Title

Permalink
https://escholarship.org/uc/item/3jj568gj

Journal
Clinical Infectious Diseases, 67(8)

ISSN
1058-4838

Authors
Wood, Lianna F
Brown, Bryan P
Lennard, Katie
et al.

Publication Date
2018-09-28

DOI
10.1093/cid/ciy265

Peer reviewed
Feeding-Related Gut Microbial Composition Associates With Peripheral T-Cell Activation and Mucosal Gene Expression in African Infants

Lianna F. Wood,1,4 Bryan P. Brown,2,3,a Katie Leonard,1 Ulas Karaoz,4,5 Enock Havyarimana,3 Jo-Ann S. Passmore,3,6 Anneke C. Hesseling,7 Paul T. Edlefsen,8 Louise Kuhn,3 Nicola Mulder,8 Eoin L. Brodie,4,5 Donald L. Sodora,10 and Heather B. Jaspan1,3,11

1University of Washington Schools of Medicine and Public Health, Seattle; 2Duke University, Durham, North Carolina; 3Institute of Infectious Disease and Molecular Medicine, University of Cape Town Health Sciences Faculty, South Africa; 4Earth and Environmental Science, Lawrence Berkeley National Laboratories, and 5University of California, Berkeley; 6National Health Laboratory Services, and 7Desmond Tutu TB Centre, Stellenbosch University, Cape Town, South Africa; 8Fred Hutchinson Cancer Research Center, Seattle, Washington; 9Columbia University, New York, New York; and 10Center for Infectious Disease Research, and 11Seattle Children's Research Institute, Washington

Background. Exclusive breastfeeding reduces the rate of postnatal human immunodeficiency virus (HIV) transmission compared to nonexclusive breastfeeding; however, the mechanisms of this protection are unknown. Our study aimed to interrogate the mechanisms underlying the protective effect of exclusive breastfeeding.

Methods. We performed a prospective, longitudinal study of infants from a high-HIV-prevalence, low-income setting in South Africa. We evaluated the role of any non–breast milk feeds, excluding prescribed medicines on stool microbial communities via 16S rRNA gene sequencing, peripheral T-cell activation via flow cytometry, and buccal mucosal gene expression via quantitative polymerase chain reaction assay.

Results. A total of 155 infants were recruited at birth with mean gestational age of 38.9 weeks and mean birth weight of 3.2 kg. All infants were exclusively breastfed (EBF) at birth, but only 43.5% and 20% remained EBF at 6 or 14 weeks of age, respectively. We observed lower stool microbial diversity and distinct microbial composition in exclusively breastfed infants. These microbial communities, and the relative abundance of key taxa, were correlated with peripheral CD4+ T-cell activation, which was lower in EBF infants. In the oral mucosa, gene expression of chemokine and chemokine receptors involved in recruitment of HIV target cells to tissues, as well as epithelial cytoskeletal proteins, was lower in EBF infants.

Conclusions. These data suggest that nonexclusive breastfeeding alters the gut microbiota, increasing T-cell activation and, potentially, mucosal recruitment of HIV target cells. Study findings highlight a biologically plausible mechanistic explanation for the reduced postnatal HIV transmission observed in EBF infants.

Keywords. exclusive breastfeeding; gut microbiota; immune activation; HIV susceptibility.

Breastfeeding is beneficial for the health of infants and their mothers, particularly in developing countries [1]. However, for human immunodeficiency virus (HIV)–infected women, breast milk is a vehicle for transmission of HIV. It is estimated that 14% of HIV-exposed, breastfed infants acquire HIV through this route, in the absence of antiretroviral prophylaxis [2]. Although, maternal antiretroviral therapy (ART) and infant prophylaxis have dramatically decreased mother-to-child transmission, around 160,000 infants still acquired HIV in 2016 [3]. However, since nonexclusive breastfeeding increases mortality and morbidity, counteracting the decrease in HIV transmission [4], current guidelines advise breastfeeding during maternal ART in developing settings.

Infants who consume only breast milk (exclusively breastfed [EBF]) have a lower risk of HIV acquisition compared to infants who are breastfed but also consume other foods (nonexclusively breastfed [NEBF]) [5]. This is somewhat counterintuitive, since infants fed supplemental foods likely consume lower volumes of HIV-containing breast milk than EBF infants. Previous studies have reported lower cell-free viral loads in breast milk of mothers who EBF compared to NEBF [6] and possibly decreased risk of mastitis with EBF [7]. Breast milk immunoglobulins capable of antibody-dependent cellular cytotoxicity may assist in prevention of HIV transmission [8]. An alternative hypothesis that nonexclusive breastfeeding increases translocation of microbes across the gastrointestinal mucosa into systemic circulation has shown conflicting results [9, 10]. Recently, NEBF infants in Uganda had higher levels of gut-homing T cells, although the cause of this increase was not identified [11]. To date, no study has systematically examined infant factors that influence susceptibility to HIV.

An important, previously unexplored factor in infant susceptibility to HIV via mixed feeding is the possible alteration...
of the gut microbiota, a known determinant of infant immune development [12], inducing activation of HIV-susceptible cells and triggering their recruitment to mucosal surfaces. Activated CD4 T cells and mucosal inflammation are key determinants of sexual transmission of HIV [13].

Here, we assessed the composition of the gut microbiota, activation in the peripheral blood, and oral mucosal gene expression in a longitudinal cohort study of infants recruited at birth. We compared these parameters among infants who remained EBF during follow-up to those who were NEBF and found lower microbial diversity, systemic immune activation, and oral mucosal chemokine and cytoskeletal gene expression in EBF infants. These data provide insight into the mechanisms that underlie reduced postnatal HIV transmission during exclusive breastfeeding, while identifying gut microbial changes that could be targeted to reduce HIV transmission to NEBF infants.

METHODS

Study Design

A total of 155 eligible infants were recruited within 12 hours of vaginal delivery at Site B Clinic in Khayelitsha, Western Cape, South Africa, between June 2011 and March 2013. Eligibility criteria included HIV-unexposed infants, term gestation, vaginal delivery, and mother planning to exclusively breastfeed. Maternal HIV status was confirmed from pregnancy records. All mothers signed consent forms in English or isiXhosa, approved by the University of Cape Town Human Research Ethics Committee and Western Institutional Review Board.

EBF infants were defined as infants fed breast milk only (except for prescribed medicine) from birth to the study visit. At each visit, mothers completed a detailed survey of infant foods consumed and infant and maternal health since the last study visit. Infants who received any other foods, including traditional medicines or water, were considered NEBF.

Sample Collection

At each visit, infants received a physical exam, and blood (EDTA), stool, oral cytobrush (Oral CDx Brush), and saliva samples (Salivette, Biodex CC) were collected. Samples were excluded from analysis if volumes or sample quality were insufficient (Supplementary Figure 1).

Sample Processing

Peripheral blood mononuclear cells were isolated (Ficoll, Sigma) within 8 hours of collection, slowly cooled to −80°C in dimethyl sulfoxide (Sigma) + 10% fetal bovine serum (Biochrom), and stored at −80°C for less than 2 years before analysis.

Peripheral blood mononuclear cells were thawed with DNase, incubated overnight with Brefeldin A (Sigma), then washed and stained with Live/Dead-Violet (Invitrogen), anti-CC chemokine receptor type 5 (CCR5)-fluorescein isothiocyanate (FITC) (2D7, BD Biosciences), anti-cluster of differentiation (CD)4-Brilliant Violet (BV)650 (OKT4, BioLegend), anti-CD39-phycoerythrin (PE)-Green (eBioA1, eBioscience), anti-CD14-PE-TexasRed (RM052, Beckman Coulter), anti-human leukocyte antigen-antigen D-related (HLA-DR)-PE-Cy5 (LN3, eBioscience), anti-a4b7-allophycocyanin (APC) (A4B7, NHP Reagent Resource), and anti-CD25-APC-Cyanine7 (Cy7) (M-A251, BD Biosciences). Samples were washed, permeabilized (Cytofix/Cytoperm, BD Biosciences), and stained with anti-CD3-Allexa680 (UCHT1, BD Biosciences) and anti-tumor necrosis factor (TNF)-PE-Cy7 (Mab11, BD Biosciences). Cells were resuspended in 1% paraformaldehyde and acquired on an LSRII (BD Biosciences). Post-acquisition compensation and analysis were performed using Flow Jo v10.0.7r2 (Treestar LLC). Samples with fewer than 1000 live/CD3+ cells were excluded from analysis, and cell subsets were excluded if the parent population had fewer than 100 cells.

Buccal samples were immediately placed in RLT lysis buffer (Qiagen) and stored at −80°C. RNA was isolated (RNeasy kit, Qiagen) and then quantified (Nanodrop, Thermo Scientific).

Stool samples were collected from diapers and stored at 4°C for no more than 6 hours, then placed at −20°C. Stools were thawed and treated with mutanolysin (Sigma Aldrich), lysozyme (Sigma Aldrich), and lysostatin (Sigma Aldrich), then disrupted with a bead-beater [14]. DNA was extracted (MioBio Powersoil DNA kit). The extracted DNA was quantified (Qubit dsDNA high-sensitivity reagent, Invitrogen).

Oral Cytobrush Nanostring

RNA was diluted and analyzed on a custom nanostring chip with probes against 248 human genes (Supplementary Table 1). The lower limit of detection was defined as the average + 2*(standard error) of diluent controls or the average + 2*(standard error) for negative control probes, whichever was greater. The 161 genes detectable in at least 25% of samples when normalized by either glyceraldehyde-3-phosphate dehydrogenase or mitogen-activated protein kinase-3 (MAPK3) were used for subsequent statistical analysis.

Oral Cytobrush qPCR

RNA was reverse transcribed (SuperScript III, Invitrogen). Taqman Universal Master Mix (Applied Biosystems) and Single Tube Taqman Assays (Applied Biosystems) were used for quantitative polymerase chain reaction (qPCR) amplification on a 7500 Fast Real Time PCR System (Applied Biosystems). Fold changes were calculated using the ΔΔCt method [15] with MAPK3 as the endogenous control and the median EBF value for each gene as the reference sample.

Stool Microbiota Characterization

The V4 region of the 16S rRNA gene was amplified using universal 515F/806R primers [16]. Pooled triplicate samples were purified (Agencourt AMPure XP beads, Beckman Coulter). Equimolar amplicon pools of 96 samples were quantified...
(KAPA Library Quantification kit, Kapa Biosystems) and sequenced from both ends (Illumina MiSeq platform, v3 chemistry).

All microbial community data processing and analysis were performed using UPARSE [17] and R, using vegan [18] and phyloseq [19] packages. Paired reads were de novo clustered into operational taxonomic units at 97% sequence identity using UPARSE. Sequence alignment was performed with Ribosomal Database Project’s secondary structure-aware INFERNAL aligner [20]. Operational taxonomic unit (OTUs) were taxonomically classified using the Naive Bayes classification approach [21] and Greengenes taxonomy (v13.8) [22]. Prior to phylogenetic analysis, all sequences were trimmed and ambiguous sites removed if the posterior probability fell below 0.95. MrBayes was used for Bayesian phylogenetic inference, using a general time reversible model with a Gamma distribution of rate heterogeneity and a proportion of invariant site estimation.

Statistical Analyses
Statistical analyses were performed in GraphPad Prism v7, Stata v12.0 (StataCorp, College Station, Texas) or R [23]. All flow cytometry data were log transformed for statistical purposes; however, means were reported on untransformed data for ease of interpretation. Adjustment for multiple comparisons was performed using the Holm step-down approach [24] for immunological data and Benjamini-Hochberg [25] for microbiota data.

Microbiome Analyses
Samples with fewer than 4000 total reads were excluded from downstream analyses. Within a sample, an OTU was disregarded if it existed at an abundance below 3e-5. Across the dataset, OTUs were removed if they existed at an abundance below 5e-5. Vegan, phyloseq, and picante [26] R packages were used to visualize ordinations and calculate statistical significance of α- and β-diversity values across categories. The Wilcoxon rank-sum test was used to compare bacterial relative abundance agglomerated at a specified taxonomic rank and test for significance of α-diversity comparisons. For β-diversity comparison, we used ADONIS on community distance matrices (Weighted Unifrac and Jensen-Shannon) to compare intra- vs inter-category distance. A permutational analysis of variance (ANOVA) was used to compare nested models of constrained combinations of principal components. Mantel and partial Mantel tests were conducted using the vegan package within R by measuring Pearson correlations between community distance matrices (weighted Unifrac and Jensen-Shannon) against a distance matrix of expression frequencies of cellular markers. A third distance matrix of infant ages was used to remove spurious correlations that arose from ontogeny. In all cases, weighted UniFrac distance matrices were permuted to maintain correlation structure between that matrix and the second matrix. A total of 999 permutations were used to calculate the P values with an alpha of .05. Differentially abundant taxa were determined using the DESeq2 package [27].

RESULTS

Cohort Characteristics
All infants were exclusively breastfed at birth, but only 44 (43.5%) and 17 (20%) of retained infants remained exclusively breastfed at 6 or 14 weeks of age, respectively (Table 1, Supplementary Figure 1). There were no differences in birthweight, weight gain, interim illnesses, or antibiotic use between EBF and NEBF infants, although gestational age differed at 6 weeks (39.2, EBF vs 38.7, NEBF weeks; unadjusted P = .042). There was no difference in gestational age and birth weight between infants lost to follow-up and those retained in the study.

Gut Microbiota Is Altered by Feeding Modality
At 6 weeks, EBF infants had lower relative abundance of *Streptococcus luteciae* (Figure 1 and Supplementary Figure 2). At 14 weeks, EBF infants had higher relative abundance of *Streptococcus lactarius*, as well as *Actinomyces* and *Atopobium* (adjusted P = .001, .036, and .015, respectively), and lower relative abundance of 2 *Bacteroides* OTUs. Within sample (α-diversity by Faith’s phylogenetic diversity [PD]) increased with age (Figure 1B). α-diversity was lower in EBF compared to NEBF infants, particularly at week 6, but did not reach statistical significance (P = .062). Feeding practice explained a significant

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Birth</th>
<th>6 Weeks</th>
<th>14 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean birthweight, kg (SD)</td>
<td>3.20 (0.38)</td>
<td>3.20 (0.41)</td>
<td>3.25 (0.37)</td>
</tr>
<tr>
<td>Mean gestational age, weeks (SD)</td>
<td>38.9 (1.41)</td>
<td>38.7 (1.49)</td>
<td>39.2 (1.22)</td>
</tr>
<tr>
<td>Interim illness (%)</td>
<td>...</td>
<td>4 (9.1)</td>
<td>9 (16.1)</td>
</tr>
<tr>
<td>Weight, kg (SD)</td>
<td>...</td>
<td>4.81 (0.57)</td>
<td>4.92 (0.74)</td>
</tr>
<tr>
<td>Interim antibiotic use (%)</td>
<td>...</td>
<td>0 (0)</td>
<td>2 (3.8)</td>
</tr>
</tbody>
</table>

Table 1. Relevant Infant Characteristics of the Cohort at Baseline and Follow-up Visits

Abbreviations: EBF, exclusively breastfed; NEBF, nonexclusively breastfed; SD, standard deviation.

*Fisher exact test.
proportion of the variance in microbiome composition ($\beta$-diversity) between EBF and NEBF infants by weighted UniFrac distances [28] (Adonis $P = .04; R^2 = 0.017$) or Jensen-Shannon (Adonis $P = .005; R^2 = 0.028$; Figure 1C).

HIV Target Cell Frequency and Activation in Peripheral Blood Is Lower in EBF Infants

CD4+ T-cell activation, measured by HLA-DR expression, was lower in EBF vs NEBF infants (Figure 2A and B), significantly so at 6 weeks (median 20.8% vs 33.3%; adjusted t test $P = .023$). CD4+CCR5+ and Treg cell frequencies did not differ between EBF and NEBF infants (Supplementary Figure 3).

Exclusive Breastfeeding Is Associated With Lower Mucosal Chemokine Receptors and Epithelial Cytoskeletal Protein Expression

Nanostring was performed to identify differentially expressed genes ($P < .05$) in buccal mucosa of EBF infants compared to infants who had been NEBF for greater than, or less than, 8

Figure 1. Microbiota composition and diversity differs between exclusively breastfed (EBF) and non-EBF (NEBF) infants. A. Bacterial community profiles between EBF and NEBF infants at various time points, colored by taxonomic order. Each bar represents an individual stool sample. B. Faith’s phylogenetic diversity between EBF and NEBF infants at each time point. C. Double principle coordinates analysis on relative abundance profiles of infant stool samples. Taxa colors correspond to those listed in panel A. Positioning of operational taxonomic units within the taxonomic plot correspond to and direct the positioning of samples in each sample plot. In all cases, EBF samples are colored green and NEBF samples are colored purple (see also Supplementary Figure 2). Abbreviations: CS, component scaled; EBF, exclusively breastfed; NEBF, nonexclusively breastfed.
weeks prior to sampling (Supplementary Figure 4). These differentially expressed genes included ARG1, caspase (CASP) 3, C–C chemokine ligand (CCL) 17, CCL22, C–X–C chemokine receptor (CXCR) 3, CXCR7, interleukin (IL) 18, IL7R, keratin (KRT) 5, and KRT10. Of interest, some genes remained altered after transitioning to nonexclusive breastfeeding; however, others, such as SLP1 and HLADQB1, were only altered if nonexclusive breastfeeding had begun at the previous visit. These findings were used to select a panel of genes that exhibited differential expression in EBF compared to NEBF infants via Nanostring and have plausible mechanistic contribution to HIV transmission. The 10 genes that fit both criteria were evaluated by qPCR in a larger number of infants at both 6 and 14 weeks (Figure 3). These included CCL5, IL12A and IL18, CCL22, CXCR3, CXCR7, IL7R, KRT5, KRT10, and CASP3.

At 6 weeks, few differences in expression of these 10 genes were evident (n = 26; Figure 3B). However, at 14 weeks, expression of a number of transcripts, including those for the chemokines CCL5 (unadjusted P = .03) and CCL22 (unadjusted P = .05), were downregulated in the oral mucosa of EBF compared to NEBF infants (n = 33; Figure 3). In addition, CXCR7 was downregulated in EBF infants relative to NEBF infants (unadjusted P = .0002, adjusted P = .002). Interestingly, KRT5 expression was more than 10-fold higher in NEBF infants at 14 weeks (unadjusted P < .0001, adjusted P = .0001).

Bacterial Community Composition Is Related to Peripheral Blood Immune Activation

We detected positive correlations between matrices of community compositional data and expression levels of the activation marker HLA-DR alone and coexpressed with CD25 (Pearson r = 0.202 and 0.195; P = .006 and .013, respectively; Table 2), which persisted after controlling for age in partial Mantel tests. Further, expression of CD25 alone and with CCR5 showed positive correlations with Jensen-Shannon distances (Pearson r = 0.131 and 0.143, P = .032 and .030, respectively).

We performed a constrained analysis of PCoA (CAP) on our 16S rRNA gene data, constrained by HLA-DR (Figure 4A). HLA-DR+CD4+ T-cell frequency explained a significant portion of the variability in the gut microbial community (permutational ANOVA P = .026). Linear combinations of all immunological factors measured did not significantly improve the model compared to HLA-DR alone (permutational ANOVA P = .035). Using DESeq2 [27], we found that counts of Akkermansia muciniphila in stool were...
Figure 3. Exclusively breastfed (EBF) infants have altered mucosal chemokine, chemokine receptor, and keratin gene expression. A. Fold change of all genes measured via quantitative polymerase chain reaction assay at 14 weeks. Genes in warm colors were upregulated in EBF infants vs non-EBF (NEBF) infants. Genes in cool colors were downregulated in EBF infants. Unadjusted $t$-test $P$-values are denoted below the bars; $P$-values in red remained significant after adjustment for multiple comparisons. B. Log$_{10}$ fold change of genes that differed between EBF (green) and NEBF (purple) infants, shown for both 6 weeks (solid bars) and 14 (open bars) weeks. Box plots represent interquartile ranges with medians; whiskers represent ranges. Unadjusted $t$-test $P$-values are shown for significant differences only. Abbreviations: CASP, caspase; CCL, C-C chemokine ligand; CXCR, C-X-C chemokine receptor; IL, interleukin; KRT, keratin.
6.5 log_2 fold higher in infants with high CD4+HLA-DR+ T-cell frequency (adjusted \( P = .005 \)). CD4+HLA-DR+ cell frequency was inversely related to counts of *Pantoea agglomerans*, *Bifidobacterium bifidum*, and *S. lactarius* (adjusted \( P = .008 \) for all), suggesting specific taxa may partially drive immune quiescence (Figure 4B).

### DISCUSSION

The protection against HIV transmission offered by exclusive breastfeeding is incompletely understood. Here, we show that exclusive breastfeeding is associated with shifts in gut microbiota composition, lower levels of immune activation within the peripheral blood, and less activation at the oral mucosa.

**Table 2. Correlations Between CD4+ T-Cell Marker Expression and Weighted UniFrac and Jensen-Shannon Distances Using the Mantel Test**

<table>
<thead>
<tr>
<th>T-Cell Marker</th>
<th>Weighted UniFrac Distance</th>
<th>Jensen-Shannon Distance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mantel r (Pearson)</td>
<td>P Value</td>
</tr>
<tr>
<td>CCR5+</td>
<td>0.033</td>
<td>.186</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>0.202</td>
<td>.006</td>
</tr>
<tr>
<td>CD25+</td>
<td>0.061</td>
<td>.170</td>
</tr>
<tr>
<td>CCR5+CD25+</td>
<td>0.061</td>
<td>.172</td>
</tr>
<tr>
<td>HLA-DR+CD25+</td>
<td>0.195</td>
<td>.013</td>
</tr>
<tr>
<td>CD25hiCD39</td>
<td>-0.0342</td>
<td>.617</td>
</tr>
</tbody>
</table>

Bolded \( P \) values are statistically significant.

Abbreviations: CCR5, CC chemokine receptor 5; CD, cluster of differentiation; HLA, human leukocyte antigen.
We propose that the increased diversity in gut microbes with nonexclusive breastfeeding induces immunologic changes that contribute to lower HIV transmission during exclusive breastfeeding.

Buccal mucosa represents roughly 60% of the oral mucosal surface area [29], and localized simian immunodeficiency virus (SIV) exposure of the buccal mucosa of primates results in systemic SIV infection [30]. Therefore, buccal mucosal barrier integrity is likely crucial in prevention of oral HIV transmission. In HIV-exposed women, the presence of elevated genital inflammation, the site of viral exposure, increases the risk of HIV acquisition [31]. The mechanism by which inflammation mediates increased HIV risk is likely complex but is thought to be mediated by recruitment and/or activation of HIV target cells, paired with compromise of mucosal barrier function, thus allowing ready access of virions to target cells [32]. In the genital tract of women at high risk for HIV with elevated mucosal cytokine levels, Arnold et al found significantly elevated cytoskeletal proteins, suggesting epithelial barrier compromise as a potential mechanism for cytokine-associated increased mucosal HIV risk [33]. Here, we observed increases in chemokines and chemokine receptors as well as cytoskeletal proteins in the buccal mucosa of NEBF infants, suggesting analogous mechanisms are at play.

Among EBF infants in our cohort, we observed relative immune quiescence compared with NEBF infants. Immune quiescence in systemic circulation confers protection from HIV transmission [34]. While immune activation is important in protection against most pathogens, HIV preferentially infects activated CD4+ T cells [35]. In highly HIV-exposed adults, differences in circulating activated CD4+ T-cell frequencies of under 1% were associated with protection from HIV infection [34]. Here, we found 12% fewer HLA-DR+CD4+ T cells in EBF infants compared to NEBF infants. Whether T-cell activation measured in blood indicates similar activation at the mucosa or whether it is an independent risk factor for HIV transmission remains to be determined. Although we did not measure HIV target cells at the mucosa, higher buccal mucosal transcript levels of chemokines capable of attracting T cells in NEBF infants suggest that these cells are likely homing to this site. Consistent with this, increased CD4+CCR5+ cells expressing the mucosal homing marker β7 were present in Ugandan NEBF infants, providing evidence that nonexclusive breastfeeding increases HIV target cells at mucosal sites [11].

Feeding practices are known to be one of the key determinates of infant gut microbial community structure [36, 37]. The gut microbiota is known to drive immune development in animal models [38, 39], and alterations in early infant gut microbiota have profound, lasting effects on T-cell development in mice [40]. Here, in human infants, we demonstrate that exclusive breastfeeding is associated with lower activation of CD4+ T cells and that the level of T-cell activation correlates with shifts in gut microbiota. The relative abundance of a few key taxa was strongly correlated with CD4+HLA-DR+ T-cell frequency, further implicating the gut microbiota in mediating the observed changes in T-cell activation. Relative abundance of Akkermansia muciniphila, a mucin-loving bacterium that is believed to be a healthy commensal in the adult gut but has been found more frequently in the gut of formula-fed infants [41], was strongly positively associated with levels of T-cell activation. Conceivably, its mucin-degrading properties could allow dysbiotic bacteria to encounter host cells more readily due to a compromised mucous layer, thereby causing inflammation in the NEBF infant gut. On the other hand, certain taxa were strongly related to relative immune quiescence, which may have potential as therapeutic interventions. These bacteria include P. agglomerans, a gram-negative symbiont that supports Lactobacilli [42] and is currently under study as an immune modulator for various indications [43], and Bifidobacterium bifidum, which, in combination with other bacteria, shifted the α and β diversity closer, albeit not completely, toward that of EBF infants in a recent randomized control trial [44]. Finally, high relative abundance of S. lactarius was significantly associated with both exclusive breastfeeding and immune quiescence, further confirming the relationships between gut microbiota, peripheral immune activation, and feeding practices.

Our study has several limitations. It is possible that differences exist in the fecal microbiota of HIV-exposed- and -unexposed infants [45], and our study includes only unexposed infants for logistic and ethical reasons. However, our findings regarding exclusive breastfeeding are generalizable beyond only the potential for HIV transmission. The cohort had a high loss to follow-up, which may have introduced bias. However, there were no baseline differences between infants who were retained and those who were lost to follow-up.

Nonetheless, these data provide plausible mechanistic insights into the observed reduction in HIV susceptibility with exclusive breastfeeding compared to nonexclusive breastfeeding. Indeed, our findings support a model in which breast milk, when given exclusively, may regulate the composition of gut microbiota, stimulating growth of bacteria that support the development of T-cell subsets that are antinflammatory. Exclusive breastfeeding may also maintain an intact epithelial barrier with low levels of chemokine and chemokine receptor expression and cellular turnover, thus avoiding recruitment of activated HIV target cells to the oral mucosa. In turn, EBF infants have fewer circulating, and likely mucosal, activated HIV target cells, which reduces their susceptibility to HIV infection. Future interventions to mitigate the risks of nonexclusive breastfeeding could include manipulation of microbial communities through anti-, pre-, or probiotics. Given the relationships between P. agglomerans, S. lactarius, and B. bifidus and T-cell quiescence, one potential intervention could be
to trial these as biotherapeutics to mitigate T-cell activation during mixed feeding.

Supplementary Data
Supplementary materials are available at Clinical 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

Financial support. This work was supported by the National Institute of Child Health and Human Development (grant K08 HD090201), the National Institute of Environmental Health Sciences (grant F30 ES022535), and the National Science Foundation (grant NSF 1106401). Additional funding was provided by the University of Washington Center for AIDS Research, a National Institutes of Health (NIH)–funded program (grant AI027757) supported by the following NIH institutes and centers: National Institute of Allergy and Infectious Diseases, National Cancer Institute, National Institute of Mental Health, National Institute on Drug Abuse, National Institute of Child Health and Human Development, National Heart Lung and Blood Institute, National Institute on Aging, National Institute of General Medical Sciences, and National Institute of Diabetes and Digestive and Kidney Diseases; the Harry Crossley Foundation; the Südafrikanse Akademie vir Wetenskap en Kuns; and Graduate Research Opportunities Worldwide, supported by the NSF and the US Agency for International Development.

Potential conflicts of interest. All authors have reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References
34. Bégaud E, Chartier L, Marechal V, et al. Reduced CD4 T cell activation and in vitro susceptibility to HIV-1 infection in exposed uninfected Central Africans. Retrovirology 2006; 3:35.