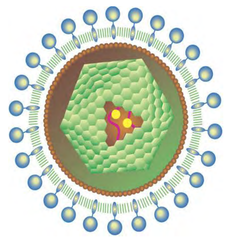
Gut Microbiota enriched in HIV-Infected Subjects Induce Inflammatory Cytokines but not T Regulatory Cells

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Abstract

Background: We co-exist with an abundant microbiota that cooperate with and help regulate our immune system. However, during HIV infection the microbial community in the gut is altered to a distinctive composition which is not consistently restored to that of seronegative subjects by ART. Recently, our group illustrated that in contrast to uninfected subjects, the microbial community in HIV-infected subjects exhibits increased phylogenetic diversity and is Prevotella-rich and Bacteroides-poor. Here we seek to understand the impact of the change in gut microbiota on HIV infection by characterizing innate and adaptive immune responses, including T regulatory cells, to selected gut bacteria that changed with HIV infection.

Methodology: Based on our 16S rRNA sequencing from HIV+ and HIV- subjects we chose four characteristic bacteria; *Bacteroides dorei*, *Bacteroides fluxus*, which were decreased, and *Prevotellaceae copri*, *Erysipelotrichaceae biforme*, which were elevated with HIV infection. PBMCs of 6 HIV-positive and 6 HIV-seronegative subjects were incubated with lysate of these bacteria. Levels of the anti-inflammatory cytokine, IL-10, were evaluated by ELISA, and T regulatory cells were enumerated by flow cytometry after 3 days. Statistical significance was determined using Mann-Whitney T tests.

Results: Levels of T regulatory cells increased with all bacteria compared with the unstimulated control, although levels were not significantly different in regard to HIV status. Median levels of T regulatory cells from PBMC cultured with *B. fluxus* (4.14) and *B. dorei* (3.94) were greater compared to that of *P. copri* (2.73) and *E. biforme* (2.55). Although these values did not reach statistical significant ($p = 0.08$). In regard to IL-10 production, higher levels were observed in PBMCs stimulated with *B. fragilis*, species that is known to stimulate IL-10 production by Tregs using a molecular factor called polysaccharide A (PSA). Stimulation with *B. fluxus*, a species that we predicted to have this same immune phenotype based on the presence of PSA encoding genes in its genome, also resulted in high IL-10 levels.. These levels were significant when comparing *B. fluxus* to non PSA producers *B. dorei* ($p = 0.008$), to *E. biforme* ($p = 0.001$) and *P. copri* ($p = 0.0007$). Additionally, *B. fragilis* compared to *B. fragilis* with a PSA gene knockout was also significant ($p = 0.008$). Helios, a maker of thymically derived Tregs, was significantly reduced ($p = 0.04$) in PBMC stimulated with *B. fluxus* compared to *E. biforme*. IL-10 production was confirmed to not be derived from monocytes.

Conclusions: These results illustrate that HIV infection is associated with a distinct microbial signature that is more pro-inflammatory. Furthermore, Bacteroides species which are lost during HIV infection are better adapted for inducing T regulatory cells than *P. copri* and *E. biforme* that are enriched in the gut during infection. Within the Bacteroides species, genomic PSA operons predict high IL-10 production from Tregs. By evaluating bacterial mediated immune responses these studies will provide important new understanding about the mechanisms and functional aspects of these bacteria in regulating the immune system and their role in HIV disease.

Background

- Commensal bacteria in the human gastrointestinal tract play multiple roles including, but not limited to: aiding in digestion, providing colonization resistance for pathogens and stimulation of the immune system
- Our group illustrated that in contrast to uninfected subjects, the microbial community in HIV-infected subjects exhibits increased phylogenetic diversity and is Prevotella-rich and Bacteroides-poor[1] (Fig. 2).
- These highly characteristic changes in the gut community may play a role in gut-linked disease that increase in prevalence with HIV infection.
- Previously we showed that *E. biforme* had a significantly higher TNF-α/IL-10 ratio compared to the other bacteria isolates[1].
- The use of anti-retroviral drug therapy does not consistently restore the gut microbiota to that of a seronegative subject[1, 2].
- *In vivo* studies in mice indicate that *Bacteroides fragilis* (*B. fragilis*) polysaccharide A (PSA) intermediates the levels of $CD4^+$ T cells, including Tregs, and inflammatory gut response to pathogens[3].

Schematic of Treg interaction with symbiotic bacteria

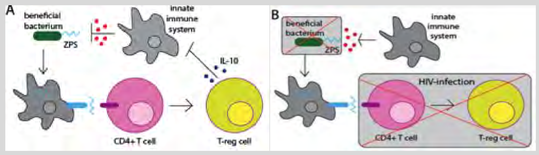


Fig. 1. Certain symbiotic bacteria, such as *B. fragilis*, require CD4+ T cell interacting molecular factors for persistence in the gut. By inducing production of the anti-inflammatory IL-10 cytokine from Treg cells, *B. fragilis* can limit the activity of both effector T-cells and innate immune cells (panel A). Although both effector and regulatory T-cells are specifically targeted and killed by HIV, innate immune cells are not. The loss of CD4+ T cells with HIV infection may thus result in killing of beneficial bacteria by innate immune cells (panel B).

Methodology

- Based on our 16S rRNA sequencing from HIV+ and HIV- subjects we chose four characteristic bacteria; *Bacteroides dorei*, *Bacteroides fluxus*, which were decreased, and *Prevotellaceae copri*, *Eubacterium biforme*, which were elevated with HIV infection.
- To predict which bacteria can produce PSA we BLASTed the genes in the *B. fragilis* 9343 PSA operon against protein coding genes from 8065 bacterial genomes.
- To test the role of PSA in generating Tregs and IL-10 production, *B. fragilis,* which expresses PSA, was modified with a PSA gene knockout (*B. fragilis* KO).
- PBMCs from HIV-infected and non infected subjects were incubated with lysate of these bacteria. Levels of anti-inflammatory cytokine, IL-10, was evaluated by ELISA, and percentage of Tregs was enumerated by flow cytometry at 3 days.
- To determine if generated Tregs were thymically derived or peripherally induced, cells were stained for Helios and Ki-67, markers of thymic derived Tregs and proliferation, respectively.
- To verify that IL-10 production was not monocyte derived, monocytes were depleted from PBMC by adherence to plastic or by CD14 magnetic bead depletion prior to the bacterial stimulation.
- Statistical significance was determined using Mann-Whitney T tests or ANOVA.

Prevotella are enriched and Bacteriodes are depleted in HIV-Infected subjects

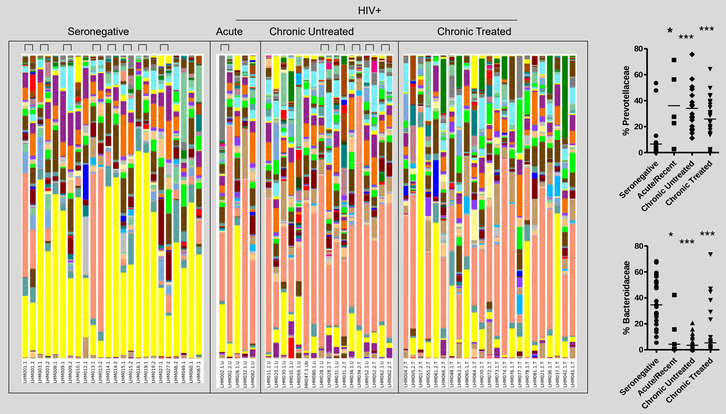


Fig 2. Prevotella (salmon) are increased with HIV infection while the Bacteroides (yellow) are reduced. Percent Prevotella and Bacteroides are indicated in the panels on the right.

PSA operon of *B. fragilis* and predictive PSA operon of *B. uniformis*

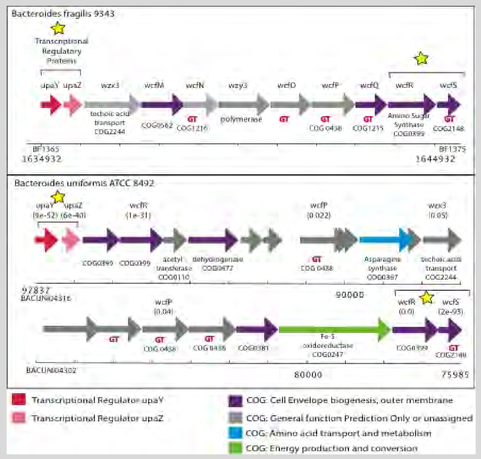


Fig. 3. The PSA locus of *B. fragilis* strain 9343 has been identified using transposon mutagenesis and the essential genes within it determined.[4] These include wcfR, which encodes the protein that synthesizes an amino sugar, and the glycosyltransferase (GT) wcfS in an adjacent position. Furthermore, there is a conservation of upaY and upaZ transcriptional regulators upstream. We have begun to predict which bacteria can produce PSA by BLASTing the genes in the *B. fragilis* 9343 PSA operon against protein coding genes from 8065 genomes. This figure illustrates that *B. uniformis*, a species that is also depleted with HIV infection, has a predicted PSA operon in its genome. Genes in the *B. uniformis* operon that are related to those in the *B. fragilis* operon are noted (e-values from BLAST are in parentheses below the gene name) and genes universally conserved across PSA-producing *B. fragilis* strains are noted with a star. Our immune assays include *B. fluxus*, which we predict to be a PSA-producer with these methods, and *B. dorei*, which does not have the PSA operon in the genome of any of the 5 strains evaluated.

PSA encoding bacteria induce IL-10 producing Tregs

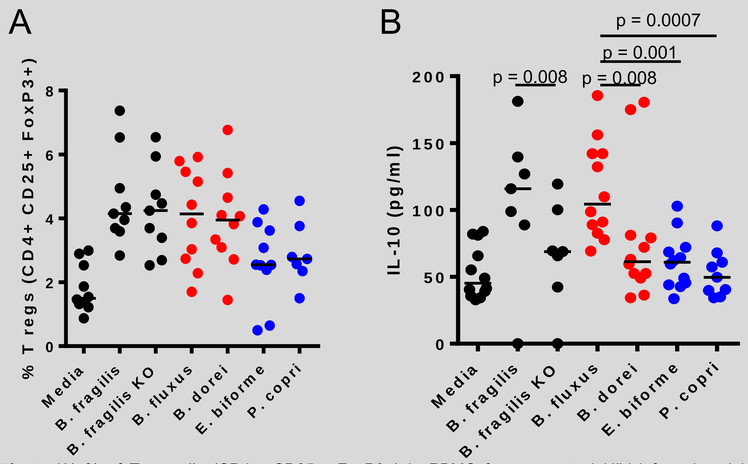


Fig 4. (A) % of Treg cells (CD4+, CD25+, FoxP3+) in PBMC from untreated HIV infected and HIV negative subjects (combined), after 3 days of culture with bacteria. Bacteria that decreased in relative abundance with chronic, untreated HIV infection are red, those that increased are blue, and no difference is black. (B) Levels of IL-10 in supernatant from cultured PBMC as determined by ELISA.

Helios expression on Tregs cultured with *B. fluxus* is lower compared to *E. biforme* indicating they are more peripherally induced

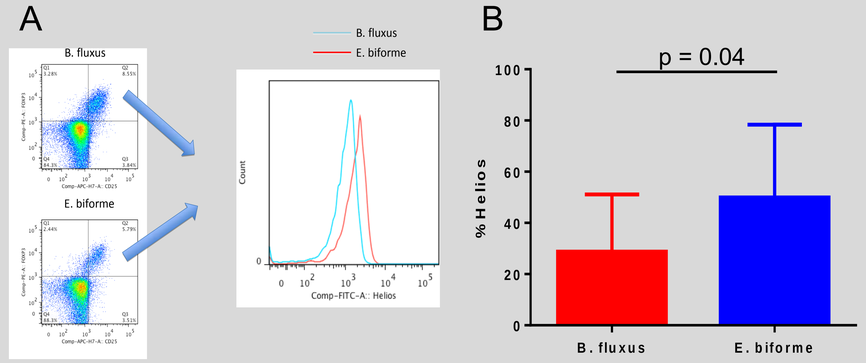


Fig 5. To determine if Tregs generated with bacterial lysates are more peripherally induced or thymically derived, Treg (CD4+, CD25+, FoxP3+) cells were subjected to Helios staining. A) A representative sample. B) Summary of Helios staining from 4 subjects.

Tregs exhibit limited proliferation

Bacteria-induced IL-10 in PBMC is not monocyte derived

Fig 6. CD4+ CD25+ FoxP3+ Tregs were subjected to Ki-67 staining to determine proliferation potential. There was no significance between conditions.

Fig 7. IL-10 production in PBMC and PBMC depleted of monocytes as determined by ELISA. There was no significance in regard to monocyte depletion.

Conclusions

- HIV infection is associated with a distinct microbial signature that is Prevotella-rich and Bacteroides-poor.
- Genomic analysis indicates that other bacteria that decrease with HIV infection produce PSA.
- Bacteroides species lost during HIV infection are better adapted for inducing T regulatory cells than *P. copri* and *E. bioforme* that are enriched in the gut during infection (Fig 4A); there was no difference in the generation of Tregs in regard to HIV status.
- IL-10 production is higher in PBMC stimulated with bacteria that have PSA (*B. fragilis* and *B. fluxus*) compared to bacteria without PSA.
- PSA gene knockout in *B. fragilis* did not induce a higher number of CD4+ CD25+ FoxP3+ (Tregs) T cells compared with wildtype *B. fragilis* (Fig 3A), but IL-10 production was significantly lower in the KO (Fig 4B).
- Tregs generated by stimulating PBMC with *B. fluxus* are more peripherally induced than thymically derived as indicated by Helios staining (Fig 5).
- Bacteria-induced Tregs do not proliferate (Fig 6).
- Bacteria-induced IL-10 in PBMC is not monocyte derived (Fig 7).
- Evaluating bacteria-mediated immune responses will provide new insights on how gut bacteria regulate the immune system and their role in HIV disease.

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