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Microbial Translocation Does Not Drive Immune Activation in Ugandan Children Infected With HIV

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Objective. Immune activation is associated with morbidity and mortality during human immunodeficiency virus (HIV) infection, despite receipt of antiretroviral therapy (ART). We investigated whether microbial translocation drives immune activation in HIV-infected Ugandan children.

Methods. Nineteen markers of immune activation and inflammation were measured over 96 weeks in HIV-infected Ugandan children in the CHAPAS-3 Trial and HIV-uninfected age-matched controls. Microbial translocation was assessed using molecular techniques, including next-generation sequencing.

Results. Of 249 children included, 142 were infected with HIV; of these, 120 were ART naive, with a median age of 2.8 years (interquartile range [IQR], 1.7–4.0 years) and a median baseline CD4⁺ T-cell percentage of 20% (IQR, 14%–24%), and 22 were ART experienced, with a median age of 6.5 years (IQR, 5.9–9.2 years) and a median baseline CD4⁺ T-cell percentage of 35% (IQR, 31%–39%). The control group comprised 107 children without HIV infection. The median increase in the CD4⁺ T-cell percentage was 17 percentage points (IQR, 12–22 percentage points) at week 96 among ART-naïve children, and the viral load was <100 copies/mL in 76% of ART-naïve children and 91% of ART-experienced children. Immune activation decreased with ART use. Children could be divided on the basis of immune activation markers into the following 3 clusters: in cluster 1, the majority of children were HIV uninfected; cluster 2 comprised a mix of HIV-uninfected children and HIV-infected ART-naïve or ART-experienced children; and in cluster 3, the majority were ART naive. Immune activation was low in cluster 1, decreased in cluster 3, and persisted in cluster 2. Blood microbial DNA levels were negative or very low across groups, with no difference between clusters except for Enterobacteriaceae organisms (the level was higher in cluster 1; $P < .0001$).

Conclusion. Immune activation decreased with ART use, with marker clustering indicating different activation patterns according to HIV and ART status. Levels of bacterial DNA in blood were low regardless of HIV status, ART status, and immune activation status. Microbial translocation did not drive immune activation in this setting.

Clinical Trials Registration. ISRCTN69078957.

Keywords. HIV; children; microbial translocation; immune activation; sequencing; pediatrics; Africa.

In 2016, 160 000 children acquired human immunodeficiency virus (HIV) infection, approximately 90% of whom were in sub-Saharan Africa [1]. Untreated HIV infection results in chronic immune activation [2, 3] and associated poorer

immune recovery, increased mortality, and increased morbidity [4, 5]. Immune activation influences CD4⁺ and CD8⁺ T cells, monocytes, and dendritic cells and is associated with increased expression of proinflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor (TNF) [2, 6], polyclonal B-cell activation, and hypergammaglobulinemia [7, 8]. Immune activation can persist, albeit at lower levels, despite viral suppression with antiretroviral therapy (ART) [2, 9]. Because immune activation pathways may differ with regard to drivers and impact, depending on setting and age group, quantifying HIV-related immune activation involves measuring levels of a broad range of cellular, soluble, and inflammatory markers (eg, T-cell activation, TNF, and C-reactive protein [CRP]) [10].

The causes of immune activation in children are poorly characterized. One potential driver is microbial translocation. It is postulated that depletion of intestinal CD4⁺ T cells during the early

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stage of HIV infection allows increased microbial translocation from the gut, driving a chronic immune response [11, 12]. These microbial products include immunostimulants such as lipopolysaccharide (LPS), a component of gram-negative bacterial outer membranes, and 16S ribosomal DNA (rDNA), which is common to all bacterial species. Increased levels of circulating LPS are associated with higher levels of immune activation in HIV-infected adults [4, 12]. Higher levels of LPS and 16S rDNA are associated with poorer immune restoration in patients receiving ART [11].

However, the evidence that microbial translocation drives immune activation is inconsistent, and quantification has proved challenging. 16S rDNA polymerase chain reaction (PCR) analysis can be contaminated by artifacts from endogenous/exogenous bacteria [13, 14], and without sequencing the PCR product it is impossible to exclude contamination. Some studies have not found an association between microbial translocation and immune activation [15, 16]. Other studies have been cross-sectional or retrospective [17–20]. Translocation may be due to, rather than a cause of, severe HIV disease [21]. There are few data from Africa. One large African longitudinal study (performed in Uganda) found no significant increase in microbial translocation during untreated disease progression in adults; a high CRP level was associated with mortality without a high LPS level [22, 23]. Data in children are limited and lack consistent associations, such as between 16S rDNA/LPS and T-cell activation or inflammatory markers [24–28].

Although microbial translocation is an attractive hypothesis, further longitudinal studies are needed, particularly among children in sub-Saharan Africa, since microbial translocation in HIV-infected children has not been consistently associated with cellular/soluble pathways of immune activation [24–27, 29]. We therefore investigated the impact of microbial translocation on cellular/soluble immune activation pathways and vascular damage in Ugandan HIV-infected children, compared with findings in age-matched, HIV-uninfected controls, using a panel of molecular techniques, including next-generation sequencing (NGS) of bacterial 16S rDNA in blood. We hypothesized that if microbial translocation drove immune activation in this cohort, we would detect significant differences in levels of plasma bacterial DNA between children with and those without immune activation.

METHODS

Study Population

We included HIV-infected children enrolled in Uganda in CHAPAS-3, a trial comparing the toxicity and efficacy of first-line ART drugs (abacavir, zidovudine, or stavudine, given with lamivudine and either nevirapine/efavirenz; clinical trials registration ISRCTN69078957) [30]. Eligible children were ART naive or were ART experienced and had been receiving a stable stavudine-containing first-line ART regimen for >2 years with a viral load (VL) of <50 copies/mL. Age, ART, weight, height,

and comorbidities were recorded longitudinally. Hematologic parameters, biochemical parameters, and CD4⁺ T-cell counts were measured at weeks 6, 12, and 24 and every 24 weeks thereafter; VL was measured in stored samples at 48 and 96 weeks (Supplementary Materials). At baseline and week 12 visits, whole-blood specimens were collected and, within 2 hours, were separated into cell pellets and plasma by centrifugation at 1500×g for 15 minutes and stored at –80°C. For flow cytometry, whole-blood specimens were evaluated within 4 hours of collection.

The trial showed good clinical, virological, and immunological responses in all randomized groups, with a low level of toxicity [30]. For this substudy, we included all 120 ART-naive and 22 ART-experienced children recruited at the Joint Clinical Research Centre (Kampala, Uganda). A total of 143 HIV-uninfected controls were recruited for a single cross-sectional assessment from community well-child clinics (Kampala). These children had their HIV-uninfected status confirmed and were age matched (±1 year) at a ratio of 1:1 to enrolled HIV-infected children during the recruitment phase. After clinical assessment of each child, a 10-mL frozen plasma specimen was collected and stored without cell pellets [31]; 109 had a sufficient volume of plasma for evaluation by assays used in this study. For microbial translocation assays, these cross-sectional samples plus longitudinal samples collected from HIV-infected children at baseline (before ART initiation, for ART-naive children) and weeks 12 and 72 were used. For markers of immune activation, samples collected at weeks 0 and 96 were used.

Microbial Translocation

Because broad-range 16S rDNA PCR analysis is vulnerable to contamination, we used a quantitative PCR (qPCR) panel targeting bacteria previously implicated in translocation (eg, Enterobacteriaceae species [32]), known gut bacteria (eg, *Bifidobacterium* species), and bacteria that could be gut derived or skin contaminants (eg, *Staphylococcus aureus*). Bacterial DNA was extracted from plasma and cell pellets (to detect phagocytosed bacteria); qPCR was performed to detect *Lactobacillus* species, *Bifidobacterium* species, *Fusobacterium* species, Enterobacteriaceae organisms, *S. aureus*, *Streptococcus pyogenes*, and *Staphylococcus* species, and then broad-range 16S rDNA PCR was performed, as previously described (Supplementary Materials) [33]. qPCR sensitivity varied between assays, from 0.1–1 to 1.3–13 colony-forming units (CFUs) per PCR run, using species-specific standards (Supplementary Table 1).

For broad-range 16S rDNA PCR, bacterial load (measured as the number of CFUs per PCR run) was quantified using standards of known concentrations (derived from *Escherichia coli*), and samples with equal or higher cycle thresholds than those of negative controls were considered to have negative results. Assay sensitivity was 5–50 CFUs per PCR reaction. CFUs and a ranking scheme of test results (ie, strong positive to negative), rather

than copy numbers, were used because presentation of a single 16S gene copy number can lead to overinterpretation, owing to variance of such data even within species ([Supplementary Materials](#)). NGS was performed according to the Illumina protocol, with modifications for samples with a low biomass level ([Supplementary Materials](#)).

Immune Activation and Inflammatory Markers

Because pathways between microbial translocation and markers of inflammation, cellular immune activation, and vascular damage are not well characterized in this population, we selected a broad range of markers. Coagulation factor III (Tissue Factor), and D-dimer levels were quantified using commercial enzyme-linked immunosorbent assay kits (R&D Systems, Technoclone) according to the manufacturers' instructions. Levels of the following 17 biomarkers were assessed using MesoScale Discovery in accordance with the manufacturer's protocols and were read using a QuickPlex SQ analyzer (MesoScale Discovery; [Supplementary Table 2A](#)) [31]: IL-1 receptor antagonist (IL-1RN), high-sensitivity CRP, TNF, interleukin 10, interleukin 6, the chemokines CXCL8 (IL-8) and CCL2 (also known as monocyte chemoattractant protein 1), angiopoietin 1, angiopoietin 2, E-selectin, P-selectin, the transmembrane glycoprotein ICAM-3, thrombomodulin, serum amyloid A, the glycoprotein SICAM-1, the cell adhesion molecule VCAM, and the growth factor VEGFA.

For cellular markers, 2 flow cytometry panels were used. First, the presence of HLA-DR⁺ and CD38⁺ was assessed in CD4⁺/CD8⁺ T-cell populations, to quantify activated (ie, double-positive) T cells [3]. Second, proliferation of CD4⁺ T cells was quantified using Ki67 in naive (CD45RA⁺), recent thymic emigrant (CD31⁺), and memory (CD45RA⁺-CD31⁻) T cells. Flow cytometry was performed using the FACSCalibur platform (Becton-Dickinson; [Supplementary Materials](#) and [Supplementary Table 2B](#)) [31].

Statistical Analysis

Baseline characteristics were compared between ART-naive and ART-experienced HIV-infected children and matched control groups, using the χ^2 or Fisher exact test (for binary data) and the Mann-Whitney *U* test (for continuous data). Paired baseline and week-96 levels of cellular, inflammation, cardiovascular injury and thrombogenesis markers were compared for HIV-infected groups, using Wilcoxon signed rank tests. Associations between markers of immune activation and microbial translocation were assessed using Spearman rho correlation analysis ([Supplementary Materials](#)).

Given the large number of baseline and week-96 laboratory parameters (age-associated CD4⁺ and CD8⁺ T-cell counts, viral load, inflammation biomarker levels, and immune activation and proliferation marker levels), we identified clusters of children with more-similar phenotypes, using principal

components analysis (based on the correlation matrix), followed by hierarchical clustering of the first 5 principal components by use of Ward's distance. We used baseline data from HIV-uninfected controls for baseline and week-96 analyses, assuming minimal change over time. Microbial translocation markers measured before randomization and at week 72 were compared across clusters. Pelleted samples were only available from HIV-infected children and were therefore not used in clustering.

All statistical analyses were performed with R, version 3.2.1. No formal adjustment was made for multiple testing, but results were interpreted on the basis of the strength of associations.

Ethical Considerations

The study was approved by UCL Research Ethics Committee (5019/001) and the Ugandan National Council for Science and Technology (HS1559). Written informed consent was obtained for storage and use of samples from caregivers, with participant assent obtained when appropriate.

RESULTS

This study recruited 285 children: 142 HIV-infected children (120 were ART naive, and 22 were ART experienced) and 143 HIV-uninfected controls, of whom 109 had sufficient plasma specimens for all assays (89 were age matched to ART-naive children, and 20 were age matched to ART-experienced children; [Supplementary Materials](#)). Two controls (age matched to ART-naive children) were excluded because they were statistical outliers with high levels of immune activation ([Supplementary Materials](#)). HIV-infected children had low and comparable toxicity rates in each randomized arm [30]. There were 3 deaths (due to measles, measles/pneumonia, and Kaposi sarcoma), all among ART-naive children, after 25, 38, and 56 weeks of ART. Four ART-naive patients were lost to follow-up by week 72; 1 only had baseline samples, and the others were followed up for >24 weeks. Samples were available for microbial translocation/immune activation assays for most children at each time point ([Supplementary Table 3](#)).

At enrollment, the ART-experienced group had received ART for a median of 4 years (interquartile range [IQR], 2.6–4.3 years). ART-naive children were younger, had lower CD4⁺ T-cell counts and percentages, and lower levels of anthropometric markers at baseline and showed greater gains over time, compared with ART-experienced children ([Table 1](#)). The ART-naive group was approximately 6 months younger than controls; ART-experienced children and controls were similar in age. Both HIV-infected groups had lower CD4⁺ T-cell percentages than controls. The ART-naive group had lower anthropometric values than controls at baseline, whereas values were similar between the ART-experienced group and HIV-uninfected controls.

Table 1. Characteristics of Antiretroviral (ART)–Naive and ART-Experienced Human Immunodeficiency Virus (HIV)–Infected Children and Age-Matched HIV-Uninfected Controls

Characteristic	HIV-Infected Children			HIV-Uninfected Children			
	ART Naive (n = 120)	ART Experienced (n = 22)	P	Matched to Naive Group (n = 87)	P (vs ART-Naive Group)	Matched to ART-Experienced Group (n = 20)	P (vs ART-Experienced Group)
Male sex	59 (49)	10 (45)	.82	40 (46)	.67	12 (60)	.37
Age, y	2.8 (1.7–4.0)	6.5 (5.9–9.2)	<.001	3.3 (2.4–4.4)	.004	6.3 (5.7–8.9)	.89
Baseline CD4 ⁺ T-cell count, cells/mm ³	922 (637–1451)	1188 (928–1813)	.02	1361 (1039–1728)	<.001	1010 (856–1343)	.11
Baseline CD4 ⁺ T-cell percentage	20 (14–24)	35 (31–39)	<.001	38 (34–43)	<.001	40 (35–45)	.06
Baseline viral load, copies/mL	386 800 (166 500–1 800 000)	<100	NA	NA		NA	
Change in CD4 ⁺ T-cell count from baseline to wk 96, cells/mm ³	314 (–38–657)	–157 (–438–59)	<.001	NA		NA	
Change in CD4 ⁺ T-cell percentage from baseline to wk 96	17 (12–22)	3 (0–6)	<.001	NA		NA	
Viral load <100 copies/mL at wk 96	84/111 (76)	19/21 (91)	.16	NA		NA	
Baseline weight-for-age z score	–2.0 (–3.2 to –0.9)	–1.5 (–2.2 to –0.4)	.05	–0.6 (–1.2–0.1)	<.001	–1.0 (–2.1 to –0.1)	.57
Baseline height-for-age z score	–2.5 (–3.5 to –1.3)	–1.5 (–2.1 to –1.1)	.02	–0.8 (–2.1–0.1)	<.001	–1.3 (–2.5 to –0.3)	.52
Weight-for-age z score change from baseline to wk 96	1.0 (0.2–1.9)	–0.1 (–0.2–0.0)	<.001	NA		NA	
Height-for-age z score change from baseline to wk 96	0.8 (0.2–1.3)	–0.1 (–0.3 to –1.4)	<.001	NA		NA	

Data are no. or proportion (%) of children or median (interquartile range). Categorical variables were compared using the χ^2 or Fisher exact test, and continuous variables were compared using the Mann-Whitney *U* test.

Abbreviation: NA, not applicable.

As expected, since ART-experienced children had virological suppression at enrollment, more ART-experienced children (91%; 95% confidence interval [CI], 70%–99%) than ART-naive children (76%; 95% CI, 67%–83%) had a VL of <100 copies/mL at week 96, but the difference was not significant ($P = .16$).

Microbial Translocation

Most plasma samples from all groups tested negative by qPCR (Figure 1), with the exception of Enterobacteriaceae organisms and *S. aureus*. Even in positive samples, levels were low. There was no significant difference between groups or over time in proportions of positive samples or in those testing weakly or strongly positive ($P > .05$; Supplementary Figure 1A and 1B), except in proportions positive for Enterobacteriaceae organisms between ART-naive and age-matched controls at baseline (23% [95% CI, 16%–32%] and 37% [95% CI, 27%–48%], respectively; $P = .03$). Because pellet samples were only available for HIV-infected children, comparisons to controls were not possible. The proportions positive for *S. aureus* and Enterobacteriaceae organisms were higher for pellets than plasma samples in both HIV-infected

groups: among ART-naive children, 52% of pellets (95% CI, 42%–61%) and 10% of plasma specimens (95% CI, 5%–17%) were positive for *S. aureus*, and 69% (95% CI, 59%–77%) and 23% (95% CI, 15%–32%), respectively, were positive for Enterobacteriaceae species; among ART-experienced children, 64% of pellets (95% CI, 41%–83%) and 23% of plasma specimens (95% CI, 8%–45%) were positive for *S. aureus*, and 59% (95% CI, 36%–79%) and 36% (95% CI, 18%–62%), respectively, were positive for Enterobacteriaceae species.

Results of broad-range 16S rDNA PCR of plasma samples were similar across groups and time points and revealed no difference between HIV-infected and HIV-uninfected children at baseline (median value, 100 CFU equivalents [IQR, 23–204] and 102 CFU equivalents [IQR, 46–148] among ART-naive children and their age-matched controls, respectively [$P = .45$], and 133 CFU equivalents [IQR, 77–185] and 104 CFU equivalents [IQR, 53–152] among ART-experienced children and their age-matched controls, respectively [$P = .25$]; Supplementary Figure 1C). Pellets had higher median bacterial loads than plasma samples (1190 CFU equivalents [IQR, 440–2290] vs 113 CFU equivalents [IQR, 33–200]; $P < .0001$), but bacterial loads

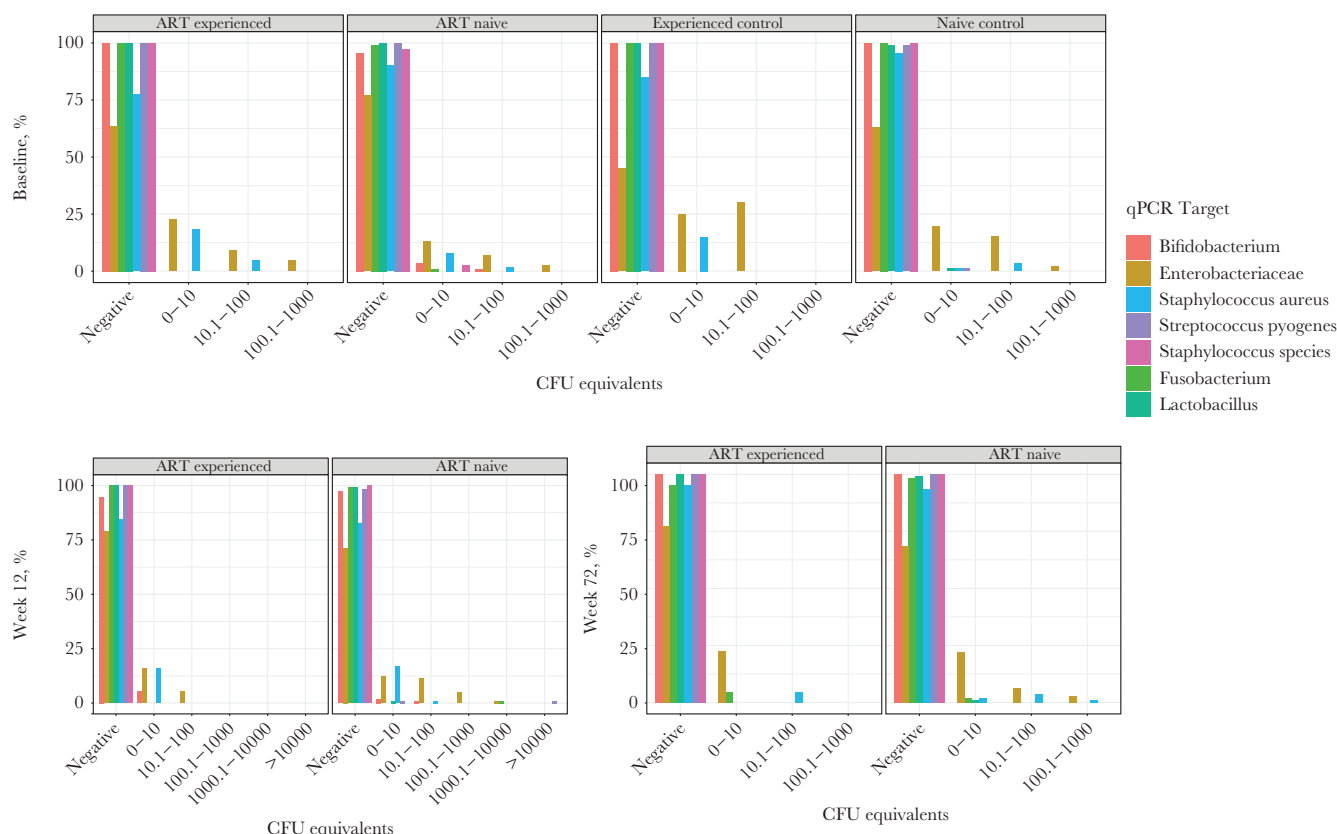


Figure 1. Quantitative polymerase chain reaction (qPCR) results over time ranked by negative to strong positive. Colony-forming unit (CFU) equivalents as compared to cycle threshold values of standards with a known CFU quantity and categorized from negative to strong positive. For each category, the total percentage of positive samples (ie, those with a result > 0) is equal to 100% minus the percentage of negative samples, shown at the left of each subpanel. ART, antiretroviral therapy.

in pellets were similar in ART-naïve and ART-experienced children ($P = .65$).

For NGS, 168 of 655 samples (26%; 105 of 140 pellets [75%] and 63 of 515 plasma samples [12%]) had sufficient quantities of the amplified 16S library to ensure successful sequencing. None of the 113 plasma samples from HIV-uninfected controls were sequenced successfully, as an insufficient quantity of the library was produced. After removing operational taxonomic units (OTUs) seen in negative experimental controls (Supplementary Materials), OTU numbers were sparse and dominated by Enterobacteriaceae organisms and staphylococci in both HIV-infected groups. Veillonellaceae, Clostridiaceae, Bacteroidaceae, and Bifidobacteriaceae organisms were also represented in low frequencies in both groups (Figure 2). Principal coordinates analysis (performed by the weighted Unifrac method [34]) revealed no clustering by ART-naïve or ART-experienced status or by time point (Supplementary Figure 2).

Immune Activation Markers

Levels of most cellular and humoral markers of immune activation, cardiovascular injury, and disordered thrombogenesis decreased significantly over time in the ART-naïve group after ART initiation (Supplementary Table 4), except for D-dimer,

angiopoietin 1 and 2, serum amyloid A, CXCL8, and IL-1RN (IL-1RA). For ART-experienced children, there were significant although less marked decreases in proliferating recent thymic emigrants (as measured by the CD45RA⁺CD31⁺Ki67⁺ cell percentage) and proliferating memory cells (as measured by the CD45RA⁻CD31⁻Ki67⁺ cell percentage), ICAM-3, interleukin 6, CCL2, VCAM, angiopoietin 2, and VEGFA. Compared with controls, all markers were higher in ART-naïve children at baseline ($P < .001$), except for IL-1RN, angiopoietin 1, and coagulation factor III. Although ART-experienced children also had significantly higher levels of immune activation markers than controls in most assays ($P < .0001$), the differences were smaller. For several cardiovascular injury and disordered thrombogenesis markers (angiopoietin 1, E-selectin, ICAM-1, VEGFA, D-dimer, and thrombomodulin), there were no differences between ART-experienced children and controls with regard to levels of 3 inflammatory markers (CXCL8, serum amyloid A, and TNF) and markers of 1 cellular immune activation pathway (double-positive activated CD4⁺/CD8⁺ T cells). There was minimal correlation between immune activation and microbial translocation markers at early or late time points among HIV-infected ART-naïve children and among HIV-infected ART-experienced children, and if any association was observed

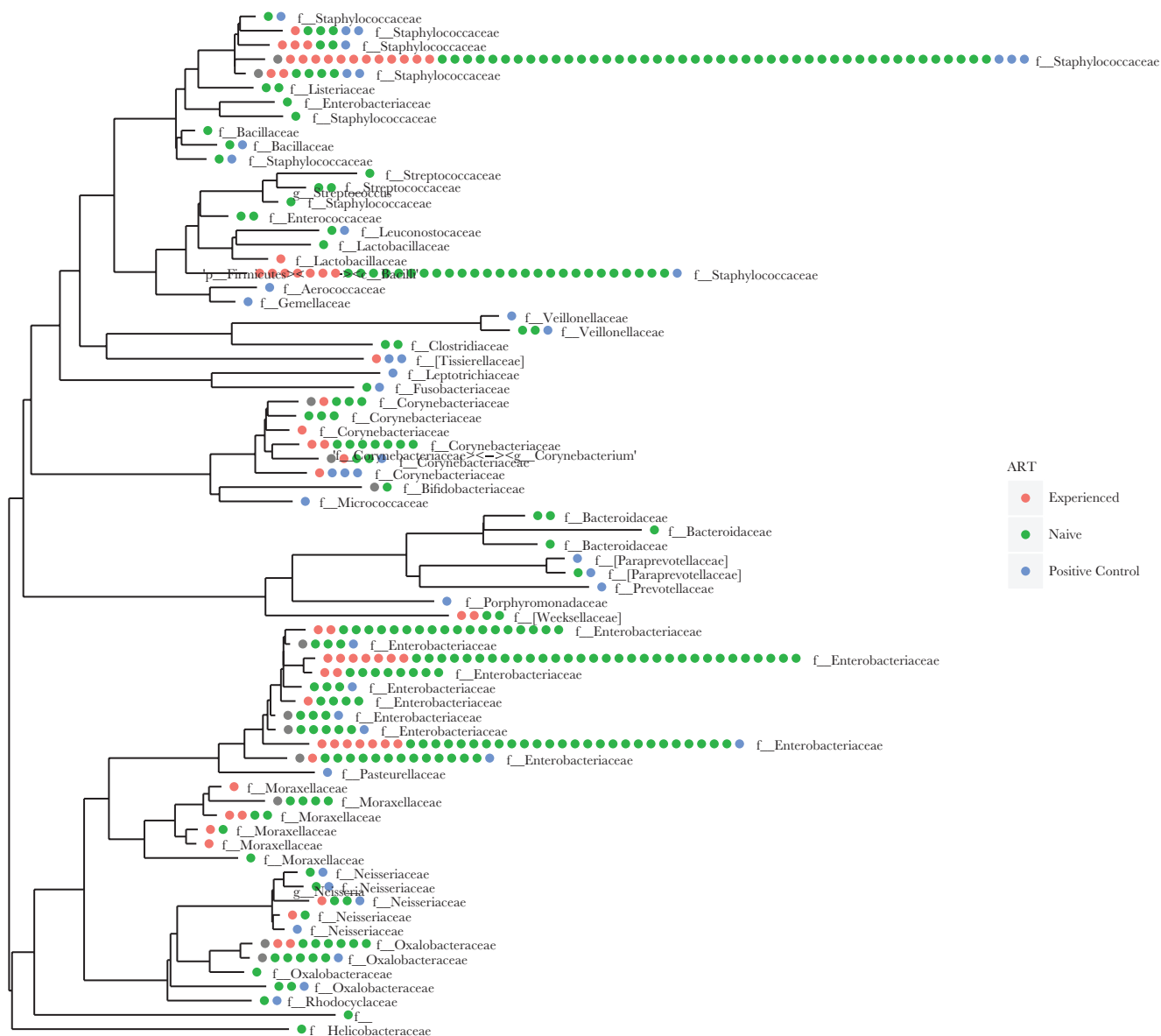


Figure 2. Results of next-generation sequencing. Positive controls are mock communities (Supplementary Materials). Phylogenetic tree, by antiretroviral therapy (ART) group (experienced or naive). Negative control operational taxonomic units (OTUs) were removed (rarefied), showing sparse OTUs, including Staphylococcaceae among other families. OTUs derived from both ART-experienced and ART-naive samples are distributed across the phylogenetic tree.

(eg, in the ART-experienced group, there was an association between 16S rDNA detection and the TNF level at week 96), it was inconsistent across time points (Supplementary Figure 3).

Immune Activation Clusters

Based on values of the 19 markers of immune activation at enrollment (for all children) and week 96 (for HIV-infected children), children could be grouped into 3 distinct clusters that reflected different intrinsic phenotypes. Cluster 1 ($n = 109$) mostly comprised ART-experienced and HIV-uninfected children, cluster 2 ($n = 33$) included HIV-uninfected controls and children from both HIV-infected groups, and cluster 3 ($n = 107$) mostly included ART-naive children (Table 2 and Supplementary

Figure 4). Cluster 1 had low levels of markers of immune activation at baseline and (for HIV-infected children) at week 96. The factors distinguishing cluster 2 were persistent immune activation at week 96, particularly in biomarkers of inflammation, cardiovascular injury, and disordered thrombogenesis, with less persistence of cellular markers (Supplementary Table 5, Figure 3, and Supplementary Figure 5). Cluster 3 had high levels of immune activation at week 0, which decreased by week 96 (Supplementary Figure 5). Of note, VL suppression at week 96 was less common in HIV-infected children in cluster 2 (3 of 13 [23%]) than those in cluster 3 (80 of 98 [83%]; $P < .0001$).

Markers of microbial translocation were low and similar across clusters, except for Enterobacteriaceae organisms: 48 of

Table 2. Characteristics and Microbial Translocation Markers, by Cluster Group

Characteristic	Cluster 1 (n = 109)	Cluster 2 (n = 33)	Cluster 3 (n = 107)	P
Percentage of all children	44	13	43	
ART status at baseline, no. (%)				<.0001
Experienced	16 (15)	3 (9)	3 (3)	
Naive	6 (5)	10 (30)	104 (97)	
HIV uninfected	87 (80)	20 (61)	0 (0)	
Viral load among HIV-infected children				
Baseline	100 (100–245)	606 275 (10 6390–1 998 000)	336 520 (141 390–949 650)	<.0001
Wk 96	100 (100–100)	171 (110–61 190)	100 (100–110)	<.0001
Virological suppression ^a at wk 96				<.0001
Yes	20 (19)	3 (3)	80 (78)	
No	2 ^b (7)	9 (31)	18 (62)	
HIV uninfected	87 (81)	20 (19)	0 (0)	
Viral load data missing	0 (0)	1 (10)	9 (90)	
Bacterial data				
Baseline	109	33	107	
I-FABP level, pg/mL	118 (59–222)	100 (57–165)	86 (40–138)	.008
16S rDNA positivity	99 (92)	28 (85)	84 (79)	.32
<i>Bifidobacterium</i> positivity	0 (0)	0 (0)	6 (6)	.02
<i>Staphylococcus aureus</i> positivity	9 (8)	4 (12)	10 (9)	.68
<i>Streptococcus pyogenes</i> positivity	0 (0)	1 (3)	0 (0)	.13
<i>Fusobacterium</i> positivity	1 (1)	0 (0)	0 (0)	.99
Enterobacteriaceae positivity	48 (44)	7 (21)	21 (20)	<.0001
<i>Staphylococcus</i> positivity	1 (1)	1 (3)	1 (1)	.51
<i>Lactobacillus</i> positivity	1 (1)	0 (0)	0 (0)	.99
Wk 72	109	33	106	
I-FABP level, pg/mL	150 (80–234)	159 (99–194)	146 (82–195)	.83
16S rDNA positivity	101 (93)	30 (91)	103 (96)	.18
<i>Bifidobacterium</i> positivity	0 (0)	0 (0)	0 (0)	
<i>Staphylococcus aureus</i> positivity	3 (3)	0 (0)	5 (5)	.26
<i>Streptococcus pyogenes</i> positivity	0 (0)	1 (3)	0 (0)	.13
<i>Fusobacterium</i> positivity	1 (1)	1 (3)	1 (1)	.51
Enterobacteriaceae positivity	48 (44)	3 (9)	31 (29)	.001
<i>Staphylococcus</i> positivity	0 (0)	0 (0)	0 (0)	
<i>Lactobacillus</i> positivity	0 (0)	0 (0)	1 (1)	.55

Data are no. (%) of children or median (interquartile range). Categorical variables were compared using the χ^2 or Fisher exact test, and continuous variables were compared using the Mann-Whitney *U* test.

Abbreviations: HIV, human immunodeficiency virus; I-FABP, intestinal fatty acid binding protein; rDNA, ribosomal DNA.

^aDefined as <100 copies/mL.

^bViral loads were 935 and 2140 copies/mL.

109 children (44%) in cluster 1 were positive for these organisms, compared with 7 of 33 (21%) and 21 of 107 (20%) in clusters 2 and 3, respectively ($P < .0001$), a difference that remained at week 72 (Table 2). There was no evidence of differences across clusters in the proportions with positive results of the other microbial assays ($P > .05$).

DISCUSSION

In this large, prospective study, with follow-up for >2 years and inclusion of HIV-uninfected controls, we aimed to evaluate the relationship between immune activation and microbial translocation in HIV-infected and uninfected children in Uganda. We used a wide range of cellular/soluble biomarkers to assess immune activation and comprehensively evaluated blood-stream bacterial DNA, using specific and broad-range PCR

analyses alongside NGS. Plasma bacterial DNA was detected at very low levels, with minimal differences between HIV-infected and uninfected children or between ART groups or over time except for Enterobacteriaceae organisms, which were found in a higher proportion of controls than ART-experienced children at baseline. Where bacterial DNA was present, there was no association with levels of immune activation.

Immune activation decreased over time in most ART-naive children, and markers of immune activation tended to be lower in ART-experienced than ART-naive children, making them more similar to age-matched controls, as expected. Cluster analysis identified a group of ART-naive children (cluster 3) with immune activation decreasing over time along with viral load, but it also identified a group (cluster 2), comprising ART-naive, ART-experienced, and HIV-uninfected children, with high

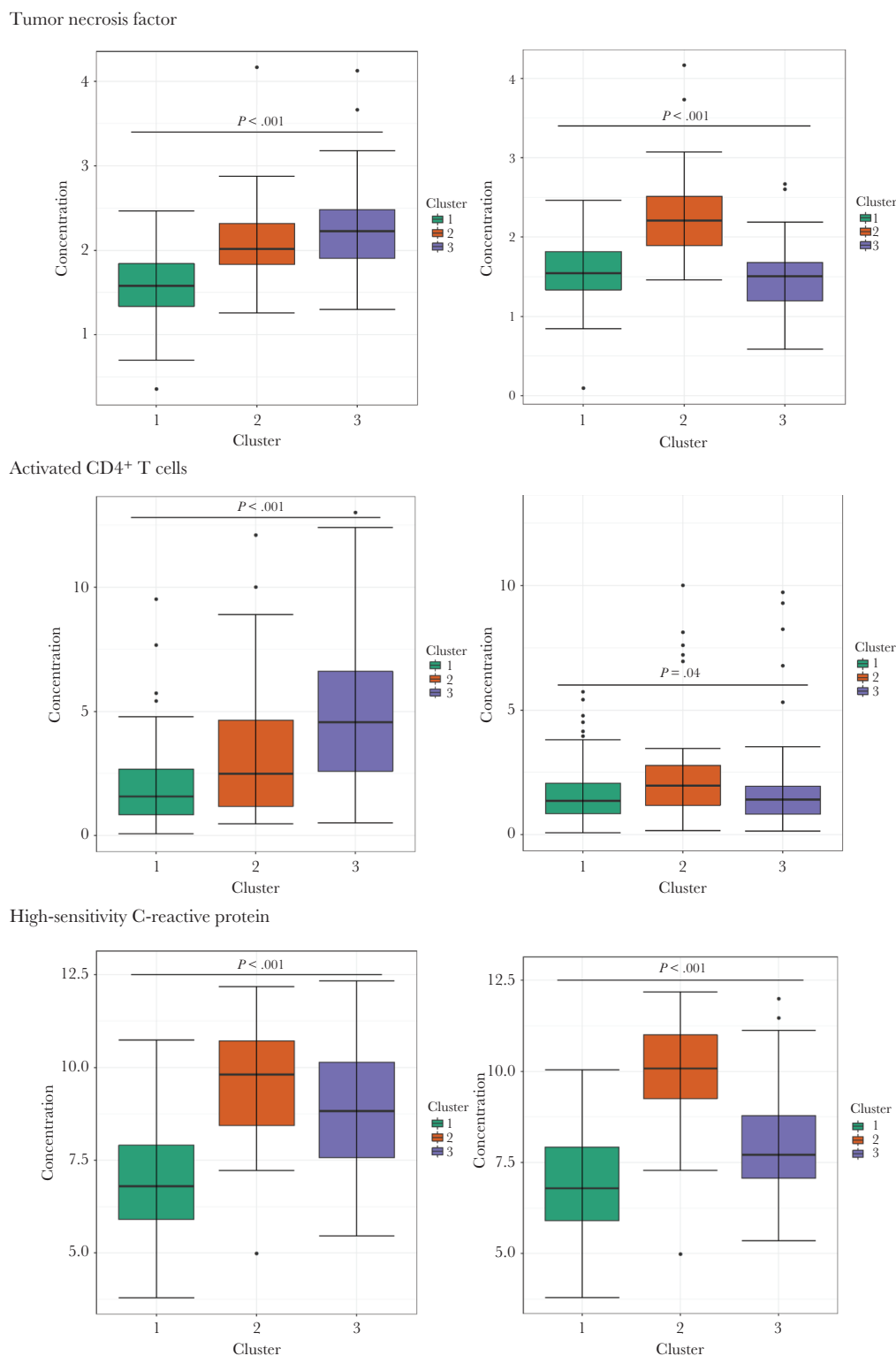


Figure 3. Box plots of log concentrations of tumor necrosis factor, activated CD4⁺ T cells (defined as, T cells double positive for HLA-DR and CD38), and high-sensitivity C-reactive protein, by cluster group, at baseline (week 0; left column) and week 96 (right column). *P* values were determined by the Kruskal-Wallis test. For concentrations of the complete set of biomarkers, see [Supplementary Figure 5](#).

levels of immune activation. In both groups there was minimal association with microbial translocation. This suggests that, in a Ugandan setting, there may be causes of immune activation beyond either microbial translocation or HIV itself.

Techniques used for detecting microbial translocation are contentious. Previous studies have used LPS, soluble CD14 (a marker of monocyte activation), lipoteichoic acid (a component of gram-positive cell walls), lipopolysaccharide-binding

protein (an acute-phase protein that binds LPS), and endotoxin core antibody (an antibody to LPS) as proxy markers of microbial translocation during HIV infection, with divergent, inconsistent results in comparison to clinical outcome, immune activation, and each other [11, 12, 16, 22, 24, 27–29, 35, 36]. Given the challenges in reproducibility and heterogeneity in previous studies [22, 29, 35, 37], we did not use LPS and instead focused on comprehensive evaluation of bacterial DNA. Broad-range 16S rDNA PCR has also shown conflicting results (Supplementary Materials). Even with sequencing, prior studies have not conclusively identified gut bacterial DNA in the bloodstream at levels greater than those of potential contaminants [11, 20, 32, 35]. By using a panel of molecular microbial techniques, including a modified broad-range 16S rDNA PCR assay used clinically for >15 years [13] and NGS, we combined sensitivity (from the qPCR assays) with specificity (from NGS) to identify bacterial species in the bloodstream that might have been derived from the gut.

The most consistently detected bacterial species across all groups was *S. aureus*, which could have been derived from the gut or a skin contaminant (despite efforts to minimize contamination). Without sequencing, it is difficult to extrapolate the degree to which previously demonstrated high frequencies of 16S rDNA positivity may have been due to skin contamination by *S. aureus* or other skin-colonizing bacteria, rather than to gut translocation. Using NGS, bacteria were identified that could be consistent with microbial translocation from the gut, such as *Veillonella* species and *Fusobacterium* species. However, they were found at very low levels in both ART-naïve and ART-experienced children at baseline (Supplementary Figure 2).

Enterobacteriaceae organisms were found in HIV-infected individuals during 2 previous studies that used sequencing methods [20, 32]. Although in this study, Enterobacteriaceae species were detected using both qPCR and NGS, the frequency of detection by qPCR was consistently low over time (and lower than was considered clinically significant in previous use of the assay [38]) in both HIV-infected groups, indicating that the results may have been due to contamination. Enterobacteriaceae species are unlikely to be driving immune activation, because the ART-naïve group experienced a diminution of immune activation over time and because positivity rates were higher in HIV-uninfected controls than cases at baseline (37% vs 23%; $P = .03$). Furthermore, given minimal differences between other groups, the assay's inherent variability, and its vulnerability to contamination, these results may be false positives. However, the possibility remains that findings reflect levels of microbial translocation in both HIV-infected and uninfected groups that are low but not linked to immune activation. Given this possibility, despite previous provisos, the detection of Enterobacteriaceae organisms in more controls than HIV-infected children merits further investigation.

We were unable to sequence DNA in any plasma samples from HIV-uninfected controls, likely because of insufficient DNA present despite similar sample volumes, which may mean that there is a biologically significant difference in the quantities of bacterial DNA between HIV-infected and uninfected groups, as found in previous studies that used broad-range 16S rDNA PCR (without sequencing) [11, 20]. We did not have pellets available for controls, which in general yielded higher quantities of DNA for each assay, possibly because of detection of phagocytosed bacteria or due to the process of pelleting concentrating bacterial DNA. In future studies, pellets may be more useful in evaluating microbial translocation than plasma samples. However, it is important to emphasize that, even in pellets, despite lower immune activation, ART-experienced children had levels of bacterial DNA similar to those in ART-naïve children. Future studies must consider the challenges associated with interstudy comparisons, as described in the Supplementary Materials.

From this comprehensive evaluation of bacterial DNA in the bloodstream of HIV-infected and HIV-uninfected children in Uganda, there is little evidence that microbial translocation is occurring at biologically significant levels or that it is driving immune activation, because markers of immune activation (along with viral load) fell significantly over time during ART but no difference was seen in levels of microbial DNA. This conclusion supports the finding that trials aiming to modify microbial translocation have not yet demonstrated clinically relevant improvements in HIV infection [39, 40]. Therefore, alternative modifiable causes of immune activation should be considered in this setting [9, 41], including HIV itself, via proteins such as Nef and gp120 [42, 43]; release from inhibition by regulatory mechanisms, such as the activity of T-regulatory cells [44]; other infections (both acute and chronic); and malnutrition. These may be more important than microbial translocation in driving immune activation.

Coinfections and malnutrition are of particular interest because children in cluster 2 had immune activation regardless of HIV/ART status. Acute and chronic infections significantly associated with immune activation during HIV infection include cytomegalovirus infection, malaria, tuberculosis, *Candida* infection, herpes zoster, and visceral leishmaniasis [45–49]. The association between these infections and immune activation in HIV-uninfected African children merits further investigation because there may be an opportunity to improve health outcomes beyond specifically targeting HIV. Malnutrition and environmental enteropathy may, independent of HIV, also drive immune activation [50].

Study limitations include the availability of baseline pellets only for HIV-infected children; these samples had higher yields of bacterial DNA and were more likely to be sequenced successfully by NGS. In future studies, the advantages of cell pellets over plasma specimens for bacterial NGS should be considered. Samples from only 1 time point were available for control

children, so their results were used twice in the clustering analysis, based on the assumption that they had minimal changes over time. Acute infection was an exclusion criterion for control children, but some acute infections may have been missed. However, for HIV-infected children, the study was relatively large, including 142 children followed for 96 weeks.

In conclusion, based on comprehensive characterization of bacterial DNA in the bloodstream of HIV-infected children in Uganda as compared to controls from the same community, microbial translocation appeared to be low in HIV-infected children, regardless of receipt of ART and over time, whereas immune activation decreased over time in children commencing ART. A small but significant cluster of children had persistent immune activation regardless of HIV/ART status. Drivers of immune activation other than microbial translocation in both HIV-infected and HIV-uninfected children merit further investigation in African settings.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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