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Research Paper

Fecal Microbiota Composition Drives Immune Activation in HIV-infected Individuals

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ABSTRACT

The inflammatory properties of the enteric microbiota of Human Immunodeficiency Virus (HIV)-infected individuals are of considerable interest because of strong evidence that bacterial translocation contributes to chronic immune activation and disease progression. Altered enteric microbiota composition occurs with HIV infection but whether altered microbiota composition or increased intestinal permeability alone drives peripheral immune activation is controversial. To comprehensively assess the inflammatory properties of HIV-associated enteric microbiota and relate these to systemic immune activation, we developed methods to purify whole fecal bacterial communities (FBCs) from stool for use in *in vitro* immune stimulation assays with human cells. We show that the enteric microbiota of untreated HIV-infected subjects induce significantly higher levels of activated monocytes and T cells compared to seronegative subjects. FBCs from anti-retroviral therapy (ART)-treated HIV-infected individuals induced intermediate T cell activation, indicating an only partial correction of adaptive immune cell activation capacity of the microbiome with ART. *In vitro* activation levels correlated with activation levels and viral load in blood and were particularly high in individuals harboring specific gram-positive opportunistic pathogens. Blockade experiments implicated Tumor Necrosis Factor (TNF)-α and Toll-Like Receptor-2 (TLR2), which recognizes peptidoglycan, as strong mediators of T cell activation; This may contradict a previous focus on lipopolysaccharide as a primary mediator of chronic immune activation. These data support that increased inflammatory properties of the enteric microbiota and not increased permeability alone drives chronic inflammation in HIV.

1. Introduction

Individuals with Human Immunodeficiency Virus (HIV)-infection display increased gut and systemic immune activation (French et al., 2009; Liovat et al., 2012). Chronic immune activation influences disease progression and persistence by enhancing viral replication and has also been linked with cardiovascular disease (Nou et al., 2016), chronic obstructive pulmonary disease (Crothers, 2007; Fitzpatrick et al., 2013) and frailty (Desquilbet et al., 2007). Chronic immune activation is thought to be mediated in part by translocation of bacterial products, such as lipopolysaccharide (LPS) and peptidoglycan (PGN) following an HIV-associated breakdown of gut mucosal barrier function (Brenchley and Douek, 2008, Brenchley et al., 2006). Elevated levels of plasma LPS correlate with monocyte and T-cell activation, propagation of HIV infection and T cell depletion (Brenchley and Douek, 2008, Marchetti et al., 2013, Ericsen et al., 2016, Ancuta et al., 2008). It is unclear whether compositional changes of the gut microbiota or other factors, such as damage caused by the virus itself, effect bacterial translocation and chronic immune activation in the periphery.

Culture-independent surveys of stool and mucosal biopsies have established that the enteric microbiome in HIV-infected individuals is compositionally distinct from HIV negative subjects (Lozupone et al., 2013, Lozupone et al., 2014, Mutlu et al., 2014, Vujkovic-Cvijin et al., 2013, Paquin-Proulx et al., 2017, McHardy et al., 2013, Dinh et al., 2015, Perez-Santiago et al., 2013, Noguera-Julian et al., 2016, Dillon et al., 2014, Pinto-Cardoso et al., 2017). However, results of many of these studies are confounded by microbiome differences in men who have sex with men (MSM). Specifically, several reports have linked stool microbiomes relatively high in the genus Prevotella and low in Bacteroides with HIV, but recent reports have demonstrated that a Prevotella rich microbiota is prevalent in MSM regardless of HIV status (Noguera-Julian et al., 2016; Kelley et al., 2017). In cohorts not confounded by MSM, relatively subtle differences in fecal microbiota composition have been found with HIV in the absence of CD4+ T cell

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counts indicative of AIDS (Noguera-Julian et al., 2016; Monaco et al., 2016), although more pronounced changes may be evident in mucosal biopsy of individuals not on anti-retroviral therapy (ART) (Vujkovic-Cvijin et al., 2013). Several studies demonstrated correlations between specific enteric bacteria and immune activation markers in the gut and blood of HIV-infected subjects (Dillon et al., 2016a; Vujkovic-Cvijin et al., 2013). However, correlations can be driven by indirect factors and do not establish causality. To establish whether correlative bacteria may be direct drivers of inflammation, *in vitro* stimulations of host peripheral blood mononuclear cells (PBMC) and lamina propria mononuclear cells (LPMC) with heat-killed cultured bacteria that differ with HIV has been utilized (Lozupone et al., 2013; Dillon et al., 2016a). However, these investigations have been limited because they 1) focused on individual bacterial species that represent a very small component of the complex microbiome, 2) obtained these organisms from culture collections rather than patient samples, 3) selected these species as HIV-associated in cohorts confounded by MSM, and 4) never showed that levels of activation induce by bacteria *in vitro* were related to *ex vivo* immune activation seen in the study population.

To determine whether gut microbiota with particularly pro-inflammatory components may drive high immune activation in individuals with HIV, we developed a method for purifying the whole set of intact microbial cells from stool. We stimulated PBMC and LPMC with these heat-killed fecal bacterial communities (FBCs) to establish the immune-modulatory properties of their collective components, which could include LPS, PGN, capsular components or other metabolic products. We then compared the results from stimulation with FBCs from MSM, men who have sex with woman (MSW) and women with and without HIV and ART. Immune assays with bacterial components and PBMC are relevant to HIV disease since it is translocation of microbial components to the periphery and not necessarily active growth of translocated bacteria in blood (*e.g.* bacteremia) that is thought to drive systemic immune activation in individuals with HIV. We then related our *in vitro* measurements of FBC-induced innate and adaptive immune activation to measurements of immune activation and viral load in the blood of our study population, demonstrating that microbiota with more pro-inflammatory components may be an essential driving factor in chronic immune activation. This system allowed us to further identify microbes and signaling pathways that may be driving factors of chronic inflammation in HIV infected individuals.

2. Methods

2.1. Study Subjects

Stool samples were obtained from seven HIV-infected males who were ART naïve (no prior treatment with antiretrovirals) and eleven HIV-infected males who were ART experienced (antiretroviral treatment with a minimum of three antiretroviral drugs for ≥12 months prior to study entry with plasma HIV-1 RNA below the limit of detection for >6 months). Male HIV-infected individuals enrolled in the study were determined to be MSM using a behavioral questionnaire. Control cohorts included low-risk HIV-seronegative heterosexual males ($n = 6$), low risk females ($n = 5$) and high-risk MSM ($n = 13$) who were recruited from a high-risk cohort assembled for a study of a candidate HIV-1 preventative vaccine (Hammer et al., 2013). Designation of high-risk could have been related to a number of different behaviors including 1) a history of unprotected anal intercourse with one or more male or male-to-female transgender partners 2) anal intercourse with two or more male or male-to-female transgender partners and 3) being in a sexual relationship with a person who has been diagnosed with HIV. All enrolled individuals live in Metropolitan Denver. Individuals were excluded during recruitment if they were pregnant, weighed <110 pounds, or had received antibiotics within the prior 30 days.

2.2. Ethics Statement

Written informed consent was obtained from healthy HIV-seronegative individuals and from HIV-infected individuals and the study protocol was approved by the Colorado Multiple Institution Review Board (COMIRB No: 14-1595). All subjects were adults.

2.3. Sample Collection and Stool Isolate Preparation

Stool samples were collected by the patient, both on a sterile swab and with a sterile scoop within 48 h of a clinic visit. Patients immediately stored the samples in a cooler with −20 °C freezer packs. After delivery to the clinic, the swab was subsequently stored at −80 °C to await DNA extraction for 16S rRNA targeting sequencing. To separate the microbiota from the rest of the fecal material, stool was subjected to density gradient according to the previously described method (Hevia et al., 2015) with some modifications. Specifically, 2 g of feces was homogenized in 40 ml sterile PBS by aggressive vortex for ~1 min. The homogenized fecal sample was passed through a 100 μm filter. 80% Histodenz (Sigma, St. Louis MO) was prepared in PBS and was sterilized by autoclave at 121 °C for 15 mins. 20 ml of filtered homogenized fecal sample was overlaid on 5 ml of the 80% Histodenz solution in 2 separate tubes and centrifuged at 10,000*g* for 40 min at 4 °C. The interphase layers corresponding to the microbiota were transferred to a 50 ml tube and were washed, resuspended, overlaid on 5 ml of 80% Histodenz solution and centrifuged again at 10,000*g* for 20 min at 4 °C. The top layer was discarded and the microbiota layers were extracted to a new tube and washed with 10 ml PBS and centrifuged at 10,000*g* for 20 min. The visible pellet comprised of white bacteria. The bacteria pellet was resuspended in 25 ml PBS and the presence of bacteria was confirmed by viewing a small aliquot on a glass slide. The number and viability of bacterial cells were determined using the BD Cell Viability and counting Kit (BD Biosciences) and samples that were <5:1 bacteria to debris were excluded. Since we determined that fresh (not previously frozen) sample tended to produce FBCs with less debris, we modified our stool collection procedure; the portion of fecal sample used to produce the FBCs was shipped in an insulated tube so that it remained cold but did not freeze during shipping, and processed the samples immediately upon arrival when possible. Isolated FBCs were resuspended at 500,000 bacteria per μl, subjected to multiple freeze/thaw or autoclave treatment to prevent bacterial growth, and aliquoted and stored at −80 °C. The bacterial composition of the FBCs was evaluated by 16S rRNA sequencing and compared to autologous whole stool (Fig. 2).

2.4. DNA Sequencing

16S rRNA targeted sequencing was conducted according to earth microbiome project standard protocols (http://www.earthmicrobiome.org). DNA was extracted from the fecal swab and from a 250 μl aliquot of the stool isolate prep using the standard PowerSoil protocol (Qiagen) with one modification; the vortexing step was replaced with bead-beating for 1 min. PCR amplification of the extracted DNA, along with water controls, was conducted with barcoded primers targeted the V4 region of the 16S rDNA gene (515F-806R; FWD:GTGCCAGCMGCCGCGGTAA; REV:GGACTACHVGGGTWTCTAAT). Amplified DNA was quantified using a PicoGreen assay (Invitrogen) and equal amounts of DNA from each sample were pooled and cleaned using the UltraClean PCR Clean-up protocol (Qiagen). The final DNA pool was sequenced using the Illumina MiSeq platform (San Diego, CA) using the V2 2 × 250 kit. All sequences and associated metadata have been deposited at the European Nucleotide Archive (ERP107331).

2.5. Sequence Data Analysis

Raw sequences were assigned to samples based on their barcodes using Qiime 1.9 (Caporaso et al., 2010). The libraries were denoised