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Breast milk cellular HIV-specific interferon γ responses are associated with protection from peripartum HIV transmission

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Abstract

Objective—Breast milk is a major route of infant HIV infection, yet the majority of breast-fed, HIV-exposed infants escape infection by unknown mechanisms. This study aimed to investigate the role of HIV-specific breast milk cells in preventing infant HIV infection.

Design—A prospective study was designed to measure associations between maternal breast milk HIV-specific interferon-γ (IFN-γ) responses and infant HIV-1 detection at 1 month of age.

Methods—In a Kenyan cohort of HIV-infected mothers, blood and breastmilk HIV-*gag* IFN-γ ELISpot responses were measured. Logistic regression was used to measure associations between breast milk IFN-γ responses and infant HIV infection at 1 month of age.

Results—IFN-γ responses were detected in breast milk from 117 of 170 (69%) women. IFN-γ responses were associated with breast milk viral load, levels of macrophage inflammatory protein (MIP) 1α, MIP-1β, regulated upon activation, normal T-cell expressed, and secreted and stromal-cell derived factor 1 and subclinical mastitis. Univariate factors associated with infant HIV infection at 1 month postpartum included both detection and breadth of breast milk IFN-γ response (P=0.08, P=0.04, respectively), breast milk MIP-1β detection (P=0.05), and plasma (P=0.004) and breast milk (P=0.004) viral load. In multivariate analyses adjusting for breast milk viral load and MIP-1β, breast milk IFN-γ responses were associated with an approximately 70%

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Conflicts of interest
There are no conflicts of interest.

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reduction in infant HIV infection [adjusted odds ratio (aOR) 0.29, 95% confidence interval (CI) 0.092–0.91], and each additional peptide pool targeted was associated with an approximately 35% reduction in infant HIV (aOR 0.65, 95% CI 0.44–0.97).

Conclusion—These data show breast milk HIV-*gag*-specific IFN-γ cellular immune responses are prevalent and may contribute to protection from early HIV transmission. More broadly, these data suggest breast milk cellular responses are potentially influential in decreasing mother-to-child transmission of viruses.

Keywords

breastfeeding; breast milk cytotoxic T lymphocytes; cytokines; early postnatal transmission; infant; MIP-1β; pediatric; sub-Saharan Africa

Introduction

Vertical transmission of HIV accounts for a large percentage of new infections in many areas of the world. In the absence of antiretrovirals, breast milk transmission is estimated to account for 30–50% of infant HIV infections in Africa [1]. However, even without antiretrovirals for the prevention of mother-to-child HIV transmission (PMTCT), many breastfeeding infants of HIV-infected women remain uninfected, despite ongoing viral exposure [2]. Better understanding of the mechanism(s) of protection from vertical transmission may help in development of HIV vaccine.

Maternal antibodies in breast milk protect the infant against bacterial and viral pathogens. Breast milk also contains molecules and cells that mediate innate and adaptive immune responses including αβ and γδ T cells, chemokines, cytokines, monocytes/macrophages and B cells [3–11]. The quantity and function of breast milk HIV-specific $CD8^+$ T cells have been characterized in small studies of HIV-infected women. These cells present in the breast milk of the majority of infected women predominantly display an effector memory phenotype and express mucosal homing receptors, demonstrate evidence of compartmentalization from peripheral blood, and recognize HIV-*gag* more frequently than *env* [9,10,12,13]. The biologic relevance of HIV-specific T cells in breast milk remains unclear; their presence may represent a marker of antigen exposure or could provide a protective mechanism against infection of the infant through cell-mediated lysis of virally infected cells present in breast milk [14]. To test the hypothesis that breast milk cellular immune responses in early milk may reduce vertical transmission of viruses, we evaluated HIV-*gag*-specific interferon-γ (IFN-γ) ELISpot responses in paired blood and breast milk specimens in a cohort of women enrolled in a perinatal HIV transmission cohort in Nairobi, Kenya, and examined the relationship between HIV-*gag*-specific IFN-γ responses and early postnatal HIV transmission.

Participants and methods

Cohort

A perinatal HIV transmission cohort of HIV-infected women and their infants was studied from 1999 to 2005 in Nairobi, Kenya, as described elsewhere [15,16]. Women were recruited in pregnancy, provided written informed consent for participation and storage of specimens, and received zidovudine prophylaxis for PMTCT [17]. Clinically apparent mastitis was evaluated and recorded at each study visit. Breast milk and peripheral blood were collected 1 month postpartum. Infant blood was collected within 48 h of birth and at 1 month. This study was approved by Kenyatta National Hospital Ethics and Research Committee and University of Washington Institutional Review Board.

Specimen collection and preservation

Approximately 30 ml of breast milk was collected by manual expression. The cellular component was isolated from the supernatant and lipid layer by centrifugation (20 min, 710*g*). Breast milk supernatant was stored at −70°C and breast milk cells (BMCs) were washed in RPMI-1640 medium (Sigma, St Louis, Missouri, USA) and lymphocytes were counted on the basis of morphology and cryopreserved in freezing medium containing 10% dimethyl sulfoxide–90% fetal calf serum (FCS) (both from Sigma). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient purification and cryopreserved as above.

Viral load measurements

The Gen-Probe (Gen-Probe Inc., San Diego, California, USA) assay was used to measure HIV RNA in blood plasma and breast milk supernatant as previously described [18,19].

Interferon γ ELISpot assays

The IFN-γ ELISpot assay was used for determination of HIV-*gag*-specific responses as previously described [12] with modifications. The technician performing the assays was blinded to HIV transmission status of the women. Cryopreserved PBMCs or BMCs were thawed, washed, and incubated for 4 h at 5% CO_2, 37°C in RPMI 1640 medium supplemented with 10% FCS, and a 1% solution containing penicillin, streptomycin, and fungizone (R10; all from Sigma). Rested viable cells were added to duplicate wells, with mean concentrations 0.26×10^5 BMCs (±SD =0.38) and 1×10^5 PBMCs per well. Duplicate wells were stimulated with either peptide pools, 10 μg/ml phytohemagglutinin (PHA; Murex Biotech Limited, Dartford, UK) as a positive control, or R10 medium as negative control. Four pools of overlapping 15-mer peptides spanning clade A HIV-*gag* (provided by Dr Tomas Hanke, Oxford, UK) were used for antigen-specific stimulation (Supplemental Digital Content 1, http://links.lww.com/QAD/A257) [20,21]. Assays were considered valid if the PHA response was more than 100 spot-forming units (SFUs) and wells were visibly free from contamination. HIV-specific responses (HIVSFU) were defined as the mean SFU from peptide-stimulated wells minus the mean SFU of negative control wells. Positive responses were defined as HIVSFU per 10^6 cells at least 50 and peptide SFUs at least twice the negative control SFUs [22].

Infant HIV diagnosis

Infants were diagnosed with HIV infection as previously described [16]. Briefly, an infant was considered HIV infected if either HIV-*gag* DNA was detected from blood spotted onto filter papers by PCR [23] or HIV RNA was detected in plasma with the Gen-Probe HIV Viral Load Assay. Infection was considered peripartum if HIV DNA or RNA was undetectable in the birth specimen but detectable at 1 month.

Breast milk subclinical mastitis and chemokines

Subclinical mastitis, defined as sodium (Na^+)-to-potassium (K^+) ratio of more than 1 in whole breast milk [24], and breast milk concentration of macrophage inflammatory protein 1 alpha (MIP-1α), MIP-1β, regulated upon activation, normal T-cell expressed, and secreted (RANTES), and stromal-cell derived factor 1 (SDF-1) have been previously reported [25].

Statistical methods

All statistical analyses were performed using StataSE v11 (College Station, Texas, USA). Viral load, ELISpot HIVSFU, and chemokine levels were $\log_{10}$-transformed to normalize the data. Spearman's correlation was used to test for independence between viral load and immune responses in breast milk. Fisher's exact test, the independent *t*-test, and the Mann–

Whitney *U*-test were used to compare proportions and continuous variables between women with and without breast milk responses and Spearman's correlation was used to test for independence between correlates of breast milk responses and response magnitude. Logistic regression was used to build univariate and multivariate models of transmission. HIV-specific IFN-γ immune responses were modeled separately as dichotomized (detected/not detected) and continuous covariates (number of peptide pools recognized or response magnitude). Response magnitude was summarized for each subject as the sum of HIVSFU in all pools, the maximum HIVSFU of all pools, and the mean HIVSFU in all pools. For multivariate models, covariates were evaluated for co-linearity before inclusion. For all transmission analyses, the outcome of interest was infant infection at 1 month of age; thus, analyses were restricted to infants who were negative for HIV DNA and RNA within 48 h of birth. To enable comparability of odds ratios and confidence intervals, the sample size for both the univariate and multivariate analyses was restricted to infants with all covariates available (n =148). Final multivariate models were restricted to covariates that retained significance when they were adjusted individually for plasma or breast milk viral load. To evaluate effect of input cells per well, analyses were re-run with breast milk ELISpot assays restricted to those with a minimum cells per well of 0.1×10^5 and yielded similar results, indicating misclassification of ELISpot results from assays with low cell input numbers does not bias overall results (data not shown). We used the Holm Test to evaluate P values with nonindependent multiple comparisons [26]. All reported P values are two-tailed.

Results

Cohort characteristics

Detailed clinical characteristics of HIV-infected women enrolled in the perinatal cohort in Nairobi, Kenya, are described elsewhere [3,15]. The study enrolled 510 pregnant HIV-1 seropositive women, HIV-1 infection status was available on 474 infants (93%), and 348 (73%) women were breastfeeding their infants (Fig. 1). Paired PBMC and BMC specimens were cryopreserved from 248 (71%) of these women; however, sufficient cell numbers from breast milk were available from 183 (74%). Thus, 248 PBMC and 183 paired BMC ELISpot assays were evaluated. Transmission analyses were further restricted to the 148 women with BMC ELISpot, viral load, and chemokine data available who delivered HIV-negative infants. As in the parent cohort, the women were young, moderately immunosuppressed (median CD4 cell count 459 cells/μl) and had high HIV viral loads (mean 4.7 $\log_{10}$ copies/ml plasma). Cell-free HIV RNA was detected in breast milk of 170 of 180 (94%) of women at 1 month postpartum with a mean of 2.9 ±SD 1.1 $\log_{10}$ copies/ml (Table 1).

Prevalence and magnitude of HIV-specific interferon γ responses in breast milk

The ELISpot assay was used to quantify HIV-specific IFN-γ production in response to stimulation with HIV-*gag* peptides. ELISpot assays that failed to meet validity criteria [14 (6%) PBMC and 13 (7%) BMC assays] were excluded from subsequent analyses. HIV-*gag*-specific responses were detected in breast milk from 117 of 170 (69%) women and in blood from 205 of 234 (88%). Of 170 women with valid paired blood and breast milk assays, 111 (66%) had concordant assays: 102 were concordant positive (92%) and nine were concordant negative (8%). Women with discordant assays were more likely to have a positive response in blood (44/59, 75%) than a positive response in breast milk (15/59, 25%; P =0.0002). Of the valid assays, the median background BMC response was 426 SFUs/10^6 cells [interquartile range (IQR) 166–1174 SFUs/10^6] and the median PHA response was 9575 SFUs/10^6 cells (IQR 3200–28823 SFUs/10^6). The median magnitude of positive breast milk responses to peptide pools 1–4 were 1400 HIVSFUs/10^6 cells (IQR 400–6314), 875 (IQR 350–2080), 825 (IQR 450–1680), and 933 cells (IQR 370–1600), respectively. These

analyses are presented in detail in Supplemental Digital Contents 2 and 3, http://links.lww.com/QAD/A257.

Correlates of breast milk HIV-specific interferon γ responses

Breast milk and blood plasma viral loads were highly correlated, with viral load in breast milk approximately 2 $\log_{10}$ lower than that in plasma, as previously shown (Fig. 2a) [27]. We evaluated several potential correlates of breast milk HIV-specific IFN-γ responses including immunologic status, HIV plasma viral load, breast milk viral load, chemokines, and subclinical mastitis (Table 2). We did not observe a correlation between the magnitude of PBMC IFN-γ responses and plasma viral load (Fig. 2b and Table 2); however, breast milk HIV viral load was weakly associated with breast milk responses (Fig. 2c and Table 2). Breast milk MIP-1α, MIP-1β, RANTES, and SDF-1 levels were significantly correlated with the magnitude of breast milk IFN-γ responses (P <0.05 for each). Women with subclinical mastitis were more likely to have detectable breast milk responses (43%) compared with women without subclinical mastitis (22%, P =0.02), and the magnitude of the breast milk IFN-γ responses was weakly correlated with the Na: K ratio (ρ =0.24, P =0.003).

Breast milk HIV-specific interferon γ responses and peripartum transmission

Univariate correlates of infant HIV infection included the number of *gag* peptide pools recognized in breast milk ELISpot [odds ratio (OR) 0.69, 95% CI 0.49–0.98], HIV viral load in breast milk and blood, and the detection of MIP-1β in breast milk (P <0.05 for each; Table 3). The magnitude of IFN-γ responses was not associated with infant HIV infection in either breast milk or blood when defined by sum of responses (P >0.05 for both), or alternatively defined by mean or maximum response (data not shown). There was a trend for reduced infection in infants born to women with any detectable breast milk IFN-γ response (OR 0.41, 95% CI 0.16–1.1). Detection of MIP-1β was associated with decreased odds of infant infection (OR 0.22, 95% CI 0.047–0.99). Because MIP-1α, RANTES, and SDF-1 were associated with breast milk IFN-γ responses, we also evaluated whether there was an association between these cytokines and infant HIV infection at month 1, and none were significant (data not shown).

We evaluated the effect of breast milk IFN-γ responses on peripartum transmission in multivariate analyses adjusting for factors associated with transmission. We constructed two complementary models that included detection of MIP-1β and breast milk viral load but differed in terms of categorization of breast milk IFN-γ responses: model 1 included detection of any breast milk IFN-γ response, whereas model 2 included a number of *gag* pools recognized in breast milk. In model 1, detection of a breast milk IFN-γ response was associated with a 71% decrease in infant infection (OR 0.29, 95% CI 0.092–0.91). In model 2, infant infections were decreased by approximately 35% for each additional peptide pool recognized (OR 0.65, 95% CI 0.44–0.97). Breast milk HIV RNA viral load and MIP-1β detection remained significantly associated with infant infection in the multivariate models. Results were similar when alternatively adjusting for plasma viral load (data not shown).

To test for effect of modification of breast milk IFN-γ responses by HIV viral load, we examined transmission data stratified by breast milk viral load above and below the median (Fig. 3a). Among women with high breast milk HIV viral load, those with detectable IFN-γ responses in breast milk were at approximately 74% lower odds of transmitting HIV compared with those who lacked detectable responses (OR 0.26, 95% CI 0.077–0.86; P =0.03). This effect was not significant in the low viral load stratum (OR 0.54, 95% CI 0.072–4.1; P =0.5), which experienced fewer transmissions. Similarly, in women with high breast milk viral load, each additional peptide pool recognized was associated with an

approximately 48% reduction in the odds of infant infection (OR 0.52, 95% CI 0.32–0.84; P =0.008). The number of pools recognized was not significantly associated with infant infection in the low breast milk viral load stratum (OR 1.0, 95% CI 0.58–2.0; P =0.8) (Fig. 3b).

Definitive diagnosis of late in-utero, intrapartum, and early breastfeeding transmission was not possible in this cohort. However, we can estimate an intrapartum transmission rate based on the number of infants who became infected after birth whose mothers were not breastfeeding (7/102, 6.9%). If we apply this estimate obtained to our transmission analysis of 148 mother–infant pairs, we could predict that 10 intrapartum transmissions may be misclassified as early breast milk transmissions. Assuming the misclassification of transmission route is distributed equally among women who had and who lacked breast milk IFN-γ responses, we evaluated the associations, which remained significant (data not shown).

Lastly, to address the possibility that ingested maternal breast milk cells could persist in the infant and reduce risk of infection, we compared HIV-specific IFN-γ responses in children born to women with detectable and undetectable breast milk responses. Among HIV-exposed uninfected infants, there was no significant difference in frequency of responses in children born to women with detectable breast milk responses (8/54, 15%) versus those born to women with undetectable responses (1/19, 5.3%, P =0.4) at 1 month of age. We also did not find a difference in set-point HIV viral load between HIV-infected infants born to mothers with detectable and undetectable breast milk responses (data not shown).

Discussion

To our knowledge, this is the first demonstration of an association between breast milk cellular immune responses and protection from vertical transmission. Breast milk responses were independently associated with an approximately 70% lower odds of infant HIV infection at 1 month of life. Importantly, the breadth of breast milk response most significantly influenced transmission: infant infection was reduced by a third for each additional HIV-*gag* peptide pool targeted, which suggests that a broad response against multiple epitopes may be more protective against peripartum transmission than a narrow response. The level of breast milk MIP-1β, an effector molecule secreted by IFN-γ-producing $CD8^+$ T cells [28,29], was also independently associated with protection from infection. Lastly, detection of these responses in women was not associated with detection of responses in HIV-exposed uninfected infants, or reduced viral replication in HIV-infected infants, suggesting their effect is concentrated in breast milk. Together, these data suggest a role for breast milk cell-mediated immunity in preventing vertical transmission of viruses and may explain why only a minority of infants exposed to HIV through breast milk become infected.

Breast milk IFN-γ responses could potentially decrease transmission by several mechanisms. Breast milk HIV-specific cytotoxic T cells (CTLs) may facilitate destruction of HIV-infected cells or lead to decreased production of infectious virus; this could occur either in the breast and/or at infant mucosal surfaces. This mechanism would be best validated by measuring the association between breast milk IFN-γ responses and cell-associated viral load. We observed correlations of breast milk IFN-γ with breast milk HIV-1 RNA, suggesting antigenic induction of IFN-γ responses rather than IFN-γ reduction of viral levels; however, we did not measure breast milk proviral DNA in this study. Our study supports a second potential mechanism by which breast milk HIV-specific $CD8^+$ T cells could reduce transmission through secretion of HIV co-receptor binding chemokines that block infection. $CD8^+$ T-cell secretion of MIP-1β has been associated with slower HIV

disease progression [30,31]. We previously found breast milk MIP-1β levels to be associated with reduced risk of overall postpartum transmission during the first year of life [25]. We found MIP-1β detection in breast milk was associated with lower risk of infant HIV transmissions between delivery and 1 month postpartum, independent of breast milk viral load and breast milk IFN-γ responses. A large proportion of activated $CD8^+$ T cells secrete both IFN-γ and MIP-1β [29,32], consistent with the correlation observed in our study between breast milk IFN-γ responses and MIP-1β levels. MIP-1β could protect against HIV entry by binding to CCR5 on target cells in either the breast milk and/or the infant gut. A third mechanism by which breast milk cellular immune responses may protect transmission is via passive transfer of immune cells to the infant. Animal studies have suggested that ingested breast milk cells can cross the neonatal gut and modify immune responses within the infant [33–35]. We addressed this hypothesis by evaluating HIV-specific immune responses in exposed uninfected infants and found no difference in the percentage of responses in uninfected children of mothers with breast milk responses versus those without. We also found no evidence to support the hypothesis that ingested breast milk cells in the infant circulation may affect HIV replication: HIV set-point was similar between HIV-infected infants of mothers with detectable and those with undetectable breast milk immune responses.

Three main factors were identified as correlates of breast milk IFN-γ responses: breast milk HIV viral load, chemokine levels, and subclinical mastitis (Na : K ratio). The association between breast milk viral load and breast milk HIV-specific IFN-γ responses was significant, although a low ρ value suggests viral load alone is not responsible for expansion of antigen-specific breast milk cells. Notably, breast milk HIV-specific responses were detected in some women with undetectable breast milk viral load in our study, which is consistent with the finding of a previous study [9]. The association between subclinical mastitis and breast milk IFN-γ responses could be explained by several mechanisms, including exudation of peripheral blood lymphocytes into the milk secondary to increased membrane permeability, proliferation of HIV-specific T cells in response to increased breast milk viral load, or increased homing of T cells to the milk in response to increased chemokine production during inflammation. As previously reported in this cohort, subclinical mastitis was associated with higher breast milk viral load and also with higher MIP-1α and MIP-1β chemokine levels [25].

This work complements our previous study examining breast milk HIV-specific responses in 53 women from the same cohort using recombinant vaccinia virus (rVV) expressing HIV-*gag* and *env* [12]. We note several important distinctions from our earlier work. First, overall prevalence of both breast milk and blood responses was greater in the current study. We hypothesize that the use of peptide pools resulted in higher sensitivity to detect low-level responses as opposed to rVV vectors, which require infection, antigen processing, and presentation. Also, the current study was designed to have adequate power to detect an effect of immune responses on transmission and focused on infections occurring during the first month of life, when the rate of transmission, the cellularity of breast milk, and, therefore, the relevance of protective breast milk responses were expected to be greatest.

Limitations of this study include inability to definitively distinguish intrapartum transmission from early breast milk transmission, low cell numbers recovered in breast milk, and the inability to evaluate late transmission events due to the combined effect of decreased breast milk cellularity and rarity of late infections. Colostrum and early milk are highly cellular, whereas the number of cells in mature breast milk are approximately 1 $\log_{10}$ lower than those in early milk [14,36–38]; we, thus, speculated that if breast milk T-cell responses were associated with protection from HIV transmission, their effects would be most discernable in the early postpartum period. Although HIV DNA and RNA were undetectable

in all infants at birth, some transmissions could have occurred either late *in utero* or intrapartum; since breast milk T cells would not be expected to affect these routes of transmission, our data may underestimate the protective effect of HIV-specific T-cell responses. Although the measurement of IFN-γ secretion in response to one HIV protein does not fully describe the anti-HIV cellular immune response present in milk, HIV-*gag* peptide pools were used exclusively due to limited cellularity; previous studies have correlated breadth of IFN-γ *gag* responses with viral control [39–44]. Finally, because PBMCs include CD4 T and natural killer cells, we can determine neither the source of IFN-γ nor the proportion of $CD8^+$ T cells capable of MIP-1β and IFN-γ secretion. Regardless of the cellular source, HIV-specific IFN-γ-secretion in the breast milk was associated with reduced HIV transmission.

In conclusion, in this large study, HIV-infected mothers frequently had HIV-specific IFN-γ responses detected in breast milk that were associated with decreased infant HIV infection. Cellular immune responses in breast milk may play a role in protecting infants from mucosal exposures to viral pathogens. More generally, these data support the relevance of the HIV-*gag*-specific responses, and the breadth of responses, in the development of an HIV vaccine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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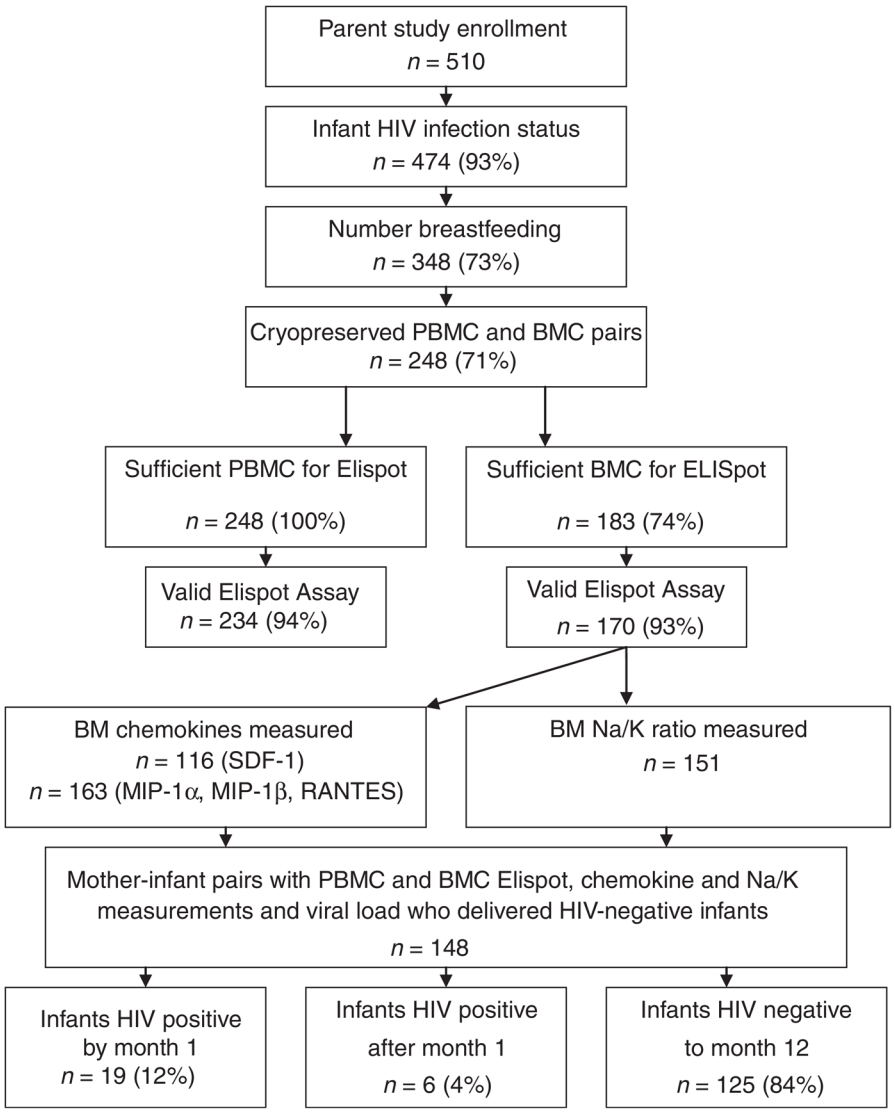

Fig. 1. Participant flow

Cohort numbers are based on available test results, focusing on breastfeeding women whose infants were HIV uninfected at birth. Chemokine and mastitis data are reported in [25]. BMCs, breast milk cells; PBMCs, peripheral blood mononuclear cells.

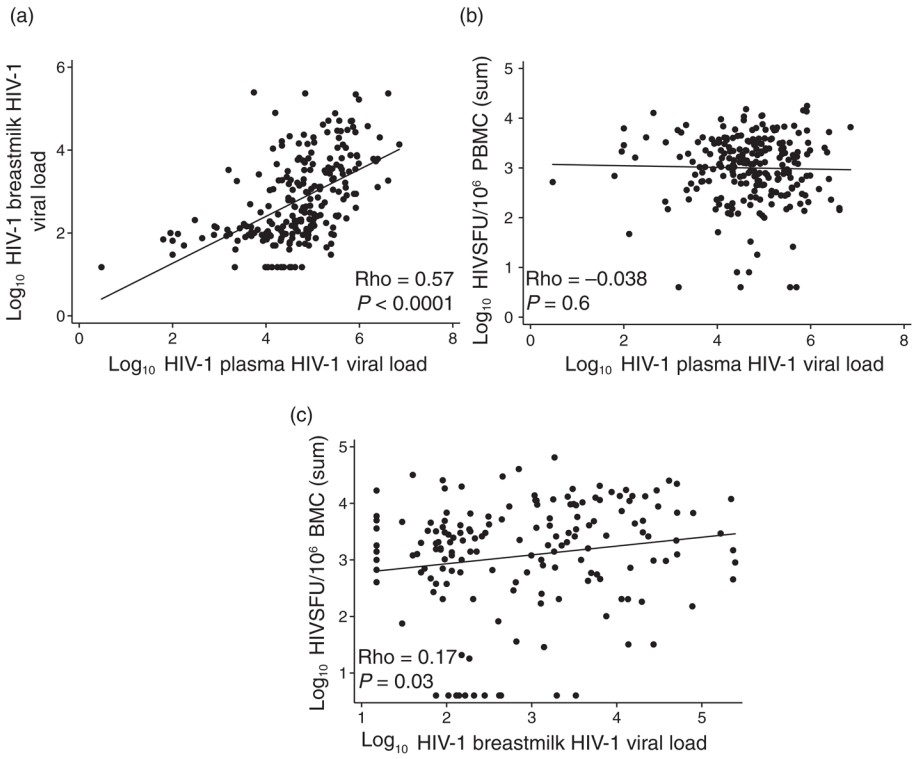

Fig. 2. HIV-specific interferon-γ responses and viral replication

Scatter plots show the correlation between (a) plasma and breast milk HIV viral load, (b) plasma viral load and HIV-specific IFN-γ responses in peripheral blood mononuclear cells (PBMCs), and (c) breast milk viral load and HIV-specific IFN-γ responses in breast milk cells (BMCs). Correlation coefficients and *P* values were generated using Spearman's correlation. IFN, interferon.

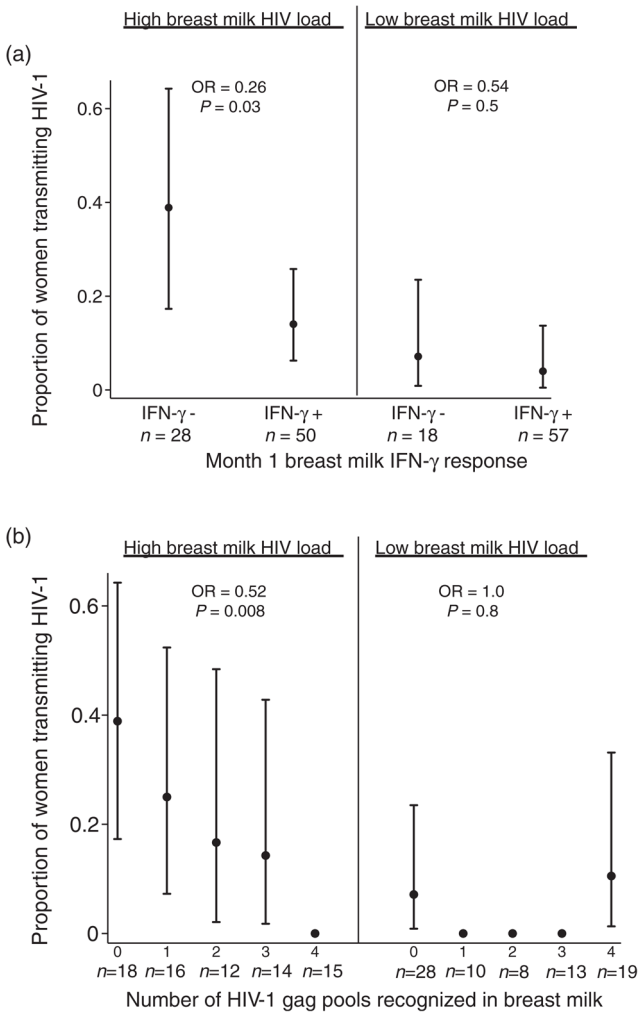

Fig. 3. Stratified analysis of early peripartum transmission and breast milk HIV-specific interferon-γ responses

Markers show the proportion of infants with HIV infection at 1 month of age, stratified by breast milk viral load and (a) HIV-*gag*-specific response detected in breast milk and (b) the number of peptide pools eliciting a positive HIV-*gag*-specific response in breast milk. Bars show exact 95% confidence intervals calculated from the binomial distribution. The number of mother–child pairs is shown for each category (*n*). No confidence intervals are shown for categories where no transmission occurred. *P* values are shown for logistic regression within strata.

Table 1

Characteristics of 183 women with paired breast milk and blood ELISpot assays 1 month postpartum.

	N (%)[a]	Mean (±SD) or median (IQR)
Age (years)	183	24 (21–27)
Parity	182 (99)	1 (1–2)
32 weeks gestation (baseline)		
CD4 cell count (cells/μl)	177 (97)	459 (313–618)
CD4 percentage	177 (97)	22 (17–29)
CD4: CD8 ratio	177 (97)	0.47 (0.33–0.71)
Plasma HIV RNA (log_{10} copies/ml)	176 (96)	4.7 (±0.80)
Month 1 postpartum		
CD4 cell count (cells/μl)	148 (81)	562 (392–755)
CD4 percentage	148 (81)	24 (17–30)
CD4 : CD8 ratio	148 (81)	0.52 (0.30–0.73)
Plasma HIV RNA (log_{10} copies/ml)	182 (99)	4.7 (±0.92)
Breast milk HIV RNA (log_{10} copies/ml)	180 (98)[b]	2.9 (±1.1)

[a] Number of women with available data per test (%). Specimens and/or test results were not available for all women at all time points.

[b] Number of women with detectable breast milk viral load =170 (94%).

Table 2

Correlates of breast milk interferon-γ responses.[a]

	IFN-γ response[b]				
	n	**Detected**	**Not detected**	***P***	**IFN-γ-response[b] magnitude**
Immunologic/virologic					
CD4 cell count (cells/μl)	139	556 (379–744)	539 (408–717)	0.9	ρ =0.030, P=0.7
CD4%	139	23 (16–29)	22 (18–30)	0.9	ρ =0.033, P=0.7
CD8 (cells/μl)	139	1108 (879–1465)	1204 (797–1448)	0.9	ρ =0.046, P=0.6
CD8%	139	50 (41–56)	45 (37–61)	0.6	ρ =0.081, P=0.3
$\log_{10}$ plasma HIV RNA load	169	4.7 (±0.92)	4.8 (±0.96)	0.5	ρ =−0.0095, P=0.9
$\log_{10}$ breast milk HIV RNA load	167	3.0 (±1.1)	2.7 (±0.91)	0.08	ρ =0.17, P=0.02
Chemokine levels in breast milk ($\log_{10}$ pg/ml)					
MIP-1α	162	1.4 (±0.39)	1.3 (±0.37)	0.07	ρ =0.30, P=0.0001
MIP-1β	163	1.8 (±0.44)	1.6(±0.48)	0.02	ρ =0.28, P=0.0003
RANTES	163	2.4 (±0.47)	2.1 (±0.56)	0.0005	ρ =0.30, P=0.0001
SDF-1	116	2.4 (±0.71)	2.1(±0.72)	0.07	ρ =0.20, P=0.03
Mastitis in the first month postpartum					
Clinical mastitis	156	11 (12/109)	8.5 (4/47)	0.8	*c*
Subclinical mastitis[d]	151	43 (46/106)	22 (10/45)	0.02	ρ =0.24, P=0.003[e]

The values are given as mean (±SD), median (IQR), or percentage (*n*/*N*). IFN, interferon; IQR, interquartile range; MIP, macrophage inflammatory protein; RANTES, regulated upon activation, normal T-cell expressed, and secreted; SDF, stromal cell-derived factor.

[a] Correlates were evaluated among 170 women with valid breast milk HIV-specific IFN-γ assays.

[b] IFN-γ responses were evaluated as dichotomous (detected/not detected) and continuous (magnitude) variables. IFN-γ response magnitude is summarized by the median of the mean sum of pooled peptide responses; log10-transformed values were used in correlation analyses.

[c] Not applicable.

[d] Subclinical mastitis is defined as breast milk Na : K >1.

[e] Correlation based on Na : K ratio.

Table 3

Correlates of early breast milk HIV transmission.[a]

	OR for infant HIV infection (95% CI)	*P*
Univariate model (*n* =148)		
Breast milk HIV RNA load, 1 month postpartum	2.0 (1.2–3.1)	0.004[b]
Plasma HIV RNA load, 1 month postpartum	2.7 (1.4–5.2)	0.004[b]
HIV-*gag* response detected in breast milk	0.41 (0.16–1.1)	0.08
HIV-*gag* response detected in blood	1.1 (0.23–5.3)	0.9
No. of HIV-*gag* pools recognized in breast milk	0.69 (0.49–0.98)	0.04
No. of HIV-*gag* pools recognized in blood	0.74 (0.53–1.0)	0.09
Sum HIVSFU in breast milk[c]	1.0 (0.62–1.6)	0.97
Sum HIVSFU in blood[c]	1.5 (0.68–3.4)	0.3
MIP-1β detected in breast milk	0.22 (0.047–0.99)	0.05
Multivariate model 1 (*n* =148)[d]		
HIV-*gag* response detected in breast milk	0.29 (0.092–0.91)	0.03
MIP-1β detected in breast milk	0.15 (0.024–0.95)	0.04
Breast milk HIV RNA load, 1 month postpartum	2.5 (1.5–4.2)	0.001
Multivariate model 2 (*n* =148)[d]		
No. of HIV-*gag* pools recognized in breast milk	0.65 (0.44–0.97)	0.03
MIP-1β detected in breast milk	0.13 (0.021–0.80)	0.03
Breast milk HIV RNA load, 1 month postpartum	2.3 (1.4–3.8)	0.001

95% CI, 95% confidence interval; HIVSFU, spot-forming unit; MIP, macrophage inflammatory protein; OR, odds ratio.

[a] Analysis restricted to 148 breastfeeding mother infant pairs with viral load, ELISpot, and chemokine data available.

[b] Univariate *P* values remain significant after Holm–Bonferroni test for multiple comparisons.

[c] Similar estimates were obtained when alternatively using maximum and mean to summarize the magnitude of peptide pool responses.

[d] Covariates that retained significance after adjustment for HIV breast milk or plasma viral load were included in the final multivariate models.