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Maternal plasma and breastmilk viral loads are associated with HIV-1-specific cellular immune responses among HIV-1-exposed, uninfected infants in Kenya

Short title: Maternal HIV levels and IFN-γ responses

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List of Abbreviations:

HIV: Human Immunodeficiency Virus

ZDV/NVP: Zidovudine/Nevirapine

3TC: Lamivudine

HAART: Highly Active Antiretroviral Therapy

ELISpot: Enzyme Linked Immunospot Assay

EU: Exposed Uninfected

SFU: Spot Forming Unit

PBMC: Peripheral Blood Mononuclear Cell

IQR: Intraquartile Range

ARV: Antiretroviral

IFN-γ: Interferon-gamma

R10: Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal calf serum

PHA: Phytohemagglutinin

PBS: phosphate-buffered saline

GEE: Generalized estimating equation

Summary:

Infants exposed to maternal HIV-1 provide an opportunity to assess correlates of HIV-1-specific IFN-γ responses and may be informative to the development of HIV-1 vaccines. HIV-1-infected women with CD4 counts 200-500 cells/mm^3 were randomized to short-course ZDV/NVP or HAART between 2003-2005. Maternal plasma and breastmilk HIV-1 RNA and DNA were quantified during the first 6-12 months postpartum. HIV-1 *gag* peptide stimulated ELISpot assays were conducted in HIV-1-exposed, uninfected infants (EU), and correlates were determined using regression and generalized estimating equations. Among 47 EU infants, 21 (45%) had ≥1 positive ELISpot result during follow-up. Infants had a median response magnitude of 177 HIV-1-specific SFU/10^6 PBMC (IQR: 117-287) directed against 2 (IQR: 1-3) *gag* peptide pools. Prevalence and magnitude of responses did not differ by maternal ARV randomization arm. Maternal plasma HIV-1 RNA levels during pregnancy (p=0.009) and breastmilk HIV-1 DNA levels at one month (p=0.02) were associated with higher magnitude of infant HIV-1-specific ELISpot responses at one month postpartum. During follow-up, concurrent breastmilk HIV-1 RNA and DNA (cell-free virus and cell-associated virus, respectively) each were positively associated with magnitude of infant HIV-1-specific responses (p=0.01). Our data demonstrate the importance of antigenic exposure on the induction of infant HIV-1-specific cellular immune responses in the absence of infection.

Introduction

Globally an estimated 370,000 children are newly infected with HIV-1 each year, the majority as a result of mother-to-child transmission(1). Infants born to HIV-1-infected mothers consume large volumes of breastmilk containing HIV-1, but despite this exposure ~80% of these breastfeeding infants remain uninfected(2). It is possible that these infants escape infection due to natural resistance, either through genetics, innate immunity, or acquired immunity, which protects them from acquiring HIV-1.

The discovery of HIV-1-specific cellular immune responses in individuals exposed to HIV-1 but who remain uninfected (EU) has been of particular interest as adaptive immunity may protect against acquisition of infection. Among HIV-1-infected adults, HIV-1-specific cellular immune responses are associated with control of viral replication and viral clearance(3, 4) and slower HIV-1 disease progression(5-11). In the pre-antiretroviral era, waning of these responses correlated with disease progression(12-14). HIV-1-specific cellular immune responses have been reported in varied HIV-1 EU populations, including commercial sex workers(15-17), HIV-1-discordant couples(18-20), and infants born to HIV-1-infected women(21-23). $CD4^+$ and $CD8^+$ HIV-1-specific responses have been observed in EU infants, with prevalence ranging from 3-56%(24-27) and 0-47%(22, 27-31), respectively, resulting in controversy around the detection of these responses and their potential protective role. However, vaccine development relies on understanding the induction of immune responses, and so it remains important to identify the correlates of presence and magnitude of HIV-1-specific immune responses in EU individuals. Historic cohorts of infants of HIV-1-infected mothers who breastfeed offer a natural human challenge study because they are continuously exposed to HIV-1 from their mothers. With both viral source and recipient identifiable, mother-infant cohorts provide a unique opportunity to investigate correlates of infant cellular immune responses.

We hypothesized that factors associated with exposure to increased levels of HIV-1 antigen would increase induction of HIV-1-specific immune responses. To test our hypothesis,

we compared the prevalence, magnitude, and breadth of infant HIV-1-specific T cell responses between breastfeeding HIV-1 EU infants born to women randomized to short-course zidovudine/nevirapine (ZDV/NVP) or highly active antiretroviral therapy (HAART) [ZDV/lamivudine (3TC)/NVP], both shown to impact the levels of HIV-1 cell-free virus exposure in breastfeeding infants. Additionally, we examined maternal systemic and breastmilk HIV-1 viral levels as correlates of infant HIV-1-specific responses.

Materials and Methods

Study population and sample collection

This study was a Phase II clinical trial conducted at the Mathare North City Council Clinic in Nairobi, Kenya between 2003 and 2005 and was approved by the Institutional Review Boards of the University of Washington and Kenyatta National Hospital (ClinicalTrials.gov number, NCT00167674). Methods for recruitment, randomization, and follow-up for this trial, along with results of the primary study, have been previously described(32, 33). Briefly, 60 HIV-1-positive pregnant women and their infants were followed for 1 year postpartum. Enrolled women had CD4 cell counts between 200 and 500 cells/mm^3. At 34 weeks gestation, women were randomized to either ZDV/NVP or HAART. In the ZDV/NVP arm, women received ZDV from 34 weeks gestation until delivery and a single dose of NVP at labor, and infants were administered a single dose of NVP within 72 hours of delivery, in accordance with Kenya national guidelines at the time. In the HAART arm, ZDV, 3TC, and NVP were given to women at 34 weeks gestation until 6 months postpartum. Also per 2003-2005 national guideline, all women were advised to stop breastfeeding 6 months after delivery, and women in the HAART arm were advised to discontinue taking HAART after breastfeeding cessation.

Maternal blood specimens were collected at 32 weeks gestation, within 2 days of delivery, then 2 weeks, 1 month, and every 3 months after delivery for HIV-1 RNA levels.

Breastmilk was obtained 1-3 times per week for the first month, then 3 and 6 months postpartum for breastmilk cell-free HIV-1 RNA and cell-associated HIV-1 DNA levels. Blood samples collected from infants at delivery and then at 1, 3, 6, 9, and 12 months of age were used to determine HIV-1 infection status and for enzyme-linked immunospot (ELISpot) assays.

Laboratory Methods

The processing of breastmilk specimens has been described elsewhere(34). Briefly, breastmilk samples were separated into supernatant and cells after discarding the lipid layer. Plasma and breastmilk HIV-1 RNA levels were determined using the Gen-Probe HIV-1 viral load assay (Gen-Probe Inc., USA), as previously described(34, 35), with a lower limit of detection of 200 copies/ml and 100 copies/ml for plasma and breastmilk samples, respectively. Infant filter paper blood specimens were tested to determine HIV-1 status by HIV-1 DNA PCR(36). HIV-1 DNA from breastmilk cells was extracted using the QIAmp DNA mini kit (Qiagen, USA) and quantified using real-time PCR as previously described(33, 34). The lower limit of detection was 1 copy/reaction, and HIV-1 DNA levels were normalized to the number of cells tested (number of β-actin copies). CD4 counts were measured from blood samples using flow cytometry (FACScan, Becton Dickinson, USA).

Infant HIV-1 *gag*-specific T cell responses were assessed using an established IFN-γ ELISpot assay protocol on fresh peripheral blood mononuclear cells (PBMC). Briefly, 96-well nitrocellulose plates (Millipore, USA) were coated with 7.5μg monoclonal antibody to IFN-γ (Mabtech, Sweden) for 2 hours at 37°C. Antibody was removed by washing the plates with RPMI-1640 and then blocked with R10 (RPMI-1640 containing 20mM L-glutamine with 10% fetal calf serum) (all Sigma, USA) for 30 minutes at room temperature. Freshly isolated infant PBMC were then added in duplicates with 2 x 10^5 PBMC/well. Each infant PBMC sample was stimulated with R10 media alone as a negative control, 10μg/ml phytohemagglutinin (PHA) as a

positive control, or 20µg/ml HIV-1 *gag* peptide pools. Seven peptide pools of overlapping 15-mers spanning HIV-1 p55 were derived from the clade A consensus sequence and were provided by the NIH AIDS Research and Reference Reagent program. Cells were stimulated overnight in a humidified incubator at 37°C with 5% CO_2 and were removed from the plates by washing with phosphate-buffered saline (PBS) containing 0.05% Tween-20. Biotinylated anti-IFN-γ antibody was applied for 3 hours at room temperature, followed by washing, and then streptavidin alkaline phosphate (Mabtech) was added for 1.5 hours at room temperature. After washing, alkaline phosphatase (Mabtech) was added for approximately 10 minutes or until spot forming units (SFU) were visible in the PHA wells. The reaction was stopped by washing the plates under running water, and plates were dried overnight before being read on a CTL ImmunoSpot Core Analyzer (Cellular Technology Ltd., USA).

Statistical Methods

HIV-1-specific SFU was defined as the average number of spots in duplicate wells minus the background response (defined as the mean SFU in the negative control wells). ELISpot responses were considered positive if experimental wells had ≥50 HIV-1-specific SFU/10^6 PBMC and more than twice the background response. Assays were excluded if PHA wells had <100 SFU/10^6 PBMC. Prevalence, breadth and magnitude of ELISpot responses were evaluated by 1) including all valid assays or 2) excluding assays in which the background SFU > 100/10^6 PBMC. Infants were defined as being positive responders if they had ≥1 peptide pool with a positive response. HIV-1 *gag*-specific immune responses were examined both as a dichotomous (using the pre-defined cut-offs above) and continuous (magnitude of responses) variable. Magnitude of responses was defined as the summed magnitude of HIV-1-specific SFU/10^6 PBMC across all peptide pools.

Viral loads below the limit of detection were recoded to the mid-point between zero and

the limit of detection for that assay. Because a high percentage (55%) of breastmilk HIV-1 RNA assays were below the limit of detection, breastmilk HIV-1 RNA was modeled as a dichotomized covariate (detected/not detected). Infant HIV-1-specific IFN-γ responses were compared between the two randomization groups at each visit. ELISpot prevalence was compared using Pearson's Chi-squared tests or Fisher's exact tests, and magnitude and breadth of responses were compared using Mann-Whitney U tests. Linear regression was used to assess correlates of magnitude of ELISpot HIV-1-specific responses (background subtracted) in all infants at specific time points. Generalized estimating equation (GEE) models with a Poisson link and exchangeable correlation structure were used to examine associations between maternal viral load and infant ELISpot responses over time. All regression models were adjusted for treatment arm and constructed with robust standard errors. Sensitivity analyses were performed in which samples with undetectable HIV-1 DNA levels and fewer than 10,000 cells tested were excluded from regression models. Stata version 11.2 (College Station, USA) was used for all analyses.

Results

Study population and characteristics

Of 60 mother-infant pairs, 3 infants acquired HIV-1 during follow-up and were excluded from the ELISpot analyses; 47 (78%) infants had ELISpot data at ≥1 visit. Among the selected mother-infant pairs, median age and CD4 cell count at 32 weeks gestation did not differ between trial arms (Table 1). While plasma HIV-1 RNA levels were similar between the two groups at 32 weeks gestation, women randomized to ZDV/NVP had significantly higher plasma viral loads (~2 $\log_{10}$ copies/ml higher) from delivery to 6 months postpartum compared to women randomized to HAART(33). Furthermore, more women in the ZDV/NVP arm had detectable breastmilk cell-free HIV-1 RNA levels at 1 month postpartum versus women in the HAART arm (82% vs. 29%, $p<0.001$). In contrast, breastmilk HIV-1 DNA levels did not differ by trial arm at any time-point. Follow-up time and number of valid assays did not differ between

infants by randomization arm. Median breastfeeding duration was similar between infants in the ZDV/NVP arm (179 days, interquartile range (IQR) 91-184) and infants in the HAART arm (182 days, IQR 155-185).

Prevalence, durability, magnitude, and breadth of HIV-1-specific IFN-γ responses and comparison by randomization arm

Ten (43%) infants in the ZDV/NVP arm and 11 (46%) infants in the HAART arm had positive HIV-1-specific IFN-γ responses at least once (Table 2), and the prevalence of positive ELISpots did not differ between randomization arms at any visit ($p>0.05$ for each visit). In the HAART arm, prevalence of positive ELISpots was low early in life and increased thereafter with the highest prevalence (43%) at 9 months of age. In contrast, prevalence of positive ELISpots among infants in the ZDV/NVP arm remained relatively constant throughout their first year of life.

The median magnitude of all ELISpot responses were similar between infants in the ZDV/NVP and HAART group overall (88 HIV-1-specific SFU/10^6 PBMC, IQR 45-187 vs. 96 HIV-1-specific SFU/10^6 PBMC, IQR 68-171, respectively) and for every time point ($p>0.05$ for each visit). When restricted to positive responders at each visit or overall, the magnitudes of responses were not different by treatment arm ($p>0.05$); however, statistical power for comparisons was limited. The median number of peptide pools recognized (breadth of response) also did not differ between randomization arms overall or at any single time point ($p>0.05$), and there were no specific pools selectively recognized in either arm (data not shown).

Of the 47 infants who had ELISpot data during the study, 21 (45%) had at least one positive HIV-1-specific response (Figure 1). Among the 21 positive responders, 13 had only 1 positive response, 5 infants (4 HAART, 1 ZDV/NVP) had positive ELISpot responses at two time

points, and 3 of these infants (2 HAART, 1 ZDV/NVP) had repeated responses to identical *gag* pools (Figure 2). The number of peptide pools that were recognized by infants with positive ELISpot responses ranged from 1 to 7, with a median of 2 pools overall (Table 2). Similar patterns of responses were observed when the analyses were restricted to assays with background responses ≤ 100 SFU/millioin PBMC. When assays with high backgrounds were removed, 17 (36%) had at least one positive response. The median magnitude of all responses were reduced to 63 HIV-1-specific SFU/10^6 PBMC, IQR 42-120 vs. 72 HIV-1-specific SFU/10^6 PBMC, IQR 52-125, in the ZDV/NVP and HAART groups, respectively. The number of pepite pools recognized remained unchanged.

Infant HIV-1-specific IFN-γ responses at 1 month of age are associated with maternal viral load

To evaluate effect of antenatal exposure of infants to maternal virus on infant ELISpot responses, we determined correlates of infant ELISpot responses at 1 month postpartum utilizing data from all infants (both negative and positive ELISpot results) and assessing all HIV-1-specific cellular responses (after subtraction of background) as a continuous variable. We utilized all infant data rather than the subset of positive responses to enhance potential analytical power because the biologic threshold for a true positive response is unknown. Maternal HIV-1 viral levels and CD4 count were evaluated as correlates of infant responses at 1 month of age. For every $\log_{10}$ increase in maternal plasma viral load at 32 weeks gestation there was a significant association for a 0.44 $\log_{10}$ increase (95% confidence interval (CI)=-0.12 - 0.76, p=0.009) in magnitude of infant IFN-γ responses (Table 3A). Thus for every $\log_{10}$ increase in viral load during gestation, infants had ~600/10^6 additional HIV-specific cells in circulation by one month of life. In contrast for every $\log_{10}$ increase in breastmilk HIV-1 DNA month 1 postpartum, there was a 0.54 (95% CI=0.11-0.97, p=0.02) $\log_{10}$ increase in magnitude of infant IFN-γ responses (Table 3A). Thus for every $\log_{10}$ increase in concurrent breastmilk viral load,

infants have ~3000/10^6 additional circulating HIV-specific cells. Similar results were found with sensitivity analyses excluding samples that had less than 10,000 cells and undetectable HIV-1 DNA (data not shown). When the analysis was restricted to assays with background ≤100 SFU/10^6 PBMC, the associations were similar: the contribution of plasma viral load during pregnancy was reduced to a trend (p=0.08) while the contribution of concurrent breastmilk viral load remained a significant correlate for detection of infant HIV-1 specific IFN-γ responses one month after birth.

Association between concurrent breastmilk HIV-1 levels and magnitude of HIV-1-specific IFN-γ responses

GEE models were developed to determine the relationship between HIV-1 exposure through different biological compartments and infant cellular immune responses longitudinally (Table 3B and Figure 2). The magnitude of infant ELISpot responses was significantly associated with the concurrent detection of HIV-1 RNA in breastmilk (β=0.84, 95% CI=0.19-1.48, p=0.01) and the concurrently measured level of breastmilk HIV-1 DNA (β=0.84, 95% CI=0.19-1.49, p=0.01). Sensitivity analyses produced similar results when excluding samples with undetectable HIV-1 DNA in which less than 10,000 cells were tested (data not shown). There was a trend for a positive association between plasma viral load at 32 weeks gestation and magnitude of subsequent ELISpot responses (β=0.35, 95% CI=-0.03-0.72, p=0.07).

Discussion

In this study, prevalence and correlates of HIV-1-specific IFN-γ responses among breastfeeding HIV-1-exposed uninfected (EU) infants born to mothers on antiretroviral therapy were evaluated. We found 45% of infants were able to generate cellular immune responses of substantial breadth and magnitude; however, most responses were transient. Our finding

confirms previous studies that detected responses in HIV-1 EU infants(22, 27, 28, 31) and is consistent with our previous study that observed 47% prevalence of at least one positive ELISpot assay using HLA-matched peptide stimulation in breastfeeding EU infants(29). We also found significant associations between maternal plasma and breastmilk HIV-1 viral levels and infant magnitude of HIV-1-specific ELISpot responses, suggesting that antigen exposure modifies the induced infant HIV-1-specific immune responses.

In contrast to our study hypothesis, we did not observe that randomization to the ZDV/NVP arm was associated with higher infant HIV-1-specific immune responses. We may have been underpowered to detect a difference between the two arms, however, the absence of a difference by treatment is consistent with our finding that breastmilk cell-associated HIV-1 DNA predicted infant IFN-γ responses. We previously demonstrated in this cohort that whereas breastmilk cell-free virus was significantly decreased in women on HAART, breastmilk cell-associated virus (as measured by HIV-1 DNA levels) remained similar to women in the ZDV/NVP arm(34), and the persistence of breastmilk HIV-1 DNA despite HAART has also been observed in a study from Botswana(37). Thus, although breastfeeding infants born to mothers on HAART had less exposure to maternal cell-free virus, there was persistent exposure to HIV-1 infected cells in breastmilk, and this may be a key determinant in generating infant immune responses.

We found a significant association between maternal pregnancy plasma HIV-1 RNA levels and magnitude of IFN-γ responses in EU infants at 1 month of age, suggesting that *in utero* exposure influences infant immune responses in the absence of HIV-1 infection. Furthermore, ongoing HIV-1 exposure through breastmilk appears to induce responses as seen by the correlation between both breastmilk HIV-1 RNA and DNA and magnitude of infant IFN-γ responses during the postpartum period. The results from this study are consistent with other EU cohorts (29, 38) (39, 40). Together these observations support the hypothesis that infant cellular immune responses are due to HIV-1 exposure and not randomly distributed false

positives. However, it should be noted that not all studies have observed associations between increased transmitter virus exposure and EU cellular HIV-1 response. Some studies of HIV-1 discordant couples and mother-infant pairs have noted inverse associations with the partner's or mother's HIV-1 viral load(41, 42). Consideration of the measures of transmitter virus (RNA, DNA), transmitter compartment (plasma, genital secretions, or breastmilk), assay (ELISpot or intracellular cytokine staining), and EU HIV-1-specific response score (positive/negative or magnitude) differ between studies and may contribute to the differences in results. These predictors of cellular immune responses in EU individuals may reveal factors to consider in vaccine design in order to effectively induce immune responses.

We observed infant ELISpot responses that were of relatively high magnitude and were comparable to levels noted after HIV-1 vaccines in trials among adults(43-45). Responses were detected in 3 infants at 1 month of age, suggesting responses can be primed very early in life; however, these responses were not maintained and subsequently disappeared in all 3 infants. The characteristics of these infant responses are analogous to what may be expected among recipients of a prime-boost vaccine (46). The lack of persistent immune responses in infants suggests that initial in utero priming of responses may not be sufficient for a sustained response, perhaps due to antiretroviral treatment decreasing maternal HIV-1 viral load in the last trimester. As for route of vaccine delivery, our data and others(19, 38) have shown that oral exposure to HIV-1 induces systemic HIV-1-specific IFN-γ responses, lending support for discussing the potential role of mucosally administered HIV-1 vaccines. Recently, CD4+CCR5+ T-cells have been noted to be prevalent in infant gut mucosa, yielding potential susceptibility to HIV-1 infection or vaccination(47).

This study benefited from the longitudinal assessment of HIV-1 EU infants to monitor durability of immune responses and to determine correlates over time, and to identify the infant's viral source and to collect detailed HIV-1 exposure data from the mothers. A limitation of this study was the relatively small number of mother-infant pairs. In the absence of a biological

threshold or gold standard for HIV-1-specific SFU, cut-offs for positive assays are arbitrary and are based on lab-based comparisons to background wells or to control individuals. By using continuous HIV-1-specific SFU instead of dichotomous data, we were able to increase analytical power and precision to discern associations.

In summary, our findings suggest that HIV-1-specific IFN-γ responses in HIV-1 EU infants are associated with maternal levels of HIV-1 in plasma and breastmilk, and that the dose of infant exposure to maternal virus during and after pregnancy influences the induction of infant HIV-1-specific responses. Associations with breastmilk viral load suggest that these responses result from HIV-1 exposure at the oral and/or gut mucosal surfaces. Our results suggest that oral induction of immune responses is possible and related to dose of antigenic exposure; however, sustained responses are rare and the relevance of isolated cellular responses to protection is uncertain. It is likely that multipronged humoral and cellular responses induced by vaccines will be required.

Conflict of Interest: The authors declare no financial or commercial conflicts of interest.

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Figure Legends

Figure 1. Detection of negative and positive HIV-1-gag-specific IFN-γ responses in HIV-1-exposed, uninfected (EU) infants during the first year postpartum. The detection of HIV-1-specific IFN-γ responses is shown for 47 EU infants born to mothers randomized to either short-course ZDV/NVP or HAART. Filled circles=detectable response. Open circles=undetectable response. No circle=not tested.

Figure 2. Infant HIV-1-specific peptide responses and maternal viral loads. Interferon-gamma (IFN-γ) ELISpot assays were conducted on freshly isolated PBMC samples from HIV-1-exposed, uninfected infants using 2 x 10^5 PBMC per well with 2 wells per peptide pool. Data from assays with background SFU ≤ 100/10^6 PBMC are depicted. Pools with ≥50 HIV-1-specific SFU/10^6 PBMC and >2x the background response were defined as positive ELISpot responses. Magnitude of HIV-1-specific peptide responses (stacked bar), plasma HIV-1 RNA (open squares, dashed line), breastmilk HIV-1 RNA (closed circles, solid line), and breastmilk HIV-1 DNA (open triangles, solid line) are shown for all infants with positive ELISpot responses. Months -1 and 0 refer to 32 weeks gestation (initiation of ARV regimen) and delivery, respectively. The mean days to delivery after the 32 weeks gestation visit was 39 (median 39 days, range 2-82 days). Data points marked with an NT indicate time-points when infants were not tested for ELISpot responses. Black reference lines indicate the lower limits of detection for HIV-1 RNA/ml in plasma (200 copies/ml, dashed line) and breastmilk (100 copies/ml, solid line) and red reference lines indicate threshold for a positive HIV-1-specific IFN-γ response (50 HIV-1-specific SFU/10^6 PBMC).