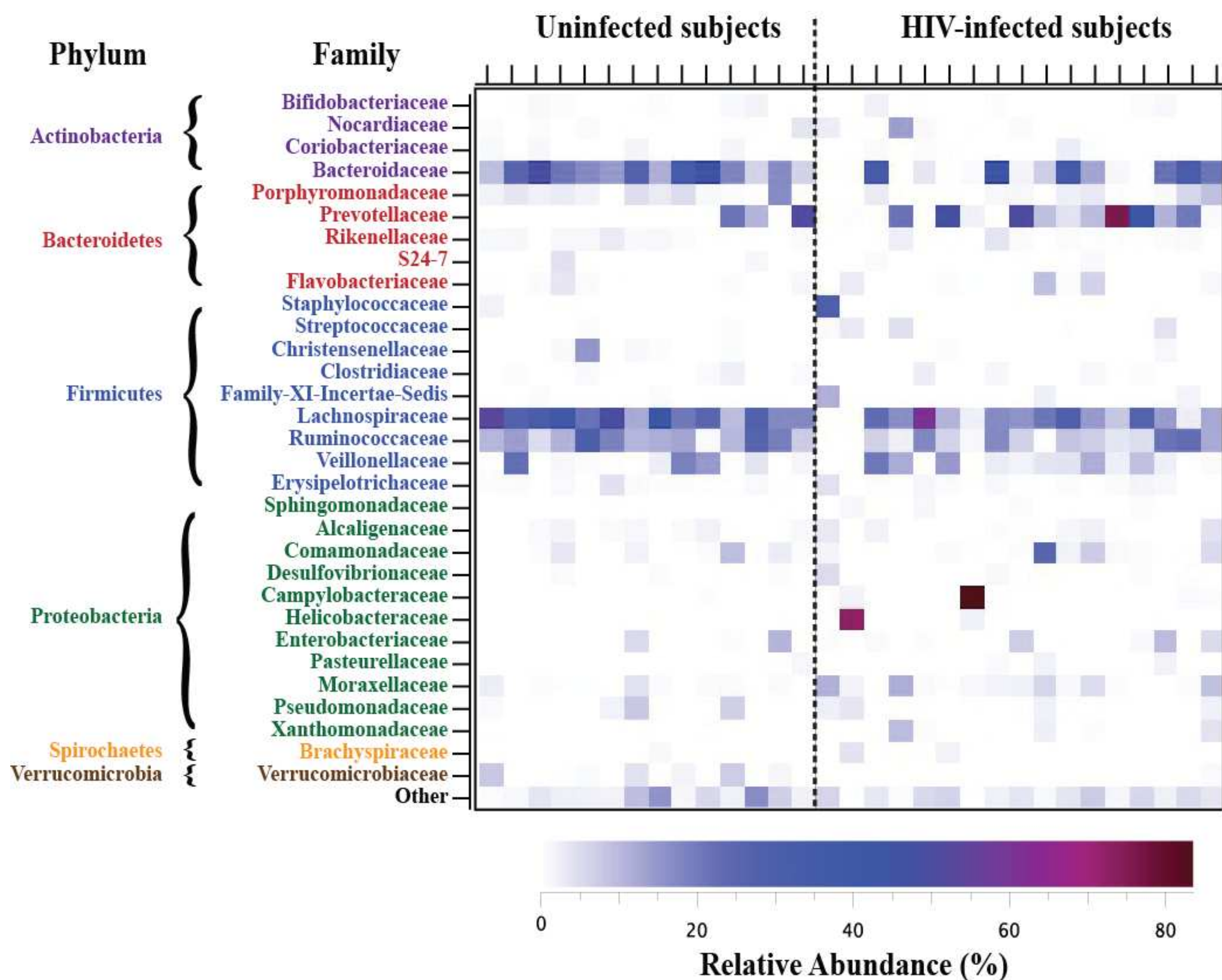
SUPPLEMENTARY FIGURES AND TABLES.

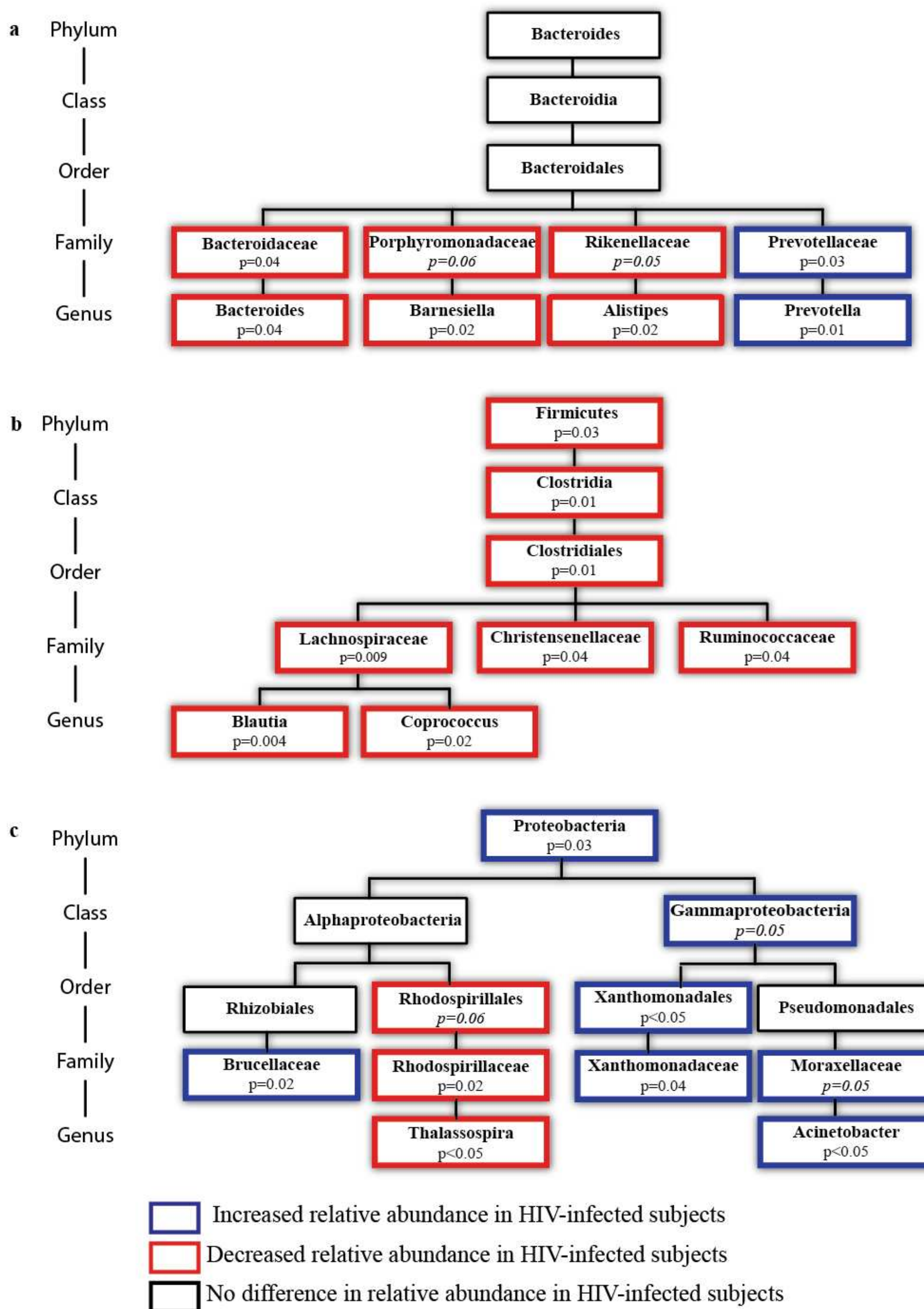


Supplementary Figure S1. Distribution of colonic mucosal tissue-associated bacterial families in all study subjects. Color levels indicate the abundance of each bacteria relative to total bacteria (Relative Abundance, %) in colonic mucosal tissue for each individual. Each line at the top of the box represents each uninfected (n=14) or HIV-infected (n=17) subject with the dotted line indicating separation between the two groups. Bacterial families are organized into alphabetical order based on Phylum. Any family not shown are grouped together as 'Other".

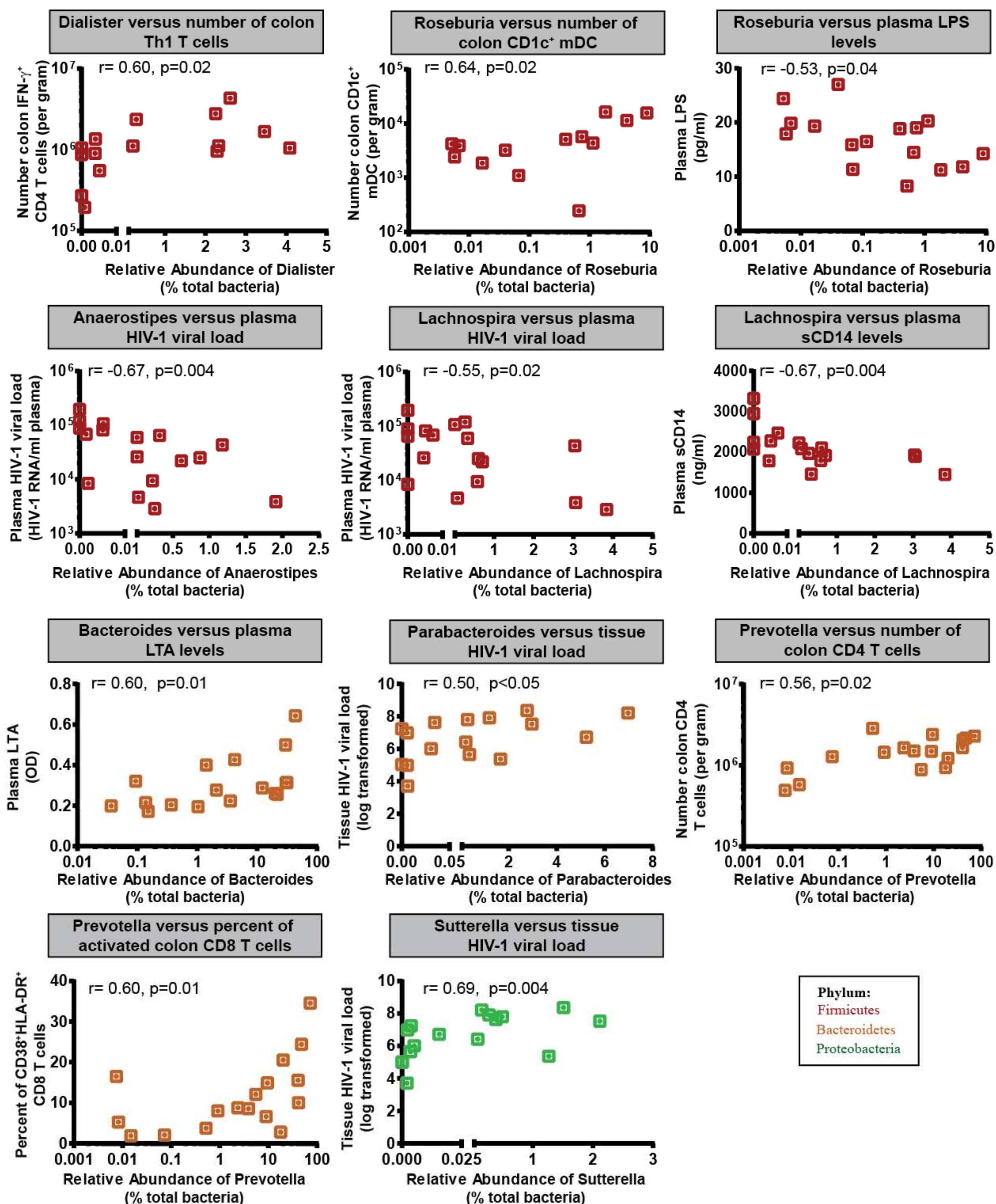
Supplementary Table S1. Relative abundance of families of bacteria within colonic mucosal tissue which are statistically different in HIV-infected subjects compared to uninfected subjects

	Uninfected subjects (n=14)	HIV-infected subjects (n=17)	Difference in relative abundance P value*
Bacteroidetes Phylum			
Prevotellaceae	0.02% (0.003-48.9%)	5.5% (0.01-74.7%)	↑ 0.03
Porphyromonadaceae	2.2% (0.02-16.6%)	0.2% (0-8.8%)	↓ *0.06*
Rickenellaceae	1.1% (0.002-3.1%)	0.09% (0-3.9%)	↓ *0.05*
Bacteroidaceae	17.2% (6.0-46.8%)	3.6 (0.04-43.8%)	↓ 0.04
Firmicutes Phylum			
Lachnospiraceae	24.5% (8.7-52.1%)	13.6% (0.09-59.9%)	↓ 0.009
Christensenellaceae	0.3% (0-15.2%)	0.001% (0-1.5%)	↓ 0.04
Ruminococcaceae	11.3% (0.8-29.3%)	6.4% (0.02-22.0%)	↓ 0.04
Proteobacteria Phylum			
Brucellaceae	0% (0-0.01%)	0.001% (0-0.6%)	↑ 0.02
Xanthomonadaceae	0.008% (0-0.4%)	0.04% (0-9.8%)	↑ 0.04
Moraxellaceae	0.6% (0.06-4.2%)	1.37% (0.06-11.9%)	↑ *0.05*
Rhodospirillaceae	0.01% (0-1.3%)	0% (0-0.2%)	↓ 0.02

Values are shown as median, range with arrows indicating an increase (↑) or decrease (↓) in relative abundance in HIV-infected subjects compared to uninfected subjects. *Statistical analysis performed using Mann-Whitney test. Italics highlight p values trending towards significant.



Supplementary Figure S2. Flow chart illustrating the altered microbiota in colonic mucosal tissue of HIV-infected individuals. These flow charts illustrate the specific bacteria that are altered in relative abundance in colonic mucosal tissue of HIV-infected subjects and their rank within the taxonomic hierarchy (Phylum, Class, Order, Family, Genus) within a) Bacteroidetes, b) Firmicutes and c) Proteobacteria phyla. Each box represents a change in the relative abundance of bacteria in HIV-infected subjects compared to uninfected subjects (blue:increase; red: decrease; black: no significant difference). Statistical analysis was performed using the Mann-Whitney test. italics: trend only.



Supplementary Figure S3. Significant associations between mucosa-associated genera and immune and virological parameters in HIV-1 infected subjects. Cartesian graphs are shown for genera that significantly associated with immune or virological paramters as illustrated in Figure 3a. Additional associations between Prevotella and activated colon T cells and CD1c+ mDC are shown in Figures 3b-c. Statistical analysis was performed using the Spearman t test.

Supplementary Data, Materials and Methods.
Dillon *et al*.

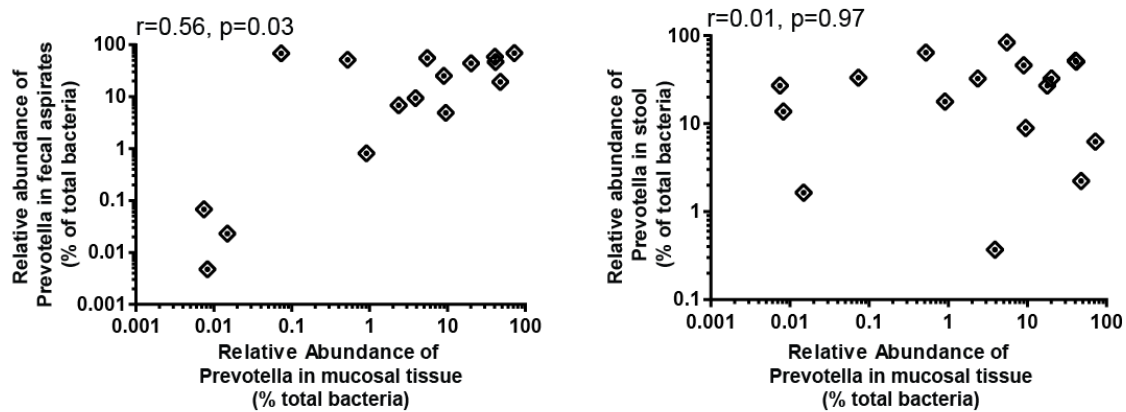
	Mucosal tissue	Fecal Aspirates	Stool
Phylum			
Proteobacteria			
Firmicutes			
Bacteroidetes			
Families in Proteobacteria phylum			
Brucellaceae			
Xanthomonadaceae			
Rhodospirillaceae			P=0.098 (decrease)
Genera in Proteobacteria phylum			
Acinetobacter			P=0.099 (increase)
Thalassospira			P=0.092 (decrease)
Families in Firmicutes Phylum			
Lachnospiraceae			
Christensenellaceae			
Ruminococcaceae		P=0.08 (decrease)	
Genera in Firmicutes phylum			
Blautia			
Coprococcus			
Families in Bacteroidetes phylum			
Prevotellaceae			
Bacteroidaceae			
Genera in Bacteroidetes phylum			
Prevotella			P=0.099 (increase)
Bacteroides			
Barnesiella			
Alistipes			

Statistically significant increase in relative abundance in HIV-infected subjects compared to uninfected subjects

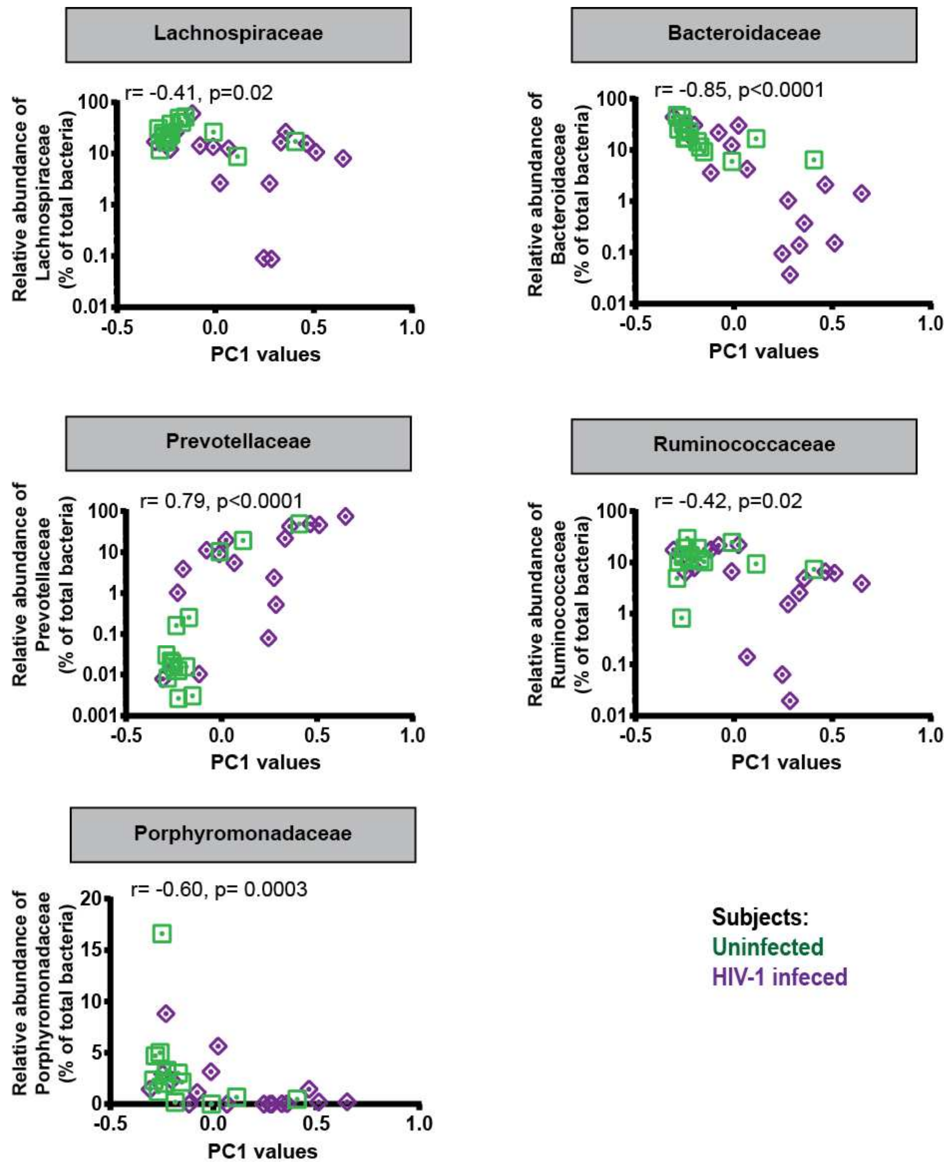
Statistically significant decrease in relative abundance in HIV-infected subjects compared to uninfected subjects

No statistically significant difference in relative abundance in HIV-infected subjects compared to uninfected subjects

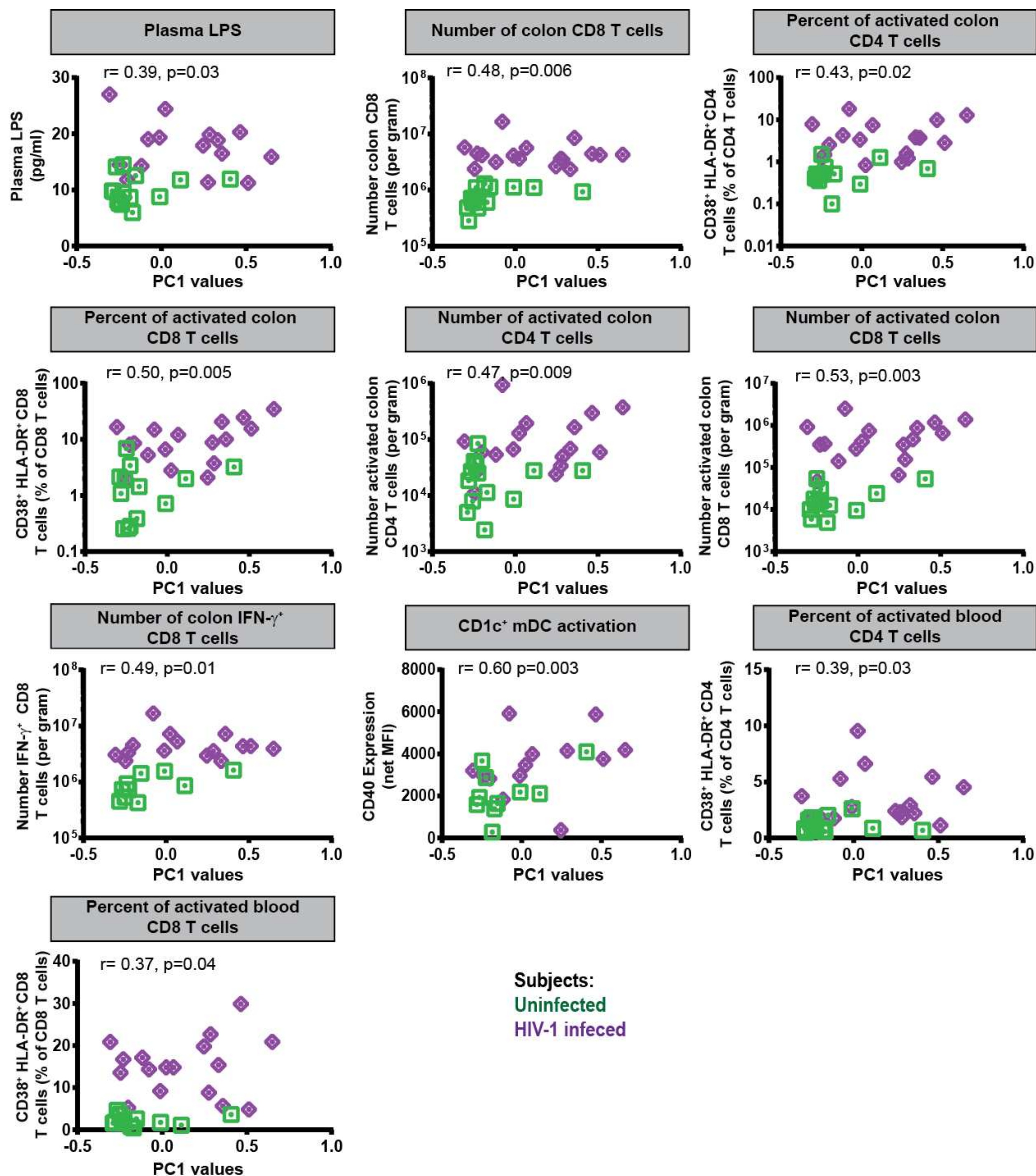
Supplementary Figure S4. Color-coded diagram illustrating differences in relative abundance of bacteria between HIV-infected and uninfected subjects in mucosal tissue, fecal aspirates and stool samples. The specific phyla, families and genera shown are based on the mucosal-adherent bacteria in mucosal tissue that are considered significantly different in HIV-infected individuals compared to uninfected subjects. Statistical analysis comparing the relative abundance of bacteria in HIV-infected and uninfected subjects was performed using the Mann-Whitney test.



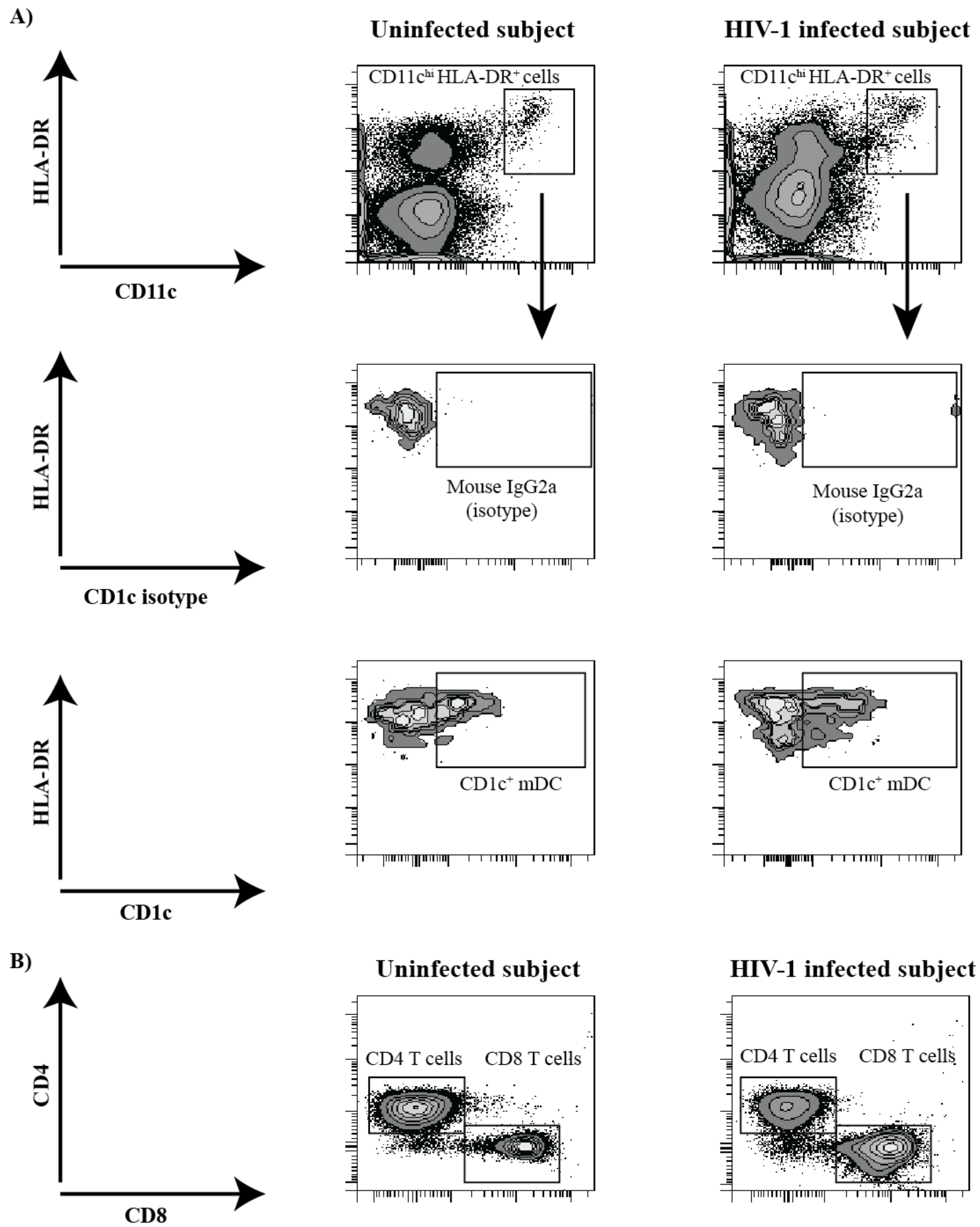
Supplementary Figure S5: Relative abundance of mucosa-associated Pretotella in HIV-1 infected subjects associates with the relative abundance of Prevotella in fecal aspirates, but not in stool samples. Correlations between the relative abundance of Prevotella in mucosal tissue and relative abundance in a) fecal aspirates (n=16) or in b) stool (rectal swabs; n=17) in HIV-1 infected subjects. Statistical analysis was performed using the Spearmen t test.



Supplementary Figure S6. Significant associations between PC1 values and bacterial families. Cartesian graphs are shown for bacterial families that significantly associated with PC1 values as illustrated in Figure 4d. Statistical analysis was performed using the Spearman t test.



Supplementary Figure S7. Significant associations between PC1 values and immunological parameters. Cartesian graphs are shown for immunological parameters that significantly associated with PC1 values as illustrated in Figure 4e. Statistical analysis was performed using the Spearman t test.



Supplementary Figure S8. Representative flow plots illustrating colon CD1c^{+} mDC and T cell populations in an uninfected and in an HIV-1 infected subject. All populations were identified from viable, CD45^{+} cells (plots not shown). A) CD1c^{+} mDC were then identified within CD11c^{hi} HLA-DR^{+} cells using an isotype control. B) CD4 and CD8 T cells were identified within CD3$^{+}\gamma\delta^{neg}$ T cells (not shown) to exclude CD4 and CD8-expressing $\gamma\delta$ T cells.

SUPPLEMENTARY MATERIALS AND METHODS

Study exclusion criteria.

The list of exclusion criteria for HIV subjects included: 1) presence of AIDS-defining condition (WHO Criteria) within 6 months and CD4 count <200 cell/µl within 3 months of clinic visit, 2) HIV-1 plasma viral load <2000 copies/ml or designation as "long-term non-progressor", 3) ART taken for more than 7 days within the past 6 months. Exclusion for all subjects included 1) medical history of bleeding disorder, 2) daily use of aspirin or NSAIDS with inability to withhold drug for 10 days, 3) medical history of rectal bleeding within the preceding year or surgical history of left-sided colonic, rectal or anal resection or anastomosis, or bleeding from prior endoscopic procedures, 4) medical history of inflammatory bowel disease, celiac disease, colitis or other intestinal inflammatory disorders, 5) any chronic or acute medical condition that would interfere with study conditions such as cancer, heart disease or diabetes, 6) acute or chronic hepatitis or treatment for viral hepatitis within the past 6 months, 7) a bacterial infection requiring antibiotic therapy within 3 months of enrollment, 8) continuous use for more than 3 days within the past 60 days of immunosuppressives, immune modulators, antineoplastic agents or probiotics, 9) vaccination of any sort within 1 week of sample collection.

Collection and storage of samples.

Rectal swaps were collected by insertion of a sterile cotton swab into the rectum at the time of study screening (typically 2 weeks prior to flexible sigmoidoscopy procedure). Swabs were immediately stored in liquid nitrogen. On the day of the clinic visit, all subjects had been fasting for at least 12hrs and underwent an enema (Sunmark McKesson, San Francisco, CA) before the flexible sigmoidoscopy procedure. A saline rinse of the surrounding area was performed prior to the collection of the pinch biopsies (fecal aspirate). The fecal aspirate was collected into sterile tubes and stored in liquid nitrogen. The subjects underwent a flexible sigmoidoscopy using 2.3mm standard forceps. Twenty biopsies were placed in HBSS for immediate tissue digestion. Biopsies for tissue HIV-1 viral load assessment and DNA isolation were placed in RNAlater (Ambion, Austin, TX) (n=4 per subject) for 20-24hrs, snap frozen and stored in liquid nitrogen. Peripheral blood samples were also processed immediately for plasma and PBMC isolation as previously described.[1]

Processing of colon biopsies for single cell suspensions.

Biopsies were washed in HBSS (Cellgro, Manassas, VA) and digested in Collagenase D Treatment media consisting of RPMI (Invitrogen, Carlsbad, CA) + 2% Bovine Serum Albumin (BSA; Sigma-Aldrich, St Louis, MO) + 1% Penicillin/Streptomycin/L-glutamine (Invitrogen, Carlsbad, CA) + 500ug/ml piperacillin/tazobactam (Zosyn; Wyeth, Madison, NY) + 1.25ug/ml amphotericin B (Fungizone; Invitrogen) + 10µg/ml DNase (Sigma-Aldrich) + 0.25mg/ml Collagenase D (Roche Indianapolis, IN) for 40min at 37°C with gentle rotation. Released cells were passed through a 70µm cell strainer and the remaining tissue washed with HBSS + 500µg/ml piperacillin/tazobactam + 1.25µg/ml amphotericin B and any additional released cells passed through the cell strainer. Collagenase-digestion and washing were performed 1-2 more times with mechanical disruption using the plunger end of a 1ml syringe until all tissue was digested. All released cells were pooled and counted with large epithelial cells and dead cells excluded.

Collection of plasma and serum and processing of PBMC.
Peripheral blood was collected into BD Vacutainer Blood Collection Tubes containing EDTA or Heparin (BD Biosciences; San Jose, CA) and whole blood centrifuged to isolate plasma. For serum collection, peripheral blood was collected into BD Vacutainer Blood Collection Tubes containing a clot activator (BD Biosciences) and left at RT for 60min prior to centrifugation. Plasma and serum were frozen at -80°C in single-use aliquots. PBMC were then isolated from heparinized blood by standard Ficoll-Hypaque (GE Healthcare, Piscataway, NJ) density gradient centrifugation, cryopreserved and stored in liquid nitrogen as previously described.[1-3]

Surface and intracellular flow cytometry analysis.
All antibodies and dyes are listed in Supplementary Table S2. To identify viable, mononuclear cells and exclude epithelial cells, cells were first gated on CD45 expression and then viable cells within this population determined. $CD1c^+$ mDC were defined within an $HLA\text{-}DR^+$ $CD11c^{hi}$ population to exclude B cells and macrophages with specific CD1c expression determined using an isotype control **(Supplementary Figure S8)**.[3,4] Activation was determined by evaluating the level of specific CD40 expression using an isotype control. Evaluation of $CD1c^+$ mDC frequency and degree of activation were only performed when there were at least 25 $HLA\text{-}DR^+CD11c^+CD1c^+$ events. CD4 and CD8 T cells were gated from within a $CD3^+\gamma\delta$ TCR^- population to exclude intestinal γδ T cells that express CD4 and CD8 **(Supplementary Figure S8)**. To determine activated colon CD4 and CD8 T cells, the combination of antibodies was altered slightly to accommodate other fluorochrome-labeled antibodies and a specific CD4 antibody was excluded from the antibody cocktail. CD4 T cells were identified as $CD3^+CD8^-$ and T cell activation evaluated by determining expression of HLA-DR in combination with CD38 or matched isotype control. Peripheral blood T cells were identified as $CD3^+CD4^+$ or $CD3^+CD8^+$.

Standard ICF assays were followed to identify cytokine-producing T cells as previously described.[5] Mitogenic stimulation drastically reduced CD4 expression therefore CD4 T cells were identified as $CD3^+\gamma\delta^-CD8^-$ and CD8 T cells as $CD3^+\gamma\delta^-CD8^+$ within viable, $CD45^+$ cells. Specific expression of IFN-γ, IL-17 or IL-22 were then determined using isotype controls.

$CD1c^+$ mDC activation was assessed by evaluating CD40 expression levels with values shown as mean fluorescent intensity (MFI) with background isotype values removed (net MFI). The percentage of $cytokine^+$ T cells are reported as net values with background isotype staining values removed. For enumeration of DC and T cell frequencies, the percentage of DCs or T cells within viable, $CD45^+$ cells was converted to an absolute number per gram based on the frequency within viable, $CD45^+$ cells, initial cell counts and biopsy weights. Similarly, the percent of activated colon CD4 and CD8 T cells as well as the percent of $cytokine^+$ CD4 and CD8 T cells were also converted to a total number per gram of mucosal tissue.

Supplementary Table S2. Antibodies and dyes used in flow cytometry protocols.

	Company	Location
Viable, mononuclear cell identification		
PerCpCy5.5 CD45	eBioscience	San Diego, CA
Aqua Live/Dead Fixable Viability dye	Invitrogen	Carlsbad, CA
CD1c^{+} mDC identification and assessment of activation		
APC BDCA-1	Miltenyi Biotec	Auburn, CA
APC mouse IgG2a isotype control		
APC-Cy7 HLA-DR	BD Biosciences	San Jose, CA
PE-Cy5 CD11c	BD Biosciences	
Biotinylated CD40	Ancell	Bayport, MN
Biotinylated mouse IgG1 isotype control		
Streptavidin ECD (PE-Texas Red)	Beckman Coulter	Fullerton, CA
Colon T cell identification and assessment of activation		
eFluor^{450} CD3	eBioscience	
AF700 CD4	BD Biosciences	
PE-Cy7 CD8	BD Biosciences	
PE γδ TCR	BD Biosciences	
APC-Cy7 HLA-DR	BD Biosciences	
AF700 CD38	eBioscience	
AF700 mouse IgG1 isotype control		
Blood T cell identification and assessment of activation		
ECD CD3	Beckman Coulter	
PE-Cy7 CD8	BD Biosciences	
eFluor^{450} CD4	eBioscience	
APC-Cy7 HLA-DR	BD Biosciences	
APC-Cy7 mouse IgG2 isotype control		
AF700 CD38	eBioscience	
AF700 mouse IgG1 isotype control		
Colon T cell identification and assessment of cytokine production		
ECD CD3	Beckman Coulter	
APC-Cy7 CD4	BD Biosciences	
PE-Cy7 CD8	BD Biosciences	
PE γδ TCR	BD Biosciences	
AF700 IFN-γ	BD Biosciences	
AF700 mouse IgG1 isotype control		
V^{450} IL-17	BD Biosciences	
V450 mouse IgG1 isotype control		
PE IL-22	eBioscience	
PE IgG1 isotype control		

DNA Extraction and Quantification for 16S rRNA sequencing.
One rectal swab, 250μl of fecal aspirate, or one pre-weighed colon biopsy were vortexed with silicon beads and extracted DNA washed and isolated using the provided filters. Samples were eluted into 50μl of elution buffer, divided into 10μl aliquots and stored at -80°C. Total 16s

rDNA was quantified in all samples by qPCR. The Probe and Primer combination used for amplification of the total 16S rDNA in each sample were: Probe: 5'-/56-FAM/CGT ATT ACC/ZEN/GCG GCK GCT GGC AC/3IABkFQ/-3'; Primer1: 5'-GGA CTA CCA GGG TAT CTA ATC CTG TT-3'; Primer2: 5'-TCC TAC GGG AGG CAG CAG T-3'. Probe, Primers and DNA were mixed with the Taqman Gene Expression Master Mix (ABI) and amplified on a CFX96 Thermocycler (Bio-Rad Laboratories, Hercules, CA) with the following cycler conditions: 56°C 2min, 95°C 8min, 40 cycles of 95°C for 30sec/56°C for 30sec/62°C for 1min with a final elongation step of 62°C for 8min. Sample concentrations were calculated by comparing sample RFUs to a clostridium plasmid eight point standard curve.

Analysis of 16S rRNA Sequences

Illumina paired-end sequencing was performed on the Miseq platform with version 2.0 of the Miseq Control Software, using a 500-cycle version 2 reagent kit. Paired-end reads were sorted by sample via barcodes in the paired reads with a python script. The sorted paired reads were assembled using phrap.[6,7] Assembled sequence ends were trimmed over a moving window of 5 nucleotides until the average quality score met or exceeded 20. Trimmed sequences with more than 1 ambiguity or shorter than 200 nt were discarded. Potential chimeras identified with Uchime (usearch6.0.203_i86linux32)[8] using the Schloss[9] Silva reference sequences were removed from subsequent analyses. Assembled sequences were aligned and classified with SINA (1.2.11) using the 244,077 bacterial sequences in Silva 111NR[10] as reference configured to yield the Silva taxonomy. Operational taxonomic units (OTUs) were produced by clustering sequences with identical taxonomic assignments. The software package Explicet[11] (v. 2.8.3) was used to display, analyze, and generate figures for microbiome data. Alpha-biodiversity indices (Good's coverage, S_{obs}, S_{chao1}, Shannon Diversity, Shannon Evenness) were estimated using resampling (1000 replicates) of sequence datasets rarified to 10000 reads/sample.[11]

Determination of colon tissue viral load.

To extract total RNA from pre-weighed colon biopsies, samples were mechanically disrupted in 0.6ml of buffer RLT (with addition of Beta-Marcaptoethanol) and homogenized. Subsequently, RNA was extracted according to the manufacturer's instructions, inclusive an incubation with 80 µl DNAse I (RNAse free DNAse set, Qiagen) for 30 minutes and finally eluted in 50µl elution buffer.

Using extracted RNA, HIV cDNA was generated using the SuperScript III first-strand synthesis kit (Invitrogen) according to the manufacturers' protocol with specific primer mf302 (PMID: 20116399). HIV RNA was quantified by real-time (RT)-PCR in an ABI 7900HT thermocycler (Applied Biosystems, Foster City, CA) with 0.005 ROX µM dye (Invitrogen) as a passive reference. A total reaction volume of 50 µl was added to each well consisting of 5 µl of cDNA template, TaqMan Environmental Mastermix 2.0 (Applied Biosystems), PCR primers mf302 and mf299 (1µM each), and probe mf348 (0.3µM) (PMID: 20116399). The PCR conditions were 2 min at 50°C, 10 min at 95°C, and 60 cycles of 15 s at 95°C and 60 s at 60°C. HIV RNA quantification standard was obtained from the DAIDS Virology Quality Assurance (VQA) Program (PMID: 8897167).

Finally, normalization was applied to the cellular input obtained with the housekeeping gene glyceraldehyde phosphate Dehydrogenase (GapDH), as determined by a separate RT-PCR reaction. To account for variation in the number of $CD4^+$ T cells in different samples, HIV RNA copy numbers were also normalized per CD4 T cell within each biopsy calculated by the percent

of all viable cells that were $CD45^{+}CD3^{+}CD4^{+}$ (by flow cytometry) and weight of each biopsy and reported as HIV-1 RNA per million CD4 T cell.

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