Probiotic supplementation reduces inflammatory profiles but does not prevent oral immune perturbations during SIV infection

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HIV/SIV infections lead to massive loss of mucosal CD4+ T cells and breakdown of the epithelial mucosa resulting in severe microbial dysbiosis and chronic immune activation that ultimately drive disease progression. Moreover, disruption of one of the most understudied mucosal environments, the oral cavity, during HIV-induced immunosuppression results in significant microbial and neoplastic co-morbidities and contributes to and predicts distal disease complications. In this study we evaluated the effects of oral probiotic supplementation (PBX), which can stimulate and augment inflammatory or anti-inflammatory pathways, on early SIV infection of rhesus macaques. Our study revealed that similar to the GI mucosae, oral CD4+ T cells were rapidly depleted, and as one of the first comprehensive analyses of the oral microflora in SIV infection, we also observed significant modulation among two genera, Porphyromonas and Actinobacillus, early after infection. Interestingly, although PBX therapy did not substantially protect against oral dysbiosis or ameliorate cell loss, it did somewhat dampen inflammation and T cell activation. Collectively, these data provide one of the most comprehensive evaluations of SIV-induced changes in oral microbiome and CD4+ T cell populations, and also suggest that oral PBX may have some anti-inflammatory properties in lentivirus infections.

Pathogenic HIV and SIV infections are characterized by early and sustained loss of mucosal homeostasis, inflammation, loss of CD4+ T cells and translocation of microbial products from the intestines into circulation and lymphoid tissues. Circulating lipopolysaccharide (LPS) and sCD14 are established biomarkers for microbial translocation and major modulators of inflammation by engaging TLRs. One proposed methodology to modulate inflammation is by regulating the microbiome of the lower gastrointestinal tract through probiotic supplementation (PBX). Indeed, PBX therapy has previously been used to improve general mucosal function and decrease the incidence of GI inflammation and disease, specifically inflammatory bowel disease, ulcerative colitis and Crohn’s disease. PBX enhances NF-kB signaling and improves epithelial barrier function, likely through TLR signaling, and multiple lines of evidence suggest PBX use in persons living with HIV (PLWH) may reduce microbial translocation and inflammation. Further studies using SIV infected nonhuman primate models also demonstrate improved immune function, reduced cellular immune activation, and decreased co-morbid conditions.

Due to a lack of global access to cART and ongoing oral immune activation in PLWH, oral complications in HIV disease are increasingly common. Oral bacterial and mycotic co-infections such as candidiasis, necrotizing gingivitis, and periodontitis are all common in PLWH, as are KSHV and EBV infection of the oral epithelia resulting in increased oral tumors and hairy leukoplakia. Much like has been shown in HIV disease, complications of the oral mucosa are also routinely found during experimental SIV infection of macaques. Although

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the mechanisms are not entirely clear for oral diseases in HIV/SIV infections, SIV-induced dysbiosis of the oral microbiome does result in increased production of cytokines and other soluble inflammatory mediators in the oral cavity, while simultaneously suppressing antimicrobial functions and epithelial development27.

So called innate lymphoid cells (ILC) are identifiable by their lack of common lineage markers (those identifying T cells, B cells, NK cells, and myeloid cells), and can be distinguished by high expression of the IL-7 receptor, CD12728–31. ILC3 in particular play a major role in modulating gut homeostasis, but can be disrupted by changes in the inflammatory microenvironment, infectious disease, and shifts in the microbiome32. HIV and SIV infections drive a perturbation of ILC3 resulting in loss of intestinal barrier integrity, increased inflammation and potential dysregulation of the local microflora. Specifically, others and we have shown that acute SIV infection causes depletion of ILC3 in the GI mucosae33–35. In PLWH, ILCs are depleted but partially restored by ART36. Importantly, we have previously shown that probiotic therapy improves IL-23 production and as a result expands ILC3 numbers in naïve macaques37.

It has been shown that HIV alters human microbiome including oral bacteria communities38. Periodontal disease, a common oral inflammatory infection in HIV infected patients, has been shown to be associated with general dysbiosis in the oral cavity, as well as with HIV disease progression39. Dysbiosis in the oral cavity and changes to the oral bacterial communities might play a role in the sustained host proinflammatory responses to the virus. Restorative therapies that could improve oral and GI function, inflammation, and microbial health could be of significant benefit to PLWH. Indeed, previous studies by our groups have demonstrated that therapy with probiotic, beneficial bacteria can improve mucosal health and dampen inflammation in healthy macaques as well as in the context of SIV infection37,38,40,41. Thus, we hypothesized that probiotic supplementation prior to SIV infection and during acute infection may enhance the oral microbiome and prevent pathogenic consequences of SIV. In this study we evaluated the effects of pre- and acute administration of oral probiotic therapy on viral kinetics, inflammation, CD4+ T cell loss, microbiome, and associated lymphoid tissues in SIV-infected rhesus macaques.

Results and discussion

Early probiotic administration during SIV infection. To determine the effects of probiotic (PBX) administration pre-SIV and during acute SIV infection, we evaluated 12 Indian origin rhesus macaques, 6 receiving oral PBX and 6 receiving oral vehicle control. Animals were given PBX for a total of 42 days: 28 days pre-virus challenge and for a subsequent 14 days following challenge (Fig. 1a). Macaques were intrarectally chal-
Challenged with SIVmac251 as described previously42, and buccal, colorectal, and lymph node biopsies in addition to blood draws were collected as indicated. No significant differences were observed in plasma viral loads longitudinally (Fig. 1b,c), nor were any differences observed in peak viral loads between the two groups (Fig. 1d).

However, previous studies have also reported that the benefits of probiotic therapy in HIV and SIV are uncoupled from viremia which is generally not affected40.

**T cell responses during PBX treatment of SIV-infected macaques.** To assess broad antiviral T cell responses we performed longitudinal ELISPOT assays on PBMC as well as tissues at necropsy. Interestingly, PBX-treated animals consistently had lower levels of both GAG (fourfold at day 112) and ENV (ninefold at Day 112) ELISPOT responses (Fig. 2a,b). Further, the total magnitude of the ELISPOT response (as measured POL, ENV, GAG) in control animals was significantly greater at necropsy in all tissues measured (PBMC, spleen, oral lymph nodes, and tonsil) (Fig. 2c).

Since SIV infection has a significant impact on memory CD4+ T cell populations, as well as CD8+ T cell activation, we next used polychromatic flow cytometry to evaluate these cell subsets in detail in PBX-treated animals. As expected, the frequency of total CD4+ T cells in circulation declined early following SIV challenge concomitant with an increase in the frequency of CD8+ T cells (Fig. 3a). Similar trends were observed in colorectal tissue where the shift was even more robust (Fig. 3b), in line with previous studies33,44. Remarkably, this study demonstrated for the first time an acute and sustained loss of CD4+ T cells in the buccal mucosa following SIV infection (Fig. 3c). However, little to no difference was observed in CD4+ and CD8+ T cell dynamics in any tissue when comparing PBX-treated to control animals, including lymph nodes (Fig. 3d). There was also little difference between the two groups when specifically examining CD28+ CD95+ central memory CD4+ T cells in any tissue (Fig. 4a). However, PBX treatment reduced Ki67 expression (measured at day 42 PBX/Day 14 post-challenge) in both bulk and CM CD4+ T cells in blood and colonic tissue (Fig. 4b). Interestingly, proliferation was not altered in the buccal by PBX. Although it is difficult to speculate, this could be due to differences in local flora, immune cell composition, but also likely due to time of exposure. The probiotics move more rapidly through the oral mucosa than they do through distal mucosae, so this could reduce some effects. Nonetheless, these data suggest that while PBX may not protect CD4+ T cell loss, it may reduce T cell activation, which is well in line with previous observations37. Thus, PBX could be a beneficial augmentive therapy to protect CD4+ T cells and reduce overall cellular activation.

**Effects of probiotic treatment on ILC3 populations during SIV infection.** We and others have previously shown that HIV and SIV infection can modulate ILC3 populations in the mucosae. Further, our
groups have demonstrated that probiotic supplementation can actually expand ILC3 numbers in some animals and could possibly function as a putative rheostat mechanism of action for controlling inflammation during lentivirus infections. In this study we quantified the frequency of Lin-NKp44+NKG2A/C− ILC3, as described...
previously\textsuperscript{34,45}, in longitudinal colorectal and buccal biopsies. Interestingly, little change was observed in ILC3 frequencies during the probiotic supplementation period (day 0 to day 28) in this study (Fig. 5). Although some
animals demonstrated minor transient increases in ILC3 day 14 (colorectal) or day 28 (oral) following probiotic supplementation, these changes were not significant. It is unclear why ILC3 might not be expanded in this context, but previous work from our laboratory has shown that colorectal ILC3 are less likely to be increased compared to those in the jejunum and there is significant animal-to-animal variation. Overall, this study suggested in contrast to our previous pilot work that PBX may only marginally impact SIV-induced changes in ILC3 frequencies, indicating that while PBX may impact T cell responses, it is unlikely to protect against loss of ILC3.

**Soluble measures of inflammation and immune activation.** To further address the impact that PBX and ILC3 modulation might be having on global inflammation during SIV infection we compared soluble inflammatory mediators in the circulation by Luminex. Animals treated with PBX had a distinctly lower inflammatory profile on day 28 (day of challenge) as indicated by both analyte expression (heatmaps) and PCA, suggesting that PBX treatment alone was sufficient to decrease soluble mediators of inflammation (Fig. 6a, Supplementary Fig. 2, Supplementary Table 2). This reduced inflammatory profile persisted from day 42 (day 14 post-challenge), and even persisted in some animals up to day 112 (day 84 post-challenge. However, PBX therapy did not appear to have any effect on microbial translocation products (Fig. 6b) which is similar to HIV studies showing improved immune function but no consistent decline in circulating bacterial products.

**Probiotic supplementation partially modulates oral microbiome during SIV infection.** To study the effects of PBX on the microbiome, we used 16s rRNA gene sequencing to profile bacterial communities in the buccal and colonic mucosa. No notable differences in community richness, evenness, Shannon diversity or over all principal components analysis were observed between groups in either oral or gut samples, consistent with previous studies of probiotic supplementation. The most abundant bacteria in the oral mucosa of all animals were of Firmicutes phyla, with lesser representation of Bacteroidetes, Proteobacteria, Fusobacteria, and Actinobacteria (not shown). The colorectal mucosa top phyla was Epsilonbacteraeota followed by Bacteroidetes and Spirochaetes (not shown). At the phyla level, no notable differences between groups or across time points were observed in either site. At the genus level for the oral mucosa, a high abundance of *Streptococcus* and *Veillonella* was observed in all samples, followed by minor abundances of *Alloprevotella*, *Carnobacterium*, *Actin-
The top genera for colorectal samples were dominated by *Helicobacter* with an abundance of fifty-five percent followed by *Prevotella*, *Treponema*, *Alloprevotella*, *Ruminococcaceae*, *Camplobacter* with abundances starting at nine percent (Fig. 7b). We performed differential abundance analysis on the PBX and placebo groups across all timepoints in the oral mucosae. In the placebo group, *Porphyromonas* and *Actinobacillus* were found to be significant between day -21 and 0 ($p = 0.012$ and 0.021), day -21 and day 28 ($p = 0.028$), and day 28 and day 42 ($p = 0.004$ and 0.001) (Fig. 7c,d). In the PBX group, *Porphyromonas* and *Actinobacillus* were significant between day -21 and day 28 ($p = 0.04$) and day 42 and day 112 ($p = 0.037$ and $p = 0.017$) (Fig. 7d). However, notable differences were not observed between groups, but it does appear that the changes observed were dampened in the probiotics group compared to the placebo group. Thus, while probiotics did not have a substantial impact in modulation of the microbiome, these changes reflect possible dampening of SIV-mediated dysbiosis in the oral microbiome. It is also important to note that this study used a general PBX formulation. Future studies using formulations more specific to individual sections of the GI tract could have more targeted impacts.

**Concluding remarks.** In summary our study presents a number of new findings relevant to SIV immunobiology. First, SIV infection clearly demonstrates an early depletion of CD4+ memory T cells in the oral mucosa. Next, PBX does not appear to protect against loss of CD4+ T cells or ILC3 but does partially reduce some measures of systemic inflammation. These data also offer one of the most in-depth characterizations of microbiome in the oral mucosa, where we demonstrated novel changes to the oral microbiome after SIV infection, which are somewhat dampened with probiotics. However, no substantial microbiome alterations were observed between...
probiotics and placebo groups. Overall, our studies indicate a role for oral PBX in modulating SIV-induced inflammation, but additional work will be required to tease out detailed mechanisms of action.

Methods

Ethics statement. All animals were housed at Biomere, Inc., Worcester, MA in accordance with the rules and regulations of the Committee on the Care and Use of Laboratory Animal Resources. Animals were kept in agreement with the American Association for Accreditation of Laboratory Care standards and The Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Biomere Institutional Animal Care and Use Committee (IACUC) and in compliance with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. Animals were fed standard monkey chow diet supplemented daily with fruit and vegetables and water ad libitum. Social enrichment was delivered and overseen by veterinary staff and overall animal health was monitored daily. Animals showing significant signs of weight loss, disease or distress were evaluated clinically and then provided dietary supplementation, analgesics and/or therapeutics as necessary. Animals were humanely euthanized using an overdose of barbiturates according to the guidelines of the American Veterinary Medical Association. All studies reported here were performed under IACUC protocols 17–02 and 16–08 which was reviewed and approved by the Biomere IACUC. When necessary, macaques were immobilized with ketamine HCl (Parke-Davis) at approximately 10 mg/kg and injected intramuscularly after overnight fasting. Blood samples were collected using venipuncture.

Animals, probiotic supplementation and SIV challenge. Sixteen experimentally naive age-matched Indian-origin rhesus macaques (Macaca mulatta) were analyzed in this study. All animals were colony-housed at Biomere, Inc. and were free of simian retrovirus type D and simian T-lymphotropic virus type 1. Animals were challenged intrarectally with SIVmac251 as described previously. Six animals received Visbiome (www.visbiome.com) for 42 days mixed with food, while six received food only (vehicle) control. The Visbiome product contains: L. paracasei (DSM 24733); L. plantarum (DSM 24730); L. acidophilus (DSM 24735); L. delbrueckii subspecies bulgaricus (DSM 24734); B. longum (DSM 24736); B. infantis (DSM 24737); B. breve (DSM 24732); and S. thermophilus (DSM 24731). No adverse events due to PBX therapy were observed in any of the animals.

Blood and tissue processing. Blood samples were collected in EDTA-treated tubes, and peripheral mononuclear cells (PBMCs) were isolated using standard density gradient centrifugation over lymphocyte separation media (MP Biomedicals, Solon, OH) and any contaminating red blood cells were lysed using hypotonic ammonium chloride solution. Processing of tissues was carried out using protocols optimized in our laboratory.
Briefly, colon and buccal tissue were cut into 1 cm² then incubated in 5 mM EDTA for 30 min before undergoing mechanical and enzymatic disruption. Samples were then gently pushed through filters before lymphocytes were isolated via blayer (35%/60%) isotonic Percoll density gradient. Lymph nodes and spleen were trimmed of excess tissue then mechanically disrupted. Manual cell counts were performed for each sample using Trypan Blue. Two million cells from each sample were then used for real-time flow cytometry staining while remaining cells were cryopreserved in a DMSO solution and stored in liquid nitrogen vapor.

**ELISPOT.** ELISPOTs were performed with Mabtech IFN-γ ELISPot Plus Kits. Peptides used were SIVmac239 GAG (Cat: 12364), ENV (Cat: 12635), and POL (Cat: 12766) obtained through the NIH AIDS Reagent Repository and aliquoted into pools. Briefly, samples were thawed and rested for four hours. Cells were then counted and aliquoted into 96 well plates at 0.25–0.2 M cells per well. Following incubation at 37 degree C and substrate development, plates were read by ZellNet Consulting (Fort Lee, NJ).

**Antibodies and flow cytometry.** Flow cytometry staining of mononuclear cells was carried out for cell surface and intracellular molecules (Supplementary Table 1). Briefly, samples were incubated with the LIVE/DEAD Aqua amine dye (Invitrogen, Carlsbad, CA), then washed before staining with surface antibodies. After staining, cells were permeabilized using Thermo Fisher Scientific Fix & Perm buffer kit (ThermoFisher Scientific, Waltham, MA) and incubated with the intracellular staining antibodies. Isotype-matched controls were included in all assays. All acquisitions were made on an LSRII (BD Biosciences) and analyzed using FlowJo (9.9.6).

**Viral load quantification.** Quantified RNA was used for RT-PCR against a conserved region of gag using gene-specific primers\(^4^\). In the first step, RNA was reverse-transcribed followed by treatment with RNase for not performed.

**DNA extraction, 16S rRNA gene sequencing and data analysis.** We extracted DNA from cryopreserved buccal and colorectal biopsies using the PowerFecal DNA Isolation Kit (Qiagen, Valencia, CA). We then prepared sequencing libraries as described by the Earth Microbiome Project and sequenced them using the Illumina MiSeq Sequencer (Illumina, San Diego, CA). 515F-806R primers were used to sequence the V3-V4 region of the 16 s SSU rRNA as previously described\(^4^\). 16 s sequencing reads were demultiplexed, trimmed and then processed using QIME2 (version 2020.2)\(^5^\). Operational taxonomic units (OTUs) were clustered at 99% similarity using the DADA2 method and assigned taxonomy with the Silva 132 classifier for taxonomic determination. All sequence reads and operational taxonomic unit (OTU) observations were included in our analyses, in order to maximize the observed diversity of the bacterial communities and was done utilizing R (v3.6.0) packages. Alpha diversity (richness, evenness (Pielou)), and Shannon diversity) was calculated by the microbiome package. Beta diversity was calculated with pairwise sample dissimilarity and unweighted unifrac ordination analysis by means of principal coordinates analysis (PCoA) using the vegan package for Ordination, Diversity and Dissimilarities. Statistical analyses were completed using the Pairwise Adonis package. Differential abundance analysis was completed using DESeq2. Beta diversity and taxonomic plots were created utilizing the phyloseq and ggplot. Alpha diversity and differential abundance plots were created in Prism 8 version 8.4.3 (GraphPad Software).

**Statistical analyses.** Statistical analyses were carried out using Prism Version 8.3.0 (GraphPad Software). Unpaired, nonparametric, Mann–Whitney U tests, Wilcoxon, and t tests were used where indicated, and P < 0.05 were assumed to be significant. Samples were grouped and heatmaps were generated in Prism versions 7.0d, 8.3.0, or 8.4.3 (GraphPad Software; www.graphpad.com/scientific-software/prism). For beta diversity, statistical significance was assessed using the pairwise permutational multivariate analysis of variance (PERMANOVA; Adonis) test in R. For taxonomic differential abundance DESeq2 was used and accounted for sequencing depth and multiple comparisons using the Benjamini and Hochberg method to control the false discovery rate (FDR) and adjusted p values of < 0.05 were considered significant.

**Multiplex cytokine analysis of plasma.** Plasma samples were analyzed for rhesus macaque cytokines and chemokines by Luminex using established protocols for non-human primates. Evaluation of analytes IL-6, IL-12p70, IL-17, TNF-α, IL-2, G-CSF, CXCL13, MIP-1β, IP-10, IL-1Ra, Perforin, IFN-α, IFN-γ, MCP-1, MIG, II-23 and MIF were included in this assay. Two million cells from each sample were then used for real-time flow cytometry staining while remaining cells were cryopreserved in a DMSO solution and stored in liquid nitrogen vapor.

**Statistical analyses.** Statistical analyses were carried out using Prism Version 8.3.0 (GraphPad Software). Unpaired, nonparametric, Mann–Whitney U tests, Wilcoxon, and t tests were used where indicated, and P < 0.05 were assumed to be significant. Samples were grouped and heatmaps were generated in Prism versions 7.0d, 8.3.0, or 8.4.3 (GraphPad Software; www.graphpad.com/scientific-software/prism). For beta diversity, statistical significance was assessed using the pairwise permutational multivariate analysis of variance (PERMANOVA; Adonis) test in R. For taxonomic differential abundance DESeq2 was used and accounted for sequencing depth and multiple comparisons using the Benjamini and Hochberg method to control the false discovery rate (FDR) and adjusted p values of < 0.05 were considered significant.
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Acknowledgements

The authors thank Michelle Lifton, Dr. James Whitney, and Dr. So-yon Lim for technical assistance, Dr. Dan Barouch for providing the SIV challenge stock, as well as Dr. Luis Giavedoni (Southwest National Primate Research Center) for assistance with Luminex assays. We also thank Dr. Angela Carville and Biomere, Inc. for animal management.

Author contributions

R.K.R. and N.K. designed the project. R.J., S.S., K.K., V.V., B.H., S.V.S., D.R., and C.M processed samples. V.V. coordinated animal studies, and R.J., K.K., C.B., L.S., and R.K.R. analyzed the data. All authors contributed to the final preparation of the manuscript.

Funding

This work was supported by R01 DE026327, R01 DE026014, and R21 AI145678 (to R.K.R.). The authors also acknowledge the CFAR/CVVR Flow Cytometry Core (P30 AI060354).

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-93918-x.

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