contributing to cardiovascular disease in PWH [79]. Furthermore, microbial translocation has been proposed to contribute to progression of liver fibrosis through the involvement of Kupffer cells, which are specialized macrophages residing in the liver [48, 81]. Specifically, the ability of LPS to stimulate an inflammatory response may account for its pathogenicity in the liver. In addition to its ability to clear LPS, the liver responds to LPS with production of cytokines and reactive oxygen intermediates.

Toxicity of antiretroviral drugs may also contribute to organ dysfunction and disease. Lipodystrophy is a known side effect of some forms of cART, and it has been associated with altered circulating levels and adipose tissue mRNA expression of proinflammatory cytokines such as IL-6 and TNF- α [82]. Further, therapeutic regimens including protease inhibitors are associated with pro-atherogenic dyslipidemia including increases in low-density lipoprotein (LDL) cholesterol and triglycerides which, in turn, may affect cardiovascular risk [83]. Drug-specific toxic effects may also include a reduction in gut bacterial diversity of PWH. Decreases in microbial richness and diversity have been observed in hypertensive patients.

HIV disrupts the balance between the host and coinfecting microbes, worsening control of these potential pathogens. PWH are more likely than the general population to have hepatitis C virus (HCV) and hepatitis B virus (HBV) coinfections which in turn increase the risk of liver fibrosis and cirrhosis. Similarly, cytomegalovirus (CMV) infection is common in PWH who may experience reactivation more often than uninfected individuals [84]. CMV is well known to induce high levels of multiple cytokines, particularly IFN- γ [85]. Blood plasma levels of IFN- γ -inducible protein 10, TNF-RII, and D-dimer are higher in people coinfecting with CMV and HIV as compared to those in HIV-monoinfected subjects, suggesting that CMV might specifically drive expression

of these biomarkers [85]. In the setting of underlying immune deficiency, CMV is associated with more rapid HIV disease progression, more AIDS-related events and a wide range of serious end-organ diseases [85]. CMV is also a risk factor for severe, non–AIDS-related adverse clinical events in HIV disease. The event most frequently associated with CMV is CVD. Given its ubiquity as a human pathogen and its ability to infect endothelial cells and smooth-muscle cells, CMV is an ideal candidate pathogen for atherosclerosis [85]. Behavioral factors such as cigarette smoking and alcohol intake, well-known risk factors for the development of AANCC in the general population, are shown to further increase the incidence of AANCC in PWH [86].

In summary, despite long-term suppressive cART, systemic inflammation and chronic immune activation persist in PWH [87, 88]. Safe treatment strategies that can be given in addition to cART are needed to enhance gut mucosa reconstitution, to reduce microbial translocation and to downregulate key pathways of inflammation.

Development of ancillary treatment strategies which, alongside ART, target immunological dysfunction in chronic treated PWH and immunocompromised individuals

Previous studies focused on inhibiting translocating microbial products through administration of the LPS-sequestering, phosphate-binding drug, Sevelamer [89, 90], nonabsorbable antibiotics such as rifaximin [91], and mesalamine, an anti-inflammatory drug used in the treatment of inflammatory bowel disease [92], but have yielded negligible improvements in inflammatory or immune parameters in treated PWH. In contrast, other therapeutic strategies involving supplementation of probiotics/prebiotics during cART resulted in increased GI tract function,

better reconstitution and functionality of intestinal CD4⁺ T cells, and reduced fibrosis in colonic lymphoid follicles in SIV-infected pigtail macaque model of AIDS [93]. Several approaches using IL-21 improved T_H17 cell restoration and promoted more-effective reduction of immune activation in blood and gut mucosal tissues of SIV-infected Asian macaques [94]. Encouraging results have also been obtained with another cytokine, namely IL-7. IL-7 is important in promoting homeostatic division of naïve T-cells and enhancing T-cell homing to the gut. In PWH, IL-7 administration induces T-cell expansions in both peripheral blood and gut mucosa [95]. Moreover IL-7 dramatically enhances the function of MAITs from both healthy individuals and HIV-infected patients in vitro [33]. Therefore, implementation of novel therapeutic strategies may promote effective immune reconstitution by restoring structural and immunological components of the GI tract. The mechanisms thought to underly the increased risk of age-associated non communicable comorbidity in treated HIV infection as well as potential entry points for intervention are summarized in the following figure:

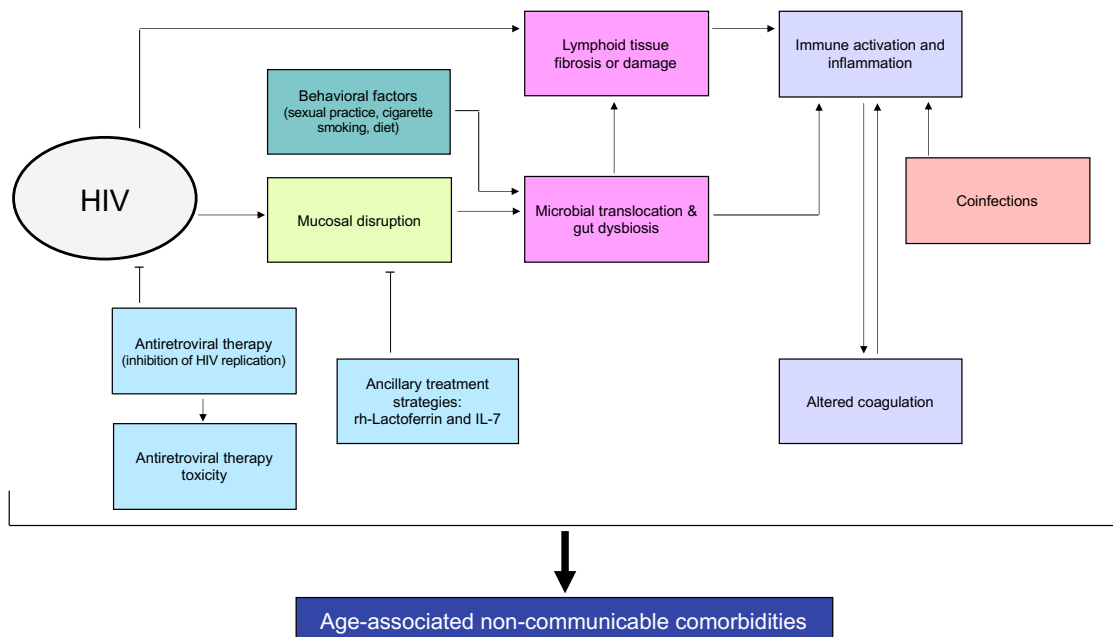


Figure 1 The multifactorial nature of age-associated non-communicable comorbidities in HIV and potential entry points for intervention.

Aims of the thesis

The overarching aim of the studies presented in this thesis is to assess how gut microbiota and mucosal immunity are affected and contribute to disease pathogenesis and progression during SIV infection of non-human primates, HIV infection in humans, and in people with idiopathic CD4⁺ lymphocytopenia (ICL). Experimental microbial dysbiosis was induced and its contribution to disease progression studied in SIV-infected rhesus macaques [**Chapter 2**]. In order to try and disentangle the contribution of sexual practice and treated HIV infection to gut dysbiosis, an extensive analysis of fecal microbiota profiles was undertaken in a carefully designed case control study which involved both PWH with suppressed viremia on cART and HIV-uninfected individuals participating in the Amsterdam AGE_hIV Cohort Study. Stored stool samples from participants were selected stratified by sex (i.e. male and female) and sexual practice (*i.e.* lifetime or recent vaginal/anal intercourse with women/men) and matched for age, sexual behavior, BMI, and birth country [**Chapter 3**].

In order to determine whether antiretroviral drugs themselves may affect the microbial composition and immune function of the gut, a series of experiments were conducted in which selected HIV protease-, integrase- or reverse transcriptase inhibitors were administered for 1-2 or 5-6 weeks to healthy rhesus macaques [**Chapter 4**]. In order to determine how early gut microbial dysbiosis is established following HIV infection and to which extent it may be restored by early cART, a study was designed in a subset of participants of the ongoing RV254/SEARCH010 cohort, enrolling persons with early acute HIV infection (Fiebig Stages 1–5) who were identified from the Thai Red Cross Anonymous Clinic in Bangkok, Thailand. Fecal microbiota composition in relation to markers of systemic inflammation and gut epithelial integrity was studied in a group

of men who have sex with men (MSM), at diagnosis of acute HIV infection and after 6 months of cART initiation, as well as in a small number of Thai MSM HIV-uninfected controls [**Chapter 5**]. In terms of possible ancillary interventions alongside cART to restore gut mucosal immunity, three clinical studies were conducted. Firstly, in a single arm clinical trial, individuals with chronic HIV infection and durable suppression of viremia, received interleukin-7 (IL-7) by subcutaneous injection, and its ability to restore Mucosa-Associated Invariant T (MAIT) cells in peripheral blood was assessed [**Chapter 6**]. Secondly, MAIT cells were studied using samples from a cohort of individuals with idiopathic CD4⁺ lymphopenia, a syndrome defined by reduced circulating CD4 T-cell counts, (<300/ μ L) in the absence of HIV infection or any other cause of immunodeficiency. MAIT cells were evaluated in six of these ICL patients enrolled in an open-label phase 1/2A, dose-escalation study of rhIL-7 [**Chapter 7**]. Finally, the immunomodulatory properties of recombinant human lactoferrin as well as its impact on fecal microbiome composition were assessed in a cross-over placebo-controlled trial of PWH with suppressed viremia on cART [**Chapter 8**].

Finally, we provide a perspective of how findings have contributed to further our understanding of the role and importance of gut microbial composition and immunity in the pathogenesis of HIV and the long-term complications of treated HIV disease. We also discuss the modifying impact of cART and ancillary interventions, and directions for future research [**Chapter 9**]. We end with a concise summary of our main findings [**Chapter 10**].

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Chapter 2

Experimental microbial dysbiosis does not promote disease progression in SIV-infected macaques

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Abstract

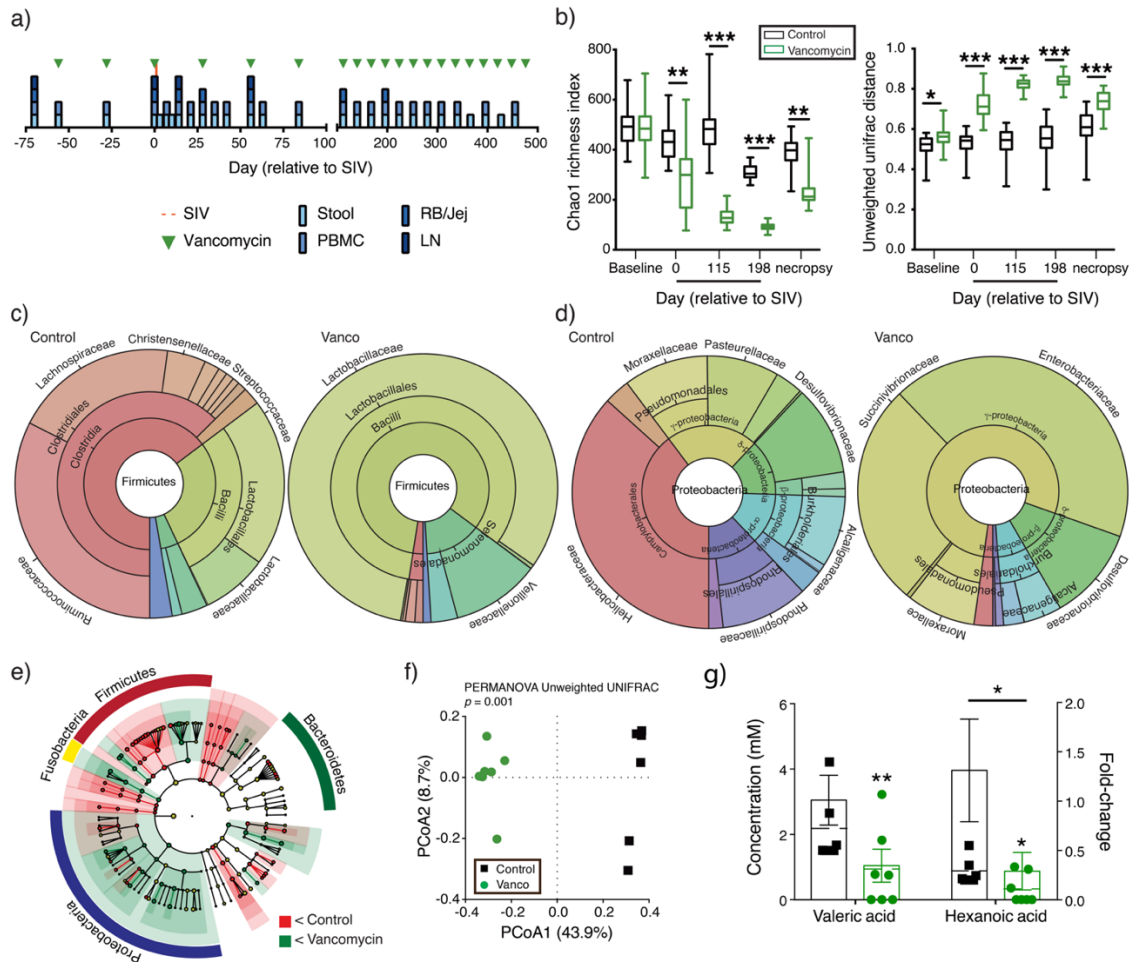
Intestinal microbial dysbiosis has been described in individuals with an HIV-1 infection and may underlie persistent inflammation in chronic infection, thereby contributing to disease progression. Herein, we induced an HIV-1-like intestinal dysbiosis in rhesus macaques (*Macaca mulatta*) with vancomycin treatment and assessed the contribution of dysbiosis to SIV disease progression. Dysbiotic and control animals had similar disease progression, indicating that intestinal microbial dysbiosis similar to that observed in individuals with HIV is not sufficient to accelerate untreated lentiviral disease progression.

Main

Intestinal microbial dysbiosis has been described in progressive HIV-1-infected individuals. This dysbiosis includes enrichment of Proteobacteria (particularly Enterobacteriaceae) and Erysipelotrichaceae, coincident with declines in Clostridia and Bacteroides[96]. Dysbiotic taxa in infected individuals correlate with persistent inflammation – a hallmark of progressive infection[97]. However, an empirical assessment of the contribution of microbial dysbiosis to disease progression is lacking. Recent data suggest that risk factors for HIV-1 can also result in microbial dysbiosis[36] and although simian immunodeficiency virus (SIV) non-human primate (NHP) models for HIV-1 infection recapitulate salient features of infection[98], dysbiosis is not consistently observed[12, 96].

In healthy NHPs, vancomycin inhibits the growth of Gram-positive bacteria, inducing a dysbiosis similar to that described in HIV-1 infection (Supplementary Fig. 1). To assess whether intestinal dysbiosis accelerates lentiviral disease progression, we treated 7 rhesus macaques (RMs) with vancomycin for 5 days, every 28 days, beginning 56 days pre-SIV infection (Fig. 1a) and infected these and 6 control animals with SIV_{mac239}. Following treatment, fecal microbiome diversity became increasingly disparate between groups, with vancomycin-treated (Vanco) animals exhibiting reduced α -diversity and increased β -diversity compared to control animals (Fig. 1b and Supplementary Fig. 2a). No differences in total bacterial DNA recovery were observed between groups (Supplementary Fig. 2b).

Fig. 1: Vancomycin treatment promotes dysbiosis in SIV-infected RMs.



a, Longitudinal study design depicting time points for vancomycin treatment, SIV-infection, and specimen sampling. RB, rectal biopsies. Jej, jejunum. b, Diversity measures Chao1 (α -diversity; left) and unweighted Unifrac (β -diversity; right) of fecal microbiome in RMs at indicated time points. Hinges span 25th–75th percentiles, center denotes mean, and whiskers represent minimum-to-maximum values. c, Krona plots depicting relative frequency of fecal Firmicute subtaxa comprising $\geq 5\%$ phylum burden at d115 p.i. to the family level. Shown taxa are collapsed to the lowest common taxon. d, Krona plots depicting relative frequency of Proteobacteria as in c. e, Cladogram depicting significantly disparate fecal taxa frequencies at d115 p.i. to the level of family where nodes and overlays in red highlight features enriched in control animals and those in green are enriched in Vanco animals. Arcs demarcate phyla of interest. f, PCoA of unweighted UNIFRAC distances measured at d115 p.i. g, Fecal SCFA at baseline and d198 p.i. Average line represents mean. P values for comparison between time points span time points; those for intergroup

analysis span compared groups. d198 p.i. mean fold-change \pm s.e.m. from baseline values are denoted on graphs. For all time points, samples were obtained from n=6 and 7 longitudinally accessed control and vancomycin-treated animals respectively, except for d-56 (n=5 and 7) and d198 (n=6 and 6) in b-f. Sequence reads per animal are an average of 1 or 2 reads per time point as listed in the NCBI sequence read archive (SRA) ([PRJNA417022](https://www.ncbi.nlm.nih.gov/sra/PRJNA417022)). α diversity indices represent the mean of 10 iterations of 1,000 sequences per sample. Statistical significance of vancomycin effect on α -diversity in b and for intergroup analysis in g was determined by unpaired, two-way t-test. Intragroup analysis in g was by paired, two-way t-test. Significance of vancomycin effect on fecal β -diversity in b and f assessed by PERMANOVA. Significantly enriched features highlighted in e were identified by LEFSE.

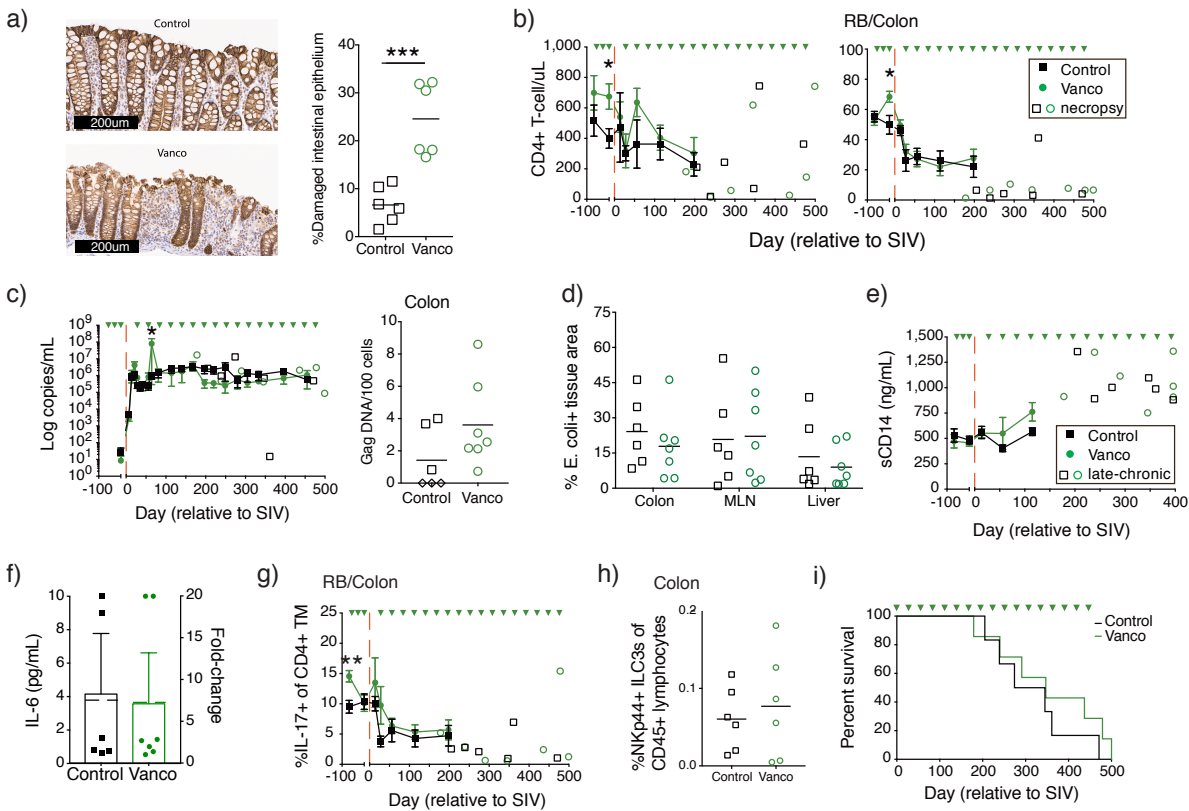
Gram-positive Firmicutes dominated the fecal microbiome irrespective of treatment; however, relative Firmicute frequencies in Vanco animals decreased (Supplementary Fig. 2c). Whereas control animals had comparable distributions of Clostridia and Bacilli, Vanco animals were enriched in Bacilli (Fig. 1c). Inversely, Vanco animals displayed increasing Proteobacteria frequencies post-infection (Supplementary Fig. 2c). Here, control animals had even distributions of Proteobacteria classes at d115 p.i., while Vanco animals were enriched for Gammaproteobacteria (Fig. 1d). Large intestine, epithelial-associated communities of Vanco animals displayed similar enrichments for Proteobacteria relative to Firmicutes (Supplementary Fig. 2d).

Of 390 operational taxonomic units (OTUs) identified, 256 features were differentially present at d115 p.i. as identified by LDA-effect size (LEFSE) algorithm (Fig. 1e, Supplementary Fig. 3, and Supplementary Table 1). Proteobacteria were enriched in Vanco animals, underpinned by differences in Gammaproteobacteria (especially Enterobacteriaceae) and Deltaproteobacteria. In Vanco animals, LEFSE confirmed an enrichment of Bacilli and a depletion of Clostridia - particularly Lachnospiraceae and Ruminococcaceae - and Erysipelotrichaceae among Firmicutes

while among Bacteroidetes, Bacteroidales and a sub-cluster of families were also moderately depleted. Principle coordinate analysis (PCoA) of returned OTUs at d115 p.i. revealed clustering of microbial communities by treatment (Fig. 1f). Longitudinal quantification of Lachnospiraceae, Ruminococcaceae, Erysipelotrichaceae and Enterobacteriaceae confirmed persistent alterations in Vanco animals (Supplementary Fig. 2e-f). Predicted metagenomic analysis revealed non-distinct fecal metagenomes at baseline (Supplementary Fig. 4); however, there was a notable enrichment for metabolic and virulence pathways in Vanco animals at d115 p.i. Short-chain fatty acids (SCFA) - microbial-produced metabolites important for GI immunity[41, 99] - were perturbed in Vanco animals, with significant disruptions from baseline observed for fecal valeric and hexanoic acids at d198 p.i. (Fig. 1g).

To assess interdependency of intestinal microbial dysbiosis and lentiviral disease progression, we first assessed the integrity of the intestinal epithelium. By immunohistochemical (IHC) staining for the tight junction protein Claudin-3, we observed reduced integrity of the colonic barrier in Vanco animals at necropsy (Fig. 2a). We next measured canonical parameters of disease progression. We found comparable: a) CD4⁺ T-cell counts and intestinal CD4⁺ T-cell frequencies (Fig. 2b and Supplementary Fig. 5a), b) plasma viral loads (Fig. 2c); c) memory CD4⁺ T-cell (TM)-associated viral DNA Fig. 2c and Supplementary Fig. 5b); and d) an equivalent depletion of intestinal CD4⁺ TM expressing the HIV/SIV co-receptor, CCR5 (Supplementary Fig. 5c), irrespective of treatment.

Fig. 2: SIV disease progression is unaffected by vancomycin-induced intestinal dysbiosis



a, IHC Claudin-3 staining in representative colon sections (left) and as mean percentage negative epithelial perimeter (epithelial damage; right) at necropsy. b, Mean, longitudinal circulating CD4⁺ T cell counts (left) and RB/Colon CD4⁺ TM frequencies (right). Green triangles indicate when vancomycin was administered. *P between groups at d0 of 0.028 for CD4⁺ T cell count and 0.021 for RB %CD4⁺ T cells. c, Mean, longitudinal plasma viral loads (left) and cell-associated viral loads from Colon CD4⁺ TM at necropsy (right). Samples below the limit of detection reported as open diamonds. *P = 0.042 at d28. d, Mean percent IHC positive E. Coli tissue area from necropsy of colon (lamina propria), MLN, and liver. e, Mean, longitudinal, circulating sCD14 concentrations. f, Individual plasma IL-6 concentrations at baseline and d198 p.i. Average line represents mean. d198 p.i. mean fold-change ± s.e.m. from baseline values denoted on graphs. g, Mean, longitudinal frequencies of RB/Colon T_H17. ** P = 0.005 between groups at baseline. h, Mean frequency of colon NKp44⁺ ILC3 at necropsy. i, Survival curve depicting time to death. Samples were longitudinally accessed from n = 6 and 7 for control and vancomycin-treated animals respectively, except for: d198 (n = 6 and 6); in c, where time points ≥d198p.i. depict mean viremia for all surviving animals; in g, at d-68 and

d14 (n = 6 and 6), d0 (n = 5 and 6), d28 (n = 3 and 4), and d115 (n = 5 and 7); and in h (n = 6 and 6). Longitudinal lines represent mean and include \pm s.e.m. where individual data points are not shown. Statistical significance between groups was assessed by unpaired, two-way t-test. Survival significance in i was assessed by Mantel–Cox.

We next assessed whether intestinal microbial dysbiosis may impact immune activation by measuring phenotypic and functional markers of T-cells by flow cytometry. Isolated differences in cell-surface activation markers were observed in intestinal TM during acute infection (Supplementary Fig. 5c-d). Although stimulated jejunal CD8⁺ TM from Vanco animals displayed significant reductions in mitogen-induced IFN γ and TNF α at several timepoints (Supplementary Fig. 6a-c), these changes were not paired with differences in inhibitory PD-1[100] nor polyfunctionality[101] (Supplementary Fig. 6d-e), suggesting that intestinal microbial dysbiosis did not modulate immune activation.

To assess whether intestinal microbial dysbiosis increases microbial translocation, we measured *E. coli* in the colonic lamina propria, mesenteric lymph nodes (MLN), and livers of our animals by IHC and levels of plasma soluble CD14 (sCD14) and IL-6 by ELISA. Although both groups displayed evidence of translocation and elevated IL-6 (**Fig. 2d-f**), there were no differences. We further considered that intestinal microbial dysbiosis may predispose emergence of opportunistic infections by hindering the development or maintenance[41] of IL-17-expressing CD4⁺ TM (T_H17), CD8⁺ TM (T_C17) and NKp44⁺ innate lymphoid cells type-3 (ILC3s)[12]. Despite differing Clostridia frequencies, we observed equivalent post-infection depletions of these subsets irrespective of treatment (**Fig. 2f-g, Supplementary Fig. 6f**).

In accordance with a lack of evidence for exacerbated immune dysfunction or microbial translocation, survival curves were comparable between groups, with control animals exhibiting a mean survival of 310 days p.i. and vancomycin-treated animals, 346 days p.i. (Fig. 2h; Mantel-Cox $p=0.33$, Hazard Ratio=0.6042 compared to control, 95% CI 0.192-1.902). Findings documented at necropsy included thrombosis, lymphadenopathy, and secondary infection (Supplementary Table 2); however, outcomes and blood cell counts (Supplementary Fig. 7) did not differ by group.

SIV-infection of RMs recapitulates key aspects of HIV-1 infection[98]. Although the gut microbial communities of RMs and humans share several dominant taxa, they differ substantially beyond the genus classification[102]. We cannot discount that underappreciated differences in viral, host, or commensal identity may uniquely contribute to lentiviral disease progression in human and NHPs; however, largely parallel disease progression between the two argues that shared mechanisms are responsible. Our data does not preclude a role for intestinal dysbiosis in lentiviral transmission[103], nor does it exclude a contribution of alternative dysbioses to lentiviral disease progression. Our data do, however, demonstrate that neither significant upheaval of the intestinal microbiome nor enrichment for Proteobacteria at the expense of Clostridia are sufficient for accelerating disease progression.

Conceivably, immune dysfunction resulting from viral replication may overshadow intestinal dysbiosis in HIV-infected individuals with HIV infection, and microbial translocation may be saturated. Importantly, intestinal dysbiosis may contribute to inflammation observed in highly active antiretroviral therapy (HAART)-treated individuals. This premise is supported by improved

disease progression in HAART-treated RMs receiving additional microbiome-mediated therapeutics. Thus far, however, similar efficacy has not been achieved in placebo-controlled human trials. Whereas probiotic treatment in humans with HIV-infected promotes modest improvements in T-cell activation and CD4 T cell reconstitution, preliminary attempts toward fecal microbial transplantation have shown limited engraftment [104, 105].

In conclusion, we demonstrate that experimentally induced intestinal dysbiosis in SIV-infected RMs does not accelerate disease progression. Dysbiotic and control animals exhibited comparable viremia, target-cell depletion, immune activation, microbial translocation, and ultimately, survival rates. These data indicate that intestinal microbial dysbiosis in untreated lentiviral infection does not necessarily contribute to disease progression. Further studies are required to unravel the circumstances surrounding intestinal dysbiosis in HIV-infected patients and to determine whether components of specific dysbiosis contribute to HIV transmission or therapeutic immune reconstitution.

Methods

Animals and Vancomycin Treatment

Thirteen healthy rhesus macaques (*Macaca mulatta*), aged 6-15, were assigned to a control (n=6, 5/6 male) or vancomycin treatment (n=7, all male) group, with sample size based on previous studies of experimental manipulations of disease progression in the macaque model. Groups were stratified by weight and genotype (Mamu-A*001, -A*002, -B*017, and -B*029) and animals sampled as mixed populations. Vancomycin-treated animals received vancomycin hydrochloride (Pfizer, 10 mg/kg, p.o.) for 5 days approximately every 28 days, beginning 56 days prior to

infection. All animals were infected with 3000 TCID₅₀ SIV_{mac239} i.v. at d0 and were followed until clinical endpoints were met.

The NIAID Division of Intramural Research Animal Care and Use Program, as part of the NIH Intramural Research Program, approved all experimental procedures (protocol LVD 26). The Program complies with all applicable provisions of the Animal Welfare Act and other federal statutes and regulations relating to animals.

Animals were housed and cared for at the NIH Animal Center, under the supervision of the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited Division of Veterinary Resources and as recommended by the Office of Animal Care and Use Nonhuman Primate Management Plan. Husbandry and care met the standards set forth by the Animal Welfare Act, Animal Welfare Regulations, as well as The Guide for the Care and Use of Laboratory Animals (8th Edition). The physical conditions of the animals were monitored daily. Animals in this study were exempt from contact social housing due to scientific justification, per IACUC protocol, and were housed in non-contact, social housing where primary enclosures consisted of stainless steel primate caging. Animals were provided continuous access to water and offered commercial monkey biscuits twice daily as well as fresh produce, eggs and bread products twice weekly and a foraging mix consisting of raisins, nuts and rice thrice weekly. Enrichment to stimulate foraging and play activity was provided in the form of food puzzles, toys, cage furniture, and mirrors.

Sample Collection

Blood, tissue, stool and luminal swabs were collected as previously described, with each measurement resulting from a single sample per timepoint[106]. Sampling occurred in random order. Neither the investigators nor the animal handlers were blinded to group allocation in order to ensure multi-lateral supervision of design and palliative treatment. Animals were sedated with Ketamine HCL at 10 mg/kg i.m. for longitudinal blood sampling or with Telazol at 3-4 mg/kg i.m. for tissue timepoints. For jejunal biopsies, animals were further anesthetized with isoflurane gas by intubation, to effect. Successful anesthetization was monitored by response to stimuli.

Endpoint criteria included presentation of any of the following: (a) loss of 25% body weight from baseline weight when assigned to the protocol, (b) major organ failure or medical conditions unresponsive to treatment, (c) complete anorexia for 4 days or an inability to feed or drink sufficient nutrients to maintain body weight without assistance for 7 days, (d) distress vocalization unresponsive to treatment or intervention for 7 days, or (e) tumors arising from other than experimental means that grew in excess of 10% of body weight, impaired movement, or ulcerated. Euthanasia was initiated at clinical or experimental endpoints using protocols consistent with the American Veterinary Medical Association (AVMA) guidelines. Animals were first sedated with Telazol at 4 mg/kg i.m., followed by Pentobarbital at 80 mg/kg to achieve euthanasia.

Immune Phenotyping and Functional Assessment

Polychromatic flow cytometry and cell sorting were performed on stained mononuclear cells as previously described[106]. Antibodies against the following antigens were used for staining at

predetermined concentrations: CD4 (clone OKT4), CD8 (SK1), IFN γ (4S.B3), IL-17 (eBio64DEC17), IL-21 (3A3-N2), IL-22 (IL22JOP), TNF α (MAb11), and PD-1 (J105) from eBioscience; CCR5 (3A9), CD3 (SP34-2), CD8 (SK1), CD16 (HI149), CD20 (2H7), CD23 (M-L233), CD45 (D058-1283), CD123 (7G3), and HLA-DR (L243) from BD; c-Kit (104D2), CD1a (HI149), CD11c (3.9), CD14 (M5E2), CD34 (561), CD95 (DX2), and IL-2 (MQ1-17H12) from Biolegend; CD127 (eBioRDR5) and CD218a (H44) from Thermo; NKp44 (2.9) from Miltenyi; and CD28 (CD28.2) from Beckman Coulter. CD4⁺ and CD8⁺ TM were defined as CD95⁺ singlet, clean, live, CD3⁺ lymphocytes. NKp44⁺ ILC3s were defined as CD45⁺ singlet, clean, live, lineage-negative (CD1a, CD3, CD8, CD11c, CD14, CD16, CD20, CD23, CD34, CD123), NKp44⁺CD127⁺CD218⁺ lymphocytes. Positive/negative gating based on clearly grouped populations, historical-determined expression, and the use of internal controls (Supplementary Fig. 8). A threshold of 100 collected events in the parent population was utilized for all subset expression analysis.

16S isolation and analysis

DNA was isolated from 250 mg of stool using the PowerLyzer PowerSoil DNA isolation kit (MO BIO) substituting stool for soil during the initial processing steps. Samples were homogenized on a Precellys 24 (Bertin Technologies). The Biomek NK^P (Beckman Coulter) was used to facilitate automated, high-throughput processing via the PowerSoil manufacturer's protocol, substituting column binding with PowerMag magnetic beads and a magnetic plate (Alpaqua) on Corning 96 Deep-Well Assay Blocks (Fisher Scientific) to improve DNA retention throughout processing. 16S DNA was amplified from 50-100ng total DNA using barcoded universal primers spanning basepairs 515(F)-806(R) of the 16s rRNA V4 region. 16S amplicons were subjected to Agencourt

AMPure XP PCR Purification (Beckman Coulter #A63882). >1ng 16S amplicons quantified using the 16S MiSeq (Illumina) platform. Returned FASTQ, paired-end reads were processed using QIIME[107] (v1.9.1) through the NIAID/NIH Microbiome Analysis Platform, Nephele (Office of Cyber Infrastructure and Computational Biology/NIAID/NIH <http://nephele.niaid.nih.gov>), and reads were truncated before 3 consecutive bases having Phred quality scores below 20. OTUs were classified by open reference using SILVA_99, and data were rarefied at 1000 reads per sample. First-run, incomplete 16S miSeq timepoints were re-run in full with banked stool and duplicate returns averaged per timepoint as listed in the NCBI Sequence Read Archive (SRA) under project number PRJNA417022. For metagenomic analysis, OTUs were reclassified by closed reference using Greenegenes_99, and metagenomic profiles predicted by PICRUSt [108]

Immunohistochemistry and quantitative image analysis

Formaldehyde fixed, paraffin-embedded (FFPE) tissue sections, 5µm thick, were placed on Fisher-Plus microscope slides, deparaffinized in xylenes, rehydrated through graded ethanol and subjected to HIER with 0.01% citraconic anhydride (Sigma-Aldrich; 125318), then cooled and washed in double distilled H₂O (ddH₂O), as previously described[26, 109]. IHC for E. coli was performed on an intelliPATH autostainer (Biocare Medical): following incubation with blocking buffer (0.25% casein in tris-buffered saline (TBS) with 0.05% Tween-20) for 10 minutes and an endogenous peroxidase block of 1.5% (v/v) H₂O₂ in TBS (pH 7.4) for 10 minutes, slides were incubated with a rabbit polyclonal E. coli antibody (Dako, B0357; 3.8µg/ml) diluted in blocking buffer for 1 hour at room temperature and detected using a biotin-free anti-rabbit Polink-1 HRP polymer system (Golden Bridge International, Inc.) for 30 minutes at room temperature in conjunction with DAB (3,3'-diaminobenzidine; Vector Laboratories) for 2-5 minutes. IHC for

claudin 3 was performed as above with the following modifications: slides were stained manually with a rabbit polyclonal claudin 3 antibody (Thermo Scientific, RB-9251-p; 1 µg/ml) for 1 hour at room temperature. The slides were developed as described above. All slides were washed in ddH₂O, counterstained with hematoxylin, mounted with Permount (Fisher Scientific) and scanned at high magnification (×200) using a whole-slide scanning microscope (Aperio AT2 System, Aperio Technologies), yielding high-resolution data from the entire tissue section. For *E. coli* quantification, representative regions of interest (500 × 500 µm) were extracted from the whole-tissue scans and the percent area of the anatomical site of interest (lamina propria of the GI tract, T-cell zone of the LN) was quantified using Photoshop CS5 or CS6 (Adobe Systems, San Jose, CA) and Fovea (Reindeer Graphics, Asheville, NC) image analysis tools as previously described[26, 109]. Claudin 3 staining was used to visualize epithelial tight junctions so as to quantify epithelial barrier integrity (percent damage) as previously described[26, 109].

ELISAs

Concentrations of sCD14 and IL-6 were quantified from plasma using a commercially available ELISA kits (R&D DC140 and HS600B, respectively), according to the manufacturers' protocols.

Short-chain fatty acids

Fecal short-chain fatty acid concentrations were determined by gas chromatography (GC; Thermo Trace 1310 with TG-WAXMS A GC, 30 m, 0.32 mm, and 0.25 µm columns), by Creative Proteomics (division of Creative Dynamics; Shirley, NY). Fecal pellets were homogenized in 17% (w/w) water suspension and titrated to pH 2.0 using 5M HCl. Fecal debris were pelleted and removed by centrifugation. The remaining supernatant was spiked with internal standard 2-

Ethylbutyric acid. Samples were injected into the GC flame ionization detector and calibrated against a cocktail of standardized SCFAs – sodium propionate and acetic, butyric, isobutyric, valeric, isovaleric, hexanoic, and heptanoic acids. Peaks were assigned using the Chromeleon 7.2 software (Thermo), based on retention times of individual SCFA components. Chromeleon automatically determined mM concentrations of sample peaks using peak integration.

VL and cell-associated viremia

Plasma viral RNA (vRNA) levels were determined by qRT-PCR (ABI Prism 7900 sequence detection system; Applied Biosystems). vRNA was reverse transcribed and cDNA amplified (45 cycles/default setting) with AmpliTaq Gold DNA polymerase (PCR core reagents kit; Perkin-Elmer/Roche) utilizing primer pairs corresponding to SIV_{mac239} gag gene sequences (forward, nucleotides 1181-1208, and reverse, 1338-1317). Cell-associated viral DNA within sorted CD4TM cells was measured as previously[110]. Briefly, qRT-PCR was performed on sorted, lysed cells using the Taq DNA polymerase kit (Invitrogen) and the SIV_{mac239} forward primer GTCTGCGTCATYTGTTGCATTC, reverse primer CACTAGYTGCTCTGCACTATRTGTTTTG, and probe sequence CTCRTCAGTYTGTTTCACTTTCTTTCTGCG.

Statistical Analyses

Statistical analyses of viremia, single parameter lymphocyte phenotype and function, and single microbial taxa frequency were performed using Prism (v7.0, GraphPad Software Inc.). Multiple t-tests (unpaired, two-way, with no correction for multiple tests or assumption of consistent standard deviations) were used for comparisons between groups; paired t-tests were used for intra-group

comparisons. Averaged longitudinal data are presented as arithmetic mean \pm standard error of the mean (SEM). Significance of polyfunctional cytokine expression was assessed using the Spice (v5.35; NIAID) permutation-test on relative expression values. Frequencies of microbial taxa are calculated relative to all returned bacterial OTUs mapping to non-mitochondrial (Rickettsiales Mitochondria) taxa. Microbial diversity was assessed by Chao1, Shannon, and unweighted and weighted Unifrac with statistical significance assessed by unpaired, two-way t-test for alpha indices and PERMANOVA (R-vegan function Adonis) for Unifrac distances. The empirical identification of differentially abundant bacterial taxa between groups was determined using the LEFSE algorithm[111] one-against-all multi-class analysis, normalized to 10^6 reads. PCoA plot of d115 p.i. Unweighted UNIFRAC distances generated by XLSTAT (Addinsoft v2017.5). PICRUST-predicted metagenomic profiles were analyzed by STAMP[112], with significance assessed by Welch's t-test with Storey's FDR (reported *P <0.05, effect size > 0.5). No data that met minimum threshold requirements as outlined in the Online Methods were excluded.

Reporting Summary

Further information on experimental design is available in the [Nature Research Reporting Summary](#) linked to this article.

Data Availability

The 16S MiSeq data (Figs. 1-2 and Supplementary Fig. 1) were deposited in the NCBI SRA under project number PRJNA417022. Other data available from this study are available from the corresponding author upon reasonable request.

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Chapter 3

HIV-associated gut dysbiosis is independent of sexual practice and correlates with noncommunicable diseases

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Abstract

Loss of gut mucosal integrity and an aberrant gut microbiota are proposed mechanisms contributing to chronic inflammation and increased morbidity and mortality during antiretroviral-treated HIV disease. Sexual practice has recently been uncovered as a major source of microbiota variation, potentially confounding prior observations of alterations in the gut microbiota of persons with HIV (PWH). To overcome this and other confounding factors, we examined a well-powered cohort of antiretroviral-treated PWH and seronegative controls matched for age, body-mass index, sex, and sexual practice. We report significant gut microbiota differences in PWH regardless of sex and sexual practice that include *Gammaproteobacteria* enrichment, *Lachnospiraceae* and *Ruminococcaceae* depletion, and decreased alpha diversity. Men who have sex with men (MSM) exhibit a distinct microbiota signature characterized by *Prevotellaceae* enrichment and increased alpha diversity, which is linked with receptive anal intercourse in both males and females. Finally, the HIV-associated microbiota signature correlates with inflammatory markers including suPAR, nadir CD4 count, and prevalence of age-associated noncommunicable comorbidities.

Introduction

Effective antiretroviral therapy (ART) has prolonged survival and shifted the morbidity spectrum for persons with HIV (PWH) from AIDS toward age-associated noncommunicable comorbidities including cardiovascular, osteoporotic, metabolic, hepatic, and renal disease, conditions that occur with increased incidence compared to age-matched HIV-uninfected individuals¹. A key contributor to the current disease spectrum is HIV-associated inflammation and immune activation that persists in chronically treated HIV infection², the etiology of which remains incompletely defined. Mounting evidence supports the concept that the gut microbiota plays a vital role in maintaining immune homeostasis and can, when its composition becomes aberrant, spur pathological, chronic inflammation in a variety of conditions³. Numerous studies have suggested that the gut microbiota contributes to HIV-associated inflammation, though illumination of its role in this process is hampered by an incomplete understanding of the precise identities of microbiota members that are altered in PWH.

The microbiota of the gastrointestinal tract is a tightly regulated community of microorganisms that has dramatic effects on host physiology and immune processes. For example, *Akkermansia muciniphila* can promote intestinal mucosal homeostasis through modulation of mucus thickness⁴. Abundance of certain *Lachnospiraceae* and *Ruminococcaceae* species in the gut induces expansion of regulatory T cells that can down-regulate harmful inflammatory responses⁵. Furthermore, short-chain fatty acid (SCFA) metabolites, derived from commensal bacteria in these clades and others, promote intestinal barrier integrity via their role in epithelial cell energy metabolism and induction of regulatory T cells⁶. Derangement of abundances of these microbes can thus impact the aforementioned protective physiological phenomena. Furthermore, enrichment

of invasive, translocative *Gammaproteobacteria* in mice can be sufficient to cause chronic systemic inflammation⁷. Several components of the gut immune barrier that are responsible for regulating composition of the gut microbiota are abnormal in HIV infection, including Paneth cells⁸, macrophages⁹, epithelial cells^{10,11}, and T_H17 cells¹², giving rise to the possibility of an altered microbiota that results from HIV infection. Additionally, these gastrointestinal barrier components are important for preventing translocation of microbial products into the circulation, a process that has been postulated to contribute to the chronic inflammation that spurs age-associated noncommunicable comorbidities in PWH. Indeed, in vitro studies have shown a higher pro-inflammatory capacity of gut bacteria from treated PWH¹³ compared to seronegative participants, and prophylactic antibiotic treatment that alters the microbiota also reduces levels of intestinal inflammation¹⁴. Importantly, our understanding of which gut bacteria are responsible for these phenotypes, and whether homeostasis-promoting or pro-inflammatory gut bacteria are differentially abundant in PWH, remains incomplete.

Studies of the gut microbiota in HIV infection exhibit great heterogeneity with regard to the characteristics of participants investigated, cohort sizes, type of sampling method used, sequencing depth, and level of bacterial taxonomic classifications reported. Despite this, some common features in microbiota composition of PWH have been reported¹⁵⁻²⁴. These include an over-representation of *Gammaproteobacteria* within the *Enterobacteriaceae* and *Desulfovibrionaceae* families, of which several are known as having pro-inflammatory properties and have been linked to plasma levels of innate immune activation markers in PWH^{15,16}. In addition, a decline in the frequencies of *Lachnospiraceae*, *Ruminococcaceae*, *Rikenellaceae* and *Bacteroides* - bacterial taxa linked with anti-inflammatory properties and maintenance of gut homeostasis - have been

reported in PWH with varying degrees of consistency¹⁵⁻²⁴. Studies have also described an increased abundance of *Prevotella* in PWH as compared to HIV-uninfected controls^{18,20-22}, though these taxonomic shifts have been observed with less consistency.

Recent seminal studies have reported an increased abundance of *Prevotella* and depletion of *Bacteroides* in the gut microbiota among men who have sex with men (MSM) as compared to men who have sex with women (MSW), independently of HIV-1 infection status²⁴⁻²⁶. Because the HIV-infected population in many resource-rich settings is predominantly comprised of MSM, selection of seronegative controls from the general population without matching for MSM could therefore have confounded prior studies examining the impact of HIV infection on the gut microbiota. Studies for which an uninfected control population was not deliberately selected from the MSM population thus likely measured the impact on the gut microbiota of both being MSM and HIV-infected, as opposed to singly examining microbiota associations with HIV infection. These important revelations have raised the hypothesis that after stratification by sexual practice, there may be little to no evidence for shifts in the gut microbiota in PWH compared to seronegative controls, and thus that prior reports of HIV-associated shifts in the gut microbiota were in actuality capturing solely MSM-associated microbiota shifts. Alternately, MSM status and HIV infection status may both be microbiota-modulating factors that each exert unique, orthogonal effects on the microbiota, in which case it remains to be determined which taxonomic shifts described in prior reports were driven by which microbiota-modulating factor (i.e. PWH or MSM status).

In light of these recent findings and resulting competing hypotheses, a lack of well-powered studies in which PWH and controls are matched for variables that may confound microbiota analyses has

limited our understanding of the role of the gut microbiota in HIV disease. Variables previously reported to impact the microbiota - and thus to confound comparisons if cases and controls are not matched for these variables - include sex, age, sexual practice, body mass index (BMI), and immigration status, and have not been tracked in well-powered, evenly represented cohorts. To achieve a high resolution analysis of the impact of HIV infection on the gut microbiota, a large cohort is necessary in order to accommodate selection of a population that is heterogeneous enough in the aforementioned variables to appropriately represent the human population while also being carefully matched for these variables between PWH cases and seronegative controls. In the present study, we investigate the fecal microbiota profiles of a well-powered cohort of chronically HIV-infected people with suppressed viremia on antiretroviral therapy (ART) and HIV-uninfected controls, stratified by sex (i.e. male and female) and sexual practice (i.e. lifetime or recent vaginal/anal intercourse with women/men) and matched for age, sexual practice, BMI, and birth country. We present evidence for distinct and opposing microbiota signatures of both MSM status and HIV infection status, and in addition present links between gut microbiota features in HIV infection and both inflammatory markers and prevalence of noncommunicable comorbidities in this population.

Results

Clinical characteristics and demographics of participants

A cohort of matched PWH and HIV-seronegative controls (total n=160, PWH n=80, HIV-seronegative controls n=80), was assembled to include stratified subgroups of 83 MSM, 38 non-MSM males (MSW, defined as male participants that reported never having sex with men) and 39

females. When examining each subgroup individually, the female group [F= HIV-infected (PWH-F) and HIV seronegative controls (SN-F)]; the men who have sex with men group [MSM= HIV-infected (PWH-MSM) and HIV seronegative controls (SN-MSM)] and the non-MSM male group [MSW= HIV-infected (PWH-MSW) and HIV seronegative controls (SN-MSW)], there were no significant differences in age, BMI, tobacco smoking status, receptive anal intercourse within the past 6 months (RAI), lifetime partners, alcohol intake frequency, or amphetamine use among the PWH and seronegative controls (Supplementary Data 1). All participants were residents of the Netherlands and although some differed in their birth countries, birth countries did not differ significantly between PWH and seronegative controls within each subgroup (Supplementary Data 1). Ninety-one percent of the cohort completed a questionnaire regarding sexual practice during the 6-month period preceding their study visit. Sixteen percent of PWH-F, and ten percent of SN-F declared to have had receptive anal intercourse within the past six months (RAI) ($P=0.298$ Fisher's exact test). Forty-eight percent of PWH-MSM and sixty-two percent of SN-MSM engaged in RAI ($P=0.334$ Fisher's exact test, Supplementary Data 1). There were no significant differences in virologic and immunologic characteristics of PWH across subject groups (Supplementary Data 2).

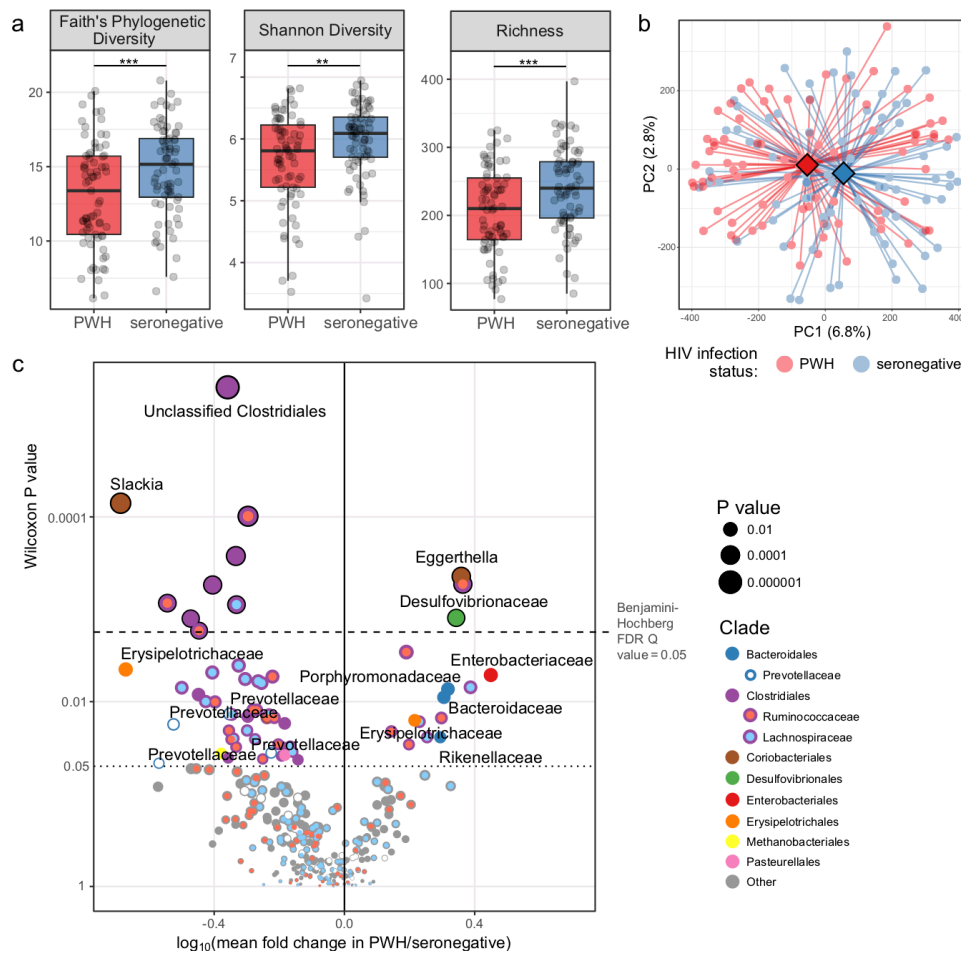
Gut microbiota differs between PWH and HIV-negative controls

Fecal samples were used for profiling of microbial communities via 16 rRNA sequencing. After filtering for quality and sequencing depth, confounder-matched pairs of PWH and seronegative controls totaling $n=142$ total subjects were compared (36 females, 72 MSM, 34 MSW). HIV infection status was significantly associated with a decrease in all common measures of alpha diversity (Figure 1A), supporting a recent meta-analysis²⁷. Principal coordinates analysis (Figure

1B) revealed clustering of PWH and HIV-seronegative controls which was verified by permutational multivariate analysis of variance ($P < 0.001$, PERMANOVA). Community composition differentiated PWH from HIV-seronegative controls ($P = 0.001$, PERMANOVA) even when stratifying by group (F, MSM, MSW) and birth country, two variables that also exhibited significant impact on the gut microbiota (Supplementary Data 3). Having selected uninfected control participants that matched each PWH participant by age, sex, sexual preference, BMI, and birth country, we employed a paired-sample statistical study design to ensure comparisons were not confounded by any of the aforementioned characteristics. The paired non-parametric Wilcoxon test was thus used to identify gut bacterial taxa that differed in abundance between PWH and seronegative controls ($n = 142$ total paired samples, Supplementary Data 4). Among the bacterial taxa enriched in PWH were members of the *Desulfovibrionaceae* (specifically, *Bilophila wadsworthia*) and *Enterobacteriaceae* families (Figure 1C), whose frequency has been variably observed as increased in PWH^{15-19,21-24} and in inflammatory bowel disease²⁸. Taxa depleted in PWH – comprising the likely origin of the reduction in alpha diversity – were predominantly *Clostridiales* members of the *Lachnospiraceae* and *Ruminococcaceae* families, an observation also in agreement with some prior studies^{16,17,20-24}. Members of these clades are known to be enriched for producers of SCFA⁶, a class of compounds that provide energy for gut epithelial cells, prevent expansion of pro-inflammatory *Proteobacteria*²⁹, and induce differentiation of immunologic tolerance-promoting T regulatory cells⁶. These data support the conclusion that PWH exhibit an altered gut microbiota as compared to HIV-uninfected controls that may include an expansion of pro-inflammatory gut bacteria and a depletion of homeostasis-promoting microbiota members. Notable, however, was the lack of observed depletion of *Bacteroides* and

enrichment of *Prevotella* taxa, which were hallmark features of some prior HIV microbiota studies¹⁸⁻²³.

Fig.1: Gut microbiota composition differs between PWH and uninfected controls.



a) Alpha diversity measures are shown for all participants, grouped as PWH and seronegative controls (n=142). b) Principal Coordinates Analysis (PCoA) plot based on the Canberra beta diversity metric with centroids depicted as diamonds for PWH and seronegative controls. Community differences were verified by PERMANOVA, P=0.001. c) Volcano plot depicting OTUs (operational taxonomic units, referring to dada2 sequence variants) in differential abundance between PWH and seronegative controls using the paired non-parametric Wilcoxon test, with pairs of PWH and controls matched for sex, sexual practice, age, BMI, and birth country. Benjamini-Hochberg false discovery rate Q value cutoff of 0.05 is shown. OTUs are colored by either their family (for *Lachnospiraceae*, *Ruminococcaceae*,

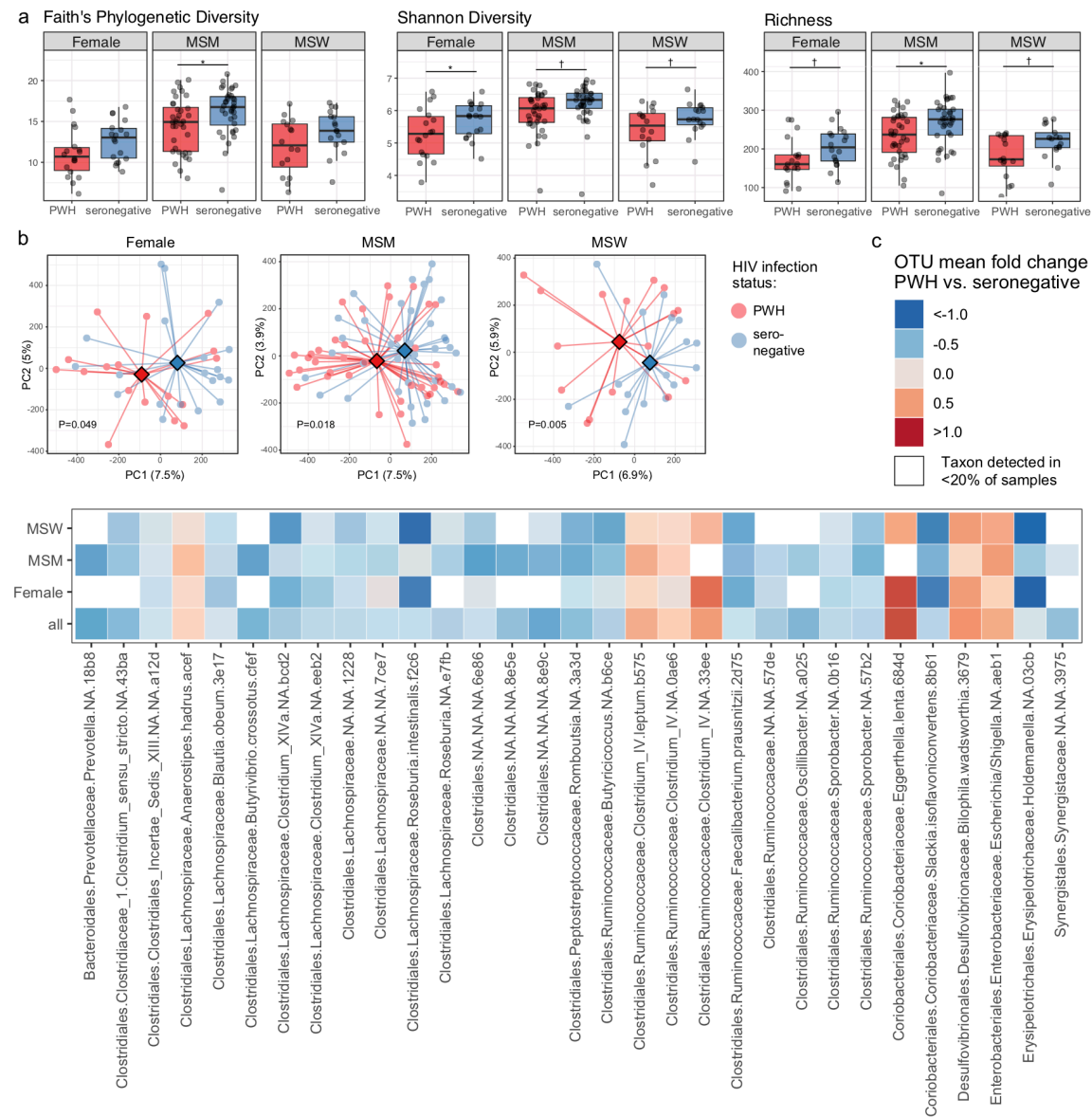
and *Prevotellaceae*) or order (all else), and families of selected taxa are denoted by text. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.005$.

Microbiota differs in PWH regardless of sex/sexual practice

To determine to what degree treated HIV infection itself and sex/sexual practice contribute to microbial community alterations in PWH, we stratified the analysis by groups: F (n=36), MSM (n=72) and MSW (n=34). Among MSM and F, PWH exhibited decreased alpha diversity by at least one alpha diversity measure (Figure 2A), with an equivalent trend for MSW. Shannon diversity, a measure of both species richness (the number of taxa detected in a sample) as well as evenness of their distribution, was reduced in PWH-F and exhibited the same pattern among PWH-MSM and PWH-MSW (Figure 2A). PWH-MSM exhibited robust decreases in species richness and Faith's phylogenetic diversity (a measure of the breadth of the phylogenetic tree that is present in a sample), while both PWH-F and PWH-MSW also exhibited lower species richness. Overall, these data suggest a decrease in alpha diversity among PWH compared to HIV-seronegative controls regardless of sex/sexual practice. Comparisons of community structure using beta diversity analyses revealed significant differences by HIV status in each of the three groups considered individually (MSM $P = 0.005$, MSW $P = 0.018$, F $P = 0.049$; Figure 2B). We found that amplicon sequence variants (ASVs) that were in differential abundance between all matched PWH and negative controls bore consistent abundance trends across all three groups (Figure 2C). Furthermore, when examining the top ASVs that differentiated PWH from controls within each group considered individually, these taxa exhibited concordance in the directionality of their abundance shifts (whether enriched in PWH or controls) across the three groups (Fisher's exact test $P < 0.0001$, Supplementary Figure 2). Together these data suggest that treated HIV

infection exerts a robust effect on the gut bacterial community across human populations that pervades sex and sexual practice.

Fig.2: Gut microbiota composition differences are evident within subgroups.



a) Alpha diversity measures are shown for the three sex/sexual practice subgroups (n=36 females, n=72 MSM; men who have sex with men, n=34 MSW; men who have sex with women), split by PWH and seronegative controls. Welch's t-test was used to test significance of differences between groups. b) PCoA plot based on Canberra beta diversity metric with centroids depicted as diamonds for PWH and seronegative controls. Community differences

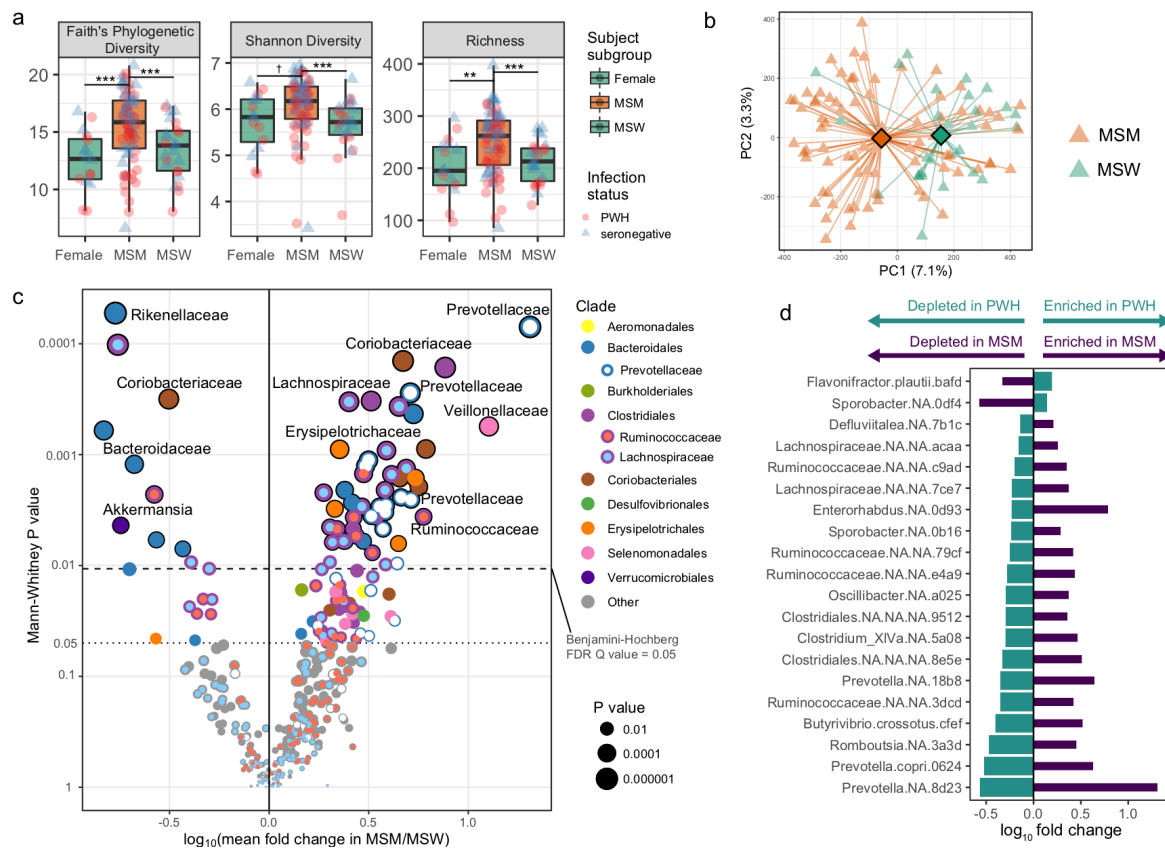
were verified by PERMANOVA (Female P=0.04998; MSM P=0.005; MSW P=0.018). c) Tile plot showing mean fold change of OTUs among all PWH vs seronegative participants, with representations of log mean fold change depicted per subgroup for significant OTUs. OTUs with Wilcoxon significance $P < 0.0125$ in the comparison of PWH vs. seronegative among all paired participants together (including all three subgroups) are shown. White squares denote OTU being present in fewer than 20% of samples within the given subgroup. † $P < 0.10$, * $P < 0.05$.

MSM-associated microbiota is distinct from that of HIV

Next we investigated the gut microbial community in MSM to understand the contribution of sexual practice to PWH-associated dysbiosis. Despite the fact that birth country of study participants was matched between PWH and seronegative controls within each group, subgroups (F, MSM, MSW) differed in their proportions of participant birth countries, with the predominant group being born in the country of recruitment, the Netherlands. Because immigration status is associated with microbiota differences³⁰, for subsequent MSM analyses we sought to mitigate confounding effects of immigration status by selecting only non-immigrant persons born in the most numerous birth country group (Netherlands). MSM exhibited significantly higher alpha diversity regardless of HIV infection status when compared to MSW and F (Figure 3A) (Supplementary Figure 1A-B), a finding supported by prior studies^{24,25}. Pronounced clustering of MSM and MSW gut microbiota community profiles was observed independently of HIV status (Adonis, $P = 0.001$), suggesting that sexual practice per se may drive variability in bacterial composition (Figure 3B). We next compared abundances of ASVs in MSM versus MSW (Supplementary Data 5), and found that MSM were characterized by significantly higher abundance of several *Prevotellaceae* members in agreement with previous reports²⁴⁻²⁶, as well as increases in *Coriobacteriaceae*, *Erysipelotrichaceae*, and *Clostridiales* (primarily *Lachnospiraceae* and *Ruminococcaceae*) members (Figure 3C). Conversely, frequencies of

Bacteroides and *Rikenellaceae* members as well as *Akkermansia muciniphila* were lower in MSM compared to MSW (Figure 3C).

Fig.3: Sexual practice exerts major impact on gut microbiota composition.



a) Alpha diversity measures are shown for the three sex/sexual practice subgroups (n = 16 female, n = 72 MSM, n = 25 MSW), with participants matched for birth country (Netherlands). Faith's phylogenetic diversity $P = 3 \times 10^{-7}$, Shannon Diversity $P = 2.3 \times 10^{-6}$, Richness $P = 3.2 \times 10^{-7}$ by Kruskal–Wallis tests. † $P < 0.10$, * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ by Mann–Whitney U-tests. Boxes denote inter-quartile range, bar denotes median, and whiskers denote range. b) Principal coordinates analysis (PCoA) plot based on Canberra beta diversity metric with centroids depicted as diamonds for MSM and MSW. Shown are participants matched for birth country (Netherlands) and infection status between the comparator groups of MSM and MSW (n = 72 MSM [36 PWH and 36 seronegative] vs. n = 22 MSW [11 PWH and 11 seronegative]). Community differences were verified by PERMANOVA, $P = 0.001$. c) Volcano plot depicting ASVs in differential abundance between MSM and MSW matched for infection status and birth country,

using the unpaired non-parametric Mann–Whitney U test. ASVs are colored by either their order or family, and families of selected taxa are denoted by text. d) A comparison of abundance trends among ASVs significantly differing in both the comparison of MSM vs. MSW and PWH vs. uninfected controls. ASVs were selected by P-value < 0.05 in both comparisons (paired Wilcoxon for PWH vs. uninfected, unpaired Mann–Whitney for MSM vs. MSW). Directionality of ASV abundances are in opposition to each other and refute the expectation that having MSM status enriches for the same taxa as does HIV infection (Fisher’s exact test $P < 0.001$).

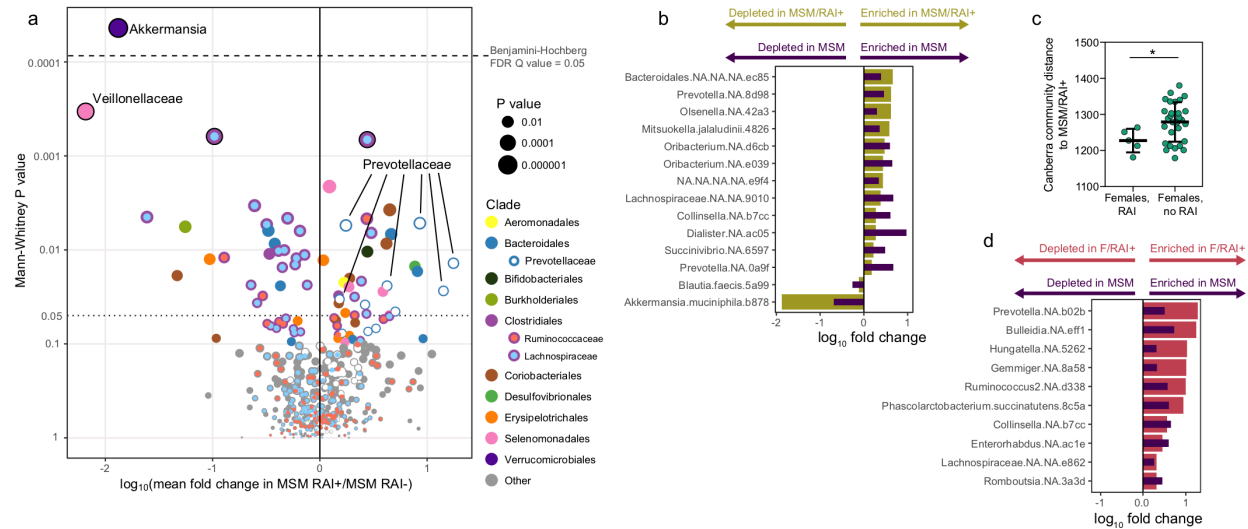
Prior studies examining the gut microbiota of PWH may have been confounded by an over-representation of MSM in the PWH grouping as compared to a seronegative control grouping drawn from the general population in which MSM may be a minority. Thus, taxa enriched in MSM due to the MSM-associated microbiota signature may have emerged as false positive observations conflated with taxa enriched in HIV infection. In order to understand whether the MSM-associated microbiota signature overlaps with and thus explains the appearance of an HIV-associated microbiota signature, we sought to address the important unanswered question of whether taxa observed to be enriched in PWH are the same as those enriched in MSM or conversely if those depleted in PWH were also depleted in MSM. Of ASVs that differed significantly between PWH vs. seronegative (total ASVs=65 with $P < 0.05$) and MSM vs. MSW (total ASVs=99 with $P < 0.05$), twenty ASVs overlapped between these lists (13.9% of the two lists). Surprisingly, these ASVs were found to exhibit opposing abundance trends among these two sets of comparisons (Figure 3D). Specifically, *Prevotella* taxa and several *Lachnospiraceae* and *Ruminococcaceae* members were enriched in MSM but depleted in PWH. These data reveal that the MSM microbiota signature is complex and unique, and importantly, mostly non-overlapping with that of HIV infection with some taxa exhibiting opposing abundance shifts as those in the HIV microbiota signature.

Sexual practice is linked with microbiota regardless of sex

To explore potential drivers of the MSM-associated microbiota signature, we investigated microbiota trends associated with sexual practice of PWH and HIV-seronegative controls during the six months prior to study visit. Ninety-one percent of MSM completed a behavioral questionnaire that provided information on recent receptive anal intercourse within the last six months (RAI). We found that out of 72 total MSM profiled, 44 reported having engaged in RAI (MSM/RAI+), and 28 reported no such recent activity (MSM/RAI-), with no significant differences in RAI activity between PWH-MSM and SN-MSM ($P=0.73$). In contrast to two prior studies^{25,26}, we observed significant community differences between MSM/RAI+ and MSM/RAI- (PERMANOVA $P=0.01$, Supplementary Figure 3). Numerous ASVs were found to be in differential abundance between MSM/RAI+ and MSM/RAI- (Supplementary Data 6), including a depletion of ASVs belonging to the clades *Clostridiales*, *Veillonellaceae*, and *Akkermansia* in MSM/RAI+, while members belonging to the *Prevotellaceae* family were enriched in MSM/RAI+ (Figure 4A). Next, we selected the top ASVs that differentiated MSM versus MSW (ASVs with $P<0.05$ in Figure 3C) and compared them to ASVs that differentiated MSM/RAI+ versus MSM/RAI- (ASVs with $P<0.05$ in Figure 4A). Among the taxa that overlapped in these two lists, there was a clear consistency in abundance trends such that taxa that were enriched in MSM vs. MSW were also enriched in MSM/RAI+ vs. MSM/RAI- and this pattern was mirrored in the depleted bacterial taxa ($P = 0.006$, Fisher's exact test, Figure 4B). The bacterial communities increased in MSM and MSM/RAI+ included *Prevotella*, *Collinsella*, and *Oribacterium* members, while *Blautia faecis* and *Akkermansia muciniphila* were depleted in both MSM and MSM/RAI+. To further examine sexual practice-specific changes in the composition of the gut microbiota, we sought to examine the role of receptive anal intercourse in female participants within our cohort.

Using the Canberra beta diversity distance metric, which quantifies the overall ecological community similarity between two microbiota samples and is well-suited for microbiota count data³¹, we quantified the similarity of gut microbiota profiles of females that reported RAI (F/RAI+, n=5) to MSM that reported RAI (MSM/RAI+), as well as that of females without RAI (n=33) to MSM/RAI+ participants. We found that females with RAI exhibited greater bacterial community similarity to the MSM/RAI+ population than did females that reported no RAI ($P < 0.05$) (Figure 4C). Consistent with the impact of RAI in males, all ASVs that shifted in both females with RAI vs. females without RAI and MSM vs. MSW did so in the same direction ($P = 0.033$, Figure 4D). Finally, we found that when comparing the overall community structure of MSM that engaged in recent condomless RAI (n=31) to that of MSM that engaged in RAI with use of a condom (n=18), there was not a significant difference in community composition ($P = 0.16$, PERMANOVA). In summary, these data reveal that sexual practice is associated with a unique microbiota signature regardless of sex and possibly regardless of condom use.

Fig.4: Recent sexual practice and lifetime sexual practice impacts gut microbiota composition regardless of sex.



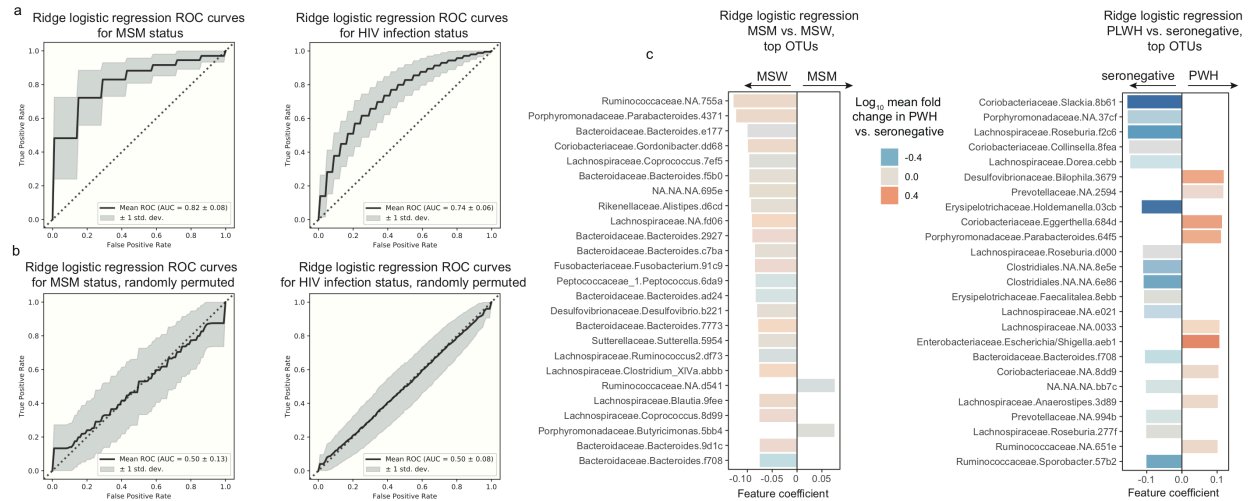
a) Volcano plot depicting OTUs in differential abundance between MSM/RAI+ (men who have sex with men that experienced receptive anal intercourse within the last 6 months prior to sampling, n=44) and MSM/RAI- (men who have sex with men that did *not* experience receptive anal intercourse within the last 6 months prior to sampling, n=28), matched for infection status. b) Overlap in abundance trends among the top 100 taxa in differential fold abundance among MSM vs. MSW (n=72 MSM, n=22 MSW) and MSM/RAI+ vs. MSM/RAI- (n=44 MSM/RAI+, n=28 MSM/RAI-). Mann-Whitney tests were performed on each OTU for both comparisons and shown are taxa that reached P<0.05 on both lists. c) Average Canberra community distances to MSM/RAI+ were calculated for each female participant sample that reported receptive anal intercourse within 6 months prior to sampling (F/RAI+, n=5) and female participants that reported no such activity (F/RAI-, n=28). F/RAI+ gut communities were significantly more similar to MSM/RAI+ than were those for F/RAI-. d) Overlap in abundance trends among the taxa that reached P<0.05 in both comparisons of MSM vs. MSW and F/RAI+ vs. F/RAI- (Fisher's exact test P = 0.077), as performed in b.

Robustness of MSM- and HIV-associated microbiota signals

Microbiota survey studies comparing diseased versus healthy individuals are highly prone to false discovery due to the high numbers of comparisons being made ($\sim 10^3$ taxa commonly compared across groups) and the subsequently high likelihood of random observations appearing significantly discriminative of cases and controls. One method of overcoming the challenge of identifying signal among the noise is to examine the consistency of taxonomic shifts across independent studies, an exercise that largely supports the shifts reported herein^{15-22,24}. Machine learning offers another attractive methodology to reduce false discovery using the concept of intra-study cross-validation. By this method, a binary classification model identifies the most discriminatory bacterial taxa between positive and negative classes (i.e. cases and controls) from a subset of samples – known as the ‘training’ set. Then, the capacity for the model to accurately predict the class of the remainder of samples – ‘validation’ set of unseen samples held-out from the training of the model – is determined. Models are repeatedly trained on randomly selected training subsets and evaluated on the remaining held-out validation data. The overall capacity for the gut microbiota to accurately predict unseen samples into their correct class is quantified numerically as the area-under-the-curve of the receiver-operating characteristic curve (AUROC), which can be interpreted as the probability that, given a random pair of unseen positive and negative samples, the classifier will choose the positive sample over the negative sample as belonging to the positive class. In this manner, we utilized ridge logistic regression binary classifiers to estimate the overall robustness of the gut microbiota to predict MSM vs. MSW and PWH vs. HIV-uninfected, separately. In both cases, the microbiota was able to classify persons with significant prediction accuracy with AUROC = 0.84 ± 0.07 ($p = 0.015$) for MSM vs. MSW and AUROC = 0.73 ± 0.06 ($p = 0.017$) for PWH vs. seronegative (Figure 5A), indicating that both

HIV infection status and MSM status had robust microbiota signatures and that the MSM microbiota signature was more robust than that of HIV. This is consistent with the observation that a greater number of ASVs differed significantly between MSM vs. MSW as compared to PWH vs. seronegative (Supplementary Data 4, 5). Results were similar when using random forests binary classifiers (Supplementary Figure 4). To test whether our model was producing accurate predictions due to true microbiota differences between our comparator rather than overfitting to noise within the taxon abundance data, samples were randomly assigned to either MSM or MSW in the MSM vs. MSW comparison, or separately to PWH or seronegative for its respective comparison, and our pipeline was performed on the resulting corrupted data. When sample class labels were thus randomized, results were consistent with guessing near random chance (0.50 ± 0.13 and 0.50 ± 0.08 , for MSM vs. MSW and PWH vs. HIV-seronegative, respectively, Figure 5B), supporting the results of the true groupings and the overall conclusion that both sexual practice and HIV infection status exert influences on the gut microbiota that are non-random, robust, and consistent across samples.

Fig.5: Gut microbiota profiles robustly segregate samples by HIV infection status and sexual preference.



a) The ridge logistic regression application of machine learning was used to test robustness of differences between MSM vs. MSW ($n=72$ MSM, $n=22$ MSW), and PWH vs. uninfected ($n=71$ PWH, $n=71$ seronegative). Both MSM status and HIV infection status exhibited robust signatures by which ridge logistic regression could predict the classification status of unseen samples with high accuracy (AUROC=0.84 for MSM vs. MSW with $P = 0.015$, AUROC=0.73 for PWH vs. seronegative with $P = 0.017$). b) Sample classifications (e.g. MSM, MSW, PWH, seronegative) were randomly permuted to introduce noise into the dataset and quantify capacity for machine learning to classify persons based on spurious data. Random class label permutation demonstrated no classification accuracy to spurious data (AUROC=0.5 for both MSM vs. MSW and PWH vs. uninfected). c) Top OTU features that informed models for ridge logistic regression predictions of MSM vs. MSW and PWH vs. seronegative. Feature coefficients represent extent to which each OTU was weighted to represent the given class (positive for MSM vs. MSW indicated OTU was indicative of MSM; positive for PWH vs. seronegative was indicative of PWH). Bars are color-coded by log mean fold change in PWH vs seronegative, and show a lack of correlation between OTUs informative for predicting MSM status and those altered in abundance in PWH.

Ridge logistic regression models allow for the use of feature parameter coefficients (i.e. feature importance) that indicate the relative association of each ASV and the classes, learned by the model

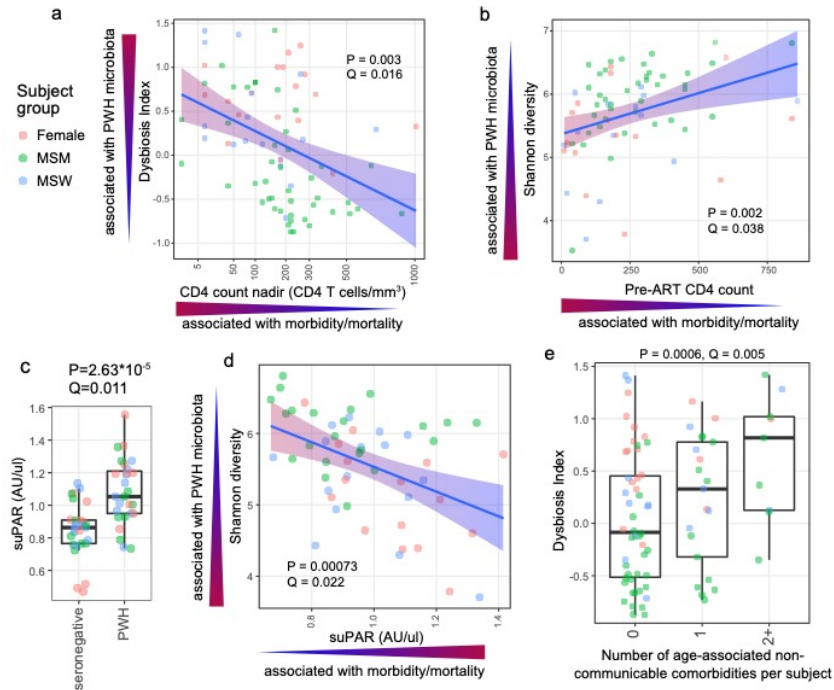
during minimization of the classification error (Supplementary Data 7, 8). ASV features that were most informative for predicting PWH vs. HIV-seronegative included *Enterobacteriaceae* and the *Desulfovibrionaceae* member *Bilophila wadsworthia* as being indicative of PWH, and *Coriobacteriaceae* and *Lachnospiraceae* members as indicative of HIV-seronegative samples (Figure 5C), observations that were largely consistent with those found to be in differential abundance by traditional statistical methods (Figure 2C) and prior studies^{15-22,24}. Interestingly, ASVs that were depleted in MSM had greater capacity to predict MSM vs. MSW status (Figure 5C), and these included *Bacteroides*, the *Alistipes* genus of the *Rikenellaceae* family which have previously been reported as depleted in HIV infection among studies for which a control population was not actively matched for sexual practice³².

HIV-associated microbiota correlates with inflammation

To understand the clinical implications of HIV-associated gut microbiota perturbations, we calculated a ‘dysbiosis index’ (DI) to collapse into a single number the shifts in bacterial taxa that were characteristic of PWH vs. seronegative participants. This was accomplished by calculating the log ratio of geometric means of taxon abundances that were enriched in PWH (having Wilcoxon $P < 0.05$) over taxa that were depleted in PWH (having Wilcoxon $P < 0.05$) as compared to seronegative controls. The resultant DI differed between HIV-infected groups and was lowest among PWH-MSM (Supplementary Figure 5), possibly due to abundance trends of certain taxa in the MSM signature being in opposition to the abundance trends for the same taxa in the HIV microbiota signature (Figure 3D), as well as the generally increased alpha diversity in MSM (both PWH and seronegative, Supplementary Figure 1A,B) which is contrary to the trend in PWH (Figure 2A). We next examined associations between DI and clinical characteristics and

inflammatory markers within PWH using linear mixed effects models with subgroup (F/MSM/MSW) included as a random effect. We found a strong, significant correlation between the DI and known duration of HIV infection, duration of antiretroviral therapy (which correlated with duration of HIV infection), as well as nadir CD4 count³³ and pre-ART CD4 count³⁴ (Figure 6A, Supplementary Data 9). As reduced alpha diversity was also a feature of the HIV-associated microbiota signature, we performed the same association analysis within PWH on Shannon diversity versus clinical and inflammatory markers to find associations with soluble CD14 (sCD14) (Supplementary Figure 6A,B) and pre-ART CD4 count (Figure 6B, Supplementary Data 10).

Fig.6: HIV-associated gut microbiota features correlate with markers of HIV disease progression, immune activation, as well as comorbidity prevalence.



Linear mixed effects models were used to control for subgroup (F, MSM, MSW) as random effects for all analyses depicted. Linear regression lines denoted in blue with 95% confidence intervals depicted as a gradient with red representing directions for both independent and dependent variables that are associated with the PWH state (e.g. high dysbiosis index, low nadir CD4 count) and blue representing the seronegative state. A) ‘Dysbiosis Index’ (DI) correlates with nadir CD4 (count/mm³), P = 0.003, Benjamini-Hochberg Q = 0.017, among PWH subjects (n=80, all in study). B) Shannon alpha diversity correlates with pre-ART CD4 count, P = 0.002, Q = 0.038. C) suPAR concentrations in plasma are strongly elevated in PWH (n=27) compared to matched seronegative controls (n=27). Q values calculated with consideration of all 1,317 plasma markers quantitated by aptamer-based SOMAlogic assay. D) Shannon alpha diversity correlates with plasma suPAR levels, P = 0.00073, Q = 0.022, n=54 PWH. E) Number of comorbidities experienced by PWH participants (n=80) by time of sampling correlates with DI, P = 0.0006, Q = 0.005.

To expand understanding of links between the HIV-associated microbiota and host immune states, we performed unbiased aptamer-based proteomic screens to quantitate 1,305 human plasma proteins on a subset of samples evenly split between studied groups (9 SN-F, 9 SN-MSM, 9 SN-MSW, 16 PWH-F, 22 PWH-MSM, and 16 PWH-MSW). Comparison of all plasma proteins between paired PWH and seronegative controls identified several inflammatory markers enriched in HIV infection (Supplementary Data 11) including myeloperoxidase (MPO), growth/differentiation factor 15 (GDF15), and secreted urokinase plasminogen activator surface receptor (suPAR), a marker of systemic immune activation recently identified as a remarkably strong independent predictor of myocardial and overall non-AIDS morbidity and mortality^{35,36}. Notably, suPAR was both significantly higher in PWH ($P = 2.6 \times 10^{-5}$, $Q = 0.011$, Figure 6C, $n=27$ matched pairs) and inversely correlated with Shannon diversity ($P = 0.0007$, $Q = 0.021$, Figure 6D) when examining all PWH queried via proteomics ($n=54$ PWH). While DI and Shannon diversity were themselves inversely correlated with each other, multivariate linear mixed effects analysis revealed that each was associated with the aforementioned markers of disease independently of the other (CD4 nadir vs. DI and Shannon: DI $P = 0.0173$, Shannon $P = 0.8147$; sCD14 vs. DI and Shannon: DI $P = 0.737$, Shannon $P = 0.0436$; suPAR vs. DI and Shannon: DI $P = 0.981$, Shannon $P = 0.0088$). Furthermore, suPAR positively correlated with the DI ($P = 0.0987$, Supplementary Figure 6C) though did not reach statistical significance, and fold enrichment/depletion patterns of ASVs that differed in abundance between PWH and seronegative controls (those comprising the DI) were associated with directionality of correlations with suPAR, such that bacteria depleted in PWH correlated inversely with suPAR levels in PWH while bacteria enriched in PWH correlated positively with suPAR levels (Supplementary Figure 6D), suggesting

a link not only with alpha diversity but also with the specific taxa that are altered in abundance in PWH.

Comorbidity prevalence is linked with HIV-associated microbiota

Having found links between clinical and inflammatory markers of disease progression with microbiota features, we sought to test whether microbiota features associated also with inflammatory comorbidity events including type II diabetes, renal disease, fibrosis (using FIB4), osteoporotic fractures, chronic obstructive pulmonary disease (GOLD 2 and higher), diagnosed myocardial infarction, cerebrovascular accident, transient ischemic attack, angina pectoris, peripheral arterial disease, and non-AIDS associated cancer, which all occurred in a subset of our treated PWH cohort at varying prevalence and to a greater degree than in seronegative controls (Supplementary Figure 7). The DI, though not Shannon diversity, was significantly associated with the number of comorbidities (0,1, ≥ 2) (Figure 6E), and this effect was observed within individual subgroups (Supplementary Figure 8). Furthermore, DI retained a significant association with comorbidity prevalence when including CD4 nadir as a co-variate in a linear mixed effects model framework (DI $P = 0.0044$, CD4 nadir $P = 0.4523$).

Discussion

The role of enteric microbiota alterations in chronic HIV infection has been the focus of increasing investigation. Chronic, systemic immune activation is a hallmark of treated and untreated HIV infection that is considered the primary driver of viral replication and disease progression^{37,38}. Presence of specific gut microbiota members in mice has been causally implicated in spurring

pathologic inflammation in experimental models^{7,39}, leading to the hypothesis that dysregulation of the gut microbiota in humans may also contribute to human inflammatory diseases such as chronic HIV infection. While many studies have found alterations in the gut microbiota of HIV-infected individuals including enrichment of bacteria known to be pro-inflammatory and a simultaneous depletion of tolerogenic, homeostasis-promoting bacteria, recent seminal studies have revealed a major shift in the gut microbiota of MSM as compared to MSW. Such reports have called into question earlier observations of microbiota dysregulation in relation to HIV *per se*^{24,40}. Of note, numerous early studies recruited primarily MSM in the PWH cohort and did not actively match cases and controls for sexual practice, causing an enrichment of MSM in PWH as compared to uninfected control comparators. Due to the existence of such confounding variables and to the tremendous heterogeneity in gut microbiota profiles within the human population, the lack of a well-powered cohort that simultaneously examines the impact of HIV infection in females and males with differing sexual practices while being carefully matched for microbiota-modulating confounding variables has hampered our understanding of HIV-associated dysbiosis and its role in HIV disease. Furthermore, a lack of precise delineation of how the MSM-associated microbiota compares or contrasts with that of HIV infection remains a barrier toward identifying gut microbiota constituents linked to HIV disease.

In this study, we dissect the differential impacts on the gut microbiota for two major sources of microbiota heterogeneity: that of MSM status and HIV infection status. We find gut microbiota shifts associated with HIV infection in our comparison of antiretroviral-treated PWH as compared to uninfected controls carefully matched for age, sex, sexual practice, birth country, and BMI. Alpha diversity shifts as well as differences in relative abundance of several taxonomic clades of

bacteria were found to have consistency across three major groups of interest: females, MSW, and MSM. Components of HIV-associated microbiota shifts correlated with clinical and inflammatory markers of HIV disease progression as well as prevalence of age-associated noncommunicable comorbidities, highlighting the need to elucidate causality in these associations. Furthermore, we characterize a robust microbiota signature associated with MSM status that is consistent with prior reports²⁴⁻²⁶. Importantly, we build upon these observations to contrast the microbiota signatures of MSM status and HIV infection status to show that the MSM-associated microbiota signature is unique from that of HIV infection, and that taxa that constitute both the MSM microbiota signature and the HIV microbiota signature exhibit opposing abundance trends. Finally, we provide evidence that the MSM-associated microbiota signature may be driven by receptive anal intercourse (RAI), a hypothesis supported by our examination of RAI in females.

Our results reveal that antiretroviral-treated HIV infection is associated with distinct gut microbiota features independently of sex/sexual practice, in the form of a reduction in alpha diversity, overall shifts in community structure as denoted by beta diversity analyses, and consistent shifts in abundance of specific bacterial taxa common across participant groups. The bacterial shifts observed in PWH included an enrichment of pro-inflammatory taxa belonging to *Enterobacteriaceae* (*Escherichia/Shigella*) and *Desulfovibrionaceae* (*Bilophila wadsworthia*) consistent with other cohorts^{16,22,24}. Notably, the *Enterobacteriaceae* family includes several pathogenic taxa such as *Escherichia*, *Shigella*, and *Klebsiella* which are well documented sources of bacteremia in PWH. Members of the *Desulfovibrionaceae* family have also been implicated in chronic inflammatory states such as inflammatory bowel disease²⁸. *Desulfovibrionaceae* encompass several species with the metabolic capability to reduce sulfate which produces the

byproduct hydrogen sulfide, a cytotoxic compound that inhibits colonocyte butyrate oxidation. Butyrate is a crucial energy source for colonocytes, an inducer of inter-enterocyte tight junction proteins that promote gut barrier function⁴¹, and augments gut regulatory T cells which constitute a lymphocyte population critical for mitigating pathologic inflammation^{42,43}. A concomitant decline in butyrate producing gut microbiota members belonging to the *Lachnospiraceae* and *Ruminococcaceae* families⁴⁴ was observed in PWH compared to HIV-seronegative controls. Indeed, abundance of *Lachnospiraceae* members in HIV-infected people has been recently found to correlate inversely with I-FABP, a marker of gut enterocyte turnover, supporting the importance of this clade in mucosal homeostasis⁴⁵, though I-FABP did not correlate with our dysbiosis index in this study. Although the enterocyte turnover that is measured indirectly by serum I-FABP may represent loss of mucosal integrity in cases of enterocyte death⁴⁶, reduced expression of tight junction proteins between enterocyte cells can also increase likelihood of microbial translocation. Tight junction expression is positively regulated by butyrate⁴¹ and thus may constitute a pathway by which the HIV-associated microbiota may impair gut barrier function independently of enterocyte death, via lessened butyrate production. Thus, an increase in production of molecular inhibitors of butyrate oxidation in combination with a decrease in butyrate-producing gut bacteria may synergize to contribute to the epithelial barrier disruption that is seen to persist in treated HIV infection¹⁰. This impairment of the function of the intestinal epithelium may permit translocation of pro-inflammatory microbiota products into systemic circulation and exacerbate pathological chronic inflammation in treated PWH⁴⁷⁻⁵⁰, providing a putative link between the described HIV-associated microbiota features and noncommunicable, inflammation-associated comorbidities.

The extent of dysbiosis in our study was found to correlate with CD4 nadir and pre-ART CD4 counts, both being measures of the severity of immune collapse during untreated infection which suggests a link between immune function and preservation of microbial community composition. Dysbiosis was tightly correlated with the prevalence of comorbidities, which themselves have previously been linked to CD4 nadir and pre-ART CD4 counts^{33,34}. It is possible that the extent of immune damage (proportional to the extent of CD4 T cell loss, i.e. CD4 nadir) dictates the degree of dysregulation of microbiota composition but is itself the cause of later age-associated comorbidities, with the effects on the microbiota contributing little to pathology. However, extent of dysbiosis was significantly correlated with comorbidities independently of CD4 nadir, suggesting that the link between dysbiosis and disease progression is not merely an innocuous consequence of prior events but may impact later comorbidity occurrence. Indeed, innate immune activation may constitute a link between the comorbidities measured herein – each of which have been linked to innate immune activation levels in PWH⁵¹⁻⁵⁵ – and the gut microbiota, which has been shown to causally impact innate immune activation states in mice^{7,39}. SuPAR is a marker of innate immune activation⁵⁶ which has been recently characterized as a robust predictor of myocardial³⁵ and overall non-AIDS clinical events (with an odds ratio of 2 per inter-quartile range for death³⁶) in PWH. It has also been described to be an accurate predictor of bacteremia^{57,58} and is increased as a result of LPS injection in humans⁵⁹ but does not increase in certain cases of sterile inflammation⁶⁰, prompting the hypothesis that its capacity to predict HIV-associated mortality may be due to its relationship with bacterial translocation. Our finding that suPAR levels are linked with features of the HIV-associated microbiota signature (i.e. overall alpha diversity and specific taxa associated with PWH) may prompt its usage as a biomarker for microbiota-mediated

strategies to alleviate HIV-associated inflammation and warrants further study into the impact of HIV-associated gut bacteria on this pathologic inflammatory pathway.

We report that MSM harbor a markedly different gut microbiota composition than that of MSW. Though HIV infection status exhibited a strong microbiota signature, the effect of MSM on the microbiota was more pronounced than that of HIV status by both numbers of ASVs that were in differential abundance and by robustness as assessed via machine learning analyses. We find overlap between bacterial community shifts in MSM and MSM/RAI+, supporting the idea that RAI represents a driving factor for microbiota differences observed between MSM and MSW. The lack of bacterial community differences between condomless MSM/RAI+ and MSM/RAI+ with condom suggests that the RAI-associated microbiota may not be an effect of semen but perhaps is linked with other practices associated with RAI, for example rectal douching and use of lubricants. Interestingly, the females who reported engaging in RAI bore compositional similarities to MSM/RAI+, indicating that RAI may be linked to a microbiota signature regardless of gender. Although we found statistically significant results in this analysis, our sample size was limited and further study is warranted. The RAI-associated microbiota in both MSM and F was mainly characterized by an expansion in the *Prevotella* genus which has been previously associated with lubricant use⁶¹. A decline in abundance was observed for *Bacteroides* members as well as *Akkermansia muciniphila*. Both *Bacteroides* members and *A. muciniphila* are known to utilize mucus as major growth and adhesion substrates that can reside preferentially in the mucus layer of the large intestine^{62,63}. These observations prompt the hypothesis that the colonic mucus layer of MSM undergoes remodeling and possible depletion. Further investigation is warranted as to the consequences of RAI on host mucus integrity and on the microbiota, as well as the effect of these

microbial community shifts on host physiology, especially given recent studies showing that *Akkermansia* supplementation in humans promotes mucosal barrier health with the effect of reducing plasma LPS levels and ameliorating metabolic syndrome⁶⁴. Furthermore, due to the robust nature of the MSM-associated microbiota signature, we recommend that sexual preference data be systematically collected and taken into consideration in microbiota studies beyond HIV.

Earlier cross-sectional studies examining the gut microbiota in PWH that were not matched for microbiota-associated confounding variables such as MSM status (primarily having an over-representation of MSM in the PWH group) reported shifts from *Bacteroides* to *Prevotella* predominance following HIV-1 infection¹⁸⁻²³. Stratifying participants by sex/sexual practice, we found that enrichment of *Prevotella* and depletion of *Bacteroides* were not features of HIV infection per se but were instead characteristics of the MSM-associated microbiota. However, we found evidence that treated HIV infection does exert a distinct effect on the microbiota that is largely distinct from that of MSM status and is characterized by a reduction in overall diversity, enrichment of *Gammaproteobacteria*, and depletion of *Clostridiales* members, a finding strengthened by our having matched cases and controls for metadata variables reported to affect the microbiota (age, BMI⁶⁵, birth country⁶⁶ and sex/sexual practice²⁴⁻²⁶). Interestingly, bacterial taxa associated with MSM either did not overlap with taxa of the HIV-associated microbiota signature or when overlap did occur (as was the case for 13.9% of the total number of taxa that comprised the MSM and HIV microbiota signatures in our study) these taxa exhibited abundance trends opposite that of HIV infection, supporting the conclusion that some earlier studies examining the gut microbiota in HIV in which MSM status was not actively matched between cases and controls had a reduced capacity to discern true differences in the microbiota driven by

HIV infection and an increased risk of spurious observations linked to MSM status rather than HIV serostatus.

As with all cross-sectional human studies, one cannot rule out the effects of other uncaptured confounding factors that differ across a population. Although diet is known to affect the gut microbiota, HIV gut microbiota survey studies that have examined diet have found evidence that dietary intake in PWH does not correlate with abundances of gut microbes of the HIV-associated microbiota signature^{20,24,45} as it does in the seronegative population^{20,67}. For example, relative abundance of *Bacteroides* members correlates with meat intake in seronegative subjects^{20,67}, but this association was not found in PWH²⁰, suggesting HIV infection may have a greater impact on the abundance of certain microbes than do certain major components of diet. Furthermore, dietary patterns between PWH and seronegative participants did not differ significantly^{20,45}, suggesting that diet alone is unlikely to be the driver of the HIV-associated microbiota signature. Furthermore, given the potent impact of sexual preference on the gut microbiota, it is conceivable that sexual activities not captured in the present dataset may impact the microbiota signatures described herein, warranting further study. Longitudinal studies may also help disambiguate microbiota signals from confounding factors, though some lifestyle factors may change with diagnosis and should likely be continuously monitored. Relative longitudinal stability of the gut microbiota also was not examined in the present study, though prior studies have noted limited intra-individual dispersion among PWH^{68,69}. We cannot exclude that HIV infection induces microbiota instability in humans, which was not able to be captured in the present cross-sectional study design. An added and as-yet unaddressed caveat to the present study is the inability to tease apart the role of HIV infection from that of antiretroviral therapy, as all our PWH participants were also treated over a

long term (median of 12.5 years). Though several studies report gut microbiota shifts in untreated persons that bear similarities to those reported herein^{15-18,20,23}, the effect of antiretrovirals on the gut microbiota remains poorly understood and cannot be ruled out as a contributor to the observed microbiota shifts in the absence of studies directed at this question. Furthermore, while country of birth and immigration status were collected in our metadata, ethnicity information was not and may be an important potential confounding factor to collect in future studies.

Avenues to further elucidate the role of the microbiota in HIV disease progression include the examination of mucosal-resident communities in the context of the populations studied herein (i.e. MSM, PWH). Given the mucosal-resident nature of potentially immunostimulatory SFB and *Akkermansia* in mice and given that this community differs from that of the lumen, studies examining microbial communities of mucosal biopsies may yield important insights into the relationship between the gut microbiota and immune states in HIV infection. Non-human primate studies offer an attractive path for longitudinal analysis, though challenges in translating SIV microbiota studies exist in that SIV infection does not durably alter the macaque gut microbiota through the early phases of chronic infection⁷⁰⁻⁷². However, wild chimpanzee studies have reported microbiota instability in advanced SIV infection^{73,74}, and experimentally-infected rhesus macaques similarly exhibit microbiota alterations during advanced disease characterized by low CD4 counts⁷⁵ not unlike those described herein (i.e. *Enterobacteriaceae* bloom). Given that nadir CD4 count correlates in our study with extent of dysbiosis, it is possible that the inflammation and/or immune destruction occurring in advanced HIV and SIV disease causes microbiota disruptions that persist even after CD4 restoration via treatment, and that this altered microbiota contributes to elevated comorbidity burden in PWH. Future prospective longitudinal studies in humans and

non-human primates are warranted to further dissect the impact of the observed specific shifts in the gut microbiota on associations with chronic inflammation and comorbidity prevalence in HIV as reported herein.

Methods

Study participants and study design

The AGE_hIV Cohort Study is an ongoing observational cohort (NCT01466582) of HIV-infected participants from the HIV outpatient clinic of the Amsterdam University Medical Centers (location Academic Medical Center) and HIV-uninfected participants recruited from either the sexual health clinic or the Amsterdam Cohort Studies on HIV/AIDS at the Public Health Service in Amsterdam, the Netherlands. All participants signed informed consent prior to study procedures. Participants were 45 years or older at enrollment and attended biennial study visits. Participants were requested to complete a standardized questionnaire concerning demographics, personal and family medical history, use of medications, participation in population screening programs, substance use, quality of life, depression, sexual orientation/behavior/dysfunction, cognitive complaints, calcium/vitamin D intake, physical exercise, social behavior, and work participation/income. During each study visit, participants underwent a venous blood draw, measurements of blood pressure, height, weight, and hip/waist circumference, as well as electrocardiography, spirometry, and bone densitometry. Fecal and urine samples were also cryopreserved during each study visit. Study participants self-collected stool at their homes no more than 24 hours prior to study visits and stored specimens in a refrigerator until being brought in for their visits. None of the respondents included in the final analysis reported antibiotics usage at the time of the study visit

at which time fecal samples were collected. For this cross-sectional study, one fecal sample was analyzed per individual.

Cryopreserved fecal samples (n=169) were processed from PWH and HIV-seronegative controls that were matched for age, BMI, smoking, and immigration status within each of the three subgroups: men who have sex with men (MSM), non-MSM males (MSW), and females (F). Efficacy of matching for these variables (defined as no significant difference between PWH and seronegative participants within each subgroup) is shown in Table 1. The following numbers of samples were successfully extracted, amplified, and sequenced to sufficient depth: 83 MSM, 38 MSW and 39 females. Paired samples for which the matched sample failed extraction, amplification, or to reach sufficient sequencing depth were removed for paired statistical comparisons. Thus, for paired statistical analyses, numbers of samples were as follows: MSM (n=72), MSW (n=34), and F (n=36).

Assessment of comorbidity

Comorbidities objectively assessed during each study visit were: (1) chronic obstructive pulmonary disease (COPD) (GOLD 2 or more by spirometry); (2) advanced liver fibrosis (FIB-4 ≥ 3.25); (3) decreased kidney function (eGFR < 60 mL/min/1.73 m² using the Chronic Kidney Disease Epidemiology Collaboration [CKD-EPI] equation); (4) osteoporotic fractures (Dual-energy X-ray absorptiometry [DXA] T-score < -2.5 standard deviation (SD) for men aged ≥ 50 years and post-menopausal women or a Z-score < -2.0 for men aged < 50 years and pre-menopausal women using World Health Organization definitions and having reported a fracture 2 years before or after DXA measurement with osteoporosis as an outcome); (5) diabetes mellitus (HbA1c ≥ 48 mmol/mol or elevated blood glucose (non-fasting ≥ 11.1 mmol/L or fasting ≥ 7.0 mmol/L) or using

antidiabetic medication). Self-reported comorbidities included: (6) heart failure (diagnosed by cardiologist); (7) non-AIDS defining cancers (confirmed by pathologist, excluding non-melanoma skin cancers); and (8) atherosclerotic disease (diagnosed by specialist; myocardial infarction, angina pectoris, peripheral arterial disease, ischemic stroke or transient ischemic attack).

Self-reported diagnoses were validated using hospital records for HIV-positive participants, and general practitioners' (GP) records for HIV-negative participants who had provided consent to contact their GP. Unvalidated diagnoses were only used when validation was not possible (i.e. participants not providing consent to validate self-reported diagnoses with their GP, or documentation about a self-reported diagnosis was absent for HIV-positive participants who received care in other hospitals during follow-up). Of the 35 self-reported comorbidities, 17 (48.6%) were validated as correct, 16 (45.7%) were rejected, and 2 (5.7%) could not be validated. Prevalence of all aforementioned comorbidities were summed when comparing to Dysbiosis Index and Shannon diversity

Soluble Biomarker Quantification

Soluble CD14, soluble CD163, and I-FABP concentrations were determined in plasma samples stored at -80°C using enzyme-linked immunosorbent assay (ELISA) (DuoSet ELISAs; R&D Systems) in all 160 subjects. The SOMAscan platform was used to measure plasma concentrations of 1,317 human proteins in a subset of $n=81$ subjects ($n=27$ seronegative controls and $n=54$ PWH; 9 SN-F, 9 SN-MSM, 9 SN-MSW, 16 PWH-F, 22 PWH-MSM, and 16 PWH-MSW). For identification of plasma proteins in differential abundance between PWH and seronegative controls, we limited analyses only to paired subjects ($n=27$ seronegative, $n=27$ PWH, 9 subjects from each subject group per infection status). To mitigate subject group effects, we performed both

paired Wilcoxon tests and ran linear mixed effects models using subject group as a random effect. All SOMAscan analyte units were normalized and scaled by subtracting the mean value for each analyte and dividing by its standard deviation using the R package 'scale'.

DNA Extraction and PCR Amplification

Bacterial profiles of study participants were generated by broad-range amplification and sequence analysis of bacterial 16S rRNA genes. DNA was extracted from fecal samples using the Qiagen MagAttract PowerMicrobiome DNA/RNA EP Kit automated system in conjunction with the Eppendorf Epmotion 5075 machine. The extracted DNA was then purified using Qiagen DNeasy Blood & Tissue Kit. The V4 hypervariable region of the bacterial 16S rRNA gene was amplified using fusion primers with partial Illumina adaptors. The universal bacterial 515F forward primer (5'-GTGCCAGCMGCCGCGGTAA) and the 806R reverse primer (5'-GGACTACHVGGGTWTCTAAT) were used. PCR reactions were prepared in 100 μ L volumes containing 20 μ L of 5X Phusion® High-Fidelity (HF) buffer, 2 μ L of 10 mM dNTPs, 1 μ L of each primer (50 μ M), 0.5 μ L of Phusion® High-Fidelity DNA Polymerase, and 10 ng of template DNA. Master mixes were then split into triplicate reactions of 33 μ L and amplified separately. V4 regions of the bacterial 16S rRNA gene were amplified using PCR (98 °C for 30 seconds, followed by 25 cycles at 98 °C for 10 seconds, 57 °C for 30 seconds and 72 °C for 30 seconds, and a final extension at 72 °C for 5 minutes).

Illumina MiSeq Sequencing

Following amplification, triplicate samples were pooled and purified using Ampure XP Beads (1:1 ratio). Samples were then quantified using the KAPA qPCR-based Library Quantification kit and

pooled at equimolar concentrations. Amplicons were paired-end sequenced (2×300 bp) on an Illumina MiSeq platform with a 600-cycle kit using standard protocols.

16s rRNA Sequencing Read Data Processing

Raw fastq files were processed using the dada2 algorithm after primer trimming and trimming amplicon lengths of the forward and reverse reads to 240 bp and 190 bp, respectively. Standard parameters were used (truncQ=2, maxEE=2). Taxonomy was assigned to operational taxonomic units (OTUs), or 'sequence variants' by dada2 parlance, using the RDP training set 16. Fasta sequences for each OTU were processed through the md5sum hash algorithm (R library 'digest') to create short, unique OTU identifiers. The first four characters of each md5sum OTU identifier are displayed in graphs after taxonomic classifications so as to facilitate linking of 16s sequences to visualized results. OTUs that were present in fewer than 6 samples were removed and OTU tables were rarefied to 20,000 reads for subsequent analyses. Phylogenetic trees were constructed and alpha diversity metrics were calculated using QIIME2.

Statistical Analyses

For comparisons of overall community similarity, n-by-n matrices of the Canberra beta diversity metric (R package 'vegan') were used in conjunction with the PERMANOVA statistical test (R function 'adonis'). Stratifying by subject group within the PERMANOVA test was performed using the 'strata' argument within the 'adonis' function. Benjamini-Hochberg q values were calculated for all analyses using the 'p.adjust' function in R. Log mean fold changes were calculated by adding a pseudocount of 1 to all zero-abundance OTUs, calculating mean OTU abundance for each group, and then subtracting the log-transformed means for the two groups from

each other. For statistical comparisons that sought to identify OTUs in differential abundance between groups, OTUs were filtered out if present in fewer than 20% of samples to reduce numbers of comparisons for purposes of minimizing false discovery and to filter out rare OTUs for which statistical power to discern differences was low. To identify OTUs in differential abundance between PWH and seronegative subjects, paired Wilcoxon tests were performed for each OTU in series (R package 'exactRankTests'). Paired samples were selected for this analysis, such that each PWH subject was compared to a sample matched for age, BMI, birth country, sex, and sexual preference (n=71 PWH and n=71 seronegative). For comparing MSM vs. MSW, participants were selected such that both groups had equal proportions of PWH and seronegative, and were also matched for birth country which was predominantly the Netherlands for this comparison (n=22 MSW and n=72 MSM). Unpaired Mann-Whitney U tests were performed on each OTU to test differences between these two groups. To determine overlap in OTUs that were characteristic of MSM versus those that were characteristic of PWH, the following was performed. All OTUs that passed a $P < 0.05$ cutoff in the two aforementioned sets of statistical comparisons (PWH vs. uninfected, and MSM vs. MSW) were selected. Those OTUs that overlapped between the resulting lists were then further sub-selected. Fisher's exact test was used to determine whether concordance of abundance trends (same sign of log mean fold changes) among the resulting list of overlapping taxa had occurred by random chance, using an expectation that 50% of taxa should have identical directionality of abundance difference (i.e. same sign of log mean fold changes) between two random lists as the null hypothesis. The same framework was used to determine consistency of microbiota differences between MSM vs. MSW and MSM/RAI+ vs. MSM/RAI- (n= 45 MSM/RAI+ and n=33 MSM/RAI-). First, Mann-Whitney U tests were performed on each OTU for MSM vs. MSW, MSM/RAI+ vs. MSM/RAI-. OTUs that reached $P < 0.05$ from each of these

lists were selected, and those OTUs that overlapped between the resulting lists were then further sub-selected, and Fisher's exact test was used as described above. The same was performed to test significance of the overlap among MSM vs. MSW and F/RAI+ vs. F/RAI- (F/RAI+, n=5 and F/RAI- n=33). To test significance of overlapping trends for taxa that differentiated PWH vs. seronegative in each sex/sexual practice subgrouping (female, MSM, MSW), the top 15 taxa for each subgrouping list of paired Wilcoxon tests were selected. Fisher's exact test was used as above with the null hypothesis representing the number of taxa that would by random chance exhibit the same directionality of abundance shift in all three groups (25% of taxa). For testing associations involving inflammatory markers, clinical characteristics, prevalence of age-associated noncommunicable comorbidities, and the dysbiosis index (detailed below) or alpha diversity, linear mixed effects analyses were performed using the R packages 'lme4' and 'lmerTest'. For all such analyses, subgroup (female, MSM, MSW) effects were incorporated into each model as a random effect on intercept (1|Subject Group). When examining associations between two independent variables simultaneously (e.g. dysbiosis index and Shannon diversity) on outcome variables (e.g. clinical, inflammatory marker, or comorbidity prevalence data), both independent variables were treated as fixed effects.

Dysbiosis Index Calculation

Dysbiosis index ('DI') was calculated as the log ratio of geometric means of taxon abundances that were enriched in PWH (having paired Wilcoxon $P < 0.05$ and log mean fold change > 0 in the comparison of PWH vs. HIV-seronegative controls) over taxa that were depleted in PWH (having paired Wilcoxon $P < 0.05$ and log mean fold change < 0 in the comparison of PWH vs. HIV-seronegative controls), as follows:

$$DI = \log_{10} \left(\frac{\sqrt[n]{x_1 x_2 \dots x_n}}{\sqrt[m]{y_1 y_2 \dots y_m}} \right)$$

Where each x denotes read counts for taxa enriched in PWH and n is the total number of such taxa, while each y denotes read counts for taxa depleted in PWH and m is the total number of these taxa. Taxon read counts (from a total rarefaction of 20,000 reads per sample) were used with an added pseudo-count of 1, in order to accommodate the geometric mean by removing zeroes.

Data visualization

Data were visualized using R packages ‘ggplot2’, ‘ggthemes’, ‘RColorBrewer’, ‘viridis’, and were formatted for presentation using R packages ‘ape’, ‘reshape2’, ‘plyr’, and ‘grid’.

Machine Learning Analyses

In order to reduce variables for machine learning analyses, OTUs with a mean relative abundance below 0.1% were removed from the OTU table. The OTU table was standard-scaled and log-transformed with a pseudo-count of 1. Ridge logistic regression, which reduces the chance of overfitting the training data by penalizing large model weights, was used to classify samples into one of the two binary groups (MSM vs. MSW, or PWH vs. seronegative), with the processed OTU table as the input, predictor data and either MSM and HIV serostatus as the binary response variable. The same subject samples were used for machine learning analyses and traditional statistical analyses described above. PWH participants were matched to seronegative controls for age, BMI, birth country, sex, and sexual preference ($n=71$ PWH and $n=71$ seronegative). For MSM vs. MSW, persons were selected such that both groups had equal proportions of PWH and seronegative, and were matched for birth country which was predominantly the Netherlands ($n=22$

MSW and n=72 MSM). Stratified randomized permutation cross-validation (RPCV) was employed to obtain an accurate estimate of the classification of samples, as well as the ability for the learned OTU associations to be able to classify unseen data. RPCV was used with 500 iterations and 70% of samples used for training and the remaining 30% were used to test the trained model. The area under the curve (AUC) of the receiver operating characteristic (ROC) curve was used as the measure of model performance on the test set. To demonstrate that the results of the classifier was not just due to random noise within the OTU table, for each iteration of the CV, the response variable was randomly permuted to corrupt the data, and a separate model was trained on the corrupted data in parallel with the model trained on the unpermuted data. The mean-AUC of the corrupted model across all folds was taken as the null hypothesis of the power of the microbiota OTU data to discriminate between the two binary classes and was used to determine the significance of the AUC results of the classifier on the unpermuted data. An alpha of 0.05 was used to determine significance of the performance of the classifiers over the 500 CV iterations.

Performing multiple tests on the same source dataset violates assumptions of the standard T-test (i.e. test and null distributions are inferred from the models trained on the same predictor data), thus an empirical P value defined by Ojala et al. was used to test if a classifier has found a significant class structure in the data. The empirical P value was defined as:

$$p = \frac{|\{D \in \hat{D} : e(f, D) \geq \bar{e}(f, D')\}| + 1}{k + 1}$$

where D' is the permuted dataset, and $e(f, D)$ is the error of the function f learned on dataset D .

The empirical P value thus is taken to represent the fraction of classifiers that performed worse than the averaged permuted classifier performance. Random Forests, a bootstrapped ensemble

method of random forest classifiers, was also used to predict samples but underperformed as compared to ridge logistic regression.

Data Availability

16s rRNA sequencing data are available at the NCBI Short Read Archive under accession numbers PRJNA589036 [<https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA589036>] (BioProject) and SRP229524 [<https://trace.ncbi.nlm.nih.gov/Traces/sra/?study=SRP229524>] (SRA).

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Chapter 4

Antiretroviral Therapy Administration in Healthy Rhesus Macaques is Associated with Transient Shifts in Intestinal Bacterial Diversity and Modest Immunological Perturbations

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Abstract

Gastrointestinal immune system competency is dependent upon interactions with commensal microbiota, which can be influenced by wide-ranging pharmacologic interventions. In SIV-infected Asian macaque models of HIV infection, we previously noted that initiation of antiretroviral therapy (ART) is associated with a specific imbalance (dysbiosis) of the composition of the intestinal bacteriome. To determine if ART itself might contribute to dysbiosis or immune dysfunction, we treated healthy rhesus macaques with protease-, integrase- or reverse transcriptase inhibitors for 1-2 or 5-6 weeks and evaluated intestinal immune function and the composition of the fecal bacterial microbiome. We observed that individual antiretrovirals (ARVs) modestly altered intestinal T-cell pro-inflammatory responses without disturbing total or activated T-cell frequencies. Moreover, we observed significant disruptions in bacterial diversity coupled with perturbations in the relative frequencies of bacterial communities. Shifts in specific bacterial frequencies were not persistent post-treatment, however, with individual taxa showing only isolated associations with T-cell pro-inflammatory responses. Our findings suggest that intestinal bacterial instability and modest immunological alterations can result from ART itself. These data could lead to therapeutic interventions which stabilize the microbiome in individuals prescribed ART.

IMPORTANCE

Dysbiosis of the fecal microbiome is a common feature observed in ARV-treated people living with HIV. The degree to which HIV infection itself causes this dysbiosis remains unclear. Here we demonstrate that medications used to treat HIV infection can influence the composition of the GI tract immune responses and its microbiome in the non-human primate SIV model.

Introduction

Combination ART successfully inhibits lentiviral replication by preventing the de novo infection of activated CD4⁺ T-cells. Although the widespread implementation of ART has been instrumental in reducing the morbidity and mortality of the HIV epidemic, the prolonged effects of ARV administration may not be inconsequential. In communities with widespread ART implementation, unresolved inflammation contributing to non-AIDS-related co-morbidities is the leading cause of mortality in people living with HIV [169]. While residual inflammation can itself be partially attributed to physical and immunological scarring following prolonged viral replication [170, 171], modest inflammation and immune dysfunction are similarly observed in patients who initiate ART in acute HIV infection [172-175].

Gastrointestinal complications are prevalent in ART-treated HIV-infected patients as well as in uninfected individuals taking ARVs as part of a pre-exposure prophylaxis (PreP) regimen [176], suggesting that ART might by itself contribute to inflammation. Although an estimated 70% of diarrheal-associated complications in HIV-infected, ARV-treated patients have been attributed to non-infectious causes [177-179], little is known about whether dysbiosis is a feature in these cases as high-throughput microbial sequencing is not common clinical practice. In HIV-infection, intestinal bacterial dysbiosis - an imbalance of the composition of the microbiome favoring enrichment of pathogen-associated taxa - has been shown to correlate with canonical markers of inflammation in both untreated and treated HIV infection [50, 51, 62, 125]. Although we and others have demonstrated that these described dysbioses do not promote untreated lentiviral disease progression [36, 66, 180, 181], a potential contribution of dysbiosis to co-morbidities in ART treated lentiviral-infections has not been thoroughly assessed. Intriguingly, in Asian macaque

models of HIV-1, we previously observed that the initiation of combination ART in SIV-infected rhesus macaques potentiates intestinal bacterial instability [66].

The deleterious effects of prolonged antibiotic prescription on the commensal microbiome are well-described and purported to underlie several chronic conditions [182]. Similarly, though less well-studied, the off-target effects of non-antibiotic pharmaceuticals have been shown to disrupt intestinal bacterial communities [183, 184]. Disruptions to these communities by non-antibiotics can result not only from direct interference with commensal bacteria [185], but also from interference with conserved biochemical processes [186, 187], metabolic cascades [188], and co-inhabitants of the commensal ecosystem [189, 190]. Importantly, ARVs currently prescribed to inhibit HIV replication are not specific for HIV or SIV [191]. Several reverse transcriptase (RT)-inhibitors can be incorporated into the DNA of all replicating organisms [192], protease (PR)-inhibitors target biochemically conserved aspartic proteases [193], and integrase (IN)-inhibitors target catalytic domains conserved in some bacteriophages and prokaryotic transposases [194]. PR-inhibitors in particular have been shown to alter bacterial, fungal, and protozoal cultures in vitro [195-197].

Characterizing the biochemical spectrum of off-target ARV effects is an essential step in ascribing cause to effect with regards to non-AIDS related inflammation and co-morbidities. Given the known potential for microbiome influences by ARVs, we sought to understand the influence of representative classes of ARVs on the commensal bacteria and intestinal immunity in vivo. Here, we treated healthy rhesus macaques with representative PR-, IN-, and RT-inhibitors and characterized intestinal T-cell activation and fecal bacterial stability. We demonstrate that ART modestly alters intestinal T-cell pro-inflammatory responses and bacterial diversity. These findings form the framework for a holistic comprehension of the influence of ART on systemic

immunity and by extension, inflammatory complications and co-morbidities associated with prolonged ART.

Results

Study Design. Thirty-one SIV-uninfected, healthy, rhesus macaques were assigned to control (n=6), PR-inhibitor (n=13), IN-inhibitor (n=6), or RT-inhibitor (n=6) groups and treated daily for 1-6 weeks. PR-inhibitor animals received Darunavir and Ritonavir, IN-inhibitor animals received L-870812 (L812) and RT-inhibitor animals received tenofovir (PMPA) or emtricitabine (FTC). An additional 2 animals were treated with orally administered RT-inhibitor emtricitabine (TDF) in a pilot study. No differences were observed beyond those described for PMPA administration (data not shown) and thus, this treatment was not further examined. Animals underwent peripheral blood (PBMC) and jejunal (Jej) or rectal biopsy (RB) sampling 1-2 weeks post-treatment, and for a subset of PR-inhibitor and control animals, at 5-6 weeks post-treatment (Table 1). No changes in weight were observed post-treatment. Stool was collected longitudinally for 16S bacterial sequencing.

Table 1. Characteristics of study animals.

RhDGMh	Control	Male	-	3.39	2017-11-06 (-)	3.22	2017-12-04 (-)	3.42
RhDFK9	Control	Male	-	7.32	2018-01-30 (-)	7.02	2018-02-27 (-)	7.15
RhDMEj	Control	Female	-	8.99	2018-01-30 (-)	8.65	2018-02-27 (-)	8.25
Rh37033	IN-Inhib	Male	2016-09-27	12.10	2016-10-04 (7)	11.53	-	-
RhDCKj	IN-Inhib	Male	2015-02-03	9.61	2015-02-10 (7)	9.63	-	-
RhDCVf	IN-Inhib	Male	2015-02-03	9.73	2015-02-10 (7)	9.57	-	-
RhDE1a	IN-Inhib	Male	2015-02-03	11.85	2015-02-10 (7)	11.67	-	-
RhDE1aa	IN-Inhib	Male	2016-09-27	6.21	2016-10-04 (7)	6.05	-	-
RhF64	IN-Inhib	Female	2016-09-27	7.73	2016-10-04 (7)	7.71	-	-
RhDCBC	PR-inhib	Male	2015-03-09	8.85	2015-03-16 (7)	8.56	-	-
RhDE20	PR-inhib	Male	2015-03-09	9.66	2015-03-16 (7)	9.31	-	-
RhDE2c	PR-inhib	Male	2015-03-09	8.70	2015-03-16 (7)	8.48	-	-
RhDE2w	PR-inhib	Male	2015-03-09	13.20	2015-03-16 (7)	13.08	-	-
Rh37073	PR-inhib	Female	2016-09-27	11.48	2016-10-05 (8)	11.33	2016-11-02 (36)	11.56
Rh37360	PR-inhib	Female	2016-09-27	10.83	2016-10-05 (8)	10.75	2016-11-02 (36)	11.63
RhZC08	PR-inhib	Female	2016-09-27	7.02	2016-10-05 (8)	6.95	2016-11-02 (36)	6.62
RhDF8d	PR-inhib	Male	2017-10-23	4.58	2017-11-07 (15)	4.5	2017-12-05 (43)	4.63
RhDFPn	PR-inhib	Male	2017-10-23	6.17	2017-11-07 (15)	6.06	2017-12-05 (43)	6.19
RhDGHi	PR-inhib	Male	2017-10-23	3.32	2017-11-07 (15)	3.2	2017-12-05 (43)	3.46
RhDGMp	PR-inhib	Male	2017-10-23	3.43	2017-11-07 (15)	3.45	2017-12-05 (43)	3.67
RhDFNj	PR-inhib	Male	2018-01-17	8.45	2018-01-29 (12)	8.26	2018-02-25 (39)	8.62
RhDFZc	PR-inhib	Male	2018-01-17	7.13	2018-01-29 (12)	7.37	2018-02-25 (39)	7.73
RhCL7p	RT-inhib (FTC)	Male	2015-01-20	13.43	2015-01-27 (7)	13.18	-	-
RhDCAV	RT-inhib (FTC)	Male	2015-01-20	9.37	2015-01-27 (7)	9.05	-	-
RhDCJWA	RT-inhib (FTC)	Male	2015-01-20	9.63	2015-01-27 (7)	9.58	-	-
RhCL4c	RT-inhib (PMPA)	Male	2014-09-30	11.31	2014-10-07 (7)	11.2	-	-
RhCL86	RT-inhib (PMPA)	Male	2014-09-30	11.64	2014-10-07 (7)	11.54	-	-
RhF98	RT-inhib (PMPA)	Female	2014-09-30	7.23	2014-10-07 (7)	7.1	-	-

ARV administration does not expand the activated T-cell pool in healthy macaques.

Lentiviral suppression following ART restores circulating CD4⁺ T cells; however, intestinal CD4⁺ T cells are not fully restored, and systemic lymphocytes exhibit signs of persistent activation. To determine if ART itself contributes to alterations in intestinal T-cell frequencies or activation, we quantified T-cell frequencies from intestinal biopsy specimen homogenates and PBMCs before and after ART by flow cytometry and further measured their expression of the following markers: CCR5, a lymphocyte mucosal homing marker and the HIV/SIV coreceptor; CCR7, which demarcates replenishable effector (CCR7⁻) and non-replenishable central (CCR7⁺) memory lymphocytes; and HLA-DR, a major histocompatibility complex class II (MHC-II) receptor that is commonly upregulated on activated CD4⁺ as well as CD8⁺ T cells. No differences in CD4⁺ T-cell frequencies were observed in treated animals compared to levels in controls for either the small or large intestine (Fig. 1A); however, significantly lower CD4⁺ T-cell frequencies (fold change from baseline) were observed among PBMCs from the PR inhibitor-treated animals compared to levels in controls (Fig. 1 B) for weeks 1 to 2, $P = 0.018$; weeks 5 to 6, $P = 0.005$). No significant differences in CCR5 or HLA-DR expression by CD4⁺ or CD8⁺ memory T (TM) cells were observed between treated animals and controls at any of the measured time points though reduced expression of CCR7 in RB specimens was observed for CD4⁺ TM cells in RT inhibitor-treated animals (unpaired, two-way t test, $P = 0.009$) and for CD8⁺ TM cells in IN inhibitor-treated animals ($P = 0.007$) (Fig. 1C to E).

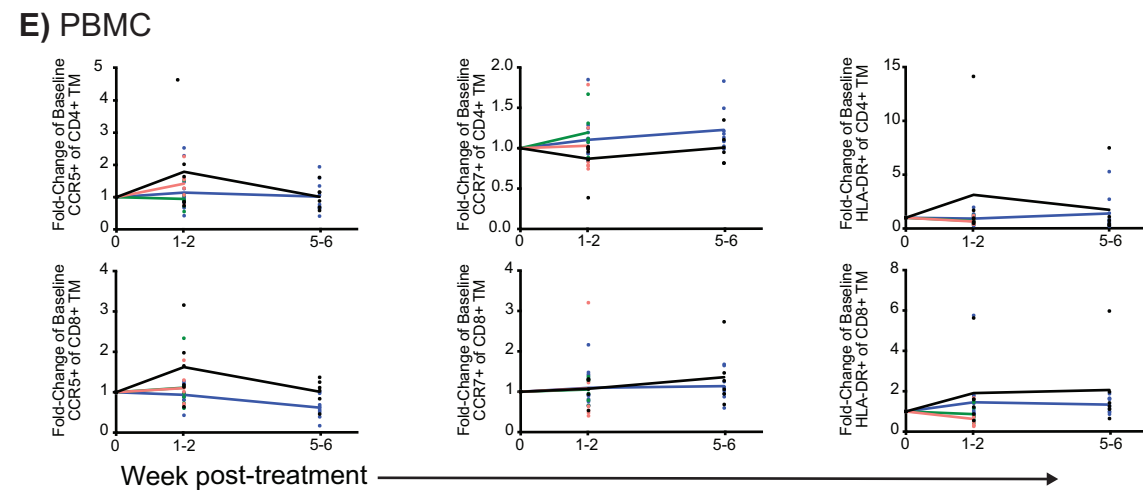
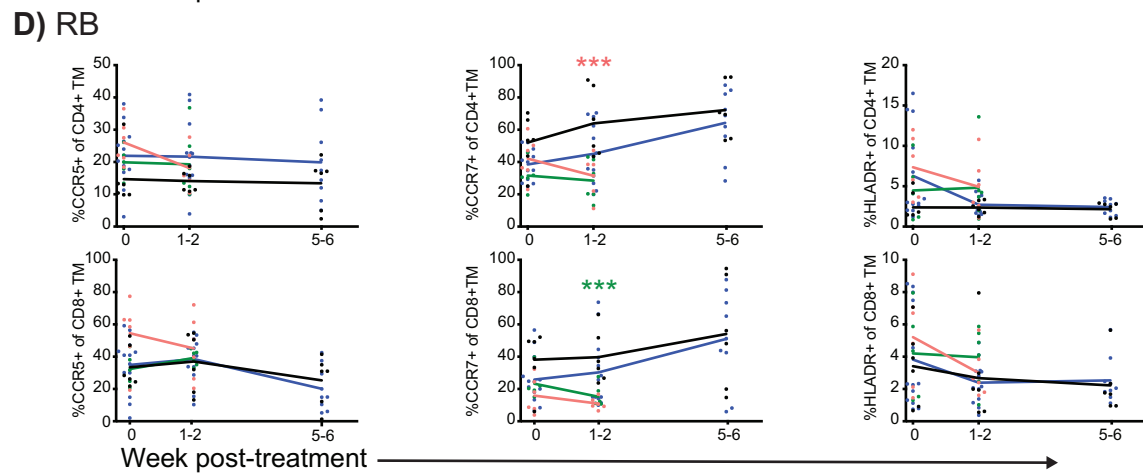
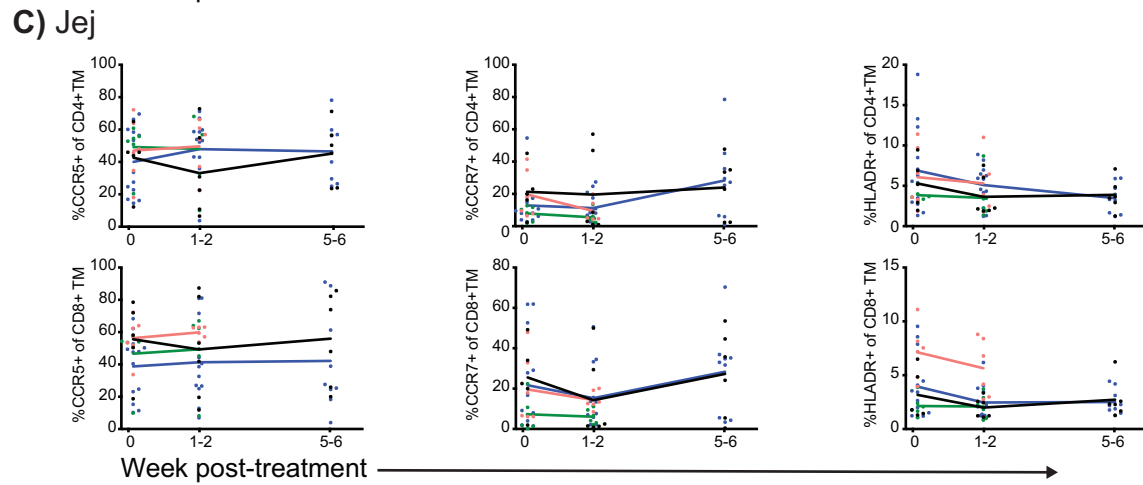
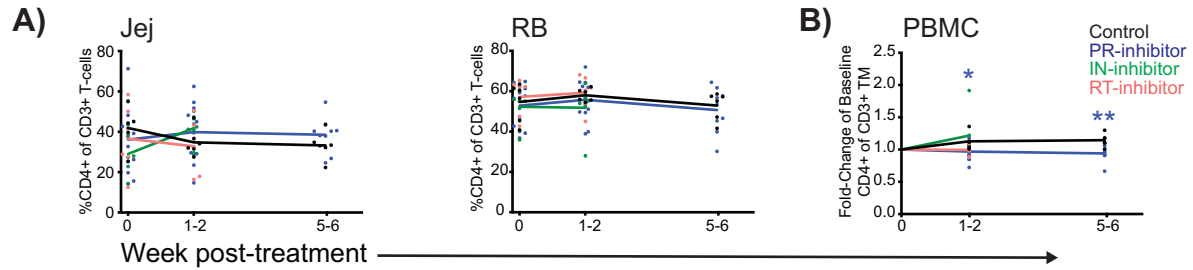


FIG 1

ART does not induce significant T-cell activation in healthy macaques. (A) Individual and mean (arithmetic) longitudinal frequencies in Jej and RB specimens of CD4⁺ T cells in control animals and animals treated with PR inhibitor, IN inhibitor, and RT inhibitor. (B) Individual and mean CD4⁺ T-cell fold change from baseline frequencies in PBMCs. Asterisks denote significance of values for the ART groups compared to those of the controls, color coded by ART treatment. (C and D) Frequency in Jej and RB specimens of CD4⁺ and CD8⁺ TM cells expressing CCR5, CCR7, and HLA-DR, as indicated. (E) Fold change from baseline frequencies of CD4⁺ and CD8⁺ TM cells expressing CCR5, CCR7, and HLA-DR, as indicated, in PBMCs. Statistical significance between groups was assessed by an unpaired, two-way *t* test as follows: in PBMCs from PR inhibitor-treated animals, fold change in frequency of CD4⁺ TM cells at weeks 1 to 2, $P = 0.018$, and at week 5 to 6, $P = 0.005$; in RB specimens from RT inhibitor-treated animals frequency of CCR7⁺ CD4⁺ TM cells at weeks 1 to 2, $P = 0.009$, and from IN inhibitor-treated animals, frequency of CCR7⁺ CD8⁺ TM cells at weeks 1 to 2, $P = 0.007$.

A modest pro-inflammatory response follows ARV administration in healthy macaques.

Residual immune dysfunction in ARV-treated individuals includes a proinflammatory milieu which is known to contribute to non-AIDS comorbidities. Although we did not observe an expansion of activated T cells following ART initiation, an increase in proinflammatory T cells from within the activated T-cell pool could contribute to inflammation. To assess proinflammatory cytokine production by intestinal T cells, we mitogenically stimulated intestinal biopsy specimen single-cell suspensions and PBMCs and measured the frequency of T cells expressing either gamma interferon (IFN- γ) or tumor necrosis factor alpha (TNF- α). Among small intestinal lymphocytes isolated from IN inhibitor-treated animals at 1 to 2 weeks post-ART, we observed increased IFN- γ -expressing CD4⁺ TM cells compared to levels in controls (unpaired, two-way *t* test, $P = 0.03$), and in PR inhibitor-treated animals at 5 to 6 weeks post-ART, we noted

increased TNF- α -expressing CD8⁺ TM cells ($P = 0.009$) (Fig. 2A). In biopsy specimens of the large intestine, the frequency of CD8⁺ TM cells expressing IFN- γ at 5 to 6 weeks of treatment initiation was significantly increased compared to that of controls ($P = 0.008$) (Fig. 2B). Among PBMCs, the fold change from baseline expression of IFN- γ among CD8⁺ TM cells was significantly higher at 1 to 2 weeks posttreatment in RT inhibitor-treated animals than in controls ($P = 0.005$) (Fig. 1C).

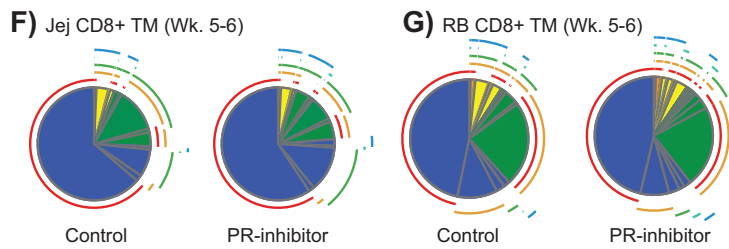
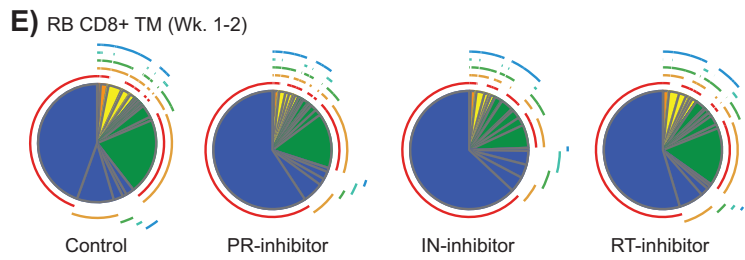
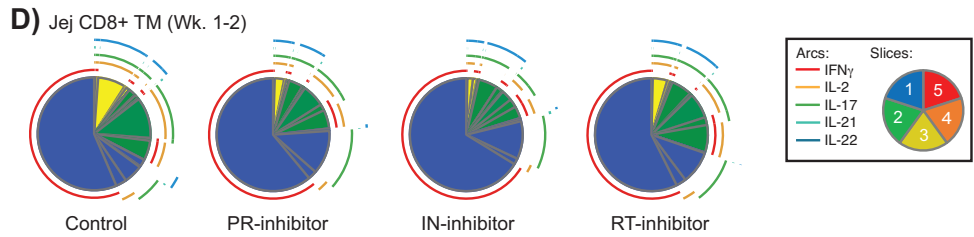
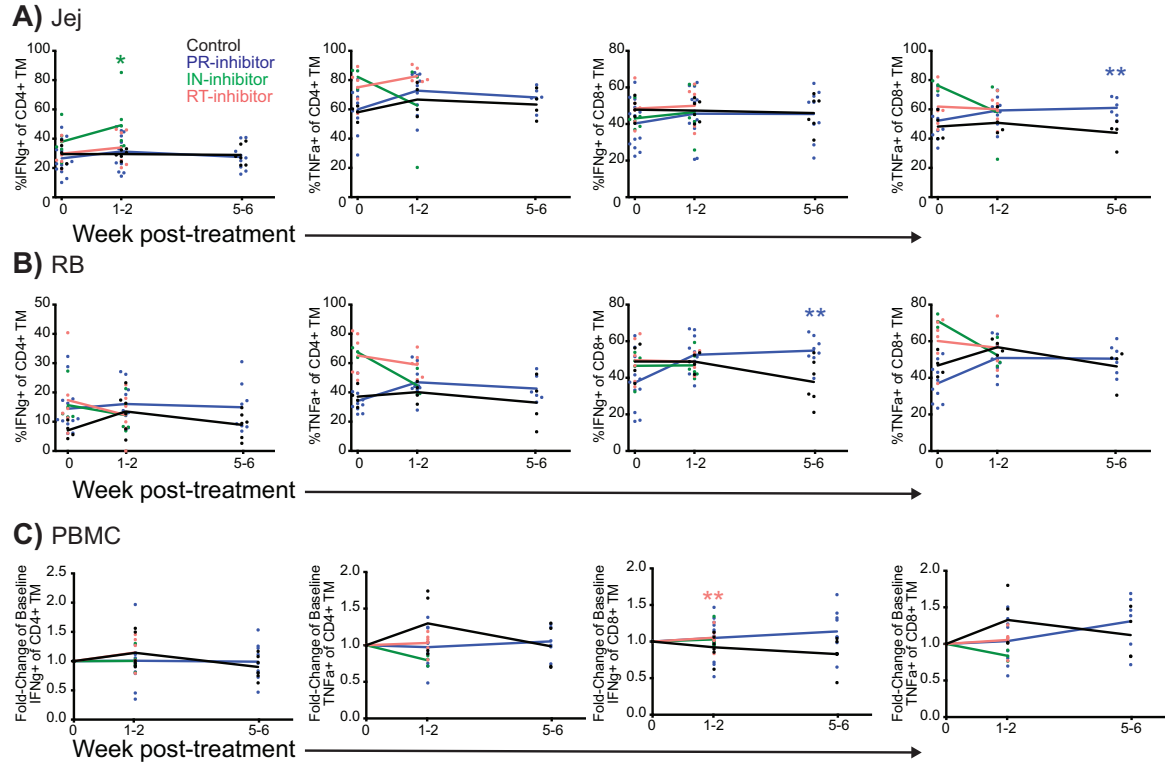


FIG 2

ART modestly alters proinflammatory cytokine expression in healthy macaques. (A and B) Individual and mean frequencies of IFN- γ - and TNF- α -expressing CD4⁺ TM cells and CD8⁺ TM cells, as indicated, in Jej and RB specimens. Asterisks denote significance of values for ART groups compared to those of the controls, color coded by ART treatment. (C) Fold change from baseline frequencies of IFN- γ - and TNF α -expressing CD4⁺ TM cells and CD8⁺ TM cells, as indicated, in PBMCs. (D to G) Pie charts depicting the relative expression levels of IFN- γ , IL-2, IL-17, IL-21, and/or IL-22 in CD8⁺ TM cells of Jej (D and F) or RB (E and G) specimens at 1 to 2 or at 5 to 6 weeks (Wk) posttreatment in the indicated groups. Pie arcs represent individual cytokines expressed, and pie slices represent the number of coexpressed cytokines, as designated in the legend. Statistical significance between groups in panels A to C was assessed by unpaired, two-way *t* test: in Jej specimens of IN inhibitor-treated animals, frequency of IFN- γ ⁺ CD4⁺ TM cells at weeks 1 to 2, $P = 0.03$; in Jej specimens of PR inhibitor-treated animals, frequency of TNF α ⁺ CD8⁺ TM cells at weeks 5 to 6, $P = 0.009$; in RB specimens of PR inhibitor-treated animals, frequency of IFN- γ ⁺ CD8⁺ TM cells at weeks 5 to 6, $P = 0.008$; PBMCs of RT inhibitor-treated animals, fold change in frequency of IFN- γ ⁺ CD8⁺ TM cells at weeks 1 to 2, $P = 0.005$. Significance for polyfunctional data in panels D to G was assessed by a Spice permutation test.

In addition to skewed frequencies of proinflammatory T cells, infected ARV-treated patients and macaques commonly display an accumulation of monofunctional cells, i.e., those capable of expressing only a single cytokine (36). To determine whether ART alone may skew functionality, we assessed the ability of intestinal T cells to simultaneously express IFN- γ , interleukin-2 (IL-2), IL-17, IL-21, and IL-22 in response to mitogenic stimulation *ex vivo*. In all cases, a dominant mono-IFN- γ response was evident, with no significant differences observed in functional profiles observed between groups or time points for either CD4⁺ (data not shown) or CD8⁺ TM cells (Fig. 2C to G).

ARV administration is associated with perturbations of the bacteriome in healthy macaques.

In people living with HIV, GI tract bacterial dysbiosis has been broadly characterized by an overall decline in bacterial richness, including a coincident enrichment for intestinal *Proteobacteria* at the expense of *Firmicutes* (12,-14). We have similarly observed that the induction of ART in SIV-infected macaques results in intestinal bacterial dysbiosis (18). To determine if the proinflammatory alterations observed in IN and PR inhibitor-treated animals (Fig. 2A to C) could be attributed to bacterial dysbiosis, we isolated fecal bacterial DNA from a subset of these macaques and measured relative bacterial frequencies by next-generation sequencing of 16S rRNA genes. Characterization of taxa of interest revealed that gross community structures were preserved following the administration of ART and compared to those of control animals (Fig. 3A). To determine if alterations in specific taxa were significantly different between control and ARV-treated animals, we next compared frequencies of all identified bacterial taxa between animals utilizing the linear discriminant analysis (LDA) effect size (LEfSe) algorithm (37). Of 402 uniquely identified taxa, 100 features (taxa identified at any phylogenetic level) were significantly different in ARV-treated animals than in controls at either 1 to 2 weeks or 5 to 6 weeks posttreatment but not at baseline or at baseline but not at either posttreatment time point (Fig. 3B and C); see also Table S1 in the supplemental material). Five differentially abundant taxa were identified in both PR and IN inhibitor comparisons: *Prevotellaceae*NK3B31 species, the genera *Blautia* of the family *Lachnospiraceae* and *Solobacterium* of the family *Erysipelotrichaceae*, and orders *Bacillales* (of which *Bacillaceae* was the only identified family) and *Burkholderiales*. Importantly, although these taxa were identified in both therapeutic comparisons at a single time point, no features were longitudinally differentially abundant within a therapeutic group.

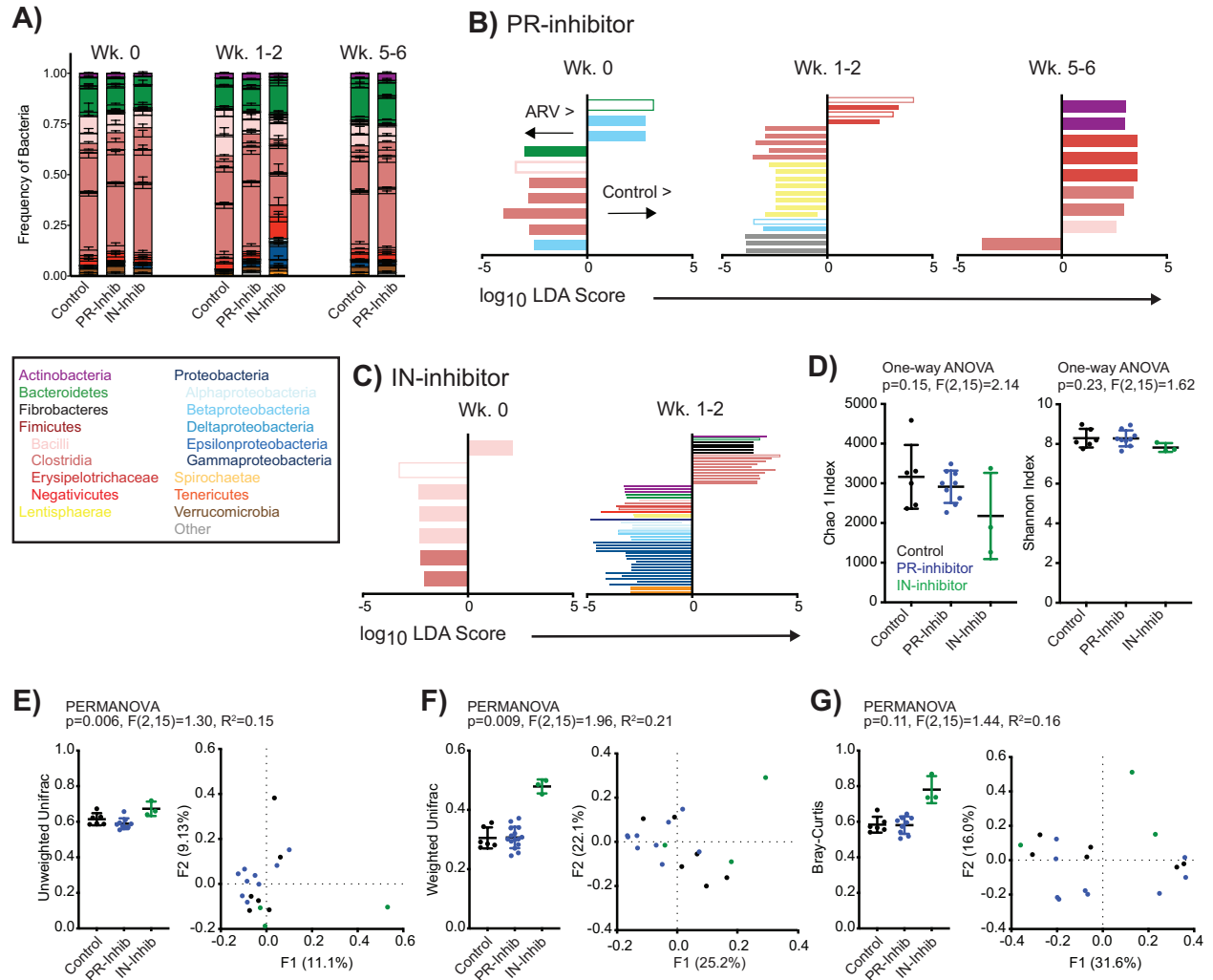


FIG 3

ART significantly alters intestinal bacterial diversity absent persistent changes in the stool microbiome. (A) Relative distribution of mean (\pm standard error of the mean) bacterial frequencies of interest within the stool of control, PR inhibitor-treated, and IN inhibitor-treated animals at designated time points. Bacteria are color coded according to the legend. (B and C) LDA scores for features identified as differentially abundant and biologically relevant by LefSe in PR inhibitor- and IN inhibitor-treated animals compared to values for the controls at week 0 but not posttreatment (Wk 0) or at weeks 1 to 2 or 5 to 6 posttreatment but not at baseline. Identified taxa are distinguished and color coded at the level of class as in panel A and differentially scored such that taxa enriched in controls are identified on the positive axis and those enriched in ARV-treated rhesus macaques are on the negative axis. Open bars denote identified taxa that were identified in both PR and IN inhibitor comparisons at any time point. (D) Individual and mean (\pm

standard error of the mean) alpha-diversity measures Chao1 and Shannon at 1 to 2 weeks posttreatment, color coded by treatment. (E to G) Beta-diversity measures unweighted UniFrac, weighted UniFrac, and Bray-Curtis analyses, as indicated, of fecal bacteria in rhesus macaques at 1 to 2 weeks posttreatment, color coded by treatment. Data are shown as individual and mean (\pm standard error of the mean) within-group measures (left) where individual data points represent the mean distance of individual animals to all other animals within the same group, and by PCoA (right). Significance was determined by LEfSe in panels B and C, by one-way ANOVA in panel D, and by PERMANOVA in panels E to G.

To quantify the influence of ART on fecal bacterial communities, we next compared indices of alpha diversity (Chao1 richness and Shannon evenness) and beta diversity (UniFrac and Bray-Curtis dissimilarity) across our treatment groups. At 1 to 2 weeks posttreatment, these analyses revealed no differences across groups for alpha diversity (Fig. 3D). Significant differences in phylogenetic beta diversity were seen across treatment groups, as measured by unweighted UniFrac (permutational multivariate analysis of variance [PERMANOVA], $P = 0.006$) (Fig. 3E) and weighted UniFrac ($P = 0.009$) (Fig. 3F). No differences were apparent by nonphylogenetic Bray-Curtis (Fig. 3G) analysis, and posttest analyses (individual PERMANOVA) did not identify a specific post-ART group as significantly different from controls. The observed differences in phylogenetic beta diversity absent changes in alpha diversity or persistent shifts in taxon abundance over time may indicate that ART induces bacterial community instability. Indeed, although temporal shifts in community beta diversity (all measures) were evident for each PR-treated animal by principal-coordinate analysis (PCoA) (Fig. 4A), these communities did not segregate by time post-ART. Temporal shifts in control animals were more subdued (Fig. 4B), with variability most evident by weighted UniFrac, and no segregation by assigned treatment group was evident at baseline (Fig. 4C). To assess bacterial community instability, we utilized an instability index whereby each index (value) is derived from the comparison of a single animal's

beta diversity measurement across two time points. For all beta-diversity measurements, the instability index comparing baseline to values at weeks 1 to 2 posttreatment was considered significantly different across treatment groups (Fig. 4D) (unweighted UniFrac one-way analysis of variance [ANOVA], $P = 0.042$; weighted UniFrac, $P = 0.035$; Bray-Curtis, $P = 0.0048$). These differences did not persist at weeks 5 to 6 post-ART.

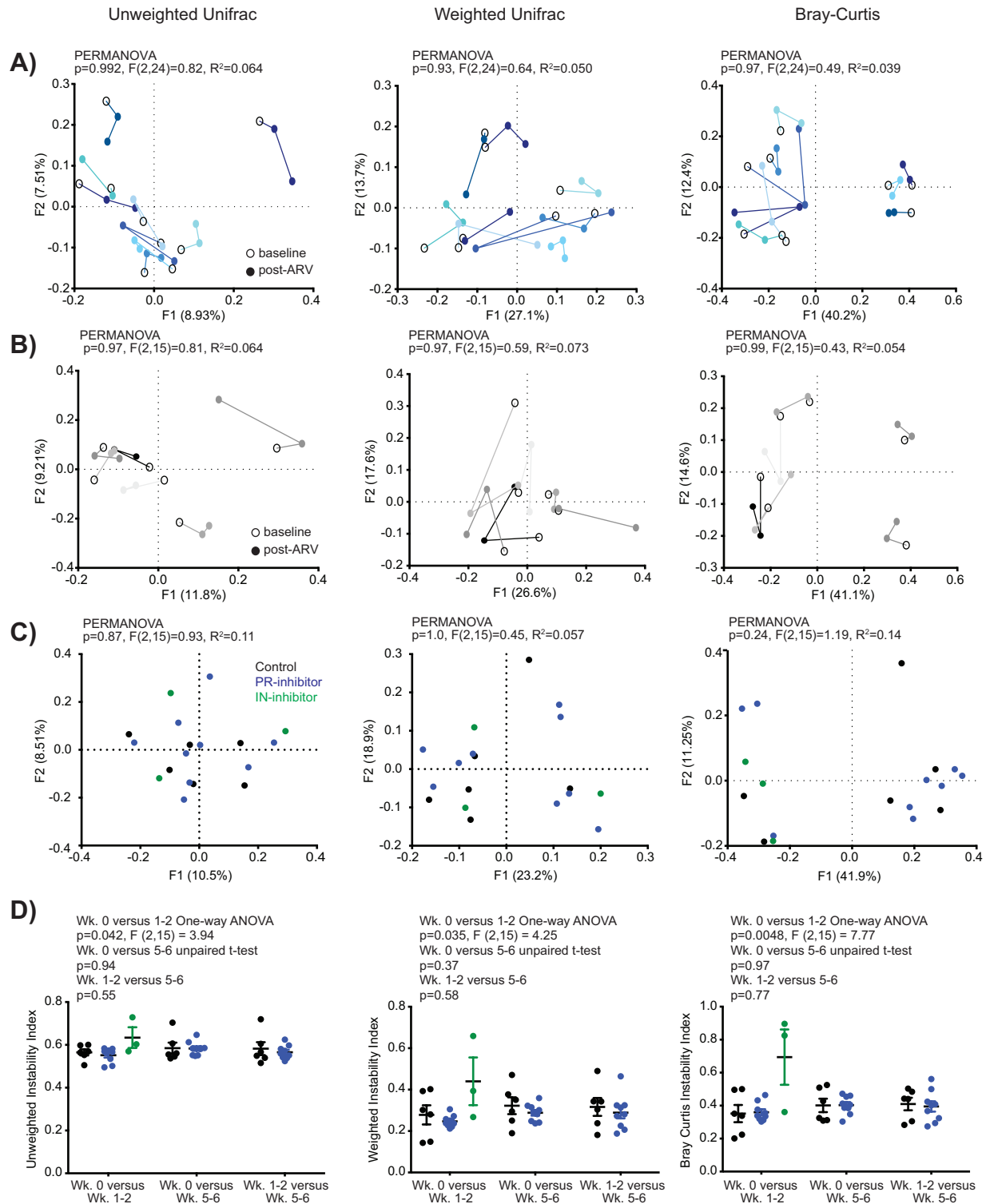


FIG 4

ART promotes transient intestinal bacterial instability in the stool microbiome of healthy rhesus macaques. (A to C) Unweighted UniFrac, weighted UniFrac, and Bray-Curtis measures, as indicated, of beta diversity by PCoA for

individual PR inhibitor-treated animals across time (A), individual control animals across time (B), and individual animals at baseline (C, color-coded by treatment group). (D) Instability indices comparing beta-diversity measures (as in panel C) across individual time points. Significance was assessed by two-way unpaired *t* test, one-way ANOVA, or PERMANOVA, as indicated.

Immunological alterations in ARV-treated animals associate with specific bacterial perturbations.

Although the differences in taxon abundances between control and ARV-treated animals were not maintained longitudinally, these differences coincided with differences in proinflammatory cytokine secretion and, thus, may be causally related. In order to investigate whether such a relationship might exist, we assessed whether *Prevotellaceae*, *Bacillaceae*, *Lachnospiraceae*, *Erysipelotrichaceae* or *Burkholderiales*, orders pertaining to the differentially abundant operational taxonomic units (OTUs) shared in both the PR and IN inhibitor LEfSe analyses (Fig. 3B and C), correlated with small or large intestine CD8⁺ TM cell IFN- γ expression at 1 to 2 weeks posttreatment (Fig. 5A). We similarly assessed whether the order *Enterobacteriales*, commonly identified as a strong correlate of immune dysfunction in HIV-infected individuals, might associate with IFN- γ expression in this time frame. Of the examined relationships, only *Enterobacteriales* (of which *Enterobacteriaceae* was the only identified family) showed a significant correlation with Jej CD8⁺ TM cell IFN- γ expression ($P = 0.0348$). No correlations were observed in relation to CD8⁺ IFN- γ expression in RB specimens (data not shown). We further extended our analysis by comparing frequencies of all identified bacterial families in all animals at weeks 1 to 2 post-ART to IFN- γ expression by intestinal T cells as well as to animal weight (Fig. 5B). Isolated associations were identified as significantly different, with the most correlations

emerging from among the *Clostridiales* and *Enterobacteriales* orders; however, no clear pattern emerged between familial abundance and IFN- γ expression or weight.

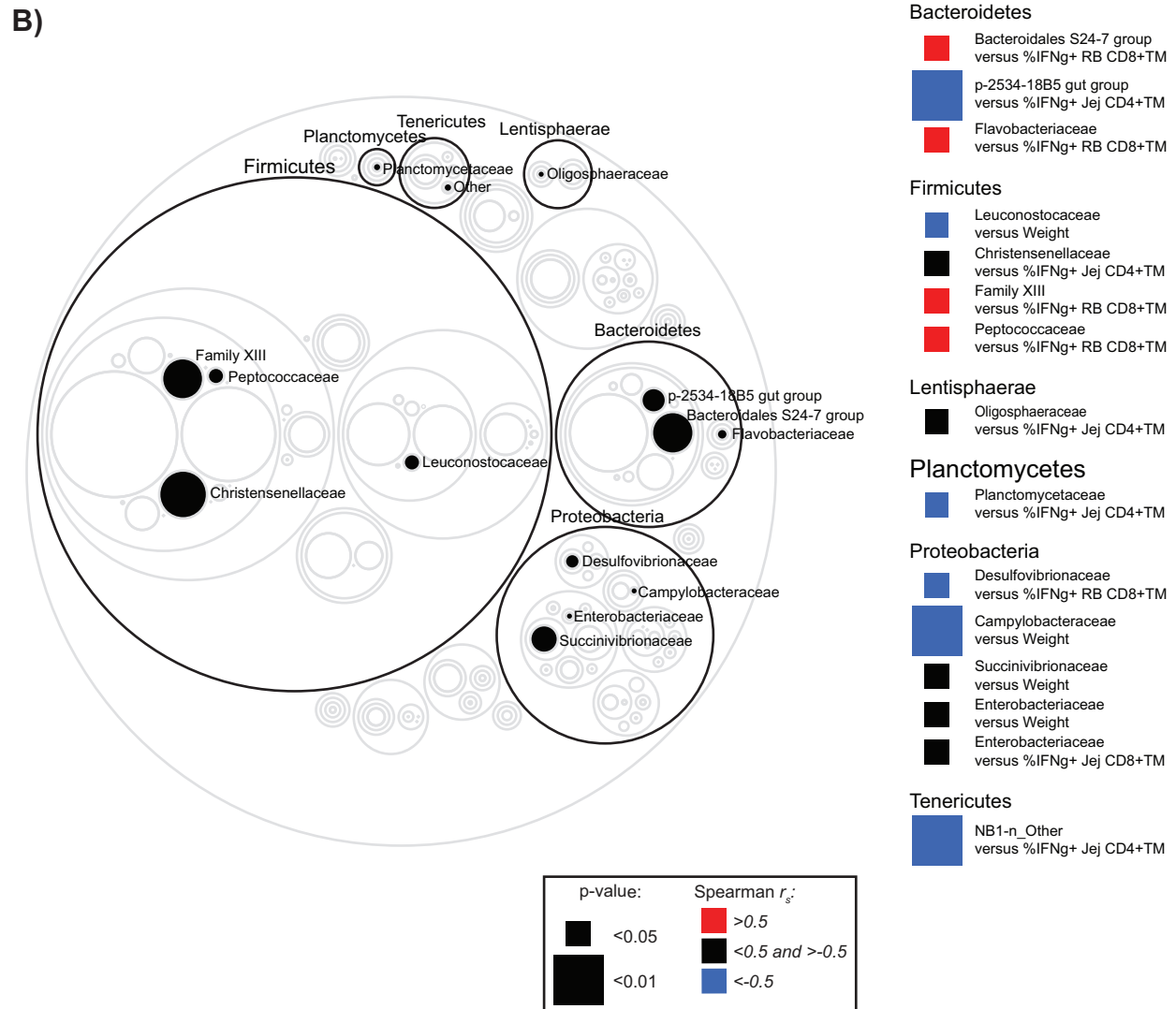
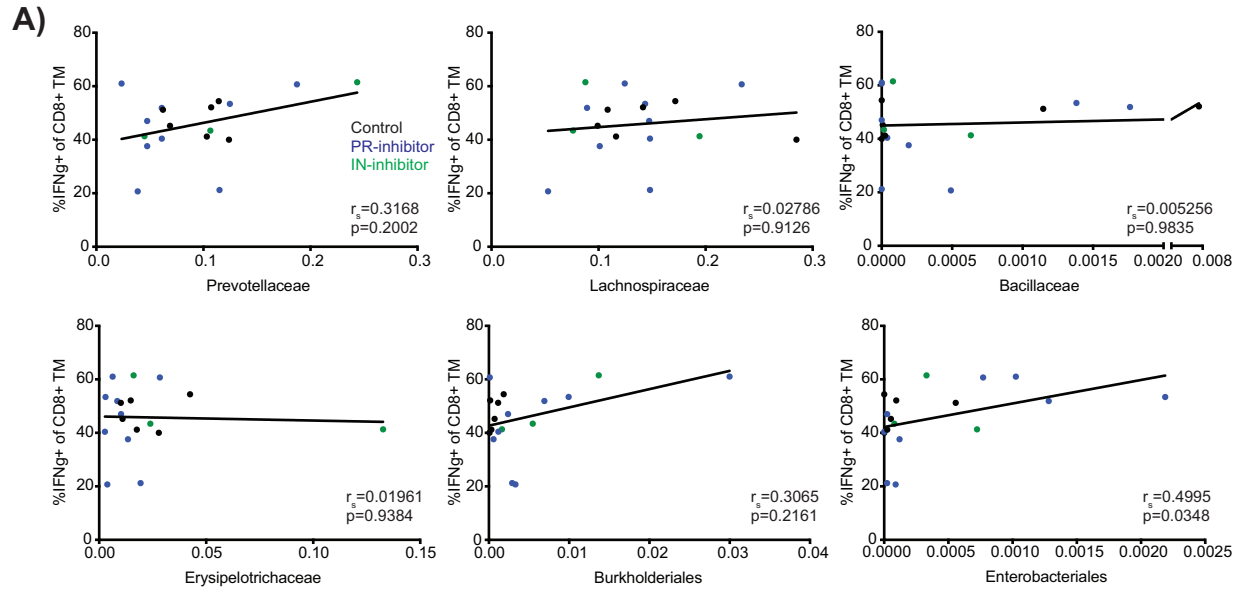


FIG 5

Associations between fecal bacterial taxa perturbed by ART and the expression of T-cell proinflammatory cytokines or animal weight. (A) Association between the frequency of IFN- γ -expressing Jej CD8⁺ TM cells and relative fecal frequencies of *Prevotellaceae*, *Lachnospiraceae*, *Bacillaceae*, *Erysipelotrichaceae*, *Burkholderiales*, and *Enterobacteriales* at weeks 1 to 2 post-ART. Lines indicate best-fit linear regressions. (B) Circle plot highlighting significant associations between bacterial families and either weight or intestinal T-cell IFN- γ expression at weeks 1 to 2 post-ART, considering all animals. Circles represent relative mean bacterial taxon size. Families of significance are filled in and identified to the right, with each family's identifying phylum encircled in black and identified above the phylum ring. Significance was assessed by Spearman correlation with resultant r_s and P values directly indicated (A) or represented as according to the color legend (B).

Discussion

Antiretrovirals have significantly improved the lifespan and quality of life for people living with HIV and have proven greater than 75% effective at preventing HIV transmission between sero-discordant partners [198, 199]. The introduction of these drugs has, without question, significantly impeded the HIV epidemic. However, prolonged treatment is not without complication for the greater than 21 million individuals accessing ARTs worldwide [200]. Non-AIDS related co-morbidities are now a leading cause of mortality for people living with HIV and include cardiovascular events, neurocognitive disorders, gastrointestinal disease, and neoplasia - all of which have been linked to persistent inflammation in treated individuals [169]. Although a causal relationship between these co-morbidities and ART is confounded by post HIV-replicative damage, gastrointestinal complications are among reported side-effects by uninfected individuals taking ARVs as PreP [198, 199]. Here we sought to determine whether ART alone might contribute to gastrointestinal inflammation and - given the intimate link between gastrointestinal

health and the composition of the intestinal microbiome - whether ART might contribute to bacterial dysbiosis in a non-human primate model of AIDS. We observed a modest induction of inflammation independent of prolonged changes in specific bacterial taxa; however, these inflammatory perturbations coincided with significant differences in bacterial diversity, suggesting that ART alone may destabilize intestinal bacterial communities.

The modest perturbations in proinflammatory responses of intestinal lymphocytes following short-term (1-2 weeks) administration of IN-inhibitor L812 and long-term (5-6 weeks) administration of PR-inhibitor regimen Darunavir-Ritonavir are not sufficient to explain the degree of immune dysfunction noted in HIV-infected, ARV-treated individuals. However, these results are in line with previous observations that attribute gastrointestinal complications to PR-inhibitor regimens in HIV-infected individuals [177, 178] and to RT-inhibitor regimens in users of PreP [198, 199]. HIV PR-inhibitors target the HIV-1 PR catalytic domain, which maintains many of the same characteristics as non-viral aspartic proteases while nucleoside/nucleotide analog RT-inhibitors (NRTIs; currently, all licensed PreP formulations) are incorporated indiscriminately into cellular DNA prior to editing and excision. In vitro, PR-inhibitors have been shown to interfere with calcium-dependent chloride conductance [201] and epithelial proliferation and survival [202]. Although the role of RT-inhibitors in epithelial cell dysfunction is more nuanced – balanced by mitochondrial toxicity and oxidative stress on one hand [203] and inflammasome-inhibition on the other [204] – direct interference by ARVs with host cellular processes can undoubtedly contribute to gastrointestinal complications.

We and others have previously reported a post-therapeutic association between bacterial dysbiosis and intestinal inflammation in HIV-infected individuals [51, 62, 125, 146] and SIV-infected macaques [66]. Many HIV-1 ARVs target biochemical processes that are conserved across

taxonomic kingdoms, with several ARVs showing bioactivity in bacterial, fungal, and protozoal culture systems [195-197]. Thus, to our surprise, we did not observe persistent changes in individual taxa in our ARV-treated, uninfected macaques (Figure 3B-C). There are several, non-exclusive, explanations for our observations. In lentiviral-infected, ARV-treated individuals and macaques, bacterial dysbiosis may be a feature of immune restoration. In SIV-infected macaques, dysbiosis does not appear to be a routine feature of disease progression [96, 102, 181, 205] but rather, has been associated with the initiation of ART [66] and the onset of end-stage disease [205]. Thus, in otherwise healthy macaques, ART may not be sufficient to induce dysbiosis. Secondly, ART-associated dysbiosis may be a feature of combination ART rather than individual ARVs. The combined effects of several ARVs may overwhelm the host cellular or bacterial capacity to mitigate independent off-target effects and consequently, result in a confluence of abnormalities that promote the prolonged dysbiosis of specific taxa. As combination ART is not currently approved for PreP, such an effect cannot account for intestinal dysfunction in uninfected individuals. However, perhaps it is not a specific dysbiosis per se that is induced by ART during lentiviral infection but rather, marked instability of the bacterial microbiome, as suggested by our data (Figure 3G and Supplementary Figure 2A-B).

Disruptions in intestinal bacterial diversity have been described pursuant to HIV infection [36, 62, 125], as well as progressive [205] and natural SIV infections [63]. Although several cross-sectional studies in people living with HIV have described specific alterations in intestinal bacterial distributions [50, 51, 62, 125], studies in SIV-infected macaques more commonly describe only temporal disturbances [66, 205]. To this end, although we did not observe persistent alterations in specific bacterial taxa after the introduction of ARVs, we observed evidence of distinct phylogenetic communities between cohorts post-treatment as well as sharp increases in the number

of differentially abundant taxa compared to control animals (Figure 3 and Supplementary Figure 2A-B). Our results are in keeping with a more generalized disruptive effect of ART. Indeed, a PCoA examination of the PR-treated group alone suggested that continued PR-treatment in these animals did not promote phylogenetic clustering of intestinal communities post-treatment but rather, was associated with community destabilization within each animal.

The potential causes for altered bacterial diversity and destabilization in in pre- and post-exposure ARV prophylaxis are unclear but likely to be varied. The stability of discrete cross-kingdom networks relies on keystone members, the disruption of which can lead to network restructuring or collapse [206]. Previous studies in SIV-infected macaques have revealed a prolonged disruption of the enteric virome [181, 205], a feature which we did not assess here. We also did not assess the influence of ART on systemic inflammation or microbial diversity, which may influence HIV acquisition. A contribution of bacterial dysbiosis to HIV acquisition has been described in females, where vaginal *Lactobacillus* non-dominance – here, increased diversity - is associated with increased genital inflammation [207, 208]. Although a contribution of PreP to genital dysbiosis and diversity has not been described, Klatt et. Al. [209] recently demonstrated that vaginal *Lactobacillus* non-dominance (*Gardnerella vaginalis* dominance) resulted in a three-fold reduced efficacy in the CAPRISA-004 topical microbicide (Tenofovir) PreP trial, and furthermore, that *Gardnerella vaginalis* can actively metabolize Tenofovir. A full metagenomic analysis of systemic ecosystems will be necessary to fully appreciate the effects of ART on immunity and immune dysfunction.

Specific immunological abnormalities within the GI tract are known to contribute directly to progressive HIV/SIV infections. T regulatory CD4⁺ T-cells (T_{regs}), Th17 cells, Tc17 cells, and innate lymphocyte type III (ILC3s), are preferentially and persistently lost from the lamina propria

of the GI tract in people living with HIV and in SIV-infected macaques and are not restored with the administration of ARVs [12, 210, 211]. The loss of these cells and altered infiltration of neutrophils into GI tract tissues [212] contribute to epithelial barrier defects, microbial translocation and inflammation. Our data suggest that ARVs themselves are unlikely to significantly contribute to immunological perturbations observed in ARV-treated, HIV-infected individuals (Figure 2) and thus, repairing these immunological perturbations might require adjuvant therapy. The administration of probiotics (live bacterial taxa which are thought to provide benefit) have been shown to impart immunological benefit to people living with HIV and in SIV-infected macaques on ART [93, 104, 106, 213, 214]. This immunological benefit might be related to probiotics stabilizing the composition of the microbiome which we observe to become disrupted after administration of ARVs.

In conclusion, we demonstrate that ARVs alone induce intestinal bacterial instability and a modestly pro-inflammatory milieu in a non-human primate model for AIDS. Importantly, given the significant alterations to the composition of the microbiome and to the GI tract immune system observed in people living with HIV and in SIV-infected macaques, ARV-induced instability to the microbiome might further aggravate these. Our work can inform an essential framework for understanding how ART itself contributes to immune reconstitution and non-AIDS related comorbidities in people living with HIV. The identification of keystone organisms or biochemical processes altered by ART is necessary for informing the design of next-generation ARVs and palliative therapeutics.

Materials and methods

Study Design. Thirty-one rhesus macaques (*Macaca mulatta*) were assigned to a control (n=6), PR-inhibitor (n=13), IN-inhibitor (n=6), or RT-inhibitor (n=6) group and treated daily for 1-6 weeks as detailed in Table 1. Groups were stratified by weight and sex and animals sampled as mixed populations. PR-inhibitor animals received Darunavir and Ritonavir (400mg Prestiza® and 100mg Norvir® oral, b.i.d.), IN-inhibitor animals received L812 (120mg oral, b.i.d.), and RT-inhibitor animals received PMPA or FTC (30 mg/kg s.c., s.i.d.).

The NIAID Division of Intramural Research Animal Care and Use Program, as part of the NIH Intramural Research Program, approved all experimental procedures (protocol LVD 26). The Program complies with all applicable provisions of the Animal Welfare Act and other federal statutes and regulations relating to animals.

Animals were housed and cared for at the NIH Animal Center, under the supervision of the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited Division of Veterinary Resources and as recommended by the Office of Animal Care and Use Nonhuman Primate Management Plan. Husbandry and care met the standards set forth by the Animal Welfare Act, Animal Welfare Regulations, as well as The Guide for the Care and Use of Laboratory Animals (8th Edition). The physical conditions of the animals were monitored daily. Animals in this study were exempt from contact social housing due to scientific justification, per IACUC protocol, and were housed in non-contact, social housing where primary enclosures consisted of stainless-steel primate caging. Animals were provided continuous access to water and

offered commercial monkey biscuits twice daily as well as fresh produce, eggs and bread products twice weekly and a foraging mix consisting of raisins, nuts and rice thrice weekly. Enrichment to stimulate foraging and play activity was provided in the form of food puzzles, toys, cage furniture, and mirrors.

Sample Collection. Blood, stool, and intestinal biopsies were collected longitudinally, with each measurement resulting from a single sample per timepoint. Biopsies were maintained in RPMI prior to processing. Mononuclear cells were isolated from blood by Ficoll gradient centrifugation. Stool was collected fresh from each animal by inserting a sterile swab 2 cm into the rectum and “swirling” to collect available sample. For RBs, fecal material was removed from the rectum and biopsies obtained with biopsy forceps. Jejunal biopsies were obtained by video-guided endoscopy. Sampling occurred in random order. Neither the investigators nor the animal handlers were blinded to group allocation in order to ensure multi-lateral supervision of design and palliative treatment. Animals were sedated with Telazol at 3-4 mg/kg i.m. and isoflurane gas by intubation, to effect, for sample collection. Successful anesthetization was monitored by response to stimuli.

Immune Phenotyping and Functional Assessment. Polychromatic flow cytometry was performed on stained mononuclear cells as previously described [106]. Antibodies against the following antigens were used for staining and gating at predetermined concentrations: CCR5 (clone 3A9) conjugated to PE, CCR7 (3D12) PE-Cy7 or Alexa647, CD3 (SP34-2) Alexa700, CD8 (SK1) APC-H7, CD20 (L27) PECy7, CD28 (CD28.2) PE-Cy5, CD45 (D058-1283) BVio786 or PE-CF594, HLA-DR (G46-6) PE or (L243) APC-H7 from BD Bioscience; CD4 (OKT4) eFluor450, CD8 (SK1) PerCP-eFluor710, IL-17 (eBio64Dec17) Alexa488, IL-21 (3A3-N2) PE, IL-22 (IL22JOP) APC or PerCP-eFluor710, IFN γ (4S.B3) eFluor450, TNF α (Mab11) PE-Cy7

from Thermo Fisher-Scientific; CD95 (DX2) PE-Cy5 or BVio650, HLAD-DR (L243) BVio711, IL-2 (MQ1-17H12) BVio650, TNFa (Mab11) BVio605 from Biolegend; and CD28 (CD28.2) ECD from Beckman Coulter. Cell viability was assessed using the Live/Dead Aqua Fixable Dead Cell Stain (Thermo Fisher-Scientific). Gating strategy shown in Supplementary Figure 4. CD4⁺ and CD8⁺ TM were defined as CD95⁺ singlet, clean, live, CD3⁺ lymphocytes. Positive/negative gating based on clearly grouped populations, historically-determined expression, and the use of internal controls. A minimum threshold of 100 collected events in the parent population was utilized for all subset expression analysis.

16S isolation and analysis. 250mg of stool was transferred to soil-grinding Percellys tubes (Bertin Technologies) and homogenized at room temperature on a Precellys 24 homogenizer at 5000 rpm in four successive 20s intervals, with 750 uL PowerBead Solution (Qiagen) and 60uL Solution C1 (Qiagen). Residual particulate was removed from the supernatant by centrifugation at 10,000 xg for 30s at room temperature. Supernatant was transferred to a 96 Deep-Well plate (Costar) and processed as follows with solution addition and supernatant transfer/removal facilitated using a Biomek NK^P (Beckman Coulter): (1) 250uL of Solution C2 (Qiagen) was added to supernatant and mixed at 1000rpm for 10 minutes at room temperature on an orbital shaker, (2) the 96-well plate was sealed with an aluminum plate sealer and centrifuged at 4500 xg for 10 minutes at 4°C, (3) 580uL of supernatant was transferred to a new 96 Deep-Well plate, (4) 200uL Solution C3 (Qiagen) was added to the supernatant and mixed at 1000rpm for 10 minutes at room temperature, (5) 0.6ng of Internal Amplification Control (GenBank accession number FJ357008.) was added to each well as an internal amplification control, (6) the 96-well plate was sealed with an aluminum plate sealer and centrifuged at 4500 xg for 10 minutes at 4°C, (7) 450uL of supernatant was transferred to a new 96 Deep-Well plate, (8) 450uL of ClearMag (Qiagen) magnetic bead solution

- 19uL beads and 431uL Binding Solution – was added to the supernatant and mixed at 1000rpm for 10 minutes at room temperature, (9) supernatant-containing plate was transferred to rest on a magnetic plate (Alpaqua Magnum FLX) and bead-bound DNA allowed to precipitate for 15 minutes at room temperature, (10) 820uL of supernatant was removed following precipitation, (11) the deep-well plate was removed from the magnet and bead-bound DNA washed with 3x500uL Solution C5-D (Qiagen) on an orbital shaker, with between-wash magnetic pelleting and supernatant removal as in steps 9-10, (12) after final washing, pelleted DNA was allowed to dry overnight at room temperature, (13) 200uL of sterile water was added to elute the DNA and mixed at 1000rpm for 15 minutes at room temperature, (14) the deep-well plate was transferred back to the magnetic plate where DNA-free beads were allowed to pellet for 10 minutes, and (15) 200uL of DNA-containing supernatant was transferred to a new deep-well plate. Eluted DNA was cleaned using the Qiagen DNeasy Blood and Tissue Kit per the manufacturer's, "Purification of Total DNA from Animal Tissues (Spin-Column Protocol)," protocol beginning at the addition of Buffer AL.

Total DNA (105 ng) was subjected to dual-Index amplification and sequencing for non-human primate fecal pellets, using barcoded universal primers spanning basepairs 515(F)-806(R) of the bacterial 16S rRNA V4 region. 16S amplicons were purified using Agencourt AMPure XP PCR Purification (Beckman Coulter) both before and after Index amplification. Indexed 16S amplicons were quantified by KAPA qPCR Library Quantification kit for Illumina Platforms (ROX Low; KAPA Biosystems), normalized to 15pM, spiked-in with 15% phiX control library (Illumina), and sequenced on the MiSeq platform (Illumina MiSeq v3 Reagent Cartridge Kit, MiSeq v3 Reagent Kit, and Flow cell and PR2 Buffer kit) utilizing the 300 Paired End Reagent Plate.

Raw Illumina FASTQ files were first demultiplexed using a custom Python script. Returned paired-end FASTQ reads were filtered and processed using QIIME (v1.9.1) [107] through the NIAID/NIH Microbiome Analysis Platform, Nephele (Office of Cyber Infrastructure and Computational Biology/NIAID/NIH; <http://nephele.niaid.nih.gov>) [215]. Before quality trimming, approximately 5 million reads were included in the 51 samples with an average of 98,654 reads (median 89,350, maximum 173,767 and minimum 12,324) per sample. Sequences with any degenerate bases (e.g., N), a Phred quality score less than 25 per base, and more than 3 consecutive low-quality base calls were filtered out. Assessment of the read quality was performed using MultiQC [216]. QIIME quality trimming resulted in approximately 3.8 million high-quality reads for all the samples with an average of 75,099 reads (median of 43,534, maximum 167,988 and minimum 11,827) per sample. On average, 76.13% of reads passed the quality filtering. All samples were included in the downstream quantitative analyses. Sequences were binned into OTUs and taxonomically assigned at 99% identity using the QIIME open reference OTU picking workflow with SortMeRNA [217]. Sequences were taxonomically classified using the Silva 99% database (release 132). Frequencies of microbial taxa are calculated relative to all returned bacterial OTUs mapping to non-mitochondrial (Rickettsiales Mitochondria) taxa. 16S miSeq data are deposited in the NCBI Sequence Read Archive (SRA) under project number PRJNA526520.

Statistical Analyses. Unpaired, two-way t-tests were used in statistical analyses of single parameter lymphocyte phenotype and function (Prism v7.0, GraphPad Software Inc.). Significance of polyfunctional cytokine expression was assessed using the Spice (v6.0; NIAID) permutation-test on relative expression values. The empirical identification of differentially abundant bacterial taxa between groups was determined using the LEFSE algorithm [111] one-against-all multi-class

analysis, normalized to 10^6 reads. Microbial alpha diversity was assessed by Chao1 and Shannon indices from 10 random subsamplings of the QIIME OTU table at a sampling depth of 11179 reads per sample, with statistical significance assessed by one-way ANOVA (Prism v7). Beta diversity was assessed by unweighted and weighted Unifrac [218] and Bray-Curtis dissimilarity indices, with statistical significance assessed by PERMANOVA (R-vegan function Adonis). PCoA plots generated by XLSTAT (Addinsoft v20.7). Instability indices were identified as beta diversity indices of single animals across treatment-relative timepoints, with statistical significance assessed by one-way ANOVA or unpaired t-test as required by number of comparisons. Spearman correlations were performed without multiple-comparison adjustments on OTUs identified as differentially abundant in both the PR- and IN-inhibitor LEFSE analyses or for bacterial families where greater than one-half of the samples had non-zero reads. Clustering was assessed by Gaussian mixture modeling on beta diversity measures, using the EM inference algorithm and the EEE mixture model run to a convergence threshold of 1×10^{-5} , selected using the BIC criterion for unweighted Unifrac (log-likelihood=43.8) and Bray-Curtis (log-likelihood=17.2) which fitted 3 components and the ICL criterion for weighted Unifrac (log-likelihood=32.0) which fitted 2 components, by XLSTAT. No data that met minimum threshold requirements as outlined in the Methods were excluded.

Data Availability. 16S miSeq data were deposited in the NCBI Sequence Read Archive (SRA) under project number [PRJNA526520](https://www.ncbi.nlm.nih.gov/sra/PRJNA526520).

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Chapter 5

Impact of Acute HIV-Infection and Early Antiretroviral Therapy on the Human Gut Microbiome

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Abstract

Background. Intestinal microbial dysbiosis is evident in chronic HIV-infected individuals and may underlie inflammation that persists even during antiretroviral therapy (ART). It remains unclear, however, how early after HIV infection gut dysbiosis emerges and how it is affected by early ART.

Methods. Fecal microbiota was studied by 16s rDNA sequencing in 52 Thai men who have sex with men (MSM), at diagnosis of acute HIV infection (AHI), Fiebig Stages 1–5 (F1-5), and after 6 months of ART initiation, and in 7 Thai MSM HIV-uninfected controls. Dysbiotic bacterial taxa were associated with relevant inflammatory markers.

Results. Fecal microbiota profiling of AHI pre-ART vs HIV-uninfected controls showed a mild dysbiosis. Transition from F1-3 of acute infection was characterized by enrichment in pro-inflammatory bacteria. Lower proportions of Bacteroidetes and higher frequencies of Proteobacteria and Fusobacteria members were observed post-ART compared with pre-ART. Fusobacteria members were positively correlated with levels of soluble CD14 in AHI post-ART.

Conclusions. Evidence of gut dysbiosis was observed during early acute HIV infection and was partially restored upon early ART initiation. The association of dysbiotic bacterial taxa with inflammatory markers suggests that a potential relationship between altered gut microbiota and systemic inflammation may also be established during AHI.

Introduction

During the acute phase of HIV-infection high levels of viral replication occur in the gut associated lymphoid tissue (GALT) [14]. This leads to a substantial depletion of gut mucosal CD4⁺ T cells with a preferential loss of T helper 17 (Th17) and T helper 22 (Th22) cells, whose main effector cytokines are IL-17, IL-21 and IL-22. These cytokines are normally enriched in mucosal tissue and play a central role in protecting the integrity of the epithelial barrier and maintaining immune homeostasis at mucosal sites [24, 26, 219]. As a consequence of this altered intestinal microenvironment there is increased permeability with microbial translocation [25, 220], contributing to chronic immune activation and inflammation [31, 221]. The etiology, however, of the persistent immune activation in chronic treated HIV-infection is still incompletely understood and is probably multifactorial encompassing residual HIV replication, co-infections, mucosal alterations and incomplete immune restoration [76, 77].

Current studies in HIV-infection, similar to other chronic inflammatory states [46, 48, 222], suggest that resident gut microbiota and their metabolic products are drivers of inflammation that can persist even during ART [223, 224]. Initial studies by Brenchley et al. described elevated levels of lipopolysaccharide (LPS) in HIV-positive people that were associated with peripheral blood T-cell activation providing an indication of a link between intestinal microbial translocation and systemic immune activation [25]. During the last few years, several studies compared the gut microbiota of HIV-infected individuals to that of HIV-uninfected controls in order to evaluate differences in microbial abundance [50, 51, 53, 55, 57, 62, 125, 127]. Many studies have included cross-sectional comparisons between HIV-infected individuals and controls and have suggested

that HIV-infection is associated with alterations in gut communities, in particular an enrichment in Enterobacteriaceae members and Erysipelotricaceae with concurrent depletion of Bacteroides and Clostridia [96]. These dysbiotic bacterial communities are often linked to key markers of inflammation and cellular activation [97] including the kynurenine pathway of tryptophan metabolism and plasma concentrations of the inflammatory cytokine interleukin-6 (IL-6) in treated HIV-infected individuals[20]. It remains unclear though how early after HIV-infection gut dysbiosis is established and how early ART-treatment may affect it.

Initiation of ART during early AHI has been previously shown to be associated with preservation of immune function, enhanced recovery of CD4⁺ T cell numbers and function in blood as well as in the gastrointestinal (GI) tract and significant reduction in HIV reservoir size [19]. Despite reversing several key features of early GI tract pathology, however, mucosal injury and inflammation may not resolve completely by early ART [175, 225].

In this study, we profiled the intestinal microbiota during AHI, F 1-5, at the time of diagnosis and 6 months after ART-initiation using HIV-uninfected individuals as controls. Furthermore, we assessed the relationship of dysbiosis with systemic inflammatory and gut epithelial integrity markers.

Methods

Study Participants and Study Design

RV254/SEARCH010 is a clinical trial (NCT00796146) that was conducted in Bangkok, Thailand, enrolling persons with early acute HIV infection (F1-5) who were identified from the Thai Red Cross Anonymous Clinic through nucleic acid testing and sequential immunoassays [29]. The

RV254/SEARCH 010 study was approved by the institutional review boards (IRBs) of Chulalongkorn University in Thailand and the Walter Reed Army Institute of Research in the United States. Participation in this study involved extensive evaluation including rectosigmoid gut biopsies, anal sample collection, and storage of peripheral blood mononuclear cells (PBMCs) and serum/plasma. Initiation of ART was voluntary and done as part of the enrollment in an accompanying protocol (clinicaltrials.gov NCT00796263), which was approved by the Chulalongkorn University IRB. For all the studies mentioned above, subjects gave written informed consent. We profiled the intestinal microbiota during AHI, F1-5, at the time of diagnosis, and 6 months after ART initiation.

Microbiome Study Methods

Rectal samples from 52 men who have sex with men (MSM) at either baseline (n = 37) or 6 months after ART (2NRTI + efavirenz) initiation (n = 31; termed post-ART) were collected, including 16 paired samples at both visits. Samples (n = 7) from HIV-uninfected age-, sex-, and risk group-matched MSM Thai volunteers were obtained from a different protocol (RV304, clinicaltrials.gov NCT01397669) at the same site.

DNA Extraction and PCR Amplification

Bacterial profiles of study participants were generated by broad range amplification and sequence analysis of bacterial 16S rRNA genes. Microbiome specimen analysis is described in the Supplementary Data and [30].

Laboratory Methods for Blood Measurements

Plasma, serum, and PBMC specimens were processed within 30 minutes of collection. Intestinal fatty acid binding protein (I-FABP) and sCD14 (R&D Biosystems, Minneapolis, MN) were measured by enzyme-linked immunosorbent assay; C-reactive protein (CRP) was measured by electrochemiluminescence (Meso Scale Discovery, Rockville, MD); high-sensitivity IL-6 was measured using the Luminex (Austin, TX) platform according to the manufacturer's instructions (Millipore, Darmstadt, Germany). All assays used cryopreserved acid citrate dextrose (ACD) plasma matching rectal sampling.

Immunohistochemistry

Rectosigmoid gut biopsies were sampled and processed as described earlier [28]. In brief, subjects underwent a routine sigmoidoscopy procedure under moderate conscious sedation. Approximately 30 endoscopic biopsies were randomly collected from the sigmoid colon using Radial Jaw 3 biopsy forceps (Boston Scientific, Natick, MA), with 20–25 processed for flow cytometry analysis within 30 minutes of collection. Biopsies from AHI pre-ART (n = 23) and post-ART (n = 14) were used for immunohistochemistry (IHC) analysis. Eight patients had sampling of gut biopsies at both visits. IHC staining and quantitative image analysis (QIA) for myeloperoxidase (MPO) were performed as previously described [27] and are detailed in the Supplementary Data.

Metabolomic Analysis

The plasma metabolic profile of a subset of 17 AHI individuals' paired samples pre- and post-ART (F1-5) was assessed. Data were acquired at Metabolon, as previously described [31], and are detailed in the Supplementary Data and [30].

Statistical Analyses

In this study we performed both cross sectional and paired analysis in order to maximize utilization of all available data. Alpha and Beta Diversity were calculated using phyloseq (version 1.19.1) package in R. Boxplots and other visualizations were created using ggplot2 package in R (<https://www.r-project.org>). A PERMANOVA test was performed on beta diversity distance matrices using the Adonis function in the vegan package with 999 permutations. Relative abundance of bacterial taxa was calculated based on the total OTUs of each sample. Subsequently, families from all samples were ranked based on their average relative abundance. The top 30 most abundant bacterial families were selected to test for differences between AHI pre- (n=37), post-ART (n=31) and HIV-uninfected controls (n=7) using Mann Whitney non parametric test. Two way p-values were calculated and statistical significance was defined as $p < 0.05$. LEfSe (Linear discriminant analysis Effect Size), based on the non-parametric Kruskal Wallis rank test, was used to detect features with significant differential bacterial abundance between AHI pre and post-ART. Paired Wilcoxon signed rank test was also applied to identify OTUs in differential abundance between AHI who had both pre and post-ART samples (n=16).

The top 20 most abundant families were imported in Spice [226] to create the pie charts for comparisons of bacterial taxa in HIV-uninfected controls (n=7), HIV+ pre-ART (n=37) and HIV+

post-ART (n=31) (Permutation test, 20.000 iterations). Statistical significance was defined as $p < 0.05$.

Associations between markers of tissue or systemic inflammation (sCD14, IL-6, CRP) and intestinal gut epithelial integrity (I-FABP) and bacterial taxa were assessed using Spearman's rho non-parametric test in AHI pre- (n=37) and post-ART (n=31).

Statistical significance of PMN infiltration in AHI pre-ART (n=37) compared to post-ART (n=31) was assessed using Prism version 8.0, and two-sided p-values < 0.05 were considered significant.

For statistical analyses and data display of plasma metabolites, any missing values were assumed to be below the limits of detection; these values were imputed with the compound minimum (minimum value imputation); data for each metabolite was also median-scaled for display. Statistical tests were performed in ArrayStudio (Omicsoft) to compare data between experimental groups; $P < 0.05$ was considered significant. An estimate of the false discovery rate (Q-value) was also calculated taking into account the multiple comparisons that normally occur in metabolomic-based studies, with $Q < 0.05$ used as an indication of high confidence in a result. Principal coordinates analysis (PCoA) was performed using ggbiplot, vegan and pairwise adonis packages in R (<https://www.r-project.org>).

Results

Mild dysbiosis observed in early acute HIV infection

Study participants were MSM with a median age of 28 years. The median CD4 T-lymphocyte count was 386 cells/ μ L at baseline and 600 cells/ μ L after 6 months of ART. Median plasma HIV

RNA was 334889 copies/mL at baseline and below 50 copies/mL after 6 months of ART (Table 1). To examine whether alteration in the bacterial composition of the fecal microbiota (dysbiosis) was established during the very early acute phase of HIV-infection, we first compared AHI participants pre-ART (n=37) to HIV-uninfected controls (n=7). HIV infection was associated with a decrease in α -diversity (p=0.003 Chao1; p=0.06 Shannon Index) (Figure 1 A). PCoA revealed clustering of AHI pre-ART and HIV-uninfected controls which was verified by permutational multivariate analysis of variance (p=0.001, PERMANOVA) (Figure 1B). We then evaluated the relative abundance of bacterial taxa belonging to the main phyla. Among the 20 top abundant families, the relative abundance of Actinomycetaceae, Coriobacteriaceae, Acidaminococcaceae and Lachnospiraceae were significantly lower in AHI pre-ART compared to HIV-uninfected controls (p<0.005) in agreement with previous data which showed a decrease in Lachnospiraceae in chronically HIV-infected individuals [50, 51, 55, 57, 125, 127]. Members of the Proteobacteria phylum, such as Enterobacteriaceae, and Moraxellaceae - previously reported enriched in chronically HIV-infected individuals [50, 51, 53, 55, 62, 125, 127] - were more abundant in HIV-uninfected controls compared to AHI pre-ART (Figure 1C). Several recent studies [50, 51, 55, 62, 127] have shown a linkage between *Prevotella* abundance and HIV-infection related inflammation, while others, have reported a *Prevotella*-rich microbiota associated with sexual practice, rather than with HIV infection status *per se* [36]. In our cohort, we corroborated this observation and found no differences in the relative abundance of Prevotellaceae in MSM HIV-uninfected controls and AHI MSM individuals pre-ART (Supplemental Figure 1). Overall these data suggest that a mild dysbiosis is present during early acute HIV-infection.

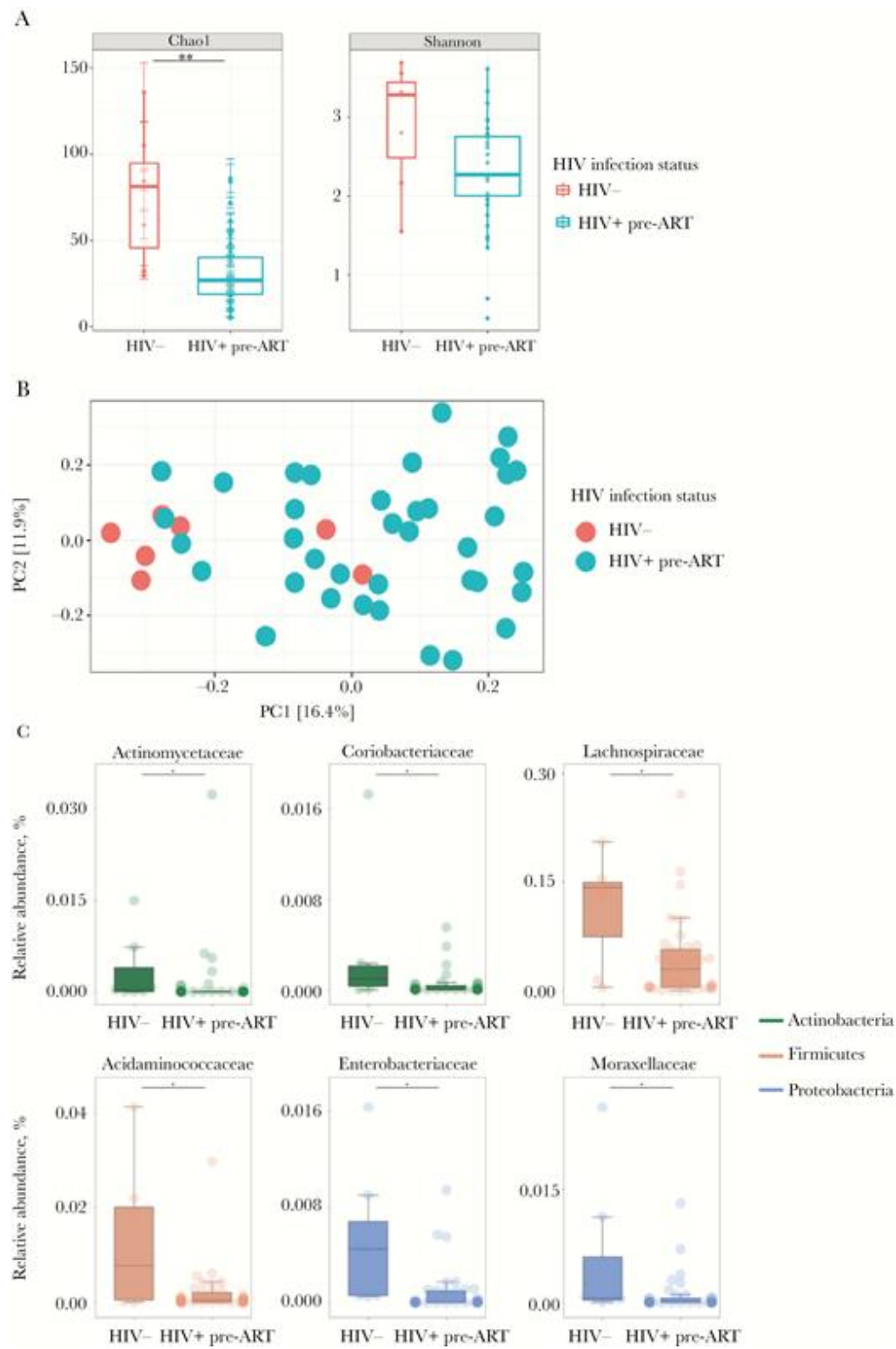
Table 1. Study Participants Characteristics

	HIV-positive individuals		HIV-seronegative controls
	Pre-ART	Post-ART	
Number of subjects	52 ^a		7
	37	31	
Fiebig Stage at diagnosis			
1	5	3	
2	10	10	
3	17	16	-
4	2	-	
5	3	2	
Median Age (IQR)	28		29
	(23.0-34.0)	(22.0-32.0)	(23.5-34.9)
Sex	Male		Male
Risk	MSM		MSM
Median CD4 count (cells/μl) (IQR)	386 (267.0-542.5)	600 (556.0-864.0)	-
Median viral load (HIV-1 RNA copies/mL) (IQR)	334889 (161770-1042075)	<50	-
cART	2NRTI + Efavirenz		-

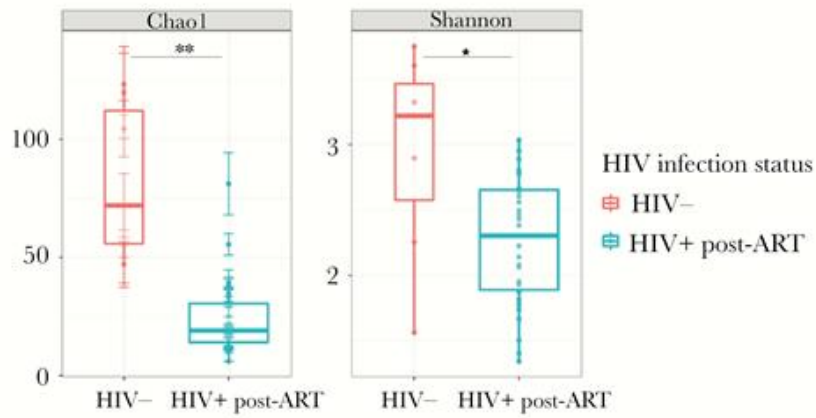
Abbreviations: ART, antiretroviral therapy; cART, combined antiretroviral therapy; IQR, interquartile range; MSM, men who have sex with men.

^aSixteen had paired pre- and post-ART samples.

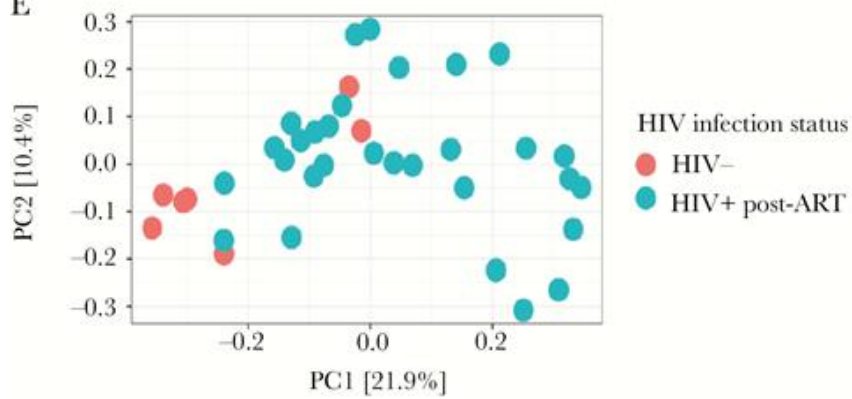
Figure 1.



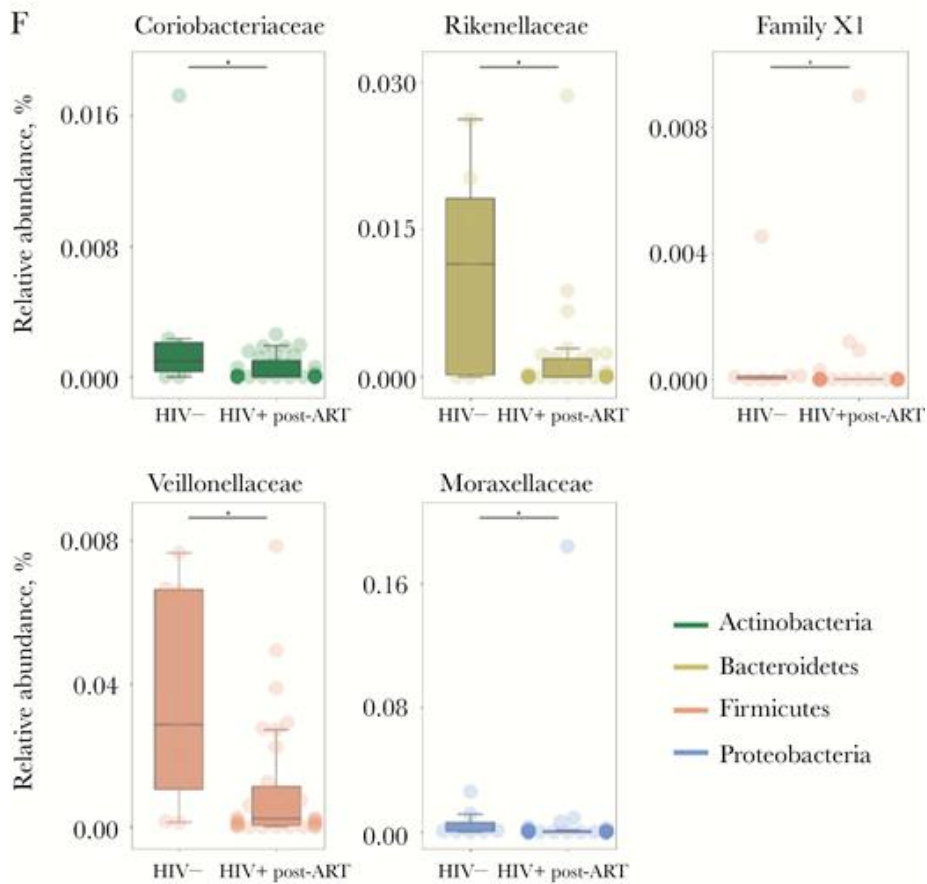
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F



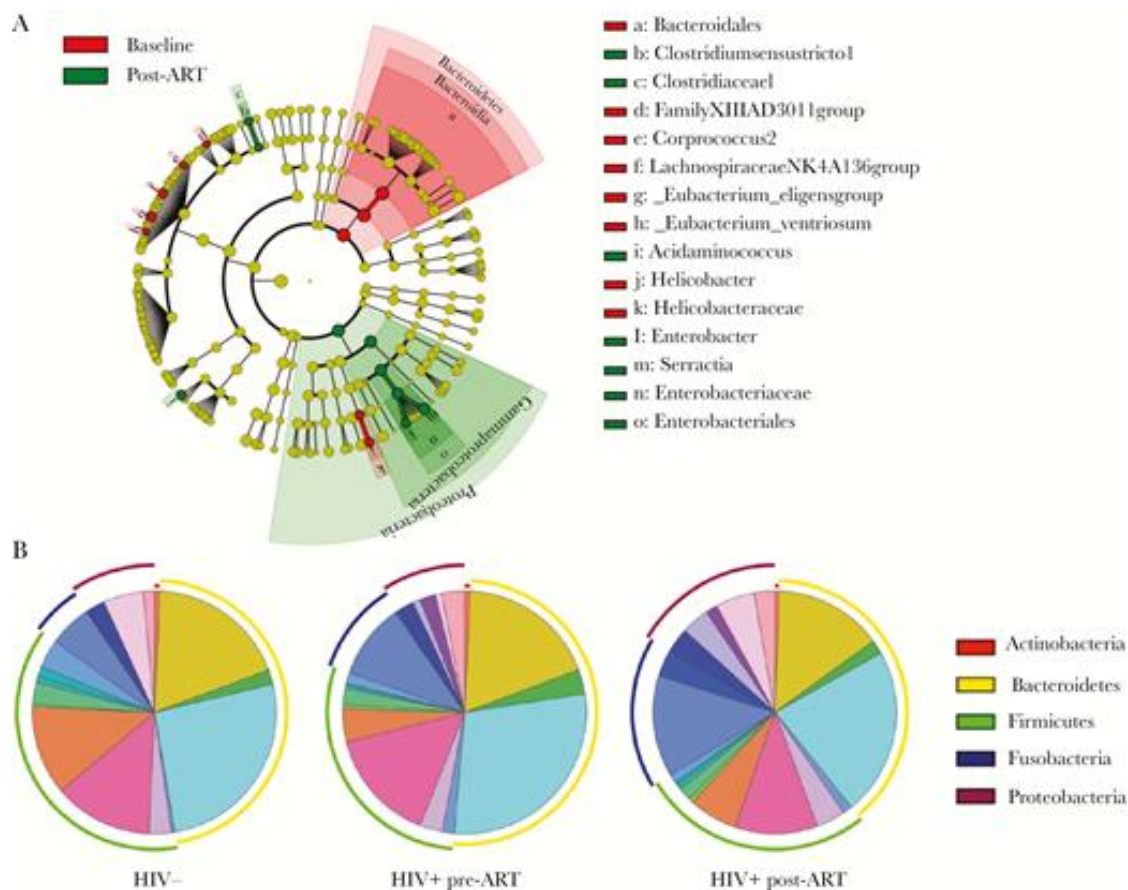
Stool bacterial community shifts in HIV-infected vs HIV-uninfected controls. A, Alpha diversity in HIV+ pre-antiretroviral therapy (ART) vs HIV-uninfected controls by Chai1 Index ($P = .003$) and Shannon Index ($P = .06$). B, Principal coordinates analysis (PCoA) shows clustering of acute HIV infection (AHI) pre-ART and HIV-uninfected controls ($P = .001$, PERMANOVA). C, Top 30 most abundant families in HIV- and HIV+ pre-ART measured and compared using the Mann-Whitney test ($P < .05$). The color of each family corresponds with the color of its respective phylum. D, Alpha diversity in HIV+ post-ART vs HIV-uninfected controls by Chai1 Index ($P = .002$) and Shannon Index ($P = .05$). E, PCoA shows clustering of AHI post-ART and HIV-uninfected controls ($P = .001$, PERMANOVA). F, Top 30 most abundant families in HIV- and HIV+ post-ART measured and compared using the Mann-Whitney test ($P < .05$). The color of each family corresponds with the color of its respective phylum.

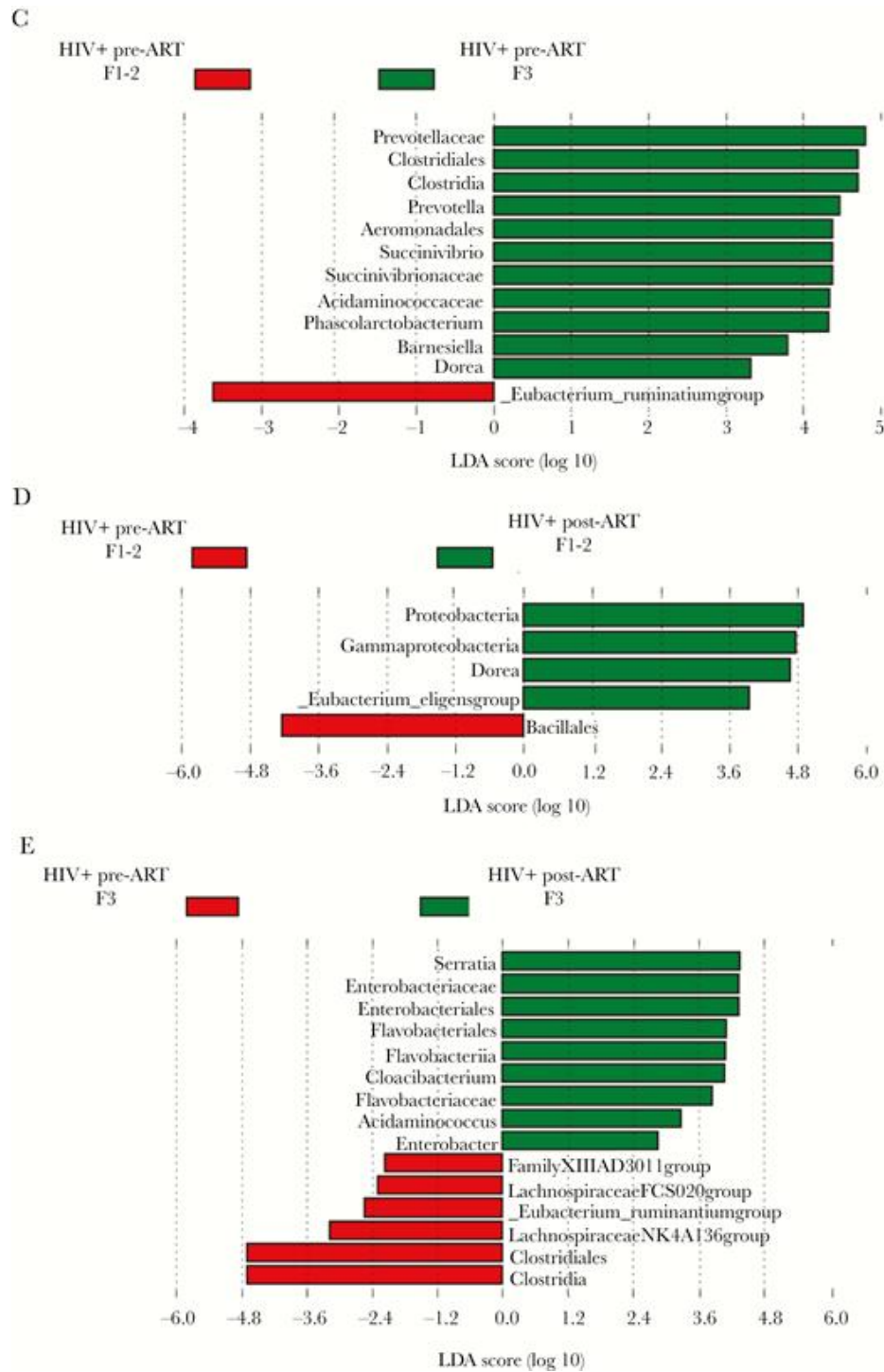
Effects of early antiretroviral therapy on gut microbiota

The effects of ART for 6 months on the fecal microbiota of acute HIV participants was then evaluated. Alpha diversity was significantly lower in AHI post-ART ($n = 31$) compared with HIV-uninfected controls ($n = 7$; $P = .002$, Chao1; $P = .05$, Shannon Index) (Figure 1D). PCoA revealed clustering of AHI post-ART and HIV-uninfected controls, which was verified by permutational multivariate analysis of variance ($P = .001$, PERMANOVA) (Figure 1E). The nonparametric Mann-Whitney test was then used to identify gut bacterial taxa that differed in abundance between AHI post-ART and HIV-uninfected controls. Among the bacterial taxa depleted in AHI post-ART were Rikenellaceae members, whose frequency has been previously observed to be reduced in chronically HIV-infected individuals [17–21], Coriobacteriaceae, family XI, and Veillonellaceae. Moraxelaceae members were still enriched in HIV-uninfected controls compared with AHI post-ART (Figure 1F). The linear discriminative analysis effect size biomarker discovery tool was subsequently used in unpaired samples to investigate the potential divergence of gut bacterial communities between the pre-ART ($n = 37$) and post-ART ($n = 31$) groups. The LEfSe algorithm identified enrichment in Enterobacteriaceae members in AHI post-ART compared with pre-ART,

whereas Bacteroidales members were more abundant in AHI pre-ART compared with post-ART (Figure 2A). Although we observed minor changes in the relative abundance of specific bacterial taxa between HIV-uninfected controls, AHI pre-ART, and AHI post-ART, overall gut microbial composition did not appear dramatically different among the 3 groups (permutation test: HIV- vs HIV+ pre-ART, $P = .6$; HIV- vs HIV+ post-ART, $P = .4$; HIV+ pre-ART vs HIV+ post-ART, $P = .3$) (Figure 2B).

Figure 2





Gut bacterial changes in HIV+ pre-antiretroviral therapy (ART) and HIV+ post-ART. A, Cladogram of linear discriminant analysis (LDA) effect size highlights discriminatory taxa, with an LDA score >2, between HIV+ pre-ART and HIV+ post-ART. B, Percentage of the top 20 most abundant families in HIV-, HIV+ pre-ART, and HIV+

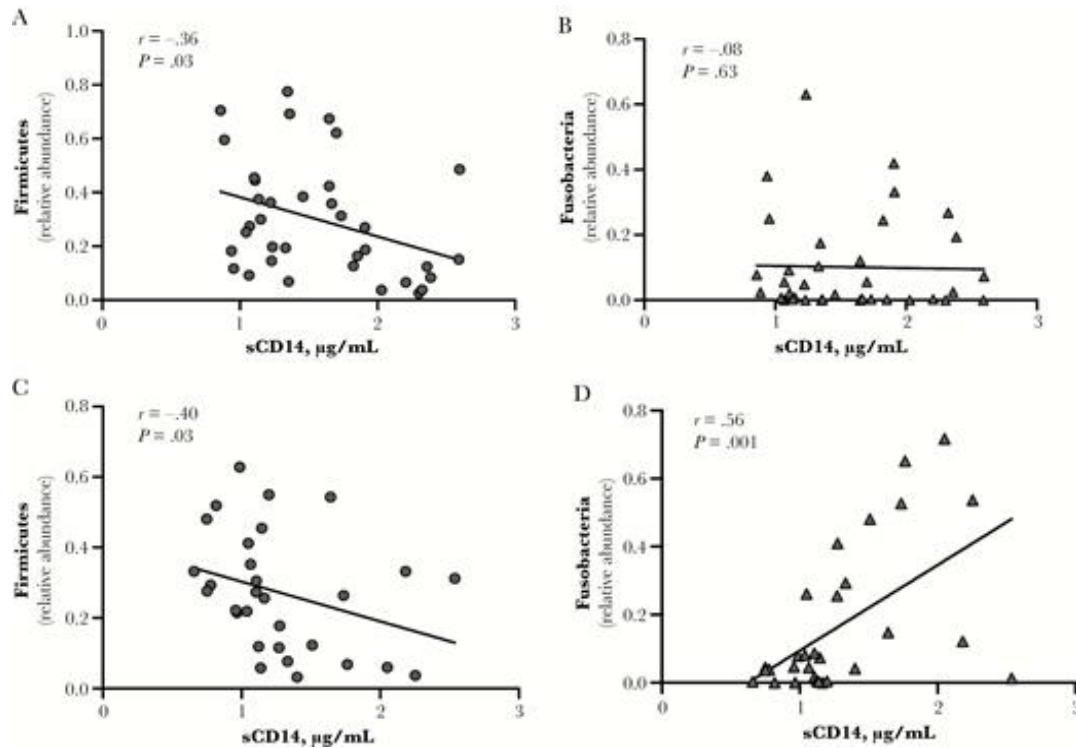
post-ART (permutation test: HIV- vs HIV+ pre-ART, $P = .6$; HIV- vs HIV+ post-ART, $P = .4$; HIV+ pre-ART vs HIV+ post-ART, $P = .3$). C, LDA scores of differentially abundant taxa among HIV+ pre-ART at F1-2 and HIV+ pre-ART at F3. D, LDA scores of differentially abundant taxa among HIV+ pre-ART at F1-2 and HIV+ post-ART at F1-2. E, LDA scores of differentially abundant taxa among HIV+ pre-ART at F3 and HIV+ post-ART at F3.

To explore if and how the microbiome changes with progression of HIV infection, we compared AHI at the time of diagnosis at Fiebig Stages 1 and 2 ($n = 15$) vs F3 ($n = 17$). Linear discriminant analysis scores of differentially abundant taxa in AHI individuals at F1-2 vs F3 pre-ART showed a higher representation of pro-inflammatory bacterial taxa, such as Succinivibrionaceae and Prevotellaceae, in F3 (Figure 2C). Comparison of F1-2 pre-ART ($n = 15$) vs F1-2 post-ART ($n = 13$) (Figure 2D), and F3 pre-ART ($n = 17$) vs F3 post-ART ($n = 16$) (Figure 2E), showed an increase in Proteobacteria members post-ART, indicating a continuous change in the microbiome under ART. Further analysis was then performed on paired samples, AHI pre-ART ($n = 16$) vs AHI post-ART ($n = 16$), to evaluate longitudinally potential microbiota-associated changes within these subjects. As assessed by Wilcoxon signed rank test, there was a significant decrease in Bacteroidetes ($P = .007$) and an increase in Fusobacteria frequencies ($P = .01$) in AHI post-ART compared with pre-ART. Proteobacteria and Firmicutes frequencies did not appear to change in AHI post-ART compared with pre-ART ($P = .5$ and $P = .7$, respectively) (Supplementary Figure 2). Overall these data reveal a slight shift in the frequencies of bacterial communities within the 3 groups, which is partially restored after 6 months of ART.

Associations of GI tissue inflammation and inflammatory markers with bacterial taxa

Given that the microbiota of AHI individuals was enriched or depleted in specific bacterial taxa, we assessed potential correlations between the relative abundance of these taxa and markers of tissue or systemic inflammation (sCD14, IL-6, CRP) and intestinal gut epithelial integrity (I-FABP) (Supplemental Table1). The median sCD14 level in AHI individuals at baseline (n=37) was 1.40 $\mu\text{g/mL}$ and decreased to 1.14 $\mu\text{g/mL}$ after six months of ART (n=31). Levels of sCD14 inversely correlated with the relative abundance of Firmicutes both pre- ($r=-0.36$; $p=0.03$) (Figure 3A) and after 6 months of ART ($r=-0.40$; $p=0.03$) (Figure 3C). No association was observed between Fusobacteria and sCD14 at baseline ($r=-0.08$; $p=0.63$) (Figure 3B), however, after 6 months of treatment their relative abundance increased and positively correlated with sCD14 levels ($r=0.55$; $p=0.001$) (Figure 3D). These data suggest that a potential relationship between altered gut bacterial communities and systemic inflammation is established during the acute phase of HIV-infection and may persist during ART.

Figure 3.



Bacterial taxa frequencies and inflammatory markers. A, Relative abundance of Firmicutes in HIV+ pre-antiretroviral therapy (ART) negatively correlated with sCD14 ($r = -.36$; $P = .03$). B, Relative abundance of Fusobacteria in HIV+ pre-ART do not correlate with sCD14 ($r = -.08$; $P = .63$). C, Relative abundance of Firmicutes in HIV+ post-ART negatively correlated with sCD14 ($r = -.40$; $P = .03$). D, Relative abundance of Fusobacteria in HIV+ post-ART positively correlated with sCD14 ($r = .56$; $P = .001$).

It has been previously demonstrated that a local tissue response consisting of infiltration of polymorphonuclear neutrophils (PMNs) in the lamina propria (LP) of the GI tract is a surrogate marker for epithelial damage and local microbial translocation [26]. Using immunohistochemistry and quantitative image analysis, we assessed the extent of PMN infiltration by measuring myeloperoxidase+ (MPO+) within the LP in AHI pre-ART and AHI post-ART. Cross sectional analysis revealed no significant changes in PMN infiltration in AHI pre- (n=23) compared to post-ART (n=14) (Mann Whitney test, $p=0.4$). Paired analysis of pre- versus post-ART (n=8) also

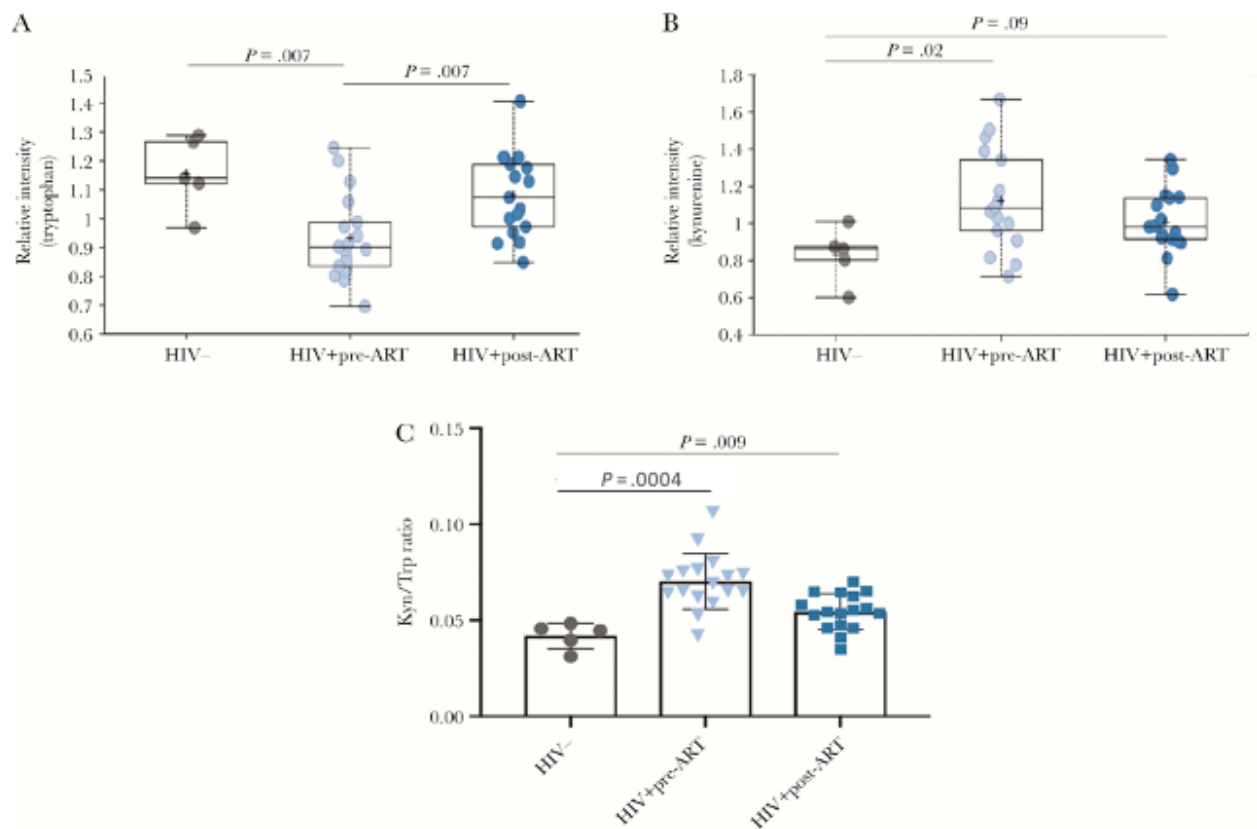
showed no significant changes in PMN infiltration (Wilcoxon rank test, $p=0.5$) as reported in Supplemental Figures 3A and 3B.

Metabolomics analysis

The plasma metabolic profile was assessed in a subset of 17 AHI participants, who had paired samples at the time of diagnosis and after 6 months of ART, and in 5 HIV-uninfected controls. PCoA revealed clustering of HIV-uninfected controls and HIV+ pre- and post-ART which was verified by Pairwise Adonis Euclidean matrices (HIV- vs HIV+ pre-ART, $p=0.01$; HIV- vs HIV+ post-ART, $p=0.04$; HIV+ pre-ART vs HIV+ post-ART, $p=0.06$) (Supplemental Figure 4). Paired *t*-test and Welch's two-sample *t*-test were used to identify biochemicals that differed significantly between experimental groups. AHI at the time of diagnosis exhibited several alterations in aminoacids metabolism when compared to the HIV-uninfected controls. Among the aminoacids found to be repressed in AHI pre-ART compared to HIV-uninfected controls tryptophan was detected ($p=0.007$, $q=0.004$) (Figure 4A). After 6 months of ART, tryptophan levels increased compared to the baseline ($p=0.007$, $q=0.003$) and no significant difference was observed between AHI post-ART and HIV-uninfected controls ($p=0.28$ $q=0.14$). Furthermore, AHI pre-ART displayed significant higher levels of kynurenine compared to HIV-uninfected controls ($p=0.02$ $q=0.07$) while no significant difference was detected between HIV+ post-ART and HIV-uninfected controls ($p=0.09$ $q=0.06$) (Figure 4B). Kynurenine is a metabolite that can be produced from tryptophan via the enzyme indoleamine 2,3 dioxygenase (IDO) under proinflammatory stimuli [227]. Interestingly, the IDO1 activity measured by the ratio of plasma concentration of the downstream product, kynurenine, to the parent compound, tryptophan, (Kyn:Trp), was significantly higher in AHI pre-ART compared to HIV-uninfected controls ($p=0.0004$) and in AHI

post-ART compared to HIV-uninfected controls ($p=0.009$). The Kyn/Trp ratio did not differ significantly between AHI pre-ART and AHI post-ART ($p=0.1$) (Figure 4C). AHI post-ART showed changes in many metabolites with links to microbial activity including several secondary bile acids and metabolites that can be generated via microbial encoded deconjugation/dehydroxylation reactions in the intestine by gut bacteria (Supplemental Table 2).

Figure 4.



Perturbation in amino acid abundance and utilization in HIV-infected vs HIV-uninfected controls. A, Relative intensity of tryptophan in HIV- vs HIV+ pre-antiretroviral therapy (ART; $P = .007$; $q = .04$) and HIV+ pre- vs post-ART ($P = .007$; $q = .03$). B, Relative intensity of kynurenine in HIV- vs HIV+ pre-ART ($P = .023$; $q = .07$) and in HIV- vs HIV+ post-ART ($P = .09$; $q = .06$). C, Kyn/Trp ratio significantly differs between HIV- and HIV+ pre-ART ($P = .0004$) and between HIV- and HIV+ post-ART ($P = .009$).

Discussion

HIV-infection is associated with an altered composition of the fecal microbiota, yet questions remain about how quickly bacterial dysbiosis is established and how antiretroviral treatment can further shape the gut microbial communities. Several recent studies have investigated the composition of gut microbiota in HIV-infected individuals and its potential role in the pathogenesis of the infection. The majority of these studies, however, were cross sectional and focused on chronically HIV-infected individuals, and controls were not always well matched for confounding variables [50, 51, 53, 55, 57, 62]. Interestingly, longitudinal studies in simian immunodeficiency virus (SIV) non-human primate (NHP) model have shown that dysbiosis is not consistently observed [12]. Furthermore, experimentally induced intestinal dysbiosis in SIV-infected macaques did not accelerate disease progression [180].

In this study, we reported differences in the composition of the gut microbiota from a longitudinal study of Thai MSM with AHI at the time of HIV diagnosis (F1-5), and after 6 months of ART with ethnicity- age- and sex-matched HIV-uninfected served as controls. Our data show a dysbiotic gut environment establishing at the earliest acute phase of HIV-infection. Many studies have shown an increase in the proportion of Proteobacteria phylum, in particular Enterobacteriaceae members, in treated and untreated chronically HIV-infected individuals compared to HIV-uninfected controls [50, 51, 53, 55, 62, 125, 127]. Interestingly, we observed a slightly higher abundance of Proteobacteria members in HIV-uninfected controls compared to AHI pre-ART which could be attributed to the fact we studied MSM with AHI with age- and sex-matched HIV-uninfected controls and that Thai individuals may have distinct genetics or dietary habits, however, the small sample size precludes definitive conclusions.

After 6 months of ART, we observed an increase in the relative abundance of Proteobacteria phylum, particularly in Enterobacteriaceae, compared to the time of AHI diagnosis, which may suggest a partial restoration of the microbiota. Furthermore, we did not find any significant correlations between Proteobacteria members and markers of systemic inflammation. These data suggest that a modest increase in the relative abundance of Proteobacteria phylum members is not necessarily associated with disease progression.

Consistent with previous observations [36, 55, 57], the relative abundance of Fusobacteria phylum members, which include many potential inflammagenic pathogens, was higher in AHI individuals at the time of diagnosis compared to HIV-uninfected controls. After 6 months of ART these bacterial taxa positively correlated with levels of sCD14, a well established marker of disease progression in AHI which we have recently reported to remain elevated despite early treatment [174, 220, 228]. This may suggest a potential relationship between altered gut bacterial communities and systemic inflammation. Gut tissue inflammation measured by MPO did not change significantly after 6 months of ART consistent with persistence of local tissue inflammation.

In addition, we investigated the composition of the gut microbiota through stages F1-3 of acute infection, which we previously found to be linked to a progressive loss of CD4, in particular Th17, that occurred predominantly in stage F3 [175]. Our findings suggested an enrichment in pro-inflammatory bacteria during the transition from F1 to F3, that seems to correspond with the progression of disease, however, our small sample size precludes definitive conclusions.

Evaluation of plasma metabolites revealed significant changes in the secondary bile acids linked with microbial activity among AHI pre- and post-ART and HIV-uninfected controls. Bile acids

are crucial metabolites in the gastrointestinal tract and contribute to maintaining intestinal immune homeostasis through cross-talk with the gut bacterial residents. The conversion of bile acids by the gut microbiome is identified as a factor impacting both host metabolism and immune responses [229].

The IDO1, the first enzyme of the kynurenine pathway, was significantly higher in AHI pre-ART compared to HIV-uninfected controls and AHI post-ART. Previous studies in chronic treated HIV-infected individuals have shown that dysbiosis characterized by an enrichment in Proteobacteria and a depletion in Bacteroidia correlated with activity of the kynurenine pathway of tryptophan metabolism [51]. We did not observe significant correlations between specific gut bacterial taxa and these metabolites, however, further analysis would be critical to better elucidate the linkage between gut dysbiosis with secondary bile acids abundance as well as with activity of the kynurenine pathway of tryptophan metabolism during HIV-infection.

It is important to note that our study had several limitations including the small sample size and the lack of HIV-controls receiving antiretrovirals, people on Pre-exposure prophylaxis (PrEP), to better decipher the role of medications in microbiome alterations. Metabolomics analysis was conducted only in the plasma and not in the gut. Despite these limitations, our study provided a unique view of microbiome composition during the earliest phases of acute infection in a highly homogeneous and well defined group of participants, with longitudinal sampling.

In summary, in this study of microbiome in early acute HIV infection we found evidence of dysbiosis which only partially restored after 6 months of ART. Overall dysbiosis was characterized by more subtle rather than profound individual changes in the bacterial structure of the gut microbiota but significant correlations between pathogenic taxa and markers of HIV-disease

progression were evident. These findings warrant further investigation on the potential role of microbiota alterations in HIV-associated inflammation as well as the potential effects of specific drugs used to treat HIV.

Data Availability

Raw reads were deposited into the NCBI Sequence Read Archive (SRA) database under the BioProject ID number PRJNA510435.

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Potential conflicts of interest. J.A. has received honoraria for participating in advisory meetings for ViiV Healthcare, Merck, AbbVie, Gilead, and Roche. The authors declare that they have no competing interests. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Author contributions. I.S., J.B., J.A., J.B. designed the study. O.S., J.D., B.I., performed experiments. O.S., A.M.O., M.Q., H.M., S.P., B.I., C.D. performed data analysis. N.P., N.C., A.S. performed samples and data collection. O.S., I.S. wrote a draft of the manuscript that was reviewed and enriched by contributions from all authors.

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Chapter 6

IL-7 treatment supports CD8+ MAIT cell restoration in HIV-1 infected patients on ART

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Summary

Chronic HIV-1 infection is associated with lower frequencies and functional impairment of mucosa-associated invariant T (MAIT) cells. We evaluated IL-7 treatment to restore MAIT cells in peripheral blood of chronically HIV-1 infected individuals on ART. IL-7 led to increased relative and absolute levels of MAIT cells and this expansion occurred primarily in the CD8⁺ subset. These results suggest that IL-7 may represent a therapeutic intervention for the restoration of MAIT cells in chronic HIV-1 infection.

Mucosa-associated Invariant T (MAIT) cells represent a subset of unconventional, innate-like T cells that are abundant in healthy individuals, constituting 1 to 10% of peripheral blood T cells ^[1] and are present in mucosa, liver, and mesenteric lymph nodes. ^[2] MAIT cells are characterized by the expression of a semi-invariant V α 7.2-carrying T cell receptor (TCR), IL-18R α , the promyelocytic leukemia zinc finger protein (PLZF), ^[1] and high levels of CD161 and the alpha chain of the IL-7 receptor (IL-7R). ^[2]

The majority of MAIT cells are either CD8 $\alpha\alpha$ or CD8 $\alpha\beta$, with smaller CD4/8 double-negative (DN) and CD4⁺ populations. ^[1] MAIT cells recognize microbial vitamin B₂ biosynthetic intermediates presented by the nonclassical MHC-1-related protein 1 (MR1), ^[3] and respond to such antigens with production of proinflammatory cytokines ^[1] and cellular cytotoxicity ^[2, 3]. During chronic HIV-1 infection, there is numerical and functional loss of MAIT cells that persists despite long-term antiretroviral therapy (ART). ^[2, 4] Approaches to restore MAIT cells may improve mucosal and anti-bacterial immunity in HIV-infected people.

IL-7 is required for the development and homeostasis of T cells.^[5] In HIV-infected patients, IL-7 administration induces T cell expansions in peripheral blood and gut mucosa.^[6] Notably, IL-7 dramatically enhances the function of MAIT cells from both healthy individuals and HIV-infected patients *in vitro*.^[2] We hypothesized that *in vivo* administration of IL-7 in chronically HIV-1 infected patients on ART may overcome the inability of long-term ART alone to restore MAIT cells. To address this hypothesis, we evaluated the numbers, phenotype, and functionality of MAIT cells before and after IL-7 administration.

HIV-1 infected patients with CD4 counts of 101-400 cells/ μ L, plasma HIV RNA < 50 copies/mL who were on ART for at least 12 months were enrolled in a single arm, open-label, multicenter study (NCT01190111) approved by the study sites Ethics committees. HIV-uninfected individuals (n=7) were recruited at the blood bank of the NIH. All study participants signed informed consent. Patients were treated with three subcutaneous injections of IL-7 (CYT107) at a dose of 10 mg/kg each. PBMCs were collected at baseline, before IL-7 administration, and at week 12 post-IL-7. The details of the clinical trial have been previously published.^[7, 8] Seven HIV-infected men with a median age of 55 years (range 43-58 years), median CD4 T-lymphocyte count of 270 cells/ μ L (range 135-364 cells/ μ L) and plasma HIV RNA < 50 copies/mL were included in this study based on sample availability. Cryopreserved PBMCs were used for immunophenotyping. The functional assays for activation of MAIT cells in the PBMC mixture with IL-12 and IL-18 or *E. coli* (ratio *E. coli* CFU:PBMC of 3) were performed for 24 hours as previously described.^[2] The antibodies used were: anti-CD4 APC-Cy7, anti-CD3 AmCyan and Alexa Fluor (AF) 700, anti-CD8 Pacific Blue and Brilliant Violet (BV) 711, anti-CD161 PECy5, anti-TNF BV650 from BD Biosciences; anti-TCRV α 7.2 PerCPCy5.5 and PECy7, anti-CD4 BV711, anti-CD8 BV570, anti-CD161 BV605, anti-GzmB FITC, anti-IFN γ BV785, and anti-perforin (Prf) BV421 from Biolegend; anti-CD69

PE-Texas Red, LIVE/DEAD Fixable Blue Dead Cells Stain (Thermo Fisher). Stained cells were fixed in Cytotfix/Cytoperm or in Transcription Factor Fixation/Permeabilization buffer (BD Biosciences and eBioscience, respectively).

Samples were acquired on an LSRII flow cytometer (BD Biosciences). Software-based compensation was performed using the compensation platform in FlowJo software versions 9.9 and 10.0.8 (TreeStar).

The Wilcoxon signed-rank test was used to assess significance. Statistical analyses were performed using Prism version 7.0a, and two-sided p-values < 0.05 were considered significant.

The frequency of MAIT cells was higher in HIV-negative controls compared to the chronically HIV-infected subjects (median (IQR) = 1% (0.87-5.12) vs. 0.5% (0.2-1.2); $p = 0.07$), consistent with previous observations^[4], however the difference did not reach statistical significance. We evaluated the percentage and absolute number of MAIT cells from baseline to week 12 post-IL-7 treatment (Fig. 1A). The percentage of MAIT cells among total lymphocytes increased significantly (median (IQR) = 1.2% (0.1-1.5) vs. 0.3% (0.1-0.7), $p = 0.01$) (Fig. 1B). Since IL-7 expands overall T cells in HIV-infected persons^[9], we examined changes in the percentage of MAIT cells within the T cell compartment. The proportion of MAIT cells within the CD3⁺ cell population trended towards an increase, although the change did not reach statistical significance (median (IQR) = 1.3% (0.27-2.12) vs. 0.5% (0.2-1.2), $p = 0.1$) (Fig. 1C). However, the absolute number of MAIT cells increased significantly at 12 weeks post-IL-7 administration compared to baseline (median (IQR) 22 (6-75.9) cells/ μ L vs. 5.2 (4.2-12.9) cells/ μ L, $p = 0.01$) (Fig. 1D). Furthermore, the absolute number of CD8⁺ MAIT cells increased after IL-7 treatment (median (IQR) 20.5 (4-33.5) cells/ μ L vs. 4 (2-11), $p = 0.01$) (Fig. 1E).

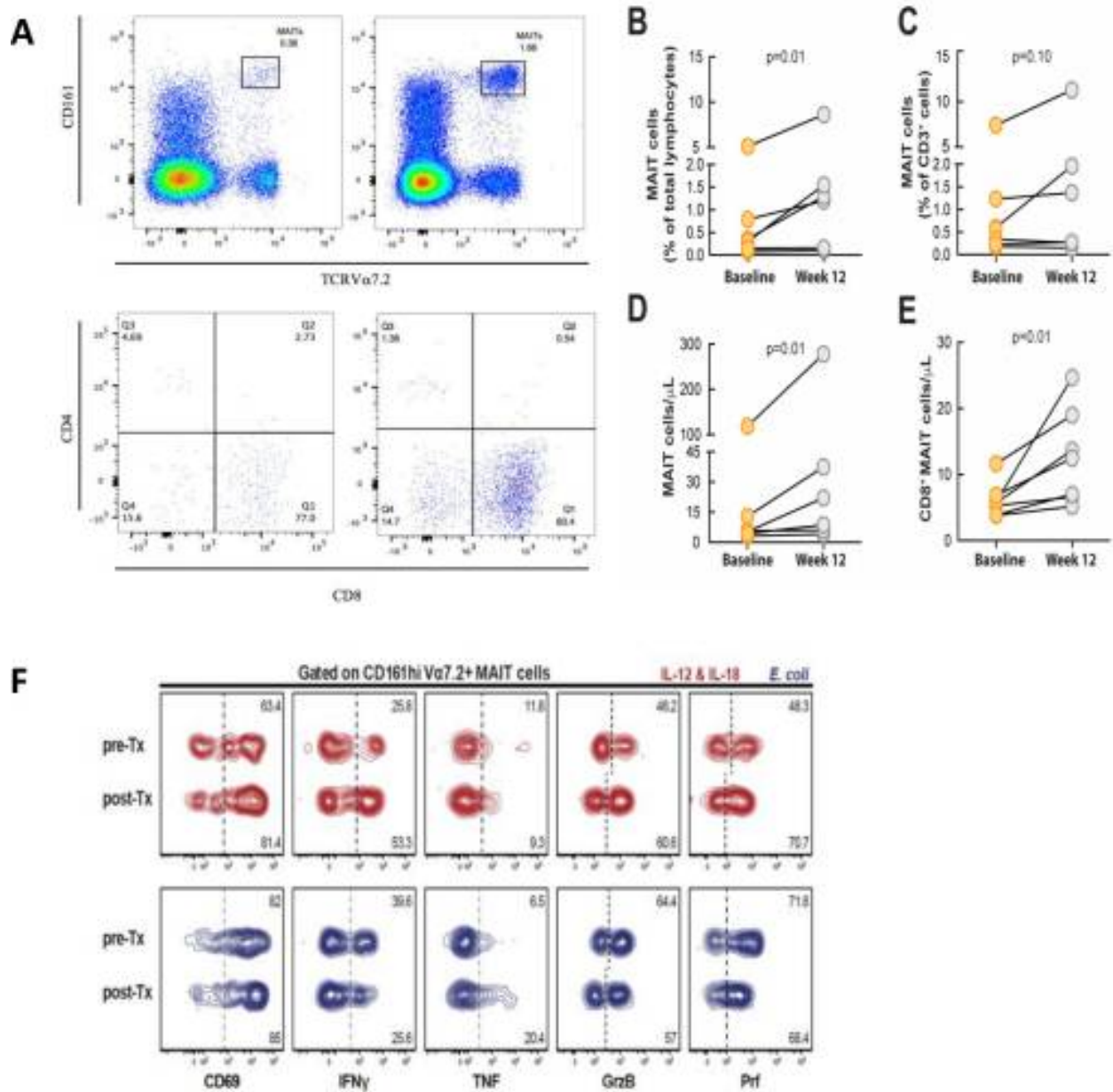


Figure 1

MAIT cell characterization and changes in the MAIT cell population after treatment with IL-7

(A) MAIT cells identified by Va7.2 and CD161 staining and flow cytometry. Expression of CD8 and CD4 in MAIT cells. (B) Percentage of MAIT cells among total lymphocytes increased ($p = 0.01$) after treatment with IL-7. (C) There was no significant increase in the percentage of MAIT cells within the total CD3+ cells. (D) The absolute number of

MAIT cells increased significantly after IL-7 treatment ($p = 0.01$). (E) There was an increase in the absolute number of CD8⁺ MAIT cells after IL-7 treatment. (F) Function of MAIT cells in a HIV-1 infected patient before and after rhIL-7 treatment after stimulation with *E. coli*.

It was previously reported that MAIT cells have reduced expression of transcription factors T-bet and EOMES in chronically HIV-infected individuals, and this was associated with loss of MAIT cells in the periphery and impaired effector function.^[2] In the same study, addition of IL-7 to MAIT cells *in vitro* enhanced their effector responses to bacterial stimulation.^[2] Due to limited sample availability, we were only able to test responses to *E. coli* stimulation in one patient, and MAIT cells in this patient maintained responsiveness and effector function after IL-7 treatment (Fig. 1F). Although we did not study MAIT cells in mucosal samples, this will be of interest for future studies to evaluate the potential significance of increased MAIT cells in mucosal immunity.

In conclusion, we found a significant increase in the frequency and number of MAIT cells in the periphery of chronically HIV-infected individuals 12 weeks post-IL-7 treatment. This is striking given that no significant restoration of MAIT cells has been seen during long-term ART.^[4] IL-7 treatment thus represents a potential therapeutic strategy to reconstitute the number of MAIT cells in chronically HIV-infected individuals. Rescue of these cells may improve immune reconstitution in HIV-infected individuals and could potentially result in a reduced risk of microbial co-infections. Future larger studies could better determine if this immunotherapy can restore both the frequency and functionality of MAIT cells in disease settings.

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Footnotes

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Chapter 7

Preserved MAIT cell numbers and function in idiopathic CD4 lymphocytopenia

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Abstract

Mucosal-associated invariant T (MAIT) cells are a conserved antimicrobial MR1-restricted T-cell subset involved in infectious and non-infectious diseases as well as in tissue repair. Chronic infectious, inflammatory or immunodeficiency diseases are often associated with persistent decline and dysfunction of the MAIT cell compartment. Herein, we investigated the MAIT cells in patients with idiopathic CD4⁺ lymphocytopenia (ICL), a syndrome characterized by consistently low CD4 T-cell counts (<300 cell/ μ L) in the absence of HIV infection or other known immunodeficiency, and by susceptibility to certain opportunistic infections. The numbers, phenotype and function of MAIT cells were preserved in ICL patients compared to healthy controls. Administration of IL-7 expanded the CD8⁺ MAIT cell subset, with maintained responsiveness and effector functions post IL-7 treatment. In summary, ICL patients maintain normal levels and function of MAIT cells that may help defend the ICL host from some bacterial infections despite the deficiency in CD4⁺ T cells.

Introduction

Mucosa-associated invariant T (MAIT) cells are unconventional innate-like T cells abundant in mucosal tissues, peripheral blood and liver of healthy individuals [23, 230, 231]. In humans, MAIT cells are predominantly CD8 $\alpha\alpha$ or CD8 $\alpha\beta$, with some CD4/8 double-negative (DN) MAIT cells and a minor CD4⁺ population [23, 232]. MAIT cells display several surface molecules including a semi-invariant V α 7.2-carrying T cell receptor (TCR), IL-18R α , high levels of CD161, and the alpha chain of the IL-7 receptor (IL-7R) [230, 233]. MAIT cells exhibit a transcriptional profile marked by the expression of promyelocytic leukemia zinc finger (PLZF) [234, 235], retinoid-related orphan receptor (ROR) γ t, eomesodermin (Eomes) and T box transcription factor 21 (Tbet) [234, 236-238].

MAIT cells recognize microbial riboflavin-related metabolites, presented by the nonclassical MHC class I-related protein 1 (MR1) [239]. MAIT cells are defined using combinations of markers, most commonly as V α 7.2⁺CD161^{hi} T cells. More recently, the development of ligand-loaded MR1 tetramers has allowed the identification of MAIT cells based on T-cell receptor specificity [240]. Upon activation, MAIT cells release proinflammatory cytokines and mediate cytolytic function against bacterially infected cells [23, 236, 238, 241]. Furthermore, high expression of receptors for IL-18 and IL-12 allow MAIT cells to respond to cytokine signals independent of their TCR [242, 243]. Thus, MAIT cells play important roles in infectious diseases, but also in non-infectious diseases, including autoimmune diseases and cancer [244]. Despite the heterogeneity of the etiology of these conditions, some of them share common features including reduced MAIT cell frequency in the blood, increased presence in the target tissues, and increased

activation and dysfunction. The loss of MAIT cells in the blood of people with HIV (PWH) seems irreversible after long-term ART treatment, which may contribute to the increased susceptibility to co-infections that occur in these individuals [22, 230, 245] .

Similar to HIV-infection, idiopathic CD4⁺ lymphocytopenia (ICL) is characterized by low CD4⁺ T cell counts (< 300 cells/ μ L), in the absence of HIV infection or any other infectious cause of immunodeficiency [246, 247]. The potential mechanisms leading to reduced circulating CD4⁺ T cells include decreased bone marrow clonogenic capability with diminished stem cell precursors, disturbed thymic T-cell maturation and increased CD4⁺ T cell activation and cycling. In addition, lymphopenia in the rectosigmoid mucosa has also been observed in ICL patients [18]. Despite this lymphopenia and in contrast to HIV infection, there is no significant systemic inflammation in ICL patients and the spectra of opportunistic infections is also different compared to PWH. People with ICL have an increased susceptibility to opportunistic infections such as cryptococcal, human papilloma virus (HPV), and mycobacterial infections, as well as autoimmune diseases such as systemic lupus erythematosus (SLE), Sjögren's syndrome, and sarcoidosis [247]. Infectious complications of ICL are managed and prevented with antimicrobials based on guidelines for HIV/AIDS patients, however, no proven therapies exist for ICL immunodeficiency.

Given the breadth of responsiveness to many microbes, MAIT-cell exhaustion and loss could lead to a higher risk of microbial co-infections in ICL patients. Thus, we sought to determine the levels, phenotype and functionality of MAIT cells in people with ICL in an effort to understand better how different T cell subsets may be affected in ICL shaping the disease pathogenesis. IL-7 has

been proposed as a potential immunotherapy in ICL [248] and we have previously shown that *in vivo* administration of IL-7 enhances the levels and function of MAIT cells in PWH [249]. IL-7 is a hematopoietic growth factor, required for T cell development, survival and homeostasis of mature T cells [250, 251]. IL-7 can also enhance the killing capacity of cytotoxic CD8 T lymphocytes [252] and the reactivity of antigen-specific T cells [253]. Thus, here we also assessed changes in size, characteristics and function of the MAIT cell population after IL-7 treatment intervention in patients with ICL.

Methods

Study participants and study design

The study participants were 30 patients referred to the NIH clinical center for ICL and evaluated under protocol 09-I-0102, Etiology, Pathogenesis, and Natural History of idiopathic CD4+ Lymphocytopenia (NCT00867269). The protocol was approved by the National Institute of Allergy and Infectious Diseases (NIAID) Institutional Review Board and all patients signed informed consent. The diagnosis of idiopathic CD4 lymphopenia was confirmed by a total CD4 count of ≤ 300 cells/ μ l, or CD4 T cell percentage lower than 20% of total T cells on at least two occasions at least 6 weeks apart. Negative testing for HIV-1, HIV-2, HTLV-1 and HTLV-2 by ELISA and Western blot and/or viral load testing was required. Patients with other primary or secondary immunodeficiency syndromes, malignancy, and immunosuppressive medications thought to cause lymphopenia were excluded. Healthy controls (HCs) (n=14) were also recruited under separate protocols approved by the institutional review board. We also evaluated samples from six patients enrolled in an open-label phase I/IIA, dose-escalation study of subcutaneous rhIL-7, of whom two received 3 μ /Kg and four received 10 μ /Kg weekly for three consecutive weeks and were samples at 12 weeks.

The study was approved by the Institutional Review Board of the NIAID, and written informed consent was obtained from all participants prior to any study procedures and in accordance with the Declaration of Helsinki.

Flow cytometry

Detailed lymphocyte subset phenotyping and evaluation of MAIT cell responses were performed using cryopreserved peripheral blood mononuclear cells (PBMCs). PBMCs were thawed and stained with LIVE/DEAD Fixable Aqua dead cell marker (DCM). Cell surface staining was performed using directly conjugated antibodies and cells were then fixed in either Cytofix/Cytoperm or Transcription Factor Fixation/Permeabilization buffer (both from BD Biosciences) as appropriate. Intracellular staining was performed using the relevant mAbs in Perm/Wash or Transcription Factor Perm/Wash buffer as appropriate (both from BD Biosciences). The antibodies used are listed in table S1. Samples were acquired on an LSRFortessa flow cytometer (BD Biosciences) equipped with 488 nm, 633 nm, 405 nm and 355 nm lasers. Single-stained polystyrene beads (BD Biosciences) were used for compensation purposes. Software-based compensation was performed using the compensation platform in FlowJo software versions 9.9 and 10.0.8 (BD).

Bacteria

The *Escherichia coli* strain D21 and *Pseudomonas aeruginosa* strain ATCC 27853 were cultured overnight at 37°C with shaking in lysogeny (Luria-Bertani; LB) broth [254]. The bacteria were then stored at -80°C in 50% glycerol/50% fetal calf serum (FCS).

MAIT cell activation assays

The MAIT cell activation assays were performed in whole PBMC cultures as previously described [255]. PBMCs were thawed and either stained with the phenotyping panel or incubated for 24h with *E. coli* (ratio *E. coli*:PBMC of 3:1), *P. aeruginosa*, (ratio *P. aeruginosa*:PBMC of 10:1), or a

combination of IL+12 p70 (10 ng/mL, Peptrotech) and IL-18 (100 ng/mL, Medical & Biological Laboratories). Microbes were mildly fixed in paraformaldehyde for 3 min, and then extensively washed in PBS before feeding to PBMCs. In all stimulations, monensin (Goli Stop, BD Biosciences) and brefeldin (Golgi Plug, BD Biosciences) were added for the last 6h of incubation. In all experiments, 6h stimulation with PMA/ion (Leukocyte Activation Cocktail with Golgi Plug, BD Biosciences) and in the presence of monensin was included as positive control.

Statistical analysis

Statistical analyses were performed using Prism software v.8 (GraphPad). Datasets were first assessed for normality of the data distribution. Statistically significant differences between samples were determined as appropriate using the unpaired t test or Mann–Whitney’s test for unpaired samples, and the paired t test or Wilcoxon’s signed-rank test for paired samples. Correlations were assessed using the Spearman’s rank correlation. Two-sided P values < 0.05 were considered significant.

Results

The median age of study participants was 52 and 50 years for the ICL and HC groups, respectively. The median CD4⁺ T lymphocyte count was 118 cells/μL for the ICL and 917 cells/μL for the HC, and the median CD8⁺ T lymphocyte count was 145 cells/μL and 479 cells/μL for the ICL and HC, respectively (Table 1). Of the 30 ICL patients included in the study, 21 had experienced infectious complications: HPV, Varicella-Zoster virus (VZV), *Cryptococcus neoformans*, *Molluscum contagiosum*, *Histoplasma capsulatum*, *Mycobacterium Avium Complex (MAC)* and progressive multifocal leukoencephalopathy (PML). Nine ICL patients had autoimmune diseases: ulcerative

colitis, vitiligo, immune thrombocytopenic purpura (ITP), sarcoidosis, psoriasis and autoimmune hemolytic anemia (Table 1). Six ICL patients, with a median age of 53 years, who received subcutaneous injections of IL-7 for 12 weeks, were also included in the study (Table 2).

MAIT cell levels are preserved in ICL patients

In order to evaluate the levels of MAIT cells in ICL patients, first we identified MAIT cells using staining with CD3, CD161, V α 7.2, and MR1 5-OP-RU tetramer (Figure 1A). There was no significant difference in the percentage of MAIT cells between ICL and HC subjects irrespective of whether MAIT cells were identified using the combination of V α 7.2 and CD161 expression, or by MR1 5-OP-RU staining (Figure 1B-C). Some ICL patients had very high MAIT cell percentages, however, the absolute count of MAIT cells was not different between ICL and HC subjects, (Figure 1D), suggesting that high MAIT cell percentages probably reflected low levels of conventional T cell subsets. Notably, the levels of MAIT cells determined by CD161 and V α 7.2, and by MR1-tetramer were similar (Figure 1A-B). Therefore, we decided to use MR1-tetramer for the identification of MAIT cells throughout the study. Next, we determined MAIT cell expression of the differentiation markers CD4 and CD8. In contrast to the pattern in the overall T cell compartment in ICL patients, there was no difference in the size of CD4⁺, CD8⁺ and CD4⁻CD8⁻ MAIT cell subsets between ICL and HCs (Figure 1E-F-G). Conventional T cell populations in peripheral blood of study participants were lower in percentage and number of CD3⁺ (p=0.0003, p<0.0001, respectively), CD4⁺ (p=0.001, p<0.0001, respectively), and CD8⁺ T cells (p=0.6, p=0.0009, respectively) in ICL compared to HC (Supplemental Figure 1), as previously reported. In order to assess any potential relationship between conventional T cells and MAIT cells we applied Spearman's rho non-parametric test, which did not show any significant correlation

between levels of lymphopenia and MAIT cells. These data suggest that MAIT cells levels are preserved in ICL subjects and levels are independent of changes in the levels of conventional T-cell populations.

Expression of activation markers by MAIT cells is unchanged in ICL patients

We next assessed the phenotype of MAIT cells in peripheral blood of ICL subjects. In contrast to the activation and exhaustion of MAIT cells in several infectious and non-infectious immunodeficiency diseases [230, 256], there was no detectable difference in the frequency of MAIT cells expressing CD25, CD38, HLA-DR and CD57 between ICL and HC subjects (Figure 2Ai-ii-iii-iv). CD56 marks a subset of MAIT cells with enhanced innate-like characteristics [232, 257] and we therefore investigated the expression of CD56 in this cohort. ICL patients tended to have higher representation of CD56⁺ MAIT cells compared to HC subjects, but this trend did not reach significance (Figure 2Aiv). Interestingly, expression of CD127 (IL7R α) on circulating MAIT cells was maintained in ICL patients compared to HCs (Figure 2Avi), unlike the lower expression of IL7R α by conventional T cells in ICL subjects [247, 258].

We next examined the transcription factor (TF) profile of MAIT cells in ICL. MAIT cells have a distinct TF profile, which is linked to MAIT cell antimicrobial effector functions, when compared to conventional CD4⁺ and CD8⁺ T cells [230]. ICL patients again had similar expression of PLZF, EOMES and T-bet compared to HCs (Supplemental Figure 2). In line with this, the expression of cytolytic proteins granzyme B (GrzB) and pore-forming protein perforin (Prf) in MAIT cells did not differ between ICL and HCs (Figure 2Bi-ii), neither did the expression of Ki-67 (Figure 2Biii). In addition, we evaluated the expression of exhaustion and inhibitory

markers including LAG-3, TIM-3 and PD-1, which did not reveal appreciable differences between ICL and HC participants (Figure 2Ci-ii-iii). Taken together, our data indicate that, as a group, ICL patients' MAIT cells are phenotypically similar to those of HCs, although there are some ICL subjects with altered MAIT cell phenotypes.

MAIT-cell responses to *E. coli*, *P. aeruginosa* and IL-12/IL-18 in ICL patients

We next investigated whether MAIT cell activation and cytokine production after stimulation with bacteria or cytokines is affected in ICL patients. We stimulated PBMCs from study participants (ICL n=30, HC n=8) with mildly fixed *E. coli*, and measured MAIT cell activation and cytokine production by flow cytometry. Overall the response patterns were similar in ICL and HC subjects, although proportions of MAIT cells that were CD69⁺IFN γ ⁺, CD69⁺TNF⁺, GrzB⁺ and the geometric MFI of the Prf within MAIT cells trended higher in ICL compared to HC subjects (Figure 3Ai-ii-iii-iv). *Pseudomonas aeruginosa* infection is known to be one of the leading causes of sepsis in patients with impaired immunity [259]. We stimulated PBMCs, from ICL (n=27) and HC (n=4) based on sample availability, with *P. aeruginosa* and again found similar responses in ICL patients and HCs (Figure 3Bi-ii-iii-iv). Moreover, we stimulated PBMCs from ICL (n=30) and HCs (n=8) with IL-18 and IL-12. Similar to bacterial stimulation, MAIT activation and cytokine production were comparable between ICL patients and HCs (Figure 3Ci-ii-iii-iv). Taken together, these data indicate that the function of MAIT cells is largely preserved in ICL.

Changes in MAIT cell levels and phenotype after treatment with IL-7

We have previously shown an increase in the number of MAIT cells in PWH after a single dose of rhIL-7 treatment [249]. Here, we assessed whether IL-7 could enhance MAIT cell levels and antimicrobial effector functions in ICL patients (n=6) who received IL-7 treatment [(n=4) 10 μ /Kg and (n=2) 3 μ /Kg]. The frequency of MAIT cells was unchanged after IL-7 treatment (Figure 4A), whereas the absolute number of MAIT cells increased significantly (p=0.03) (Figure 4B). We then evaluated numerical changes of CD8⁺, CD4⁺, and CD8⁻CD4⁻ MAIT cell subsets and found a significant expansion of CD8⁺ MAIT cells (p=0.03) (Figure 4C). On the other hand, the number of CD4⁻CD8⁻ and CD4⁺ MAIT cells was not significantly different between baseline and week 12 after IL-7 (Figure 4D, E). Interestingly, MAIT cells exhibited a significant decrease in the expression of CD56 (P=0.03) (Figure 5A) and CD57 (p=0.03) (Figure 5B) post-IL-7, while CD127 expression significantly increased (p=0.03) (Figure 5C). In line with the decreased expression of CD56 and CD57 in MAIT cells, we observed a significant decrease in Prf (p=0.03) (Figure 5D), while there were no appreciable changes in GrzB (Figure 5E), or Ki67 expression (Figure 5F) post IL-7 treatment. We next examined the effect of IL-7 treatment on MAIT cell effector responses following overnight stimulation with fixed *E. coli*, *P. aeruginosa* or IL-12⁺IL-18. MAIT cells from ICL patients post-IL-7 treatment maintained responsiveness and effector function (Supplemental Figure 3).

To assess whether changes in MAIT cells were linked with similar changes in conventional T cells, we examined T cell levels and phenotype in peripheral blood of ICL subjects before and after IL-7 treatment. In this small group of patients there were no significant changes in the frequency and number of conventional CD4⁺ T cells (Supplemental Figure 4A-B) and CD8⁺ T cells

(Supplemental Figure 4C-D) after IL-7 treatment. Likewise, the frequency of CD56⁺ CD8⁺ T cells as well as CD57⁺ CD8⁺ T cells and the expression of IL-7R were unchanged after IL-7 treatment (Supplemental Figure 5A-B-C). Further, the cytotoxic effector function (Supplemental Figure 5D-E) and Ki67 expression of T cells (Supplemental Figure 5F) did not change significantly after IL-7 treatment. This pattern supports the notion that IL-7 treatment has a strongly enforcing activity specifically on CD8⁺ MAIT cells.

Tables and Figures

Table 1. Clinical and immunological characteristics of study participants

	ICL (n=30)	Healthy Controls (n=14)
Median age (years) (IQR)	52 (30-76)	50 (24-69)
Sex	9 Female	6 Female
	21 Male	8 Male
Infectious complications*	20	-
Autoimmunity**	8	-
Median CD3 cells/μL (IQR)	336 (151-474)	1424 (1359-1534)
Median CD4⁺ T cells/μL (IQR)	118 (27-207)	917 (709-950)
Median CD8⁺ T cells/μL (IQR)	145 (57-748)	479 (415-768)

* Human papillomavirus, Varicella-zoster virus, Cryptococcus, Molluscum, Histoplasma, Mycobacterium Avium Complex, Progressive multifocal leukoencephalopathy

** Ulcerative colitis, Vitiligo, Immune thrombocytopenic purpura, Sarcoidosis, Psoriasis, Autoimmune Hemolytic anemia

Table 2. Clinical and immunological characteristics of ICL patients pre- and post- IL-7 treatment

	ICL pre-IL-7 (n=6)	ICL post-IL-7 (n=6)
Median age (years) (IQR)	53 (23-57)	54 (24-58)
Sex		5 Females 1 Male
Median CD4⁺ T cells/μl (IQR)	22 (10-233)	27 (11- 438)
Median CD8⁺ T cells/μl (IQR)	54 (10- 1311)	62 (16-1363)

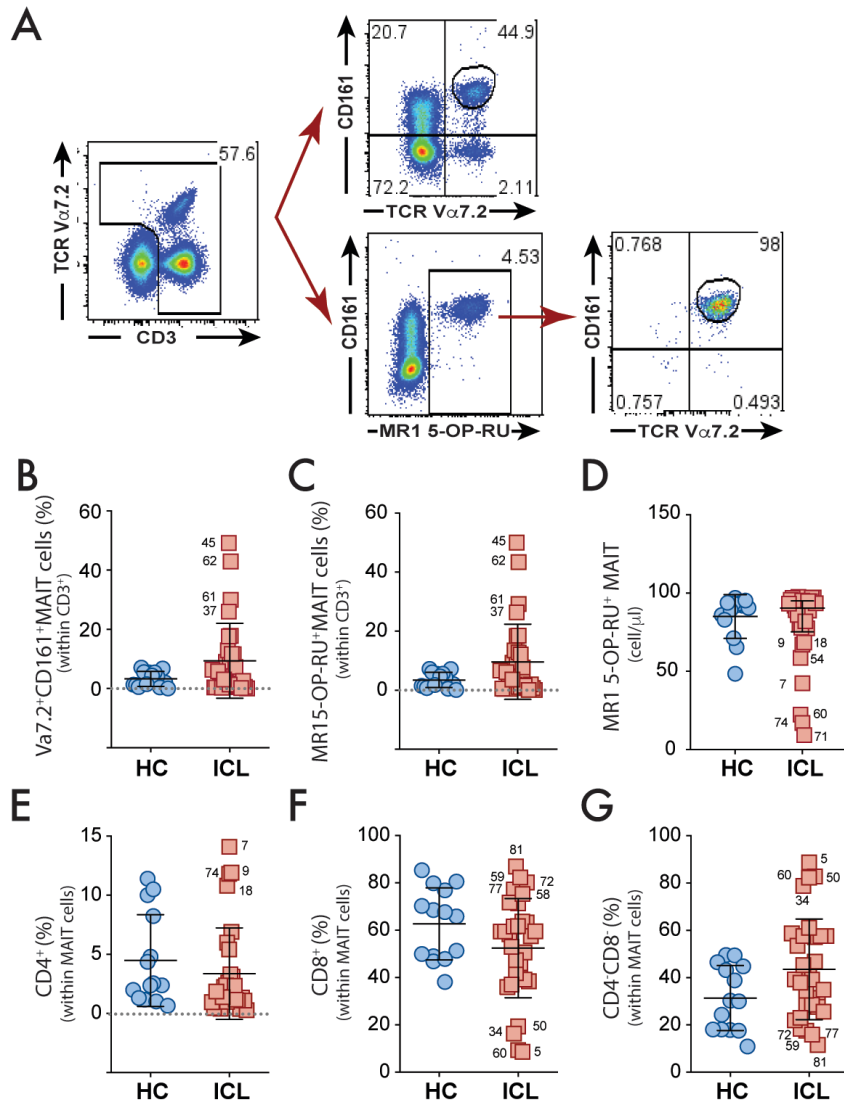


Figure 1. Levels of MAIT cells are not different in peripheral blood of ICL patients compared to HCs at steady state. (A) Flow cytometry gating strategy to identify total MAIT cells in peripheral blood of study participants. (B-D) Plots show frequency of total MAIT cells identified by CD161 and Va7.2 markers (B) or MR1 tetramer (C), and total number of MAIT cells (D) in peripheral blood of tested participants. (E-G) Frequency of MAIT sub-population namely CD4⁺ (E) CD8⁺ (F), and CD4⁺CD8⁻ (G) in peripheral blood of study participants. Scatter plots show median \pm interquartile range; n=30 ICL, n=14 HCs. Data tested by Mann-Whitney U-tests to detect differences across multiple samples. Legend: HCs healthy controls; ICL Idiopathic CD4⁺ lymphocytopenia; numbers on the plots indicate ID of patients, lying outside the IQR.

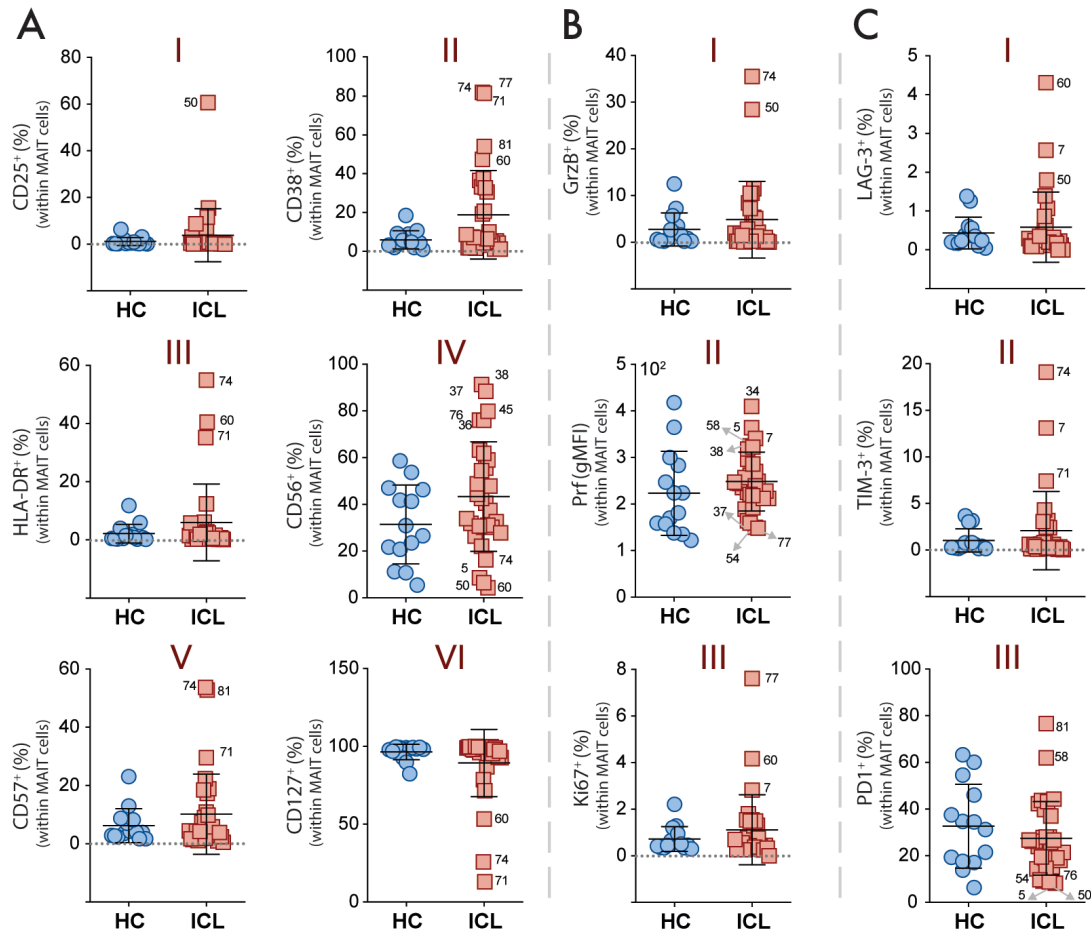


Figure 2. Immunophenotype of MAIT cells in peripheral blood of ICL patients and HCs at steady state. (AI-VI) Expression of activation markers on MAIT cells. (BI-III) Expression of inhibitory markers on MAIT cells. (CI-III) Expression of markers indicating cytotoxic capacity and proliferation. Scatter plots show median \pm interquartile range; n=30 ICL, n=14 HCs. Data tested by Mann-Whitney U-tests to detect differences across multiple samples. Legend: HCs healthy controls; ICL Idiopathic CD4⁺ lymphocytopenia; lymphocyte-activation gene 3, LAG-3; granzyme B, GrzB; perforin, Prf; programmed cell death-1, *PD-1*; T-cell immunoglobulin mucin-3, TIM-3; numbers on the plots indicate ID of patients, lying outside the IQR.

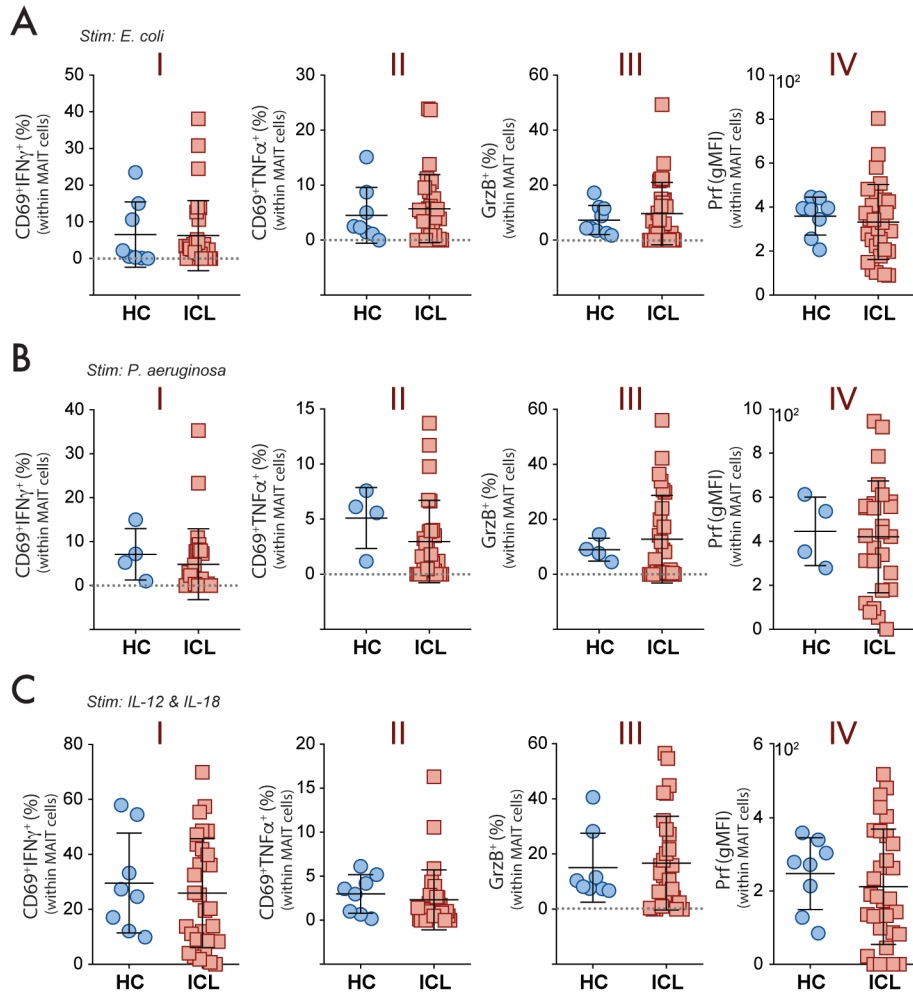


Figure 3. Function of MAIT cells tested in PBMCs from peripheral blood of ICL patients and HCs. (A-C) MAIT cell expression of CD69⁺IFN γ (I), TNF (II), GrzB (III) and Prf (IV) in PBMCs stimulated with *E.coli* (A), *P. aeruginosa* (B) or IL-12 combined with IL-18 for 24h. Scatter plots show median \pm interquartile range; n=30 ICL, n=14 HCs. Data tested by Mann-Whitney U-tests to detect differences across multiple samples. Legend: HCs, healthy controls; ICL, Idiopathic CD4⁺ lymphocytopenia; granzyme B, GrzB; interferon gamma, IFN γ ; perforin, Prf; tumor necrosis factor (TNF)-alpha, TNF α .

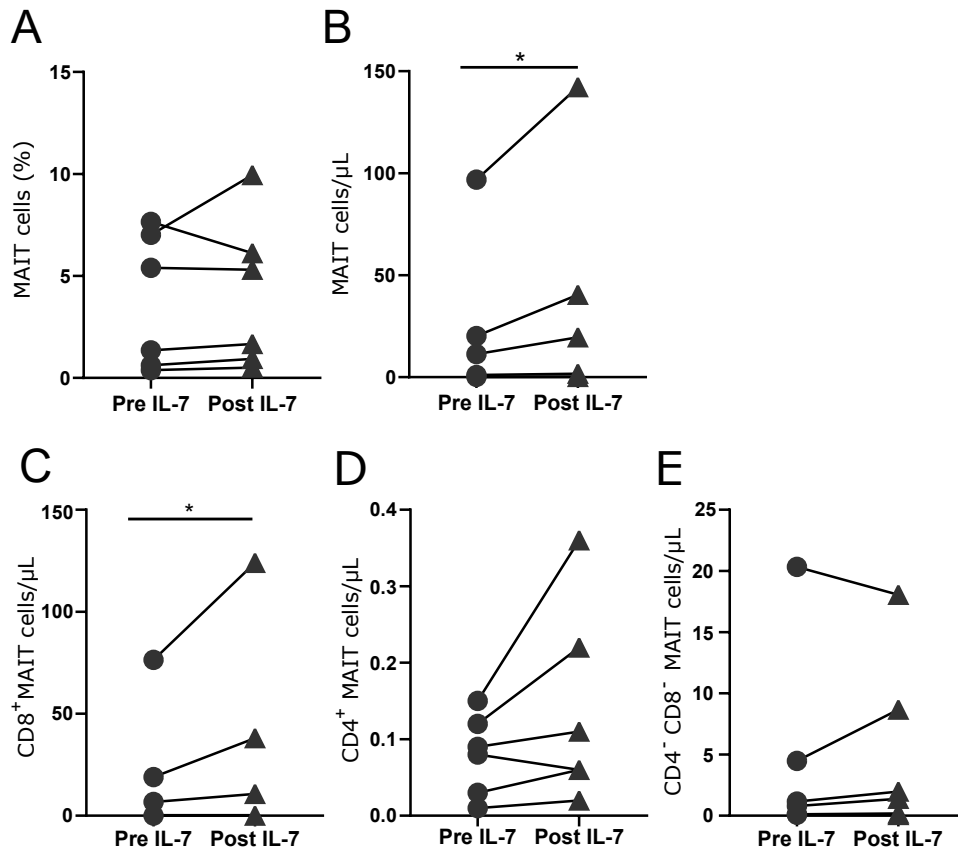


Figure 4. Effect of IL-7 treatment on levels of MAIT cells in peripheral blood of ICL patients.

(A-B) Frequency and absolute numbers of MAIT cells following 12 weeks of treatment with IL-7 in ICL patients. (C-E) Absolute numbers of CD8^+ (C), CD4^+ (D), and $\text{CD4}^- \text{CD8}^-$ (E) MAIT subpopulations following 12 weeks of treatment with IL-7 in ICL patients. Before-after plots show median; $n=6$ ICL patients. Data tested by Wilcoxon signed-rank tests; *: p -value < 0.05

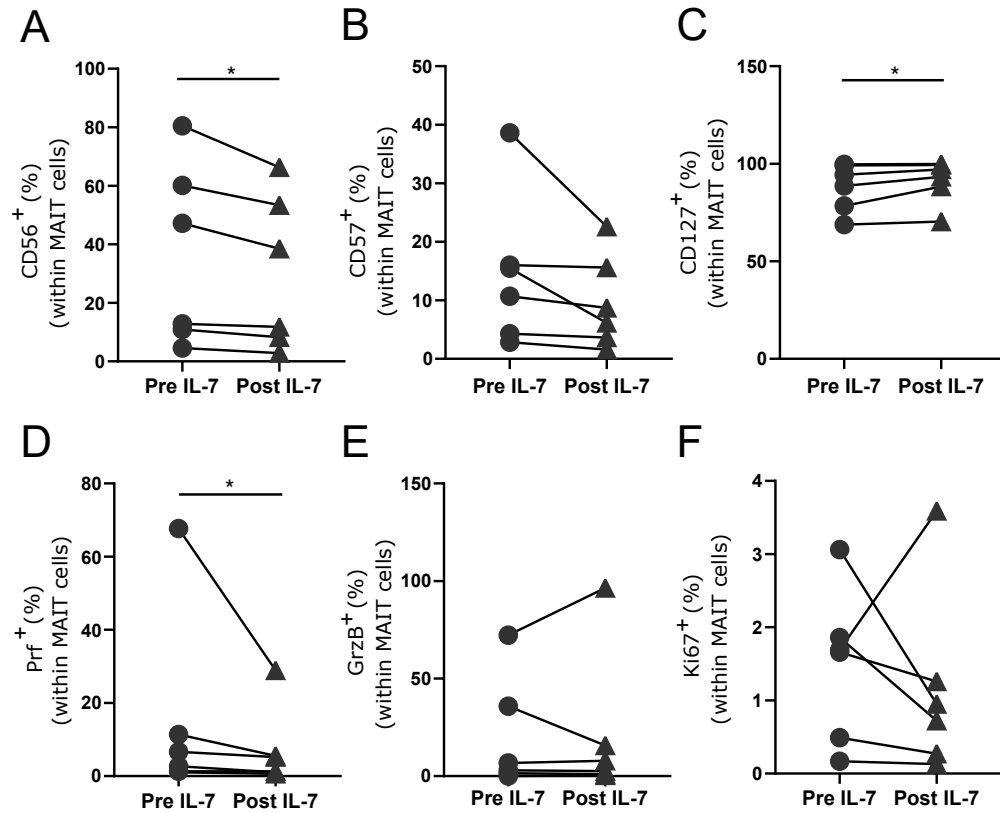


Figure 5. Phenotype and cytotoxic capacity of MAIT cells following IL-7 treatment in ICL patients. (A-C) Frequencies of MAIT cells expressing activation markers and CD127. (D-E) Frequencies of MAIT cells expressing markers indicating cytotoxic capacity. (F) Frequencies of cycling MAIT cells. Before-after plots show median; n=6 ICL patients. Data tested by Wilcoxon signed-rank tests; *: p-value < 0.05. Legend: granzyme B, GrzB; perforin, Prf.

Discussion

Idiopathic CD4⁺ lymphopenia is an immunodeficiency syndrome defined by reduced circulating CD4⁺ T cell counts, (< 300 cells/ μ L) in the absence of HIV infection or any other known cause of immunodeficiency [246, 247], increased susceptibility to opportunistic infections [247] and a lack of established treatment. The clinical manifestations of ICL are heterogeneous and can include

CD8⁺ T cell and B cell lymphopenias [247, 260]. The etiology and pathogenesis of this condition remains unclear. In this study we characterized the levels and function of the MAIT cell population to better elucidate how this unconventional T cell subset may be affected in ICL, and thus to better understand the pathogenesis of this syndrome. Interestingly, our data show preserved frequency and number of MAIT cells in the peripheral blood of patients with ICL to levels similar as those seen in HC, a pattern in sharp contrast to the decreased frequency and number of conventional T cells. Further, MAIT cells in ICL patients maintained the phenotypical and functional characteristics of MAIT cells in HCs.

The preserved number and function of MAIT cells in contrast to the loss of conventional T cells could lead us to speculate on defective mechanisms occurring during the thymic development of conventional T cells in ICL patients. Among the ICL-related causes previously explored, low CD4⁺ T cell thymic production [261], decreased response of CD4⁺ T cells to TCR stimulation [262], defective homeostatic- or antigen-driven proliferation or survival [263] and differential migration to tissues have been suggested [264]. Unlike conventional T cells, the development of MAIT cells depends on the TF PLZF. At the effector stage, MAIT cells also uniquely rely on the presence of commensal bacteria-derived signals and IL-18 for their differentiation into effector subsets [265]. Moreover, in contrast to the extensive receptor diversity of conventional T cells, the TCR repertoire of MAIT cells has limited diversity and recognizes the non-polymorphic MR1 molecules. In light of this, it can be interesting to consider retention and function of immune cells closer to MAIT cells in terms of biology and development, namely natural killer (NK) cells and innate lymphoid cells (ILCs). Previous studies, however, have shown a decrease in the number of NK cells with preserved function, measured by IFN production in response to IL-12 and IL-18, in

ICL patients [260]. A recent study has also shown depletion of the ILC population in ICL patients [266], thus suggesting that the impact of ICL, on ILCs, NK cells as well as T cells appear to be different than MAIT cells [267].

MAIT cells respond directly to microbial pathogens, immediately following activation, through secretion of pro-inflammatory and tissue-protective cytokines including IL-17, IFN- γ , TNF, and IL-22 [23, 236, 268]. In addition, MAIT cells have the capacity to directly control bacterial growth through the synergistic actions of GrzB and granulysin [269]. Innate cytokines, including IL-12 and IL-18, can also stimulate MAIT cells to produce IFN γ independently of the MR1-TCR interaction [242]. Therefore, MAIT cells perform critical functions in the immune system, particularly at mucosal sites [270, 271]. Given the breadth of responsiveness to many microbes, MAIT-cell exhaustion and loss may affect immunity to most of these microbes, which could lead to a higher risk of microbial co-infections [272]. Previous studies in chronic HIV infection have shown that MAIT cells are reduced in the periphery and the residual MAIT-cell population show severely suppressed production of IFN γ , TNF, and IL-17 [245]. Thus, MAIT cell defects may predispose PWH to an increased risk of acquiring microbial co-infections. Furthermore, common variable immunodeficiency, characterized by low levels of Igs, has been shown to be linked with activation and loss of MAIT cells [273]. Interestingly, we find that patients with ICL syndrome have an intact MAIT cell compartment. Although ICL patients have severe depletion of CD4⁺ T cells and they are often affected by opportunistic infections (mostly fungal or mycobacterial) and autoimmune diseases, their clinical course is not characterized by progression of CD4⁺ T cell depletion nor increased susceptibility to common bacterial infections. Preservation of MAIT cell number and function may thus mitigate some of the consequences of the depletion of conventional

T cells and may, at least in part, be one of the reasons of the overall stable clinical status of these patients.

In light of these observations, we sought to explore the effects of IL-7 treatment on the MAIT cell population of ICL patients. Administration of IL-7 has been previously proven to be well tolerated and successful in expanding the number of circulating CD4, CD8 T cells and tissue-resident T cells in the gut mucosa and bone marrow of ICL patients [248]. Moreover, we have previously shown a significant increase in the frequency and number of MAIT cells in the periphery of PWH post-IL-7 treatment [249]. Herein, we observed an expansion in the number of CD8⁺ MAIT cell subset after IL-7 treatment. Further, MAIT cells in these patients maintained responsiveness and effector function post IL-7 treatment. Thus, IL-7 treatment could represent a potential therapeutic strategy to further enhance the number of MAIT cells in the peripheral blood of ICL patients.

Our study has several limitations including the small sample size, and the lack of longitudinal samples and mucosal tissue samples, to better examine MAIT cell population in ICL patients. Despite these limitations, our study provides important data for further characterization of the immunologic profile of patients with ICL that helps decipher ICL etiologies and pathogenesis. The preservation of MAIT cell numbers and function in ICL underscores both the distinct ontology of MAIT cells from conventional T cells and a potential contribution of MAIT cells to mucosal defenses and the lack of increased susceptibility to some common bacterial infections in ICL.

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Conflicts of Interest

The authors declare no competing financial interests.

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Chapter 8

The Effects of Recombinant Human Lactoferrin on Immune Activation and the Intestinal Microbiome Among Persons Living with Human Immunodeficiency Virus and Receiving Antiretroviral Therapy

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Abstract

Lactoferrin modulates mucosal immunity and targets mechanisms contributing to inflammation during HIV disease. A randomized placebo-controlled cross-over clinical trial of human recombinant (rh)-lactoferrin was conducted among fifty-four HIV+ participants with viral suppression. Outcomes were tolerability, inflammatory and immunologic measures, and the intestinal microbiome. Median age was 51 years and CD4+ count was 651 cells/mm³. Adherence and adverse events did not differ between rh-lactoferrin and placebo. There was no significant effect on plasma interleukin-6 and D-dimer levels, nor on monocyte/T-cell activation, mucosal integrity, or intestinal microbiota diversity. Oral administration of rh-lactoferrin was safe but did not reduce inflammation and immune activation.

Background

Effective antiretroviral therapy (ART) has shifted the morbidity spectrum for persons living with HIV (PLWH) from AIDS toward serious non-AIDS (SNA) defining conditions, including cardiovascular disease, cancer and other end-organ diseases.[274] In addition to important behavioral factors, a key contributor to the current disease spectrum includes HIV-associated inflammation.[75, 275-277] Safe and tolerable treatment strategies that can be given in addition to ART are needed that target persistent immune activation. One potential strategy may include targeting irreversible injury to mucosal integrity during HIV infection, with translocation of microbial antigens such as lipopolysaccharide (LPS, or endotoxin).[25]

Lactoferrin is an endogenous iron-binding protein (member of transferrin family) with immunomodulatory properties that reduce inflammation.[278] Lactoferrin binds to LPS with high affinity and interferes with LPS binding of cell surface receptors (e.g., CD14) that typically

initiates broad pro-inflammatory effects.[278] Lactoferrin also promotes intestinal cell growth and cell migration, suggesting it may have potential to restore mucosal integrity and the gut barrier.[279] Lactoferrin has demonstrated the ability to suppress production and release of pro-inflammatory cytokines [280, 281] from stimulated monocytes, and to enhance secretion of anti-inflammatory cytokines from intestinal myofibroblasts.[282] A recombinant form of lactoferrin has then been shown to reduce mortality among intensive care unit patients with sepsis.[283]

There are no clinical trials of lactoferrin treatment among PLWH. We conducted a pilot study of recombinant human (rh) lactoferrin in 54 HIV positive participants receiving ART with viral suppression, to evaluate the safety, tolerability and potential anti-inflammatory effect.

Methods

Research Setting and Target Population. This study was conducted at an HIV clinic within a safety-net hospital in Minneapolis, Minnesota (Hennepin Healthcare). The protocol (NCT01830595) was approved by the institutional review board and received an IND (investigational new drug) approval by U.S. Food and Drug Administration (IND #118783). All study participants underwent a verbal and written informed consent process prior to enrollment. Eligibility criteria included PLWH of age ≥ 40 years on continuous treatment with ART and HIV RNA levels < 200 copies/mL for at least one year prior to enrollment. Participants with a gastrointestinal infection within the past month and common diseases known to influence inflammation were excluded.

Study Design and Intervention. We investigated the treatment effects of oral rh-lactoferrin (dosed 1500mg bid) versus placebo in a randomized, double-blind, cross-over clinical trial design. The

cross-over design consisted of two 3-month treatment periods separated by a 2-4 month ‘wash-out’ period. Each period consisted of 3 visits, with ‘period 1’ including baseline, month 1 and month 3, and ‘period 2’ including month 5, 6 and 8. After informed consent, participants were randomized ‘sequence’: either active rh-lactoferrin during period 1 and placebo during period 2 (A-P), or vice a versa (P-A).

Study drug (Rh-lactoferrin and matched placebo) was provided in capsule form by Ventrya Bioscience. The active drug is a recombinant form of human lactoferrin extracted and purified from milled rice flour as partially iron-saturated lactoferrin and has an amino acid sequence identical to that of human lactoferrin.

Power estimates were calculated for a composite IL-6/D-dimer score that was developed from epidemiologic data of PLWH on continuous ART to assess whether a candidate study intervention influences systemic inflammation and/or coagulation activity, and the degree to which any treatment effect would be posited to be clinically meaningful.[75] With 80% power, our goal sample ≥ 50 participants could detect an effect size in the composite IL-6/D-dimer score that corresponded to a 13% difference in clinical event risk (i.e., composite of cardiovascular disease, cancer, end-stage renal disease, end-stage liver disease or all-cause mortality).[75]

Laboratory Methods for Blood Measures. Participants were fasting for all study blood draws. Plasma, serum and peripheral blood mononuclear cell (PBMC) specimens were processed within 30 minutes of collection. Plasma soluble (s) biomarker levels were measured with ELISA and MesoScale multiplex platforms from batched cryopreserved samples. For details in biomarker measurements and immunophenotyping please see supplemental material (Supplemental Table A).

Microbiome Substudy Methods.

A subset of participants was co-enrolled into an intestinal microbiome substudy. A rectal swab specimen was collected before and after study drug exposure for both periods 1 and 2, corresponding to collection at four study visits (baseline and months 3, 5 and 8). Microbiome specimens collection and analysis are described in supplemental material.

Statistical Methods.

Characteristics and laboratory measures at randomization were summarized, and proportions with side effects and adverse events were tallied while on rh-lactoferrin and while on placebo. Treatment effects (rh-lactoferrin versus placebo) on inflammatory and immunologic outcomes were calculated using longitudinal mixed models with each participant serving as their own control. Models included indicators for drug sequence (A-P or P-A), study period (1 or 2), visit within period (0, 1 or 3) and drug (rh-lactoferrin or placebo), and tests of carryover effects were examined. For the microbiome substudy, sequence variants and OTUs were analyzed for alpha- and beta-diversity using the R package Phyloseq v1.22.3. Adonis (Vegan R project) was used to assess the effect size of rh-lactoferrin treatment in the variance. The QIIME 2.0 was used for a pairwise group comparison test and a linear mixed effect model test.

Results

Study Participants.

Supplemental Figure 1 presents the flow diagram for all consented participants through follow-up. The analysis cohort consisted of 54 participants, with 28 randomized to a rh-lactoferrin then placebo sequence and 26 randomized to the counter sequence of placebo then rh-lactoferrin. Fifty participants completed period 1, and of those 46 completed

periods 2. Participant characteristics are presented in Table 1. The median age was 51 years, with 89% of male sex at birth and 72% white. Randomized sequence groups were similar across co-morbid disease and HIV risk factors.

Table 1. Participant Characteristics at Entry Overall and by Randomized Group

Participants, No. (%)^a			
Characteristic	Total Sample (n = 54)	rh-Lactoferrin Followed by Placebo (n = 28)	Placebo Followed by rh- Lactoferrin (n = 26)
Demographics			
Age, y, median (IQR)	51 (46–56)	54 (46–60)	50 (46–53)
Male sex	48 (88.9)	26 (92.9)	22 (84.6)
Race/ethnicity			
White	39 (72.2)	21 (75.0)	18 (69.2)
African American	11 (20.4)	5 (17.9)	6 (23.1)
Hispanic	2 (3.7)	2 (7.1)	0 (0.0)
Other	2 (3.7)	0 (0.0)	2 (7.7)
Clinical characteristics			
BMI, median (IQR), kg/m ²	26.8 (24.5–32.4)	27.7 (25.7–35.2)	25.6 (23.6–28.6)
Smoker, current	20 (37.0)	7 (35.0)	13 (50.0)
Hepatitis B or C	8 (14.8)	5 (17.9)	3 (11.5)
Prior CVD	6 (11.3)	4 (14.3)	2 (8.0)
Hypertension diagnosis	18 (33.3)	10 (35.7)	8 (30.8)
Lipid-lowering therapy	17 (31.5)	11 (39.3)	6 (23.1)
Prescribed aspirin	13 (24.1)	9 (32.1)	4 (15.4)
Diabetes	5 (9.4)	4 (14.3)	1 (4.0)
HIV characteristics			
Prior AIDS	21 (38.9)	14 (50.0)	7 (26.9)
CD4 ⁺ cell count, median (IQR), cells/μL	651 (501–811)	637 (450–771)	687 (557–906)
CD8 ⁺ cell count, median (IQR), cells/μL	587 (434–903)	619 (474–928)	570 (430–799)
CD4-CD8 ratio, median (IQR)	1.0 (0.7–1.4)	1.0 (0.7–1.3)	1.0 (0.7–1.6)
HIV RNA undetectable	53 (98.1)	28 (100.0)	25 (96.2)
Tenofovir use	41 (75.9)	19 (67.9)	22 (84.6)
Abacavir use	12 (22.2)	8 (28.6)	4 (15.4)
NNRTI use	15 (27.8)	5 (17.9)	10 (38.5)
PI use	17 (31.5)	9 (32.1)	8 (30.8)

INSTI use	30 (55.6)	18 (64.3)	12 (46.2)
Clinical laboratory values, median (IQR)			
Total-C, mg/dL	179 (153–206)	177 (152–206)	180 (161–203)
LDL-C, mg/dL	106 (83–123)	105 (84–126)	106 (82–120)
HDL-C, mg/dL	46 (36–59)	47 (35–61)	42 (36–56)
Ferritin, ng/mL	104.5 (50.0–163.0)	107.5 (59.5–164.5)	93.5 (48.0–153)

Abbreviations: BMI, body mass index; CVD, cardiovascular disease; HDL-C, high-density lipoprotein cholesterol; HIV, human immunodeficiency virus; INSTI, integrase strand transfer inhibitor; IQR, interquartile range; LDL-C, low-density lipoprotein cholesterol; NNRTI, nonnucleoside reverse-transcriptase inhibitor; PI, protease inhibitor.

^aData represent No. (%) of participants, unless otherwise identified as median (IQR).

Adherence and Tolerability

The percent of participants reporting they took study drug twice every day exactly as prescribed ranged from 72-92% during follow-up visits and did not differ between groups (subjective adherence is reported in supplemental Table B). In addition, the percent of participants that noted missing ≥ 3 doses (i.e., $>20\%$ of potential doses) within the week preceding a visit ranged from 8-26%.

Supplemental Table 3 presents a summary of side effects and adverse events. Of 50 participants with side effect data available, 31 (62%) reported a side effect: 17 while prescribed rh-lactoferrin and 20 while prescribed placebo. The most common side effect was gastrointestinal related, but the frequency was again similar between those receiving rh-lactoferrin (n=15) and placebo (n=19). There were no deaths and none of the adverse events were deemed related to study medication.

Inflammation and Immune Activation Measures

Supplemental Table 4 presents median (IQR) levels of inflammatory and immunologic measurements from blood at baseline and month 5 (prior to starting study drug at beginning of period 1 and period 2, respectively). **Table 2** presents the primary treatment comparisons showing that none of the inflammatory or immunologic measures assessed significantly declined on rh-lactoferrin versus placebo (i.e., comparisons between treatments of changes in levels from pre- to post-treatment). There was no evidence of a significant carry-over effect from study drug exposure during period 1, through the wash-out phase and into period 2. We also assessed the treatment effect of rh-lactoferrin on clinical serum iron studies. The percent transferrin saturation of iron significantly increased on rh-lactoferrin versus placebo by 2.6% (95%CI: 0.2,5.0; p=0.04; Table 2). This effect did not reach statistical significance for serum ferritin, serum iron, total iron binding capacity, or hemoglobin (Table 2).

Table 2. Inflammatory and Immunologic Outcomes With 3 Months of rh-Lactoferrin Versus Placebo Treatment

	Treatment Effect of Lactoferrin Versus Placebo		
Laboratory Outcomes	P Value for Carryover Effects Between Periods	Mean (95% CI)	P Value
IL-6 and D-dimer score	.24	0.01 (-.07 to .06)	.82
Inflammation biomarkers			
IL-6, log ₂ pg/mL	.45	-0.02 (-.20 to .17)	.87
IL-6 receptor, ng/mL	.16	0.03 (-.01 to .06)	.15
IL-1b, pg/mL	.74	0.03 (-.02 to .07)	.26
IL-8, log ₂ pg/mL	.31	-0.02 (-.11 to .07)	.63
TNF- α , pg/mL	.35	0.02 (-.04 to .08)	.46
TNF-R1, ng/mL	.64	-0.00 (-.04 to .03)	.84
hsCRP, log ₂ mg/mL	.39	0.09 (-.17 to .36)	.49
Coagulation biomarkers			
D-dimer, log ₂ mg/L	.13	-0.03 (-.14 to .07)	.53
TFPI, log ₂ ng/mL	.44	0.02 (-.03 to .07)	.52
Mucosal integrity biomarkers			
Zonulin, log ₂ ng/mL	.57	0.00 (-.07 to .08)	.99
I-FABP, log ₂ ng/mL	.19	-0.04 (-.17 to .10)	.56
Monocyte biomarkers and immunophenotypes			
Soluble CD14, log ₂ mg/L	.23	0.01 (-.04 to .06)	.67
Soluble CD163, log ₂ mg/L	.24	0.09 (.02 to .17)	.02
CD14 ⁺ CD16 ⁻ , %	.08	-0.42 (-1.62 to .79)	.49

CD14 ⁺ CD16 ⁺ , %	.07	−0.36 (−1.06 to .33)	.30
CD14 ^{dim} CD16 ⁺ , %	.29	0.35 (−.35 to 1.05)	.32
T-cell immunophenotypes %			
MAITS	.51	0.01 (−.09 to .11)	.86
CD8 ⁺ PD1 ⁺	.25	−0.36 (−1.04 to .33)	.31
CD8 ⁺ A4/B7 ⁺	.96	0.33 (−.29 to .95)	.29
CD8 ⁺ KI67 ⁺	0.11	−0.48 (−1.21 to .24)	.19
CD8 ⁺ CD38 ⁺ HLA-DR ⁺	.77	−0.52 (−1.10 to .06)	.08
CD8 ⁺ CD57 ⁺ CD28 [−]	.27	−0.94 (−2.22 to .34)	.15
CD4 ⁺ PD1 ⁺	.64	−0.14 (−.78 to .51)	.67
CD4 ⁺ B7 ⁺	.96	0.30 (−.39 to .98)	.40
CD4 ⁺ KI67 ⁺	.05	−0.35 (−.92 to .21)	.22
CD4 ⁺ CD38 ⁺ HLA-DR ⁺	.97	−0.08 (−.26 to .09)	.36
CD4 ⁺ regulatory T cell	.09	0.03 (−.28 to .33)	.87
Clinical laboratory values			
Ferritin, ng/mL	.93	5.83 (−3.33 to 14.99)	.21
Serum iron, g/dL	.92	4.00 (−2.85 to 10.85)	.25
Total iron-binding capacity, mg/dL	.46	3.94 (−3.00 to 10.87)	.26
Transferrin saturation, %	.53	2.59 (.15 to 5.04)	.04
Hemoglobin, g/dL	.93	0.06 (−.08 to .21)	.41

Abbreviations: CI, confidence interval; hsCRP, high-sensitivity C-reactive protein; I-FABP, intestinal fatty acid-binding protein; IL-1b, IL-6, and IL-8, interleukin 1b, 6, and 8; MAITS, mucosal-associated invariant T cells; TFPI, tissue factor pathway inhibitor; TNF, tumor necrosis factor.

Intestinal Microbiome

Among twenty participants co-enrolled into the microbiome substudy, a subset of twelve participants had adequate specimens for microbiome analysis at all four of sentinel study visits (baseline and month 3, 5 and 8). There were no significant differences in alpha diversity between placebo/rh-lactoferrin (G1) and rh-lactoferrin /placebo (G2) groups. Beta diversity analysis did not show significant clustering of samples based on treatment group or time points. In contrast, samples from the same participant, at different time points, clustered strongly (Adonis test: G1 R^2 0.16 $p=0.001$; G2 R^2 0.10 $p=0.049$) showing stability in the diversity of the gut microbiota over time, independent of the treatment status or group sequence (Supplemental Figure 2). At both Phylum and Family levels we did not see any significant within participant changes after three months of treatment with placebo or rh-lactoferrin (Supplemental Figure 3A). At Phylum and Family level we also did not find significant changes in taxa frequencies within participants over all follow-up time points. (Supplemental Figure 3B and 3C).

Discussion

We report results from the first study of a rh-lactoferrin treatment among PLWH, given in addition to ART as a strategy to reduce systemic inflammation. In this pilot, placebo-controlled, double blind, cross-over clinical trial, we did not detect a significant treatment effect from rh-lactoferrin on blood measures of inflammation and immune activation, nor on changes to the intestinal microbiota or mucosal integrity. In fact, among a subset of twelve participants, we describe remarkable stability in the intestinal microbiota with repeated assessment over eight months. Consistent with its iron binding functionality, treatment with rh-lactoferrin did result in a small significant increase in transferrin saturation of $\sim 3\%$. Finally, the intervention was well tolerated.

Prior studies of recombinant forms of lactoferrin have demonstrated potential anti-inflammatory and immunologic benefits. In a placebo-controlled phase 2 trial of 194 patients with severe sepsis, enterally administered talactoferrin (recombinant human lactoferrin produced by *Aspergillus niger*) reduced all-cause mortality.[283] Posited mechanisms include the ability of lactoferrin to bind and modulate LPS signaling thereby reducing bacterial-mediated inflammatory response.[280] Among pregnant women with iron-deficiency anemia, treatment with bovine lactoferrin reduced plasma IL-6 levels, improved hemoglobin and increased ferritin to a greater extent when compared to ferrous sulfate supplementation.[284] In that study it was hypothesized that the anti-inflammatory effect of bovine lactoferrin improved iron homeostasis, and facilitated the release of sequestered iron that occurs in states of chronic inflammation. In addition, lactoferrin may also directly improve iron stores, and we did detect a modest increase in the percent transferrin saturation with three months of rh-lactoferrin treatment.

Differences in the potential anti-inflammatory effect of lactoferrin between prior published reports and our findings may be explained, in part, by differences in the target populations studied. Compared to HIV disease, the degree of systemic inflammation that is attributable to microbial antigen-mediated innate immune activation may be much greater during states of sepsis. Although iron sequestration is a consequence of chronic inflammation, iron deficiency per se is not a central driver of HIV-associated inflammation and our study population had normal serum ferritin levels at entry and low overall levels of inflammatory markers. Finally, it is also possible that the treatment effects of oral lactoferrin may be most apparent within the mucosal tissue effector site, and therefore an immunologic signal may not be present, or at least very modest, within the peripheral circulation. To this point, data from a murine model suggests that rh-lactoferrin may

improve T-regulatory cells within the intestinal lamina propria, but this effect was not seen within blood.[285]

Our pilot study had several limitations. The small sample size is unable to fully rule out a small but potentially meaningful treatment effect. The choice of rh-lactoferrin preparation could affect the outcome. Our immunologic assessments were limited to blood and did not directly assess the mucosal tissue. Despite this limitation, our findings suggest that rh-lactoferrin is unlikely to have clinically meaningful impact on systemic inflammation and associated end-organ disease risk among the target population of individuals with ART-treated HIV infection. We only characterized the intestinal microbiota among a limited subset of participants, though there was remarkable stability in the microbial community over time. Finally, approximately one-quarter of participants acknowledged suboptimal adherence, but sensitivity analyses restricted to optimal adherence did not change results.

In summary, rh-lactoferrin treatment among ART-treated PLWH did not result in significant changes to inflammation or immune activation within blood, nor to changes in the intestinal microbiota. Additional strategies to reduce inflammation by targeting intestinal dysbiosis and improving mucosal immunity are needed to improve the health of PLWH.

Notes

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Chapter 9

General discussion

The era of cART has led to increased survival in PWH because of the marked improvements in its potency, side-effect profile, and simplicity of use [1]. However, there is still a small but persistent gap in the lifespan between PWH and HIV-negative individuals, particularly in those in whom diagnosis of HIV infection is delayed. A higher burden of age-associated noncommunicable comorbidities (AANCC) is increasingly being reported in PWH compared to age-matched HIV-negative individuals [2, 3]. In addition to important behavioral factors, a key contributor to the current disease spectrum includes HIV-associated inflammation [4, 5]. Although root causes of HIV-associated inflammation are incompletely defined, the gastrointestinal mucosal immune disruption that follows progressive HIV and SIV infection is postulated to play a role [6]. Specifically, compromised mucosal barrier function, increased translocation of immunostimulatory microbial products from the gut lumen into the systemic circulation and dysbiosis of the gastrointestinal tract have all been importantly implicated in this process [6-9].

This thesis presents the findings of several comparative studies against the background of existing research within the context of both HIV and certain other immunodeficiencies. We assessed at what time following infection gut dysbiosis is established and whether dysbiosis influences HIV/SIV progression; the impact of HIV itself, sexual practice and antiretroviral drugs on gut bacterial communities in humans and non-human primates. Moreover, we addressed whether HIV-infection and CD4 lymphopenia disrupt components of the mucosal immune system. Finally, we evaluated new therapeutic approaches aiming at minimizing the effect of immunological dysfunction.

Characterization of intestinal dysbiosis in HIV-infection

Gastrointestinal microbial dysbiosis, an imbalance of the composition of the microbiome favoring enrichment of disease-associated taxa, can restructure tissue-specific immunity and is thought to be part of the etiology of various chronic health conditions [10, 11]. Studies consistently report disruption to this ecosystem in chronic PWH and correlation of dysbiotic bacterial taxa with canonical markers of disease progression in HIV infection. The majority of these studies, however, have mostly addressed cross sectional comparisons between chronic PWH and HIV-uninfected controls [6, 12-16]. In addition, variables previously reported to impact the microbiota, including sex, age, sexual practice, body mass index (BMI), and ethnicity, have not been tracked in well-powered, representative cohorts. Furthermore, longitudinal studies in the simian immunodeficiency virus (SIV) non-human primate (NHP) model have shown that dysbiosis is not consistently observed [17]. Therefore, our understanding of the role of the gut microbiota in HIV disease is still limited.

The RV254 cohort allowed us to investigate whether dysbiosis is established during the acute phase of HIV infection or only later during the chronic disease. Specifically, we investigated the gut microbiota in a longitudinal study of Thai MSM with acute HIV infection (AHI) at the time of HIV diagnosis (Fiebig (F) stages 1-5), and after 6 months of cART with ethnicity- age- and sex-matched HIV-uninfected MSM serving as controls. Comparisons between AHI pre-cART and HIV-uninfected controls provided evidence of a dysbiotic gut environment already established during the earliest studied phase of AHI (**chapter 5**), as previously observed during acute SIV-infection [18, 19]. Moreover, the composition of the gut microbiota through stages F1-3 of acute

infection, which seems to be linked to a progressive loss of CD4⁺-T-cells, in particular Th17 cells, highlights an enrichment in pro-inflammatory bacteria during the transition from F1 to F3, and suggests a correlation between dysbiosis and disease progression (**chapter 5**). However, not having access to PWH without dysbiosis prevented us to further investigate this aspect. We therefore turned to the animal model in rhesus macaques where animals were infected with SIV and treated with vancomycin or vehicle. Vancomycin-induced intestinal dysbiosis recapitulated salient features of HIV-ascribed dysbiosis and yet, dysbiotic animals did not display enhanced immune activation and did not progress to simian AIDS disease more rapidly (**chapter 2**), supporting the speculation that gastrointestinal microbial dysbiosis by itself is not sufficient to accelerate untreated lentiviral disease progression in human.

Notwithstanding, there are some aspects specific to HIV vs SIV infection that need further consideration and might explain the differences reported above. First, unlike the acute phase of HIV and SIV infection, where the presence of gut inflammation can result in a similar altered gut microbiota [18, 19] the magnitude of changes in gut bacterial communities during the chronic stage of SIV infection in non-human primates does not correspond to that of chronic HIV-infection in patients [11, 20, 21]. In turn, this might also result in a different impact of dysbiosis at later stages. Further, although there is vast resemblance between SIV and HIV infection, differences in viral, host-behavioral factors or commensal identity may uniquely contribute to dysbiosis, and in turn, disease progression in PWH vs SIV-infected macaques.

In line with this, the rate of disease progression for chronic pathogenic SIV infection is defined within several months in comparison to several years in HIV infection which may lead to inherent

differences in gut microbial composition [22]. Moreover, difference in environmental influences, including housing conditions, diet, and length of infection on gut microbial compositions, might limit comparisons across studies [22]. The variability of environmental conditions is not controlled in human studies and natural non-human primate infections compared with the widely used experimental AIDS model of rhesus macaques, which highly influence outcomes when analyzing gut microbial communities [22-24]. Further, as mentioned above, host-related aspects such as age, gender, ethnicity, and sexual practice, among others, can impact the gut microbiome itself and, in turn, create disparity among studies in different populations [25-27]. For instance, recent studies have described that stratification by risk factors for HIV acquisition, independent of HIV-status, removes evidence for HIV-mediated dysbiosis [28]. As a consequence, this raises the hypothesis that, after stratification by sexual practice, there may be little evidence for shifts in the gut microbiota attributable to HIV infection, and that the observed relationships between dysbiosis and immune activation in PWH may be independent.

The AGEhIV cohort provided an optimal context to try and dissect the impact that HIV-infection itself and sexual practice have on the gut bacterial communities of PWH. Similar to previous studies [12-15, 28], our cohort included chronically infected PWH with suppressed viremia on cART and HIV-uninfected controls. Unlike previous studies, PWH and HIV-uninfected controls could be elegantly grouped by sex and sexual practice and matched for age, sexual behavior, BMI, and birth country. Our data show both an HIV-associated microbiota signature as well as a MSM-associated microbiota signature, exhibiting opposing abundance trends (**Chapter 3**). Interestingly, the MSM-associated microbiota, mostly enriched in Prevotellaceae members, seemed to be driven by sexual practice. Therefore, the selection of seronegative controls from the general population,

without matching for MSM, is likely to have confounded earlier studies examining the impact of HIV infection on the gut microbiota.

Finally, in support of the hypothesis of a link between dysbiosis and disease progression in humans, pro-inflammatory taxa belonging to *Enterobacteriaceae* (**chapter 3 and 5**) and *Desulfovibrionaceae* (**Chapter 3**) were found to be enriched in PWH treated with ART. Notably, the *Enterobacteriaceae* family includes several pathogenic taxa such as *Escherichia*, *Shigella*, and *Klebsiella* which are well documented sources of bacteremia in PWH [29, 30]. Members of the *Desulfovibrionaceae* family have also recently been implicated in chronic inflammatory states such as inflammatory bowel disease [31]. This family encompasses several species with the metabolic capability to reduce sulfate which produces the byproduct hydrogen sulfide, a cytotoxic compound that inhibits colonocyte butyrate oxidation [31]. Butyrate is a crucial energy source for colonocytes and is also a robust inducer of regulatory T-cells which constitute a lymphocyte population critical for mitigating pathologic inflammation [32-34]. In addition, a concomitant decline in butyrate producing gut microbiota members belonging to the *Lachnospiraceae* and *Ruminococcaceae* families was observed in PWH compared to HIV-negative controls (**Chapter 3**). Thus, an increase in production of molecular inhibitors of butyrate oxidation in combination with a decrease in butyrate-producing gut bacteria may synergize to contribute to the epithelial barrier disruption that is seen to persist in treated HIV infection. Indeed, abundance of *Lachnospiraceae* members in PWH has been recently found to correlate inversely with I-FABP, a marker of gut epithelial barrier disruption, supporting the importance of this clade in mucosal homeostasis [35], although I-FABP did not correlate with the dysbiosis index in our study. This impairment of the function of the intestinal epithelium may permit translocation of pro-inflammatory microbiota products into the systemic circulation and thereby exacerbate

pathological chronic inflammation in treated PWH, providing a possible explanation for the observed correlation between sCD14, a marker of systemic innate immune activation, and reduced alpha diversity in PWH (**Chapter 3**). Reductions in alpha diversity are also associated with the onset of type II diabetes [36] and are seen in inflammatory bowel disease where they coincide similarly with decreased abundance of *Lachnospiraceae* and *Ruminococcaceae* [37], highlighting an analogous relationship between the gut microbiota and chronic inflammatory disease. In addition, our data show that suPAR, a marker of systemic immune activation [38] recently identified as a remarkably strong independent predictor of cardiovascular [39] and overall non-AIDS morbidity and mortality [40], is linked with features of the HIV-associated microbiota signature, i.e. overall alpha diversity and specific taxa associated with PWH (**Chapter 3**). These observations suggest suPAR may potentially be used as a biomarker for microbiota-mediated strategies to alleviate HIV-associated inflammation and warrants further study into the impact of HIV-associated gut bacteria on this pathologic inflammatory pathway.

Impact of ART treatment on Gut Microbiota Composition

Studies show that cART initiation at earlier stage of HIV-infection results in a marked decrease in the risk of AIDS-related events as well as severe non-AIDS conditions including cancers, kidney disease, and invasive bacterial infections [41-45]. As wide-ranging pharmacologic interventions, cART treatment also influences the commensal microbiota [46, 47] and its crosstalk with the gastrointestinal immune system. Evaluation of 6 months of cART treatment on the gut microbiota of PWH who started cART (2NRTI + efavirenz) at the time of diagnosis of acute HIV infection (F1-5) demonstrated a partial restoration of the gut microbiota, although it did not completely normalize the microbial composition to that of healthy individuals (**Chapter 5**). Similar to our

observations, previous studies, including short- and long-term use of cART during chronic HIV infection, have shown a partial restoration of the gut microbiota [16, 28, 48]. Further, in support of the benefits of early cART initiation, we found a strong, significant correlation between gut dysbiosis and known duration of HIV infection, duration of antiretroviral therapy as well as nadir CD4 count and pre-cART CD4 count (**Chapter 3**). Previous studies have shown strong correlations between dysbiotic bacterial taxa and markers of inflammation and epithelial dysfunction in chronic cART-treated PWH [6, 12, 13, 15]. Similar to previous studies, we found a correlation between proinflammatory bacterial taxa, whose frequencies increased after 6 months of ART, and levels of sCD14, a well-established marker of disease progression in AHI recently reported to remain elevated despite early treatment (**Chapter 5**). Further, we observed a reduced alpha diversity, in chronic cART treated PWH, which inversely correlated with plasma sCD14 levels and a positive correlation with pre-cART CD4 count (**Chapter 3**). Due to the limitation of study design in humans, we cannot surely attribute the observed microbiota shifts to the effects of antiretrovirals or to HIV-related effects.

Of note, determining the contribution of cART in the clinical setting, where everyone with HIV, according to guidelines, should be on treatment, is very difficult. Therefore, to better decipher the role of antiretroviral medications in microbiome alterations we turned again to the healthy rhesus macaques model where we treated animals with either protease, integrase- or reverse transcriptase inhibitors for 1-2 or 5-6 weeks to evaluate intestinal immune function and the composition of the bacterial microbiome (**Chapter 4**). We observed an induction of inflammation coinciding with significant differences in bacterial diversity, suggesting that antiretrovirals by themselves might destabilize intestinal homeostasis (**Chapter 4**). Different mechanisms might explain such a

phenomenon. For example, *in vitro* experiments showed that protease inhibitors interfere with calcium-dependent chloride conductance and epithelial proliferation and survival, which are essential to maintain gut homeostasis. However, the magnitude of gastrointestinal changes observed in this study is too modest to support the hypothesis that ART itself is responsible of the degree of immune dysfunction noted in treated PWH. In this context it is important to note that the lack of persistent changes in individual taxa in our ARV-treated, uninfected macaques (**Chapter 4**) is compatible with the observation that even in SIV-infected macaques, dysbiosis does not appear to be a consistent feature of disease progression. Nonetheless, one could imagine that some of the gastrointestinal side effects of protease inhibitors in PWH and of tenofovir/emtricitabine in users of preexposure prophylaxis (PrEP) may be mediated by ART induced microbiome related changes [49]. Therefore, a more conservative speculation would suggest that cART may mediate alterations in the gut, but the net effect must be framed within a more complex scenario where several factors might co-operate to ultimately define the gut phenotype associated with treated HIV infection.

Although administration of cART reduces viral loads in plasma and increases CD4⁺ T cell counts, it fails to eradicate HIV reservoirs, to fully restore the composition of the gut microbiome, gastrointestinal immunity, and to resolve gut inflammation [13, 50]. Therefore, many efforts are currently aimed at developing novel interventions that, jointly with cART, can minimize the effect of immunological dysfunction in support of mucosal health.

Alternative strategies to diminish immunological dysfunction and inflammation

A number of therapeutic strategies aimed at reducing immunological dysfunction, augmenting gut mucosal reconstitution and diminishing microbial translocation in combination with cART have been explored.

Among the strategies tested, the use of IL-7 was found to be well tolerated in humans with various lymphopenic conditions [51-54]. These previous studies show that IL-7 expanded CD4 and CD8 T cells, decreased Tregs, and helped restore CD4 T cells in the gut mucosa of lymphopenic PWH as a result of increased T-cell homing and possibly local T-cell expansion. Thus, given IL-7's ability to simultaneously stimulate thymopoiesis [55, 56], increase T-cell proliferation [57], and prolong survival of mature T cells [58], we thought to use IL-7 to target circulating MAIT cells in chronic PWH on cART. MAIT cells are a subset of unconventional, innate-like T cells that are highly abundant in mucosal tissues, liver, and peripheral blood of healthy individuals [59] but that appear to be functionally exhausted, and progressively and persistently depleted during chronic HIV-1 infection, even after long-term cART [59, 60]. Our data showed an increase in the frequency and number of circulating MAIT cells in chronic PWH on cART, 12-weeks after administration of IL-7 (**Chapter 6**). Importantly, MR1-restricted MAIT cells are believed to be a significant and evolutionarily conserved component of the T-cell response against microbes, as they respond rapidly to a range of microbes that are clinically relevant in HIV infection, including *Mycobacterium tuberculosis* and *Candida albicans* [61-64]. The decline in MAIT-cell level and function, possibly as a result of persistent exposure to microbial material, may be a serious insult to the ability to mount immune responses to microbial pathogens. Therefore, rescue of these cells may improve immune reconstitution in PWH and could potentially result in a reduced risk of microbial co-infections.

Similarly to, but in the absence of HIV infection, idiopathic lymphocytopenia (ICL) is a state of chronic immunosuppression characterized by low CD4⁺ T-cell counts (<300/ μ L), increased susceptibility to opportunistic infections, such as *Cryptococcus* and non-tuberculous *Mycobacteria* and very low numbers of CD4⁺ T-cells in the GI tract, but without evidence of systemic inflammation [65]. Recent studies showed a severe depletion of innate lymphoid cells (ILCs), a population of immune cells that play critical roles in mucosal barrier defense and tissue homeostasis, in individuals with ICL similar to PWH [17, 66]. Among our patients with ICL syndrome we did not observe a significant decrease in the frequency and absolute number of MAIT cells. Furthermore, these patients did not show alterations in MAIT cells phenotype and function when compared to HCs. Although ICL patients have severe depletion of CD4⁺ T cells and they are often affected by opportunistic infections (mostly fungal or mycobacterial) and autoimmune diseases, their clinical course is not characterized by progression of CD4 depletion nor increased susceptibility to bacterial infections. Preservation of MAIT cells number and function may thus mitigate some of the consequences of the depletion of conventional T cells and may, at least in part, be one of the reasons of the overall stable clinical status of these patients (**Chapter 7**). In light of these observation, we also characterized the MAIT cell population and determined its functionality in people with ICL after IL-7 treatment. Interestingly, *in vivo* administration of IL-7 enhanced the number of circulatory MAIT cells, specifically the CD8⁺ MAIT cells subsets after 12 weeks of IL-7 treatment (**Chapter 7**).

Another strategy which has been explored is one of interfering with translocating microbial products directly, either through administration of an LPS-binding drug such as phosphate-binding sevelamer [67] used to prevent hyperphosphataemia in patients with chronic renal failure [68], or

mesalamine an anti-inflammatory drug used in the treatment of inflammatory bowel disease [69, 70]. These approaches have however only shown negligible improvements in inflammatory or immune parameters in ART-treated PWH.

We evaluated the potential anti-inflammatory effect of another LPS-binding agent, i.e oral recombinant human (rh) lactoferrin (**Chapter 8**). Rh-lactoferrin is an endogenous iron-binding protein that has anti-microbial and immunomodulatory properties [71]. Specifically, rh-lactoferrin binds to LPS with high affinity and interferes with LPS binding of cell surface receptors (e.g., CD14) that typically initiates broad pro-inflammatory effects [72]. Prior studies of recombinant forms of lactoferrin have demonstrated potential anti-inflammatory and immunologic benefits in certain target populations, *e.g.* patients with sepsis. Posited mechanisms accounting for the treatment effect during sepsis include the ability of lactoferrin to bind and modulate LPS signaling thereby reducing bacterial-mediated inflammatory responses [73]. Among pregnant women with iron-deficiency anemia, treatment with bovine lactoferrin reduced plasma IL-6 levels, improved hemoglobin and increased ferritin to a greater extent when compared to ferrous sulfate supplementation [74]. We however did not detect a significant treatment effect from rh-lactoferrin on blood measurements of inflammation in PWH with chronic HIV infection on cART. We observed remarkable stability in the intestinal microbiota or mucosal integrity. Lactoferrin may also directly improve iron stores given its origins within the broader family of transferrin iron transport proteins, and we did detect a modest increase in the percent transferrin saturation with just three months of rh-lactoferrin treatment (**Chapter 8**). Although our immunologic assessments were limited to blood and did not directly assess the mucosal tissue immune compartment, so that we cannot rule out a limited local effect of rh-lactoferrin at the intestinal effector site, our findings

suggest that rh-lactoferrin is unlikely to have a clinically meaningful impact on inflammation-associated end-organ disease risk among the target population of cART-treated PWH.

The divergence in the anti-inflammatory effect of lactoferrin between prior studies and our own may be attributed, in part, to differences in the target populations studied. The degree of systemic inflammation that is attributable to microbial antigen-mediated innate immune activation may be much greater during states of sepsis than during treated HIV disease. Although iron sequestration may be a consequence of chronic inflammation, iron deficiency *per se* is not posited to be a central driver of HIV-associated inflammation and our study population had normal serum ferritin levels at entry. Finally, it is also possible that the treatment effects of oral lactoferrin may be most apparent within the mucosal tissue effector site, and any immunologic signal may not be present, or at least very modest, within the peripheral circulation. To this point, data from a murine model suggests that rh-lactoferrin may improve T-regulatory cells within the intestinal lamina propria [75], but this effect was not seen in blood.

Future directions

As part of the work described in this thesis both cross-sectional and longitudinal studies with limited sample sizes have been performed to assess the gut microbiota in PWH. More extensive and adequately powered longitudinal studies will be required to further dissect the impact of the observed specific shifts in the gut microbiota on associations with chronic inflammation and comorbidity incidence in HIV.

In addition, our studies as well as previous studies have observed a significant depletion in bacterial taxa involved in the production of SCFAs in PWH. These changes contribute to diminished levels

of anti-inflammatory SCFAs such as butyrate. Future metabolomics analysis should evaluate the levels of SCFAs in PWH. Currently, SCFAs are considered a promising supplementary treatment in the clinical management of active IBD patients and diversion colitis where supplementation with *Lactobacillus* strains improved intestinal inflammation and gut barrier function. Similarly, PWH on cART with increased gut *Lactobacillales* showed reduced microbial translocation and increased CD4 T cells. It remains to be established how exactly gut microbiota and the associated metabolites mechanistically influence the intestinal barrier and the consequent inflammation in HIV, and how this could be therapeutically modified.

Furthermore, the possible effect of specific antiretrovirals on the gut microbiota remains poorly understood and cannot be ruled out as a contributor to the microbiota shifts observed in our studies. Although it has been reported that the gut microbiota shifts in untreated subjects are similar to those observed in our studies, longitudinal studies focusing on the impact of antiretrovirals on the gut bacterial communities are essential to tease apart the role of HIV infection from that of antiretroviral therapy. Performing such studies however presents a challenge given the current guideline which recommends universal use of treatment regardless of an individual's CD4 count.

Of note, the studies performed in the context of this thesis have only examined the fecal microbiota. Avenues to elucidate the role of the microbiota in HIV disease progression include the examination of mucosal-resident communities in the context of MSM, PWH. Given the mucosal-resident nature of potentially immunostimulatory and given that this community differs from that of the lumen, studies examining microbial communities of mucosal biopsies may yield important insights into the relationship between the gut microbiota and immune states in HIV infection.

The assessment of IL-7 as a therapeutic intervention to restore MAIT cells in the context of HIV-infection and of the ICL syndrome, was limited to blood and did not directly evaluate the mucosal tissue immune compartment. Therefore, future larger studies including gut biopsies could better determine if immunotherapy can also reconstitute both the frequency and functionality of MAIT cells at mucosal sites in these conditions. In addition, given the commitment of MAIT cells to antimicrobial mucosal immunity, and the influence of riboflavin-synthesizing commensal bacterial species in inducing MAIT cells, the association between MAIT cells and gut dysbiosis should be assessed in both HIV infection and ICL syndrome.

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Chapter 10

Summary &

Nederlandse samenvatting

The implementation of effective combination antiretroviral therapy (cART) has led to a markedly increased life expectancy, and thereby to a growing population of middle-aged and elderly PWH. As the population of PWH continues to age, and the incidence of AIDS-related conditions has been reduced with effective cART, the spectrum and burden of age-associated non-communicable comorbidities (AANCC) has increased. A key contributor to the current disease spectrum includes HIV-associated inflammation. Among ART-treated PWH, recent data have emphasized the potential clinical consequences of abnormalities in innate immunity, including estimates of monocyte activation. One potential mechanism driving innate immune activation may include irreversible injury to mucosal integrity and effector immune sites during HIV infection, with translocation of microbial antigens such as lipopolysaccharide (LPS, or endotoxin).

This thesis describes a series of studies aimed at determining how gut microbiota and mucosal immunity are affected and contribute to disease pathogenesis and progression, during SIV infection of non-human primates, HIV infection in humans, and in people with idiopathic CD4⁺ lymphocytopenia (ICL).

Intestinal microbial dysbiosis has been described in PWH and may underlie persistent inflammation in chronic infection. In **chapter two** HIV-like intestinal dysbiosis was induced in SIV infected rhesus macaques by vancomycin administration in order to assess the contribution of dysbiosis to SIV disease progression. We found that dysbiotic and control animals exhibited comparable viremia, target-cell depletion, immune activation, microbial translocation, and ultimately, survival rates. These data indicate that intestinal microbial dysbiosis by itself does not necessarily contribute to disease progression in all lentiviral infections. Although SIV infection of

rhesus macaques recapitulates key aspects of HIV infection and the gut microbial communities of rhesus macaques and humans share several dominant taxa, they differ substantially beyond the genus classification. Therefore, we cannot discount that underappreciated differences in viral, host, or commensal identity may uniquely contribute to lentiviral disease progression in humans and NHPs.

Studies in humans and in animal models have demonstrated that the complexity of microbiota - host crosstalk is still not fully appreciated in PWH as multiple factors including genetics, diet, and sexual practice can significantly alter the composition of the gut microbiome. In **chapter three**, we examined the contribution of sexual practice and treated HIV infection to gut dysbiosis. An extensive analysis of fecal microbiota profiles was undertaken in a carefully designed case control study which involved both PWH with suppressed viremia on cART and HIV-uninfected individuals participating in the Amsterdam AGE_hIV Cohort Study. Stored stool samples from participants were selected stratified by sex (i.e. male and female) and sexual practice (i.e. lifetime or recent vaginal/anal intercourse with women/men) and matched for age, sexual behavior, BMI, and birth country. We observed significant gut microbiota differences in PWH regardless of sex and sexual practice that include *Gammaproteobacteria* enrichment, *Lachnospiraceae* and *Ruminococcaceae* depletion, and decreased alpha diversity. Men who have sex with men (MSM) exhibit a distinct microbiota signature characterized by *Prevotellaceae* enrichment and increased alpha diversity, which is linked with receptive anal intercourse in both males and females. Finally, the HIV-associated microbiota signature correlated with inflammatory markers including suPAR, nadir CD4 count, and prevalence of age-associated non-communicable comorbidities.

The gastrointestinal immune system competency, which is dependent upon interactions with commensal microbiota, can be also influenced by wide-ranging pharmacologic interventions. In **chapter four** we investigated whether ART by itself may contribute to dysbiosis or immune dysfunction. We treated healthy rhesus macaques with protease-, integrase- or reverse transcriptase inhibitors for 1-2 or 5-6 weeks and evaluated intestinal immune function and the composition of the fecal bacterial microbiome. We observed that individual antiretrovirals (ARVs) modestly altered intestinal T-cell pro-inflammatory responses without disturbing total or activated T-cell frequencies. Moreover, we found significant disruptions in bacterial diversity coupled with perturbations in the relative frequencies of bacterial communities. Shifts in specific bacterial frequencies were not persistent post-treatment, however, with individual taxa showing only isolated associations with T-cell pro-inflammatory responses. Thus, intestinal bacterial instability and modest immunological alterations can result from ART itself.

In **chapter five** we assessed how gut dysbiosis emerges early after HIV acquisition and how it is affected by early ART. A study was designed in a subset of participants of the ongoing RV254/SEARCH010 cohort, enrolling persons with early acute HIV infection (Fiebig Stages 1–5) who were identified from the Thai Red Cross Anonymous Clinic in Bangkok, Thailand. Fecal microbiota composition in relation to markers of systemic inflammation and gut epithelial integrity was studied in a group of men who have sex with men (MSM), at time of diagnosis of acute HIV infection (AHI) and 6 months after ART initiation, as well as in a small number of Thai MSM HIV-uninfected controls. We observed a mild dysbiosis in AHI pre-ART compared to HIV-uninfected controls. Transition from F1-3 of acute infection was characterized by enrichment in pro-inflammatory bacteria. Lower proportions of Bacteroidetes and higher frequencies of

Proteobacteria and Fusobacteria members were observed post-ART compared with pre-ART. Fusobacteria members were positively correlated with levels of soluble CD14 in AHI post-ART. This association of dysbiotic bacterial taxa with inflammatory markers suggests that a potential relationship between altered gut microbiota and systemic inflammation may also be established during AHI.

Ancillary interventions, alongside ART, are needed to modulate immunological dysfunction and restore the gut microbiota. In **chapter six** we evaluated IL-7 treatment to restore Mucosal Associate Invariant T (MAIT) cells, a subset of antimicrobial unconventional innate-like T cells, in peripheral blood of chronically HIV-1 infected individuals on ART. We found a significant increase in the frequency and number of MAIT cells in the peripheral blood of chronically infected PWH 12 weeks post-IL-7 treatment. This is striking given that no significant restoration of MAIT cells has been seen during long-term ART. IL-7 treatment thus represents a potential therapeutic strategy to reconstitute the number of MAIT cells in PWH. Rescue of these cells may improve immune reconstitution in PWH and could potentially result in a reduced risk of microbial co-infections.

Similar to HIV infection, people with idiopathic CD4⁺ lymphopenia (ICL), a syndrome defined by low CD4⁺ T-cell count (<300/ μ L) and an increased susceptibility to opportunistic infections, also exhibit very low numbers of CD4⁺ T-cells in the GI tract, but without evidence of systemic inflammation. In **chapter seven** we examined the levels, phenotype and functionality of MAIT cells in peripheral blood of ICL patients. We found preserved numbers, phenotype and function of

MAIT cells in ICL patients compared to healthy controls. In addition, administration of IL-7 led to an expansion in the number of MAIT cells, specifically in the CD8⁺ MAIT cells subsets.

In **chapter eight** we studied the immunomodulatory properties of recombinant human lactoferrin as well as its impact on fecal microbiome composition in a cross-over placebo-controlled trial of PWH with suppressed viremia on ART. Oral administration of rh-lactoferrin was safe but did not reduce inflammation or immune activation. We did not observe significant effects on plasma interleukin-6 and D-dimer levels, or on monocyte/T-cell activation, mucosal integrity, or intestinal microbiota diversity.

In **chapter nine** we provided general discussions in which we put our findings into perspective. Gut microbiome dysbiosis in PWH is evident since the earliest studied phase of AHI. HIV infection is linked with a specific bacterial signature which involves increased levels of proinflammatory bacteria and a decrease in bacterial taxa linked with anti-inflammatory properties and maintenance of gut homeostasis, compared to HIV-uninfected controls. Stratification by risk factor for HIV acquisition highlights a unique MSM-associated microbiota signature, providing evidences on the importance of the selection of seronegative controls from the general population. In line with the importance of early cART initiation, PWH starting therapy during early stages of acute infection, show a partial restoration of the gut microbiota. However, cART alone does not fully restore the composition of the gut microbiome, the gastrointestinal immunity, and does not resolve gut inflammation. Among ancillary intervention given alongside cART, IL-7 treatment represents a novel therapeutic strategy promoting effective immune reconstitution by restoring immunological components in people with various lymphopenia.

De beschikbaarheid van effectieve combinatie antiretrovirale therapie (cART) heeft de levensverwachting van mensen met hiv enorm verbeterd en dit heeft geleid tot een groeiende populatie van mensen met hiv van middelbare leeftijd en ouder. In deze ouder wordende populatie zijn dankzij het gebruik van cART AIDS-gerelateerde aandoeningen sterk afgenomen, maar daarbij zijn leeftijdsgebonden niet-overdraagbare comorbiditeiten in deze groep juist toegenomen. De met hiv geassocieerde ontsteking wordt gedacht belangrijk bij te dragen aan het risico op een breed spectrum van dergelijke comorbiditeiten. Recente studies hebben laten zien dat de activatie van het aangeboren immuunsysteem, waaronder monocytten activatie, bijdraagt aan het ontstaan van deze comorbiditeiten, ook bij mensen met hiv die adequaat met cART worden behandeld. Deze voortdurende stimulatie van het aangeboren immuunsysteem wordt waarschijnlijk mede veroorzaakt door microbiële antigenen zoals lipopolysaccharide (LPS of endotoxine) die het lichaam kunnen binnen dringen doordat er onomkeerbare schade aan de darmwand en de achterliggende lymfoïde weefsels is ontstaan ten gevolge van de hiv-infectie.

In dit proefschrift wordt een reeks studies beschreven die onderzoeken hoe de darmflora en de mucosale immuniteit beïnvloed worden en bijdragen aan het ziektebeeld en ziekteprogressie, zowel tijdens siv-infectie in primaten, hiv-infectie in mensen, als in mensen met idiopathische CD4+ lymfocytopenie (ICL).

In mensen met hiv wordt een onevenwichtige darmflora (dysbiose) gezien als een mogelijke oorzaak van de persisterende inflammatie tijdens de chronische infectie. In **hoofdstuk twee** wordt het effect van een onevenwichtige darmflora op het ziekteverloop tijdens siv-infectie in resusapen bestudeerd. Hiervoor werden siv-geïnfecteerde resusapen behandeld met vancomycine wat

veranderingen in de darmflora veroorzaakt die vergelijkbaar zijn met de onevenwichtige darmflora tijdens hiv-infectie. Wij vonden dat met vancomycine behandelde en onbehandelde resusapen een vergelijkbare virale load, CD4⁺ T-cel depletie, immuun activatie, microbiële translocatie en overleving hadden. Deze studie laat zien dat een onevenwichtige darmflora op zichzelf niet per definitie hoeft bij te dragen aan de ziekteprogressie tijdens lentivirale infecties. Siv-infectie in resusapen wordt gezien als een goed model voor hiv-infectie in mensen, en ook de darmflora in resusapen en mensen is vergelijkbaar voor een aantal veel voorkomende bacteriële families (taxa), maar binnen deze taxa zijn er grote verschillen tussen de aanwezige bacteriële subfamilies. Onder meer om deze reden kunnen we niet uitsluiten dat ondergewaardeerde verschillen in het virus, de gastheer en bacteriële subfamilies op diverse manieren toch zouden kunnen bijdragen aan de ziekteprogressie tijdens lentivirus infectie in mensen en resusapen.

Studies in mensen en in dier modellen hebben laten zien dat het niet geheel duidelijk is hoe de samenstelling van de darmflora en de gastheer elkaar over en weer beïnvloeden, aangezien vele factoren waaronder genetische achtergrond, dieet, en seksueel gedrag een significante verandering in de samenstelling van de darmflora teweeg kunnen brengen. In **hoofdstuk drie** hebben we gekeken naar het effect van zowel seksueel gedrag als een behandelde hiv-infectie op de samenstelling van de darmflora. In een zorgvuldig opgezette case-control studie in effectief behandelde mensen met hiv en hiv-negatieve controles die deelnemen aan de AGE_hIV Cohort Studie in Amsterdam, is een uitgebreide analyse van de bacteriële samenstelling van fecale monsters uitgevoerd. Opgeslagen fecale monsters van de deelnemers werden geselecteerd op basis van geslacht (man en vrouw) en seksuele handelingen (vaginaal/anaal geslachtsverkeer met vrouwen/mannen), en de controles kwamen overeen met de cases voor leeftijd, seksueel gedrag,

BMI en geboorteland. We vonden dat de samenstelling van de darmflora in mensen met hiv significant verschilde van de controles en deze verschillen waren onafhankelijk van geslacht en seksuele handelingen. De darmflora van mensen met hiv was onder andere verrijkt voor *Gammaproteobacteria* en verarmd voor *Lachnospiraceae* en *Ruminococcaceae*, en had een verminderde alfa diversiteit. Mannen die seks hebben met mannen hadden een karakteristieke darmflora verrijkt voor *Prevotellaceae* en met een verhoogde alfa diversiteit. Deze verschillen konden gelinkt worden aan receptieve anale geslachtsgemeenschap zowel bij mannen als vrouwen. Tot slot, de hiv specifieke samenstelling van de darmflora was gecorreleerd aan inflammatoire biomarkers waaronder suPAR, nadir CD4⁺ T-cel aantal en het voorkomen van leeftijdsgebonden niet-overdraagbare comorbiditeiten.

Het naar behoren functioneren van het mucosale immuunsysteem van de darm wordt sterk beïnvloed door de interactie met de darmflora, maar ook door medicijn gebruik. In **hoofdstuk vier** hebben we onderzocht of hetgebruik van antiretrovirale middelen op zich invloed zou kunnen hebben op de darmflora en het functioneren van het immuunsysteem. Voor dit onderzoek werden gezonde resusapen gedurende 1-2 of 5-6 weken behandeld met protease-, integrase- of reverse transcriptase remmers, en werd het effect van deze middelen op het immuunsysteem van de darm en de samenstelling van de darmflora onderzocht. Onze studie liet zien dat de individuele antiretrovirale remmers slechts een relatief klein effect op de inflammatoire T-cel response in de darm hadden en er konden geen veranderingen in de frequenties van de totale en de geactiveerde T-cellen worden aangetoond. Daarnaast vonden we wel een significante verandering in de diversiteit van de darmflora waarbij er tevens een verstoring was in de verhouding van een aantal bacteriële families. De veranderingen van de darmflora bleven echter niet aanwezig na het stoppen

van de behandeling, alhoewel er wel een associatie werd gevonden tussen het voorkomen van sommige individuele bacteriestammen en de pro-inflammatoire T-cel responsen. Wij concludeerden dat antiretrovirale middelen de samenstelling van de darmflora kunnen beïnvloeden, maar slechts beperkte effecten lijken te hebben op het immuunsysteem.

In **hoofdstuk vijf** hebben we geanalyseerd hoe snel na hiv infectie er veranderingen optreden in de darmflora en in hoeverre mogelijke veranderingen in de darmflora herstellen door het kort na infectie starten met cART. Deze studie werd uitgevoerd in een groep patiënten die deelnamen aan het RV254/SEARCH010 cohort in de Thai Red Cross Anonymous Clinic in Bangkok, Thailand. Het betreft een cohort studie waarin mensen worden geïncludeerd bij wie een hele vroege hiv-infectie (Fiebig fase 1–5) is vastgesteld. Voor deze analyse is er in een groep mannen die seks hebben met mannen, op het moment van hiv-diagnose en 6 maanden na de start van de cART en in een hiv-negatieve controlegroep, gekeken naar de relatie tussen de samenstelling van de darmflora in feces, en biomarkers van algehele inflammatie en de integriteit van de darmwand. In hiv-geïnfecteerde mannen werd vlak na de hiv-diagnose een beperkte verandering in de samenstelling van de darmflora gezien in vergelijking met de hiv-negatieve controles. Tijdens de acute fase van de hiv-infectie werd de overgang van Fiebig fase 1 naar 3 gekenmerkt door een verrijking van pro-inflammatoire bacteriën. Na de start van de cART werd een verschuiving in de darmflora waargenomen in de richting van een verminderde frequentie Bacteroidetes en een verhoogde frequentie van Proteobacteria en Fusobacteria families. De toename van Fusobacteria families in de darmflora na de start van de cART was geassocieerd met sCD14 concentraties in bloedplasma. Deze associatie suggereert dat de relatie tussen veranderingen in de darmflora en algehele inflammatie al tijdens de acute fase van de hiv-infectie ontstaat.

Naast de reguliere cART is er behoefte aan aanvullende behandelingen om de functie van het immuunsysteem en de darmflora verder te doen herstellen. In **hoofdstuk zes** evalueren wij in hoeverre een behandeling met IL-7 het aantal Mucosal Associate Invariant T- (MAIT) cellen in bloed van met cART behandelde chronisch hiv-geïnfecteerde mensen kan doen herstellen. Deze MAIT-cellen vormen een kleine populatie binnen de T-cellen en hebben een anti-bacteriële functie. We zagen een significante toename in het aantal MAIT-cellen in het bloed van chronisch hiv-geïnfecteerde mensen 12 weken na de IL-7 behandeling. IL-7 behandeling is derhalve mogelijk een behandelstrategie die verder kan worden onderzocht en kan dienen om het aantal MAIT-cellen te herstellen. Hiermee kan de immuniteit bij mensen met hiv mogelijk verder worden hersteld en daarmee het risico op andere infecties ook verder worden verkleind.

Mensen met een idiopathische CD4⁺ lymfocytopenie (ICL), een syndroom gekarakteriseerd door een laag CD4⁺ T-cel aantal in het bloed (<300/ μ L) en een verhoogde gevoeligheid voor opportunistische infecties, hebben net als hiv-geïnfecteerde mensen lage CD4⁺ T-cel aantallen in de darmen maar ze vertonen geen algehele inflammatie. In **hoofdstuk zeven** hebben we onderzoek gedaan naar de aanwezigheid, het fenotype en de functionaliteit van MAIT-cellen in bloed van ICL-patiënten. In deze patiënten vonden we dat het aantal, het fenotype en de functie van de MAIT-cellen vergelijkbaar was met gezonde controles. Het toedienen van IL-7 resulteerde in een verhoging van het aantal MAIT-cellen, met name van de CD8⁺ MAIT-cel subpopulatie.

In **hoofdstuk acht** bestuderen we de immuunmodulerende eigenschappen van recombinant humaan lactoferrine en het effect van dit middel op de samenstelling van de darmflora in een cross-over placebogecontroleerde studie bij met cART behandelde mensen met hiv en een goed

onderdrukte virale load. Orale toediening van lactoferrine was veilig, maar er werd geen effect op inflammatie en immuun activatie gezien. We zagen geen effecten van lactoferrine op de plasmaconcentraties van IL-6 en D-dimeer, op het niveau van monocyt en T-cel activatie, op de integriteit van de darmwand, en op de diversiteit van de darmflora.

In de algemene discussie (**hoofdstuk 9**) plaatsen we onze bevindingen in perspectief. Mensen geïnfecteerd met hiv hebben al tijdens de vroege fase van de acute infectie tekenen van een onevenwichtige darmflora. Een hiv-infectie veroorzaakt een specifieke verandering van de darmflora waarbij een verrijking van pro-inflammatoire bacteriën wordt gezien, met daarnaast een verarming van bacterie families met anti-inflammatoire eigenschappen welke bijdragen aan het behoud van darm homeostase. In de groep mannen die seks hebben met mannen was gedrag waarbij de kans op een hiv-infectie verhoogd is geassocieerd met een specifieke verandering in de samenstelling van de darmflora. Dit geeft aan dat de selectie van een goede hiv-negatieve controlegroep van groot belang is voor dit soort studies.

Het starten van de cART tijdens de vroege fase van een acute hiv-infectie liet zien dat de onevenwichtige darmflora deels herstelde en dit benadrukt nogmaals het belang van het vroeg starten van de hiv-behandeling. Echter het vroeg starten van de cART leidt niet tot een volledig herstel van de darmflora, het immuunsysteem en inflammatie van de darm. Naast cART, lijkt een behandeling met IL-7 een mogelijke nieuwe strategie om, naast cART, het immuunsysteem bij mensen met hiv verder te doen herstellen, maar wellicht ook bij mensen met een lymfocytopenie ontstaan door andere oorzaken.

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