Liver bacterial dysbiosis occurs in SIV-infected macaques and persists during antiretroviral therapy

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Abstract

Background: Liver disease remains a significant contributor to morbidity and mortality in HIV-infected individuals, even during successful treatment with combination antiretroviral therapy (cART). In non-human primates, SIV infection is associated with gut microbiome dysbiosis as well as bacterial translocation into the colonic lamina propria and liver via the portal vein. Here the liver microbiome was evaluated in rhesus macaques to discern the influence of SIV infection alone (SIV+) and during cART administration (SIV+cART) on liver bacterial dysbiosis and neutrophil infiltration.

Results: Dysbiosis in liver bacterial composition was observed, encompassing changes in a number of genera, during SIV infection in the absence and presence of cART. The most striking finding was an increase in the level of *Mycobacterium*, which while barely detectable in the uninfected macaques, was the most abundant genus observed in the livers of a majority SIV+ and SIV+cART macaques. Multi-gene sequencing analyses identified a species of environmental mycobacteria similar to the opportunistic pathogen *M. smegmatis*. The effect of *M. smegmatis* on host gene expression in primary hepatocytes was evaluated *in vitro* utilizing PILAM, a glycolipid cell wall component found in atypical Mycobacteria. PILAM induced an upregulation of inflammatory responses, including an increase in the chemokines associated with neutrophil chemotaxis (CXCL1, CXCL5, and CXCL6). Assessment of the macaque livers by microscopy determined that neutrophil levels were reduced in SIV+cART macaques, suggesting that the SIV infection and/or cART treatment influence the liver-associated neutrophil response.

Conclusions: A number of liver bacteria genera were altered following SIV infection even in the context of cART, possibly as a consequence of reduced neutrophil recruitment. Mycobacteria became a major component of the SIV infected macaque liver microbiome, raising the possibility that bacteria of this genus might contribute to liver disease in HIV infected patients.

Background

HIV infection continues to be a major public health concern with approximately 38 million people living with HIV at the end of 2019 [1]. With the introduction of highly effective combination antiretroviral therapy (cART), persons infected with HIV now have a life expectancy that is closer to that of the general population, particularly in higher income countries [2, 3]. Nevertheless, HIV-infected (HIV+) individuals experience a greater burden of co-morbidities, often at a markedly younger age, including cardiovascular disease, frailty, cognitive decline and liver disease [4–6]. Among these chronic health conditions, liver disease is especially prevalent, most notably non-alcoholic fatty liver disease (NAFLD), and is a leading cause of death in HIV + individuals [7, 8]. The factors that initiate hepatic inflammation and consequently contribute to the development of liver disease during HIV infection are poorly defined and are likely multifactorial. Several observational studies have linked HIV viral load to liver disease [9–11], suggesting that viral stimulation plays a role. Studies into HIV-associated immune mechanisms have implicated alteration in gene expression and subsequent induction of inflammatory cytokines and chemokines in several cell types, including Kupffer cells, hepatic stellate cells and hepatocytes [12]. However, the HIV

virus alone does not account for increased liver disease prevalence, as even persons virally suppressed with cART continue to experience high rates of liver disease [13]. Hepatotoxicity can contribute to liver disease during cART, and the use of certain nucleoside reverse transcriptase inhibitors (NRTIs) is associated with advanced liver disease [14, 15]. However, liver disease continues to affect HIV + patients despite the reduced hepatotoxicity of modern cART regimens. Taken together, this suggests that additional factors may be contributing to liver dysfunction during HIV infection.

The liver is the primary site of bacterial clearance from blood exiting the gut, prior to entering systemic circulation. In healthy individuals, this process is one of immune tolerance. However, during disease, in which the liver may experience either elevated bacterial load or exposure to dysbiotic bacteria, the liver responds in an inflammatory nature [16]. Central to this inflammatory response are Kupffer cells, resident liver macrophages that are primarily responsible for clearance of microbial products from portal blood [17]. Upon engagement of innate receptors (e.g. toll-like receptors (TLRs)) on these cells by microbial products, inflammatory and profibrotic mediators are produced, such as TNF- α , IL-12, IL-6 and TGF- β [18]. Neutrophils are recruited to the liver and aid in the clearance of bacterial products through the release of reactive oxygen species and pro-inflammatory cytokines [16, 19]. Hepatocytes are impacted by bacterial stimulation and experience altered gene expression indicative of metabolism imbalance and production of inflammatory mediators [20, 21], key features of NAFLD. Consistent with these findings, several clinical studies have established the role of microbial translocation in the context of alcoholic liver disease and in NAFLD [22]. Given that bacterial translocation is associated with HIV disease progression and systemic immune activation, and NAFLD is one of the key hepatic disruptions observed during HIV infection [23], it is conceivable that bacteria-associated liver inflammation may play a role in HIV-associated liver disease.

Understanding of the gut microbiome and immune changes during simian immunodeficiency virus (SIV) infection have advanced the understanding of gut-driven systemic immune activation and HIV disease progression [24]. The microbiome is influenced by numerous factors such as age, environment, food and antibiotics [25–29]. With regard to the liver during SIV infection, prior studies have identified elevated bacterial loads during SIV infection [30, 31], as well as in the context of cART [32], and have shown that bacteria may increase immune cell infiltration [30]. During cART-treated SIV infection, the liver microbiome has been shown to be enriched for inflammatory Proteobacteria that preferentially translocate out of the gut and into the colonic lamina propria [24]. Altogether, these studies demonstrate a role for both bacterial load and bacterial composition in liver dysfunction during SIV infection, even in the context of successful cART therapy. Importantly, characterization of hepatic bacteria to lower taxonomic levels, such as species identification, has not been reported. Here SIV-infected (SIV+) macaques are characterized with regard to their liver microbiomes using 16S rRNA gene sequencing to identify prevalent bacteria, and the results are linked with changes in immune cell subsets within dysbiotic SIV + livers. These studies help advance the knowledge of bacteria-associated liver inflammation during SIV infection by providing information regarding an association between hepatic inflammation and the presence of dysbiotic microbes.

Methods

Ethics Statement

All animal studies were directed in accordance to protocols approved by the Center for Infectious Disease Research (now Seattle Children's Research Institute; CIDR protocol DS-05 UW), and Washington National Primate Research Center, Seattle, WA (protocols 4314-01, 4213-02 and 4213-03) under the Institutional Animal Care and Use Committees (IACUCs). All rhesus macaques involved in this study were managed according to the laws, regulations, and guidelines set forth by the United States Department of Agriculture, Institute for Laboratory Animal Research, Public Health Service, National Research Council, Centers for Disease Control, the Weatherall Report titled "The use of nonhuman primates in research", and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. Nutritional plans utilized by WaNPRC consisted of standard monkey chow supplemented with a variety of fruits, vegetables, and other edible objects as part of the environmental enrichment program established by the Behavioral Management Unit. Enrichment was distributed and overseen by veterinary staff with animals having access to more than one category of enrichment. Uninfected macaques exhibiting incompatible behaviors were managed by the Behavioral Management staff and managed accordingly. SIV + macaques were kept in individual, adjoining cages allowing for social interactions with primate health observed daily by trained staff. All efforts were made to minimize suffering using minimally invasive procedures, anesthetics, and analgesics when deemed appropriate by veterinary staff. Animals were painlessly euthanized by sedation with ketamine hydrochloride injection followed by intravenous barbiturate overdose following the recommendations of the panel of euthanasia of the American Veterinary Medical Association. These macaques have been described previously [32].

Liver Tissue Collection

Liver tissue was collected at necropsy from uninfected (N = 4), SIV+ (N = 6) and SIV + cART (N = 6) adult Indian rhesus macaques (*Macaca mulatta*). Control samples from uninfected macaques were acquired from the Tissue Donor Program at WaNPRC. SIV + macaques were infected intrarectally with SIVmac239x [32]. Macaques receiving cART were administered subcutaneous tenofovir (20 mg/kg body weight) and emtricitabine (30 mg/kg) and oral raltegravir (50 mg twice daily) starting 120 days post-infection and continuing for 35–36 weeks prior to euthanasia [32]. Tissue was formalin-fixed, paraffin-embedded for microscopy or flash-frozen in liquid nitrogen and then stored at -80 °C for nucleic acid extraction.

Immunofluorescence Staining

Liver tissue was obtained at necropsy (14–55 weeks post infection), fixed in 10% formalin, and paraffin-embedded. Slides were prepared from 5µ m tissue sections and dewaxed with xylene and rehydrated with a gradient of ethanol baths. Antigen retrieval was performed with a 1% citrate buffer (Antigen Unmasking Solution, Vector Laboratories, Burlingame, CA) in a decloaking chamber at 90 °C for 30 minutes with a 10-minute cooldown. Slides were washed in a series of TBST (0.025% Triton X-100 in 1X TBS) followed by a 2-hour block step (0.1% BSA, 1% goat serum in TBST). Tissues were stained overnight at 4 °C for mouse

anti-human myeloperoxidase (MPO, polyclonal, 1:2000, Dako) and rabbit anti-human CD68 (clone KP1, 1:250, Santa Cruz Biotechnology, Dallas, TX). Slides were then washed in another series of TBST washes and incubated with secondary antibody for one hour in the dark using AlexaFluor 488 goat anti-mouse (1:500, Life Technologies, Carlsbad, CA) to detect MPO + neutrophils and AlexaFluor 594 goat anti-rabbit (1:500, Life Technologies) to detect CD68 + macrophages. After incubation, slides were washed in a series of TBST washes and mounted with Vectashield Hard set DAPI (Vector Technologies) and allowed to set. For each liver section, eight random fields were imaged at 200x magnification. Cells were counted using ImageJ.

Liver Tissue Disruption By Pulverization

Flash-frozen liver tissue was pulverized into a fine powder by ball milling with stainless balls under cryogenic conditions with liquid nitrogen (Retsch Planetary Ball Mill, Retsch Laboratory Equipment, Haan, Germany). Each sample was subjected to three cycles at 300 rpm for two minutes each. Following pulverization, the liver powder was collected and stored at -80 °C until DNA extraction.

Tissue DNA Extraction from Liver Powder

Liver powder (10–30 mg) was placed into a sterile, pre-chilled microcentrifuge tube. Genomic DNA was extracted using the NucleoSpin Tissue DNA extraction kit (Takara, Mountain View, CA) per the manufacturer's instructions, where samples were pre-lysed and allowed to incubate at 56°C for at least 1–3 hours vortexing occasionally. Samples were then lysed with provided buffer, vortexed vigorously, and incubated at 70°C for 10 minutes. Ethanol was added and samples were centrifuged in NucleoSpin Tissue Columns into a collection tube at 11,000 x g for 1 minute. After a series of washes, samples were eluted with elution buffer and collected. Following concentration determination with a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA), isolated genomic DNA was stored at -80 °C until use.

16s rRNA Gene Sequencing and Microbiome Analysis

Genomic DNA extracted from the liver (20 µL) was used for 16 s rRNA gene sequencing through Illumina according to the EMP method. In brief, a 460-bp amplicon was generated targeting the V3-V4 region of the 16 s rRNA gene. PCR amplicons were cleaned with 0.8x AMPure XP beads (Beckman Coulter, Brea, CA) before the addition of Nextera XT dual index adaptors (Illumina Inc., San Diego, CA). Indexed amplicons were cleaned using 1.1 × AMPure XP beads (Beckman Coulter), quantified using a Qubit DNA high-sensitivity assay kit (Life Technologies), and multiplexed using an equal molar ratio of DNA for each sample. 16S rRNA gene libraries were loaded on a 300-cycle MiSeq kit and sequenced using Nextera sequencing read and index primers (all from Illumina Inc.). Paired-end demultiplexed FASTQ files from the Illumina base space were imported into the QIIME2 pipeline (QIIME 2 Core 2019.10) to create a demultiplexed QIIME2 object. These objects were matched to identified amplicon sequence variants (ASVs) using the dada2 algorithm which worked to detect and correct Illumina amplicon sequence data and denoise by trimming to 145 bases to remove low-quality regions. A rooted phylogenetic tree was

constructed using the Mafft multiple sequencing alignment program and taxonomy was assigned using the Greengenes database specific to the V3-V4 region. After taxonomy was determined, results were exported from the pipeline for downstream analysis in R using the phyloseq package.

Quantification of *Mycobacterial* DNA in the Liver by qPCR

All liver DNA samples were diluted in nuclease-free water. Each sample (5 μ L) was prepared in duplicate in a 20 μ L volume reaction with the PowerUp SYBR Green Master Mix kit (Applied Biosystems, Waltham, MA) and *Mycobacterium*-specific primers (MycoARB210: TTT GCG GTG TGG GAT GGGC and MycoARB585: CGA ACA ACG CGA CAA ACCA). A 'No Template' Negative Control was included to control for contamination and non-specific amplification. A standard curve was generated by serially diluting pure *M. bovis* (BCG) DNA 10-fold, ranging from 10-0.001 ng/ μ L ($R^2 > 0.95$). PCR reactions ran one cycle at 50 °C for 2 minutes then increasing to 95 °C for 2 minutes followed by 45 cycles of 94 °C for 15 seconds, annealing at 61 °C for 30 seconds, and extending at 72 °C for 30 seconds with a final extension step at 72 °C for 7 minutes. Following the qPCR cycles, PCR reactions were subjected to a melt curve analysis to examine products formed. The concentration of *Mycobacterium* per sample was determined through a non-linear regression on the standard curve and converted to copy number based on BCG molecular weight (5.63×10^{12} mg/mole). The weight was then converted to 4.277×10^7 molecules/mole and the standard curve was plotted based on molecules where copy number was equal to 4.277×10^7 molecules/mole * log (CT) where the standard curve equation was extrapolated ($y = -0.032\ln(x) + 1.8377$). Copy number of the liver *Mycobacterium* DNA was then calculated from the standard curve equation (copy number = $e((\log(Ct) - 1.8377)/-0.032)$). Duplicates were averaged for each animal. qPCR to detect a conserved region of the 16S rRNA gene was performed as reported previously [32].

Identification of *Mycobacterium* Species by Multi-Gene Sequencing

Genomic DNA (extracted as described above) was diluted in nuclease-free water and amplified by nested PCR per the conditions outlined in Additional File 1. For each first-round PCR reaction, 500 ng (5 μ L) of gDNA was added into a 50 μ L reaction and amplified using the Platinum *Taq* DNA Polymerase reaction kit (Invitrogen, Carlsbad, CA). For nested PCR reactions, 1 μ L of the first-round PCR product was added to a 50 μ L reaction containing the nested primers and Platinum *Taq* DNA Polymerase. Each round of PCR contained a positive control of BCG DNA and a negative no template control. Following nested PCR, each reaction was examined on a 1% agarose gel. Each PCR amplicon showing the correct size was cleaned up using a Nucleospin PCR Clean-up Kit (Takara) and eluted into 30 μ L of EB buffer. Purified PCR amplicons (20 ng) were sent for Sanger sequencing using both forward and reverse nested primers in separate reactions. Following sequencing, DNA sequence quality was examined in 4Peaks software and low-quality reads from the 5' and 3' ends removed. Consensus sequences generated through the alignment of forward and reverse reads were analyzed using BLAST analysis.

Culture and Stimulation of Human Hepatocytes with *Mycobacteria* and *Mycobacterial* Antigens

Human HepaCure Hepatocytes on Matrigel overlay (350,000 hepatocytes/well) were acquired from Yecuris (Tualatin, OR) in 24-well dishes. HepaCure human hepatocytes are produced by the immunization of humanized FRG@KO mice with cadaver-derived human hepatocytes. Upon receipt, the media was immediately replenished with 500 μ L InVitro GRO Hi Medium (BioIVT, Westbury, NY) supplemented with Torpedo Antibiotic Mix (BioIVT). Cultures were incubated at 37 °C, 5% CO₂ overnight. To determine the hepatocyte response to mycobacterial pathogen associated molecular patterns (PAMPs), *M. smegmatis* purified lipoarabinomannan (PILAM, 0.1 and 10 μ g/mL, BEI Resources, Manassas, VA), or *M. tuberculosis*, Strain H37Rv, purified lipoarabinomannan (ManLAM, 0.1 and 10 μ g/mL, BEI Resources), were added to hepatocytes. Each stimulation condition was conducted in triplicate. Plates were incubated at 37 °C, 5% CO₂ for 24 hours. For live mycobacteria stimulations, *M. smegmatis* bacteria (strain MC²155) were grown to exponential phase, washed with PBS and resuspended in InVitroGRO Hi Medium without antibiotics at 350,000 bacteria/ μ L. Hepatocytes were stimulated with *M. smegmatis* (MOI 10) in duplicate for 24 hours at 37 °C, 5% CO₂. For all stimulations, conditioned media was collected and stored at -80 °C. The hepatocyte monolayer was then washed with 500 μ L pre-warmed PBS and then lysed in 300 μ L RA1 buffer containing beta-mercaptoethanol. RNA was isolated from the cell lysate following protocols from the NucleoSpin RNA isolation kit (Macherey-Nagel, Bethlehem, PA).

Transcriptomic Analysis of HepaCure Hepatocytes by Nanostring

RNA was diluted to 20 ng/ μ L in nuclease-free water and used for transcriptomic analysis using a Nanostring Inflammation Panel (Human v2) (Nanostring, Seattle, WA). Probe set-target RNA hybridization reactions were performed according to the manufacturer's protocol using 100 ng (5 μ L) of total RNA. Purified probe set-targets were processed and immobilized on nCounter Cartridges using a nCounter MAX prep station. Transcripts of interest were quantified on the Digital Analyzer for each sample. For data analysis, nCounter RCC files were imported in nSolver Analysis Software 4.0 and checked for quality control. Determination of differentially expressed genes, pathways analysis, and cell profiling was conducted using the Nanostring Advanced Analysis software per the manufacturer's instructions. For each stimulation condition, differentially expressed genes were determined by comparing the normalized count data between stimulated hepatocytes and unstimulated control hepatocytes. Heatmaps were generated in Prism version 5.0f software (GraphPad Software, Inc., San Diego, CA), showing fold change of each gene in the panel. Volcano plots were assessed using the python matplotlib package for significant genes using a threshold of 1.5-fold change ($\log_2(1.5) = 0.585$) and 0.05 adjusted p-value.

Statistics

Statistical analyses were performed using Prism version 5.0f software (GraphPad Software, Inc.). A nonparametric Mann-Whitney U test was used to compare the SIV + and SIV + cART groups to the uninfected controls. Linear regression and Spearman correlation analyses were performed. Analysis of gene expression panels was completed using nSolver (Nanostring, version 4.0.62).

Results

The Liver Microbiome during SIV Infection

The liver microbiome was evaluated in macaques that were uninfected, SIV+, and SIV-infected-cART treated (SIV + cART) through 16S rRNA gene sequencing and analysis [32]. While macaques displayed variation in the liver microbiome, some overarching similarities were observed, including the presence of *Pseudomonas*, *Bacillus*, *Stenotrophomonas*, *Massilia*, and *Delftia* in the livers of many of the macaques (Fig. 1A-C). In uninfected macaques, the highest percentage of sequences that could be classified were *Stenotrophomonas*, a diverse genus with a wide range of species. However, this genus constituted less than 25% of sequences, and the majority of sequences in the uninfected macaques fell into the 'Other' classification, which includes all genera outside of the nineteen most abundant. Therefore, the uninfected macaques had a diverse liver microbiome with no dominant pervasive genus (Fig. 1A). In contrast, in the SIV + group, there was an increase in the proportion of sequences in the *Mycobacterium* genus (Fig. 1B). In addition to *Mycobacterium* in the SIV + livers, many bacterial genera that are common in the macaque intestinal microbiome, including *Acinetobacter*, *Prevotella*, *Lactobacillus*, and *Bacillus* [24, 33], were also found to be in abundance (Figs. 1 & 2). Following cART treatment, *Mycobacterium* still remained as one of the most prevalent genera identified within the livers of the macaques (Fig. 1C). Other notable differences observed between the groups included a decreased relative abundance of the *Lactobacillus* and *Blautia* genera in SIV + macaques that persisted with cART, and an increased abundance of *Pseudomonas* in SIV + cART macaques (Figs. 1 & 2). However, *Mycobacterium* stands out, as when evaluating relative abundance, *Mycobacterium* was the most abundant bacterial genus found in the liver in SIV + and SIV + cART macaques, while it was present at extremely low levels in the uninfected macaques (Fig. 2). Importantly, although the percentage of bacteria attributed to the *Mycobacterium* genus varied within the SIV + and SIV + cART groups, *Mycobacterium* DNA was identified within every liver sample tested in each of these groups (with A14050 (SIV+), Z09068 (SIV+) and A13275 (SIV + cART) having the highest percentage of *Mycobacterium* sequences present) (Fig. 1B-C).

Differences in microbiome alpha diversity were also apparent between the macaque groups (Fig. 3A-B). Examination of microbial richness indicated that SIV+ (but not SIV + cART) macaques had high variation in the number of observed taxa compared to the uninfected macaques though mean richness was unchanged by SIV infection or cART (Fig. 3A). The SIV + group exhibited a larger range of relative abundance within the *Mycobacterium* genus when compared to the SIV + cART group (Fig. 2), and outside of mycobacteria, some SIV + macaques had a high number of observed taxa and others had a low number of observed taxa compared to the mean (Fig. 1, Fig. 3A). By contrast, SIV + cART macaques had low variation in richness, similar to the uninfected controls. However, cART treatment did not fully restore the baseline liver microbiome as the composition of the taxa in the livers of SIV + cART macaques resembled more closely the SIV + macaques, including the high prevalence of bacteria within the *Mycobacterium* genus (Fig. 1, Fig. 3A). Microbial evenness was also impacted by untreated and cART-treated SIV infection (Fig. 3B). SIV + and SIV + cART macaques exhibited a larger range in evenness score similar to the larger range in richness score. Interestingly, while mean evenness in SIV + macaque livers was similar to that in the uninfected control group, this measure of the representation by each genus tended to decrease in SIV + cART macaques, indicating a redistribution of taxa especially in the setting of

cART treated infection. Altogether, these findings support that the liver microbiome is altered during SIV infection and does not fully recover during cART.

Assessment Of Mycobacterial DNA

Since 16S rRNA gene sequence abundance is a relative estimate that reflects the abundance of other bacteria, qPCR was conducted to confirm that *Mycobacterium* was indeed increased in the liver during SIV infection in both untreated and cART suppressed macaques. Based on previous methods [34], extracted liver DNA was assessed using *Mycobacterium* 16 s rRNA gene-specific primers and *Mycobacterium* was quantified in the liver of each macaque. *Mycobacterium* were detected in all macaques; however, levels were significantly higher in both the SIV+ ($p = 0.0048$) and the SIV + cART macaques ($p = 0.0095$) when compared to uninfected macaques (Fig. 4). These data confirm the presence of *Mycobacterium* in the liver during SIV infection as seen in the 16S rRNA gene sequencing, and that drug therapy does not restore the liver microbiome to normal composition even during viral suppression.

To better understand which *Mycobacterium* are present in the liver during SIV infection, the *Mycobacterium* present in the liver were identified by multi-gene amplicon sequencing. High sequence homology in closely related mycobacteria necessitates the use of multiple genes to help discriminate at the species level. Thus, *Mycobacterium*-specific primers for both the 16S rRNA gene and the *rpoB* gene were utilized to amplify variable regions of each gene, followed by sequencing. Identification of liver mycobacteria using the 16S rRNA gene indicated the presence of non-tuberculous mycobacteria (NTM) of a few possible species, including *M. smegmatis*, *M. marinum*, or *M. goodii*, with greater than 99% sequence match (Table 1). Two of the macaques (Z09086, Z09096) yielded top BLAST hits exclusively for *M. smegmatis*. The *rpoB* gene has less sequence coverage in the BLAST database but is valuable in combination with the 16 s rRNA gene analysis. The *rpoB* gene sequencing analysis consistently yielded the identification of *M. smegmatis* in the liver of each macaque with > 99% identity matches for all liver DNA samples tested, with exception to Z09096 which did not have enough DNA template for this secondary PCR verification (Table 1). Taken together, these data suggest that *M. smegmatis* or a closely related relative is likely the specific *Mycobacterium* species present in the livers of SIV + and SIV + cART macaques.

Table 1
Identity of Liver Mycobacterium Species

SIV+	16 s rRNA Gene (% identity)	rpoB Gene (% identity)
A14049	<i>M. smegmatis</i> , <i>M. marinum</i> (99%)	<i>M. smegmatis</i> (99%)
A14050	<i>M. smegmatis</i> , <i>M. marinum</i> (100%)	<i>M. smegmatis</i> (99%)
Z09064	<i>M. smegmatis</i> , <i>M. marinum</i> , <i>M. goodii</i> (99%)	<i>M. smegmatis</i> (99%)
Z09068	<i>M. smegmatis</i> , <i>M. marinum</i> (99%)	<i>M. smegmatis</i> (100%)
Z09086	<i>M. smegmatis</i> (100%)	<i>M. smegmatis</i> (99%)
Z09096	<i>M. smegmatis</i> (99%)	QNS
SIV + cART	16 s rRNA Gene (% identity)	rpoB Gene (% identity)
A12015	<i>M. smegmatis</i> , <i>M. goodii</i> (99%)	<i>M. smegmatis</i> (99%)
A13273	<i>M. smegmatis</i> , <i>M. goodii</i> , <i>M. marinum</i> (100%)	<i>M. smegmatis</i> (99%)
A13274	<i>M. smegmatis</i> , <i>M. marinum</i> (99%)	<i>M. smegmatis</i> (99%)
A13275	<i>M. smegmatis</i> , <i>M. goodii</i> , <i>M. marinum</i> (100%)	<i>M. smegmatis</i> (99%)
QNS = DNA Quantity Not Sufficient for PCR verification		

Mycobacteria Cell Wall Components Induce Hepatocyte Inflammatory Transcriptomic Profiles

Aberrant hepatocyte inflammation and metabolism are key drivers in fatty liver disease. To explore the relationship between *Mycobacterium* and hepatocyte inflammatory responses, we stimulated primary human hepatocytes *in vitro* with purified lipoarabinomannan (LAM) and assessed gene expression using a Nanostring inflammation panel [35]. LAM is a major component of the *Mycobacterium* cell wall and a pathogen associated molecular pattern (PAMP). Different species of *Mycobacterium* express distinct LAMs: Mannosylated LAM (ManLAM) is typically found in more pathogenic *Mycobacterium* species, such as *M. tuberculosis* while phosphoinositol-capped LAM (PILAM) is found in opportunistic *Mycobacterium*, such as *M. smegmatis*. Thus, both ManLAM and PILAM were used in these experiments.

Stimulation with LAMs induced a dose dependent global change in hepatocyte gene expression (Figure 5A-B). ManLAM and PILAM both altered expression of the same genes; however, overall ManLAM impacted gene expression more strongly than PILAM (Figure 5A-B). A notable exception was *IL-7*, which was more dramatically down-regulated when hepatocytes were stimulated with PILAM (Figure 6A). Gene expression changes common between the ManLAM and PILAM stimulation include several chemokines involved in neutrophil chemotaxis (*CXCL1*, *CXCL6*, *CXCL5*) (Figure 6B), albeit with a fold change that was higher for hepatocytes stimulated with ManLAM (Figure 6C) compared to PILAM (Figure 6A). Comparison of the LAM stimulation with stimulation by live bacteria revealed that the hepatocyte transcriptome

observed under stimulation with live *M. smegmatis* (MOI 10) more closely resembled that of hepatocytes stimulated with ManLAM (*M. tuberculosis*) than PILAM (*M. smegmatis*) (Figure 6D) with an upregulation in expression of *CCL20*, *CXCL10*, *CXCL2* and *IL-8*.

Reduced Level of Liver Neutrophils is Associated with Elevated Bacterial 16S rRNA DNA

Liver phagocytes play key roles in the sensing and removal of gut-derived bacteria and bacterial products from portal vein blood, thus preventing them from entering the systemic circulation. Previously, we determined that the frequency of liver macrophages increases in SIV+ macaques but that those levels were similar to uninfected macaques in SIV+ macaques treated with cART [32]. Furthermore, we determined that the amount of bacterial 16S rRNA gene in macaque livers increases during SIV infection, but does not decrease in SIV+cART macaques, indicating that liver macrophage frequencies are not associated with levels of bacterial 16S rRNA DNA [32]. To provide further insight into immune cells changing during SIV infection, liver neutrophils (identified as MPO+ and CD68-) were enumerated in the macaque liver tissue by microscopy. Both MPO+ neutrophils (green) and CD68+ macrophages (red) were found localized throughout the liver parenchyma (Figure 7A). Hepatic neutrophils were occasionally found co-localized with CD68+ macrophages (Figure 7B), likely due to the macrophages removing the dead and dying neutrophils [36]. However, the majority of neutrophils were found to be adjacent to, but not within, liver macrophages (Figure 7C). In SIV+ macaques, there was a trend toward fewer hepatic neutrophils compared with the uninfected macaques, and in SIV+cART macaques, there were significantly fewer neutrophils (Figure 7D). It is notable that in these SIV+ macaques with a trend toward fewer liver neutrophils, we previously found an increased number of liver macrophages [32]. Furthermore, the reduction in hepatic neutrophils negatively correlated with the previously quantified liver bacteria 16S rRNA DNA levels [32] (Figure 6E), revealing an association between neutrophil deficiency and elevated total bacteria DNA in SIV+ macaques.

Discussion

Different bacterial species have the potential to be inflammatory or anti-inflammatory; thus the composition of the microbiome exerts important influences on homeostasis [22]. Like HIV infection [37], SIV infection is associated with gut and liver microbiome dysbiosis, including an enrichment for inflammatory Proteobacteria [24]. Preferential translocation of bacteria originating in the gut into the colonic lumen during SIV infection [24] suggests a model whereby bacteria travel from the gut into the colonic lumen prior to entering the liver via the portal vein. This process filters the blood from the intestine, and these liver microbial products are associated with immune activation and liver damage [30]. Here we sought to identify the liver bacteria to lower taxonomic levels and mechanistically assess the relationship between the liver bacteria and hepatic immune activation. Using bacterial 16S rRNA gene sequencing together with qPCR, we characterized the liver microbiome to the genus level and confirmed elevated levels of the most prolific genus, *Mycobacterium*, in SIV + and SIV + cART-treated macaques. Multigene PCR sequencing identified *M. smegmatis* or a closely related *Mycobacterium* species as the predominant species in SIV + and SIV + cART-treated liver samples. We evaluated the impact of *M.*

smegmatis on hepatocyte inflammation *in vitro*, finding that *M. smegmatis* PILAM induces an upregulation of neutrophil chemotactic mediators. Surprisingly, *in vivo*, elevated bacterial DNA in the livers of SIV + and SIV + cART-treated macaques was associated with reduced rather than elevated neutrophil counts. These findings suggest that the normal physiological process of neutrophil recruitment to the liver in response to bacteria and host cellular chemotactic signals may be impaired during SIV infection, thereby promoting dysbiosis and the presence of *Mycobacteria* in the liver, which does not normalize during cART. These findings provide key insights into understanding SIV-associated liver inflammation and the microbial composition that is altered during SIV infection and cART therapy.

Overall, in the livers of SIV + animals in this study, we observed a bloom of prevalent genera during infection, with *Mycobacterium* as the most abundant genus present. In HIV + patients, there is reduced diversity in gut microbiome composition that does not generally recover back to pre-HIV levels after cART treatment is initiated [37]. In the livers of SIV + macaques, we did not detect a significant drop in the alpha diversity of taxa present, but instead saw a wider variation in the number of observed taxa around the mean, suggesting variation in how individual animals respond to the infection. With the introduction of cART treatment, the bloom of bacterial populations did seem reduced, as the evenness across present genera decreased when compared to uninfected animals, yet *Mycobacterium* persisted and dominated. The finding that cART did not result in a substantial reduction in the levels of environmental mycobacteria in the liver of SIV + macaques could be due to the fact that cART treatment was still in an early stage, with treatment lasting 35 to 36 weeks before tissues were examined. Alternatively, clearance of mycobacteria may be difficult once colonization is established. Characterization of the liver microbiome over time during infection and at later time points following the introduction of cART will aid in our understanding of these findings. The low detection of *Mycobacterium* in the uninfected macaques indicates that *Mycobacterium* is likely present in the normal liver microbiome, but SIV infection allows the bacteria to opportunistically thrive to a higher prevalence. Thus, SIV infection may provide the opportunity for specific genera that are present in the liver microbiome to increase in prevalence, rather than allow for the introduction of new genera; these changes in microbial composition appear to be not reversed by cART.

In our previous publication, we evaluated changes in the liver macrophage populations that expand during SIV infection and correlate with both inflammatory (TNF- α , CCL3) and fibrosis (TGF- β) mediators [32]. Evaluating the CCL2-CCR2 chemokine network as an integral inducer of monocyte/macrophage infiltration into the liver, we observed an upregulation of both CCL2 and CCR2 in the liver in macaques during untreated SIV infection. This CCR2 expression positively correlated with the frequency of CD68 + macrophages, leading us to speculate that viral stimulation in the liver alters the immune environment through induction of CCL2, and possibly other chemokines, resulting in immune cell infiltration [32]. Liver resident macrophages (Kupffer cells) are exposed to translocated gut-derived bacterial products by portal circulation and function to sense and remove pathogens through pattern-recognition receptors (PRRs) such as TLRs. These TLRs recognize gut microbiota-derived bacterial products (e.g. LPS), triggering a response through TLR4 to produce inflammatory cytokines [17, 38]. Here, we assessed a second key phagocytic cell population, neutrophils. Neutrophils are rapidly recruited to sites of acute inflammation,

though the method of recruitment of these cells to the liver is not well known [19, 38]. However, activated neutrophils can also promote disease progression via the secretion of pro-inflammatory cytokines [19]. In HIV + and HIV + cART patients, an increase in neutrophil frequency and survival was reported; the increased survival correlated inversely with the ratio of *Lactobacillus* to *Prevotella* in the gut and *Lactobacillus* was associated with a decrease in neutrophil survival [39]. Bacterial PAMPs affect neutrophil survival differently, and all other tested bacterial species except for *Lactobacillus* significantly increased neutrophil survival after incubation with whole blood [39]. In the present study, a decreased frequency of MPO + neutrophils in the livers of SIV + and SIV + cART macaques correlated with a higher level of 16S rRNA gene. We hypothesize that this decrease of neutrophils in the liver during SIV infection results in a loss of the ability to identify and clear bacteria and bacterial products, including environmental *Mycobacterium* species. Interestingly, within the liver microbiome assessment reported herein, there was a decrease of the *Lactobacillus* genus during SIV infection.

The finding of high levels of environmental mycobacteria in the livers of the SIV + and SIV + cART macaques was unexpected. NTM, such as *M. smegmatis* are ubiquitous and inhabit a range of environmental reservoirs, including natural and municipal water, soil, aerosols, food and dust, with water being the most common source of infection [40]. Overall, water treatment processes have been shown to efficiently remove mycobacteria, indicating that mycobacteria recovered from water systems most likely contaminate post-treatment [41]. Previous studies have not identified significant levels of environmental mycobacteria in the gut microbiome in the context of HIV, nor have previous SIV studies identified mycobacteria through 16S rRNA gene sequencing in SIV + macaques. However, Sivanandham, et al. reported the presence of granulomas induced by atypical mycobacteria in the liver of SIV + pig-tailed macaques [42], and He, et al. similarly reported the presence of granulomas in the liver of SIV + African Green monkeys treated with a high fat diet although mycobacteria could not be identified by acid-fast staining [43]. It is important to note that detection of *Mycobacterium* DNA requires specialized lysis steps to rupture the cell wall [44, 45]. Here, we utilized a ball mill to mechanically disrupt liver tissue prior to DNA extraction, which likely enhanced the recovery of *Mycobacterium* DNA. Remarkably, many opportunistic infections caused by *Mycobacterium* have been identified in HIV + patients, particularly with members of the *M. avium* complex (MAC) [46]. The pathogenic potential of mycobacteria has been described beyond the gut as well, such as in the case of Lady Windermere syndrome, a polymicrobial infection including MAC that affects the lungs [47]. Generally, environmental mycobacteria do not pose a health risk to healthy individuals, but these mycobacteria can cause disease in immunocompromised individuals. Early studies investigating the connection between HIV and NTM found that in patients with HIV there was a higher chance of isolating *M. xenopi* and *M. kansasii* from cultured respiratory secretions, in addition to *M. fortuitum*, *M. terrae*, and *M. scrofulaceum* from extrapulmonary sites [48]. In fact, *M. kansasii* has been shown to cause serious pulmonary infections in patients with late stage AIDS [49, 50].

Through sequencing of the *16S* and *rpoB* genes we were able to identify the *Mycobacterium* present in the livers of the SIV + macaques as being closely related to *M. smegmatis*. *M. smegmatis* is an environmental NTM that has the potential to be an opportunistic pathogen in immune-suppressed people

[51]. The *Mycobacterium* genus comprises hundreds of species that range from pathogens with significant clinical importance, such as members of the *M. tuberculosis* complex, to environmental NTM that are prevalent in water and soil [52]. NTM are increasingly associated with opportunistic infections in immunocompromised hosts. In one case study, an immunocompromised patient with an inherited interferon-gamma receptor deficiency was diagnosed with a mycobacterial infection identified as *M. smegmatis*, which proved fatal despite treatment [51]. Interestingly, *M. smegmatis* has been shown to be pathogenic in other laboratory models; goldfish *M. smegmatis* infection induced giant cell replication and recruitment to the liver and increased mortality [53]. To delineate the effect of *M. smegmatis* on hepatocytes, *in vitro* experiments were conducted using purified PILAM, which is a component of the *M. smegmatis* cell wall. *M. smegmatis* PILAM induced an upregulation of neutrophil chemotactic mediators, CXCL1, CXCL5, CXCL6, which is similar to results obtained with *M. tuberculosis* ManLAM stimulation. Interestingly, a reduction in liver neutrophils was observed in SIV + and SIV + cART macaques in this study, which correlated with increased bacterial DNA in the liver. Taken together, our data suggest that neutrophil deficiency may ultimately enable incomplete bacterial clearance from the liver during SIV infection, thereby allowing opportunistic pathogens to thrive.

Conclusions

Liver disease is currently a major contributor to the morbidity and mortality observed in HIV+ and HIV+cART patients. Here, we identified an altered microbiome within the livers of SIV+ rhesus macaques that includes an increase in the levels of environmental mycobacteria identified as *M. smegmatis*, or a close relative. Our data raise questions regarding the presence of *Mycobacterium* in HIV+ people, including those on cART. Obtaining critical specimens, such as stool, liver and other tissues from these patients followed by optimized DNA extraction techniques is critical for determining the extent to which environmental mycobacteria are part of the microbiome during HIV infection.

Declarations

Ethics. The ethics statement concerning non-human primates is provided in the first sub-section of Methods.

Availability of data and materials. The datasets generated and analyzed during the current study are available in the SRA and Geo repositories.

Competing interests. The authors declare that they have no competing interests.

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Authors contributions.

BSF and DLS designed the study.

BSF, KAF, ATG, CF, and MPW carried out the experiments.

BSF, BJB, KAF, ATG, CF, MPW, NRK and ND analyzed the data.

MG and ATG conducted the microbiome sequencing.

BSF and KAF conducted the Nanostring analysis.

JS coordinated and oversaw the animal work.

BSF, KAF, ND, and DLS wrote the paper.

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Figures

Figure 1

Individual macaque liver 16S microbiome analysis. Amplified 16S bacterial DNA sequences from liver samples were analyzed using the QIIME2 pipeline and visualized as percentage of sequences present in individual samples for A) Uninfected macaques, B) SIV+ macaques, and C) SIV+cART macaques. Only bacteria genera representing the nineteen most abundant genera are included; other genera are compiled into the “Other” category.

Figure 2

Relative abundance of identified genera in macaque groups. Relative abundance of the most prevalent genera across all liver samples identified by 16S rRNA gene sequencing compared by condition group: Uninfected, SIV+, and SIV+cART. Plots were generated using the pyloseq package by plotting mean fraction of sequences $\pm$ SEM for each condition group per genus organized by color at the phylum level, Actinobacteria (orange), Proteobacteria (blue), Bacteroidetes (green), Firmicutes (teal).

Figure 3

Alpha diversity of the 16S rRNA gene detected in liver. Amplified 16S rRNA gene sequences from liver samples were analyzed using the QIIME2 pipeline for alpha diversity and compared by condition group: Uninfected, SIV+ and SIV+cART. A) Evaluation of richness across condition groups showing the mean (□ standard deviation, SD) genera diversity present within the condition group. B) Comparison of evenness among the condition groups, showing the mean (□ SD) representation by different taxa.

Figure 4

Hepatic Mycobacterium DNA quantitation by qPCR. Quantitative PCR was conducted with Mycobacterium specific 16S rRNA gene primers to quantify amounts of DNA present in macaque liver samples. Mycobacterium 16S rRNA DNA was quantified from a standard curve created by serially diluting pure *M. bovis* (BCG) DNA 10-fold and then converted into copy number per 750 ng template DNA based off of the molecular weight of BCG. Duplicates from each macaque were averaged and the mean plotted

by treatment group with uninfected macaques shown in black circles, SIV+ macaques in maroon squares and SIV+cART macaques in blue triangles. Statistical significance was determined using a Mann-Whitney U test with a cutoff of $p < 0.05$.

Figure 5

Mycobacterium LAM hepatocyte stimulation global inflammatory gene expression heatmap. Human HepaCure hepatocytes were stimulated with *M. smegmatis* PILAM (A) and *M. tuberculosis* ManLAM (B) at 0.1 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ for 24 hours. Extracted RNA was evaluated using the Human v2 Nanostring Inflammation Panel and compared using log2 fold change relative to the unstimulated control

hepatocytes, Genes in red indicate an up-regulation and in blue a down-regulation. All genes included in the panel are represented on the heatmap.

Figure 6

Comparison of in vitro hepatocyte transcriptomic response to mycobacterial PAMPs. Human HepaCure hepatocytes (Yecuris) were stimulated for 24 hours with live *M. smegmatis* (MOI 10), purified mycobacteria PILAM (10 ug/mL) obtained from *M. Smegmatis*, or purified mycobacteria ManLAM (10 ug/mL) obtained from *M. tuberculosis*. Expression of inflammatory genes was quantified via a Nanostring gene expression panel. A) Genes with a significant log2 fold change compared to the unstimulated control ($p < 0.05$, 1.5-fold change cut off) for each of the three stimulation conditions are displayed. Genes with significant expression changes common between stimulation conditions are placed in overlapping areas with up-regulated genes depicted in red and down-regulated genes in blue. B) Volcano plot for gene expression in hepatocytes stimulated with PILAM (10 ug/mL) for 24 hours with significant genes denoted with labels and diamond points. C) Volcano plot for gene expression in hepatocytes stimulated with ManLAM (10 ug/mL) for 24 hours with significant genes denoted with labels and diamond points. D) Volcano plot for gene expression in hepatocytes stimulated with live *M. smegmatis* (MOI 10) for 24 hours with significant genes denoted with labels and diamond points.

Figure 7

Elevation in liver bacterial DNA levels associated with neutrophil deficiency. Liver neutrophils and macrophages were assessed by immunofluorescence microscopy using MPO and CD68, respectively, in liver tissue from uninfected, SIV+ and SIV+cART macaques. A-C) MPO+ cells (neutrophils) are depicted in green, CD68+ cells (macrophages) in red, and cells expressing both markers in yellow throughout the liver. D) Neutrophils were quantified in eight randomly selected fields for each liver sample (Image J Cell Counter, mean $\pm$ SEM) in uninfected (black circles), SIV+ (maroon squares) and SIV+cART (blue triangles) groups. Statistical differences between the test groups compared to the uninfected group were evaluated by Mann-Whitney U test. E) Linear regression analysis and Spearman correlation between liver neutrophils (x-axis) and the liver bacteria 16S rRNA DNA levels reported for these macaques in [32] (y-axis) indicates a significant negative correlation ($r = -0.69$).

Supplementary Files

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