

# Association of HIV-1 Envelope-Specific Breast Milk IgA Responses with Reduced Risk of Postnatal Mother-to-Child Transmission of HIV-1

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## ABSTRACT

Infants born to HIV-1-infected mothers in resource-limited areas where replacement feeding is unsafe and impractical are repeatedly exposed to HIV-1 throughout breastfeeding. Despite this, the majority of infants do not contract HIV-1 postnatally, even in the absence of maternal antiretroviral therapy. This suggests that immune factors in breast milk of HIV-1-infected mothers help to limit vertical transmission. We compared the HIV-1 envelope-specific breast milk and plasma antibody responses of clade C HIV-1-infected postnatally transmitting and nontransmitting mothers in the control arm of the Malawi-based Breast-feeding Antiretrovirals and Nutrition Study using multivariable logistic regression modeling. We found no association between milk or plasma neutralization activity, antibody-dependent cell-mediated cytotoxicity, or HIV-1 envelope-specific IgG responses and postnatal transmission risk. While the envelope-specific breast milk and plasma IgA responses also did not reach significance in predicting postnatal transmission risk in the primary model after correction for multiple comparisons, subsequent exploratory analysis using two distinct assay methodologies demonstrated that the magnitudes of breast milk total and secretory IgA responses against a consensus HIV-1 envelope gp140 (B.con env03) were associated with reduced postnatal transmission risk. These results suggest a protective role for mucosal HIV-1 envelope-specific IgA responses in the context of postnatal virus transmission. This finding supports further investigations into the mechanisms by which mucosal IgA reduces risk of HIV-1 transmission via breast milk and into immune interventions aimed at enhancing this response.

## IMPORTANCE

Infants born to HIV-1-infected mothers are repeatedly exposed to the virus in breast milk. Remarkably, the transmission rate is low, suggesting that immune factors in the breast milk of HIV-1-infected mothers help to limit transmission. We compared the antibody responses in plasma and breast milk of HIV-1-transmitting and -nontransmitting mothers to identify responses that correlated with reduced risk of postnatal HIV-1 transmission. We found that neither plasma nor breast milk IgG antibody responses were associated with risk of HIV-1 transmission. In contrast, the magnitudes of the breast milk IgA and secretory IgA responses against HIV-1 envelope proteins were associated with reduced risk of postnatal HIV-1 transmission. The results of this study support further investigations of the mechanisms by which mucosal IgA may reduce the risk of HIV-1 transmission via breastfeeding and the development of strategies to enhance milk envelope-specific IgA responses to reduce mother-to-child HIV transmission and promote an HIV-free generation.

Recent estimates indicate that breastfeeding accounts for half of the 260,000 pediatric HIV-1 infections that occur annually (1). The risk of postnatal HIV-1 transmission can be significantly decreased with maternal antiretroviral prophylaxis or by replacement feeding; however, these strategies are often not viable in resource-limited areas (2).

Remarkably, despite chronic mucosal virus exposure, the majority of breastfed infants born to HIV-1-infected mothers do not contract HIV-1 postnatally (3, 4). The high concentration of antibodies (Abs) in breast milk gives reason to suspect that adaptive humoral immune responses are involved in natural infant protection from HIV-1 infection (5). Antibodies in milk are either transferred from the plasma by transudation or locally produced by plasma cells that have migrated to the mammary gland from other mucosal sites, in particular, the gut-associated lymphoid tissues

(6). Secretory IgA (SIgA) is the predominant milk immunoglobulin, followed by IgM and IgG (7). HIV envelope (Env)-specific antibodies of all three isotypes have been identified in breast milk, but surprisingly HIV-1 Env-specific IgG responses are higher in magnitude than HIV-1 Env-specific IgA responses and mediate the majority of the neutralization and antibody-dependent cell-mediated cytotoxicity (ADCC) activity found in breast milk (8–11). However, previous studies have reported no differences in the frequencies of detectable HIV-1 Env-specific antibody responses between transmitting and nontransmitting mothers (9, 11–13). These findings may point to the importance of milk antibody specificity and/or function in infant protection.

This study aimed to determine if there is an association between the specificity and/or function of breast milk HIV Env-specific IgG and IgA antibody responses and the risk of postnatal

mother-to-child HIV-1 transmission. Specifically, we sought to determine if the antibody responses associated with reduced infection risk in the RV144 clinical trial, including V1/V2-specific antibodies, V3-specific antibodies, and ADCC activity, also impact postnatal HIV-1 transmission (14–19). Understanding naturally elicited protective antibody responses could provide insight into future maternal or pediatric vaccine design strategies.

## MATERIALS AND METHODS

**Study cohort.** Breast milk and plasma samples were obtained from the control arm of the Breastfeeding, Antiretrovirals, and Nutrition (BAN) study (ClinicalTrials.gov number NCT00164736). This study enrolled antiretroviral-naïve, HIV-1-infected pregnant women in Malawi with CD4<sup>+</sup> T cell counts above 200 cells/μl (250 cells/μl after 24 July 24 2006) from 2004 to 2009. All mothers and infants in the control arm received single-dose nevirapine at onset of labor (postpartum for infants), followed by 7 days of zidovudine/lamivudine therapy (20). Mothers who transmitted HIV-1 to their infants during breastfeeding (*n* = 22) were included in the current study and matched in a near 1:3 ratio with nontransmitting mothers (*n* = 65) for postpartum visit and the closest peripheral CD4<sup>+</sup> T cell count. Among the transmitting women, specimens were selected from the last visit prior to infant HIV-1 diagnosis, and this postpartum visit was used as a matching criterion for selection of specimens from nontransmitting mothers. Plasma samples collected at the same visit as the milk samples were also available from 42 of the 87 total subjects (10 transmitters and 32 nontransmitters). Clinical characteristics for the study cohort are included in Table 1. There were no significant differences between transmitting and nontransmitting women in milk HIV-1 viral load, peripheral CD4<sup>+</sup> T cell count, days postpartum for sample collection, age, or gravida. High plasma viral load is known to be associated with postnatal transmission risk (21), and within our cohort a significant difference in plasma viral load was observed between transmitters and nontransmitters (Table 1) (Wilcoxon test, *P* = 0.003). Accordingly, all subsequent statistical analyses were performed with correction for plasma viral load, as well as peripheral CD4<sup>+</sup> T cell count.

**Ethics statement.** Ethical approval for the BAN study was obtained from the Malawi National Health Science Research Committee and the institutional review boards at the University of North Carolina at Chapel Hill and the U.S. Centers for Disease Control and Prevention. All study participants were adults and provided written informed consent.

**Neutralization assays.** Plasma and breast milk neutralization of HIV-1 was measured by the ability of these samples to reduce virus infection of TZM-bl cells (NIH AIDS Reagent Program; contributed by John Kappes and Xiaoyun Wu) as previously described (22). All milk samples

TABLE 1 Clinical characteristics of transmitting and nontransmitting HIV-1-infected, non-antiretroviral therapy-treated maternal cohort from the Breastfeeding, Antiretrovirals, and Nutrition Study, Malawi

| Parameter and group <sup>a</sup>                          | Value for the parameter |                 |                 | <i>P</i> value <sup>e</sup> |
|---|-------------------------|-----------------|-----------------|-----------------------------|
|   | Median                  | 25th percentile | 75th percentile |                             |
| Breast milk VL (log <sub>10</sub> copies/ml) <sup>b</sup> |                         |                 |                 |                             |
| Transmitters  | 3.45                    | 2.59            | 4.65            | 0.244                       |
| Nontransmitters   | 3.17                    | 2.45            | 3.59            |                             |
| Plasma VL (log <sub>10</sub> copies/ml)                   |                         |                 |                 |                             |
| Transmitters  | 4.84                    | 4.29            | 5.14            | <b>0.003</b>                |
| Nontransmitters   | 4.20                    | 3.80            | 4.60            |                             |
| No. of peripheral CD4 <sup>+</sup> T cells/μl             |                         |                 |                 |                             |
| Transmitters  | 324                     | 293             | 445             | 0.950                       |
| Nontransmitters   | 327                     | 273             | 446             |                             |
| Days postpartum <sup>d</sup>                              |                         |                 |                 |                             |
| Transmitters  | 48.5                    | 28              | 85              | 0.981                       |
| Nontransmitters   | 44                      | 29              | 84              |                             |
| Age (yr)  |                         |                 |                 |                             |
| Transmitters  | 25.5                    | 22              | 31              | 0.582                       |
| Nontransmitters   | 25                      | 22              | 28              |                             |
| Gravida   |                         |                 |                 |                             |
| Transmitters  | 3                       | 2               | 4               | 0.779                       |
| Nontransmitters   | 3                       | 2               | 4               |                             |

<sup>a</sup> The data represent 22 transmitting and 65 nontransmitting mothers except for breast milk VL as noted. VL, viral load.

<sup>b</sup> No target was detected in breast milk samples from 3 transmitters and 30 nontransmitters, and three additional breast milk samples from nontransmitters were below the level of quantitation (100 copies/ml).

<sup>c</sup> Wilcoxon test. A significant difference (*P* < 0.05) in plasma viral loads between transmitters and nontransmitters is indicated in boldface.

<sup>d</sup> The time point represents the last visit prior to infant HIV-1 diagnosis for the transmitting women; samples from nontransmitting women were matched by days postpartum and CD4<sup>+</sup> T cell count.

were delipidized by centrifugation at 25,000 × *g* to reduce toxicity in cellular assays as previously described (8). All assays were performed by laboratory personnel blinded to the transmission status of the women's samples. Plasma and breast milk neutralization were measured against the tier 1 HIV-1 clade C isolate MW965 (GenBank accession number U08455). Breast milk samples were also tested for neutralization against tier 1 HIV-1 clade B isolate MN (GenBank number M17449) and a tier 2 postnatally transmitted isolate, 1209BMH5 (GenBank number HM070570) (23). Plasma was tested at a starting dilution of 1:20, and delipidized milk was tested at a starting dilution of 1:10. The neutralization titer is reported as the dilution at which the relative light units (RLU) were reduced by 50% (50% inhibitory dose [ID<sub>50</sub>]) compared to the RLU in virus control wells. Samples were also tested for neutralizing activity against a nonspecific retrovirus (murine leukemia virus, SVA.MLV) to assess nonspecific inhibition of viral infection in the presence of antiretroviral drugs. Neutralization ID<sub>50</sub> titers that were less than three times the nonspecific background observed against SVA.MLV were not included in the logistic regression analysis.

**ADCC assays. (i) ADCC activity against gp120-coated target cells (ADCC-GTL assay).** A GranToxiLux (GTL) assay was used to detect ADCC activities of plasma and delipidized breast milk samples di-

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rected against gp120-coated target cells as described previously (24). CEM.NKR<sub>CCR5</sub> cells (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, from Alexandra Trkola [25]) were used as targets after being coated with recombinant gp120 from HIV-1 isolate 4403BMC5, representing a clade C postnatally transmitted virus (GenBank number HM070724) (23). Cryopreserved human peripheral blood mononuclear cells (PBMC) from an HIV-seronegative donor with the heterozygous 158F/V genotype for Fc-gamma receptor IIIa were used as the source of effector cells (26). The plasma and breast milk samples were tested after 5-fold serial dilutions starting at 1:50 and 1:10, respectively. The maximum percent granzyme B (GzB) activity was defined as the peak proportion of cells positive for proteolytically active GzB out of the total viable target cell population. The final results are expressed after subtraction of the background percent GzB activity observed in wells containing effector and target cells in the absence of plasma or breast milk. ADCC endpoint titers were determined by interpolating the dilutions of plasma or breast milk that intersect the positive cutoff (greater than the average percent GzB activity plus 3 standard deviations [SD] of 9 uninfected samples tested in parallel) using GraphPad Prism, version 5, software (GraphPad).

(ii) **ADCC against HIV-1-infected target cells (ADCC-Luc assay).** ADCC activity was determined by a luciferase (Luc)-based assay as previously described (17). CEM.NKR<sub>CCR5</sub> cells were infected with an infectious molecular clone (IMC) that encodes the HIV-1 subtype C Env from isolate 4403BMC5 in an NL4-3 isogenic backbone that contains the *Renilla* luciferase reporter gene and preserves all HIV-1 open reading frames (27). Infections were monitored by measuring luciferase activity (ViviRen Live Cell Substrate; Promega) and the frequency of cells expressing intracellular p24. Greater than 75% of the target cells were p24 positive in each assay conducted. Effector cells were the same cryopreserved PBMC used for ADCC-GTL assays, and samples were tested at the same range of concentrations. ADCC activity (percent killing) was calculated from the change in RLU amounts (ViviRen luciferase assay; Promega) resulting from the loss of intact target cells in wells containing effector cells, target cells, and plasma or breast milk samples compared to amounts in control wells containing target cells and effector cells alone according to the following formula: percent killing = [(number of RLU of target and effector well - number of RLU of test well)/number of RLU of target and effector well] × 100. We report both the maximum percent killing observed for each sample and the ADCC endpoint titer, defined as the dilution of plasma or breast milk that intersects the positive cutoff (greater than the average percent killing activity plus 1 standard deviation of 9 uninfected samples, each tested in two independent experiments).

**BAMA.** Levels of HIV-1 Env-specific IgA and IgG were determined with a customized HIV-1 binding antibody multiplex assay (BAMA) as previously described (15). Carboxylated fluorescent beads (Luminex Corporation, Austin, TX) were covalently coupled to purified HIV-1 antigens and subsequently incubated with patient samples in assay diluent (phosphate-buffered saline [PBS], 5% normal goat serum, 0.05% Tween 20, 1% BLOTTO milk) at various dilutions. For IgG, the antigen panel included biotinylated linear V3 loop peptide V3.C (KKKNNTRKSIRIGPGQTFYA TGDIIIGDIRQAHC) and proteins 4403 BMC5 gp120 (23), 1209 BMH5 gp120 (23), Con6 gp120/B (28), ConS gp140 (29), HIV-1 MN recombinant gp41 (Immuno Diagnostics Incorporated, Woburn, MA), gp70 B.CaseA2\_V1V2/169K (30), gp70 B.CaseA\_V1V2 (31), gp70 C.1086C\_V1V2 (30), and 1086C gp140 (32); for IgA, the antigen panel included biotinylated linear peptides V3.C, C1 (KKKMVEDVLSLW DQSLKPCVKLTPLCV), and Bio-C1\_104.BC (KKKMVEDVLSLW DQSLKPCVKLTPLCV) and proteins A1.con env03 gp140 (15), A244 gD-gp120 (15), B.con env03 gp140 (15), 4403 BMC5 gp120, Con6 gp120/B, ConS gp140, gp41, gp70 B.CaseA2\_V1V2/169K, gp70 B.CaseA\_V1V2, gp70 C.1086C\_V1V2, and 1086C gp140. Blank beads were used in all assays to account for nonspecific binding. HIV-specific antibodies were detected with phycoerythrin (PE)-conjugated goat anti-human IgA at 4 µg/ml (Jackson ImmunoResearch Laboratories, West Grove, PA) or PE-conju-

gated mouse anti-human IgG at 2 µg/ml (Southern Biotech, Birmingham, AL). HIV-specific SIgA was detected with mouse anti-human SIgA at 4 µg/ml (Sigma-Aldrich), followed by goat anti-mouse IgG-PE (Southern Biotech). Positive controls for SIgA binding included purified colostrum IgA and purified secretory component coupled to Luminex beads. The beads were then washed and acquired on a Bio-Plex 200 instrument (Bio-Rad Laboratories, Hercules, CA). The results were expressed as mean fluorescence intensity (MFI). A panel of negative-control samples ( $n = 15$ , except for breast milk IgA, where additional negative-control samples were included, for a total  $n = 30$ ) was included to determine baseline, nonspecific levels of binding. All assays included tracking of HIV immunoglobulin (HIVIG) standard by Levy-Jennings charts. The preset assay criteria for sample reporting were coefficient of variation per duplicate values of  $\leq 20\%$  for each sample and  $\geq 100$  beads counted per sample. All samples were analyzed at the same dilution for each antigen. For breast milk IgG samples, gp70 B.CaseA2\_V1V2/169K, gp70 B.CaseA\_V1V2, and gp70 C.1086C\_V1V2 antigens were measured at a 1:5 sample dilution; Con6 gp120/B, ConS gp140, and V3.C antigens were measured at a 1:100 sample dilution; and HIV-1 MN recombinant gp41 and 1086C gp140 antigens were measured at a 1:250 sample dilution. For plasma IgG samples, gp70 B.CaseA2\_V1V2/169K, gp70 B.CaseA\_V1V2, gp70 C.1086C\_V1V2, 4403 BMC5 gp120, and 1209 BMH5 antigens were measured at a 1:250 sample dilution; Con6 gp120/B, ConS gp140, and V3.C antigens were measured at a 1:2,500 sample dilution; and HIV-1 MN recombinant gp41 and 1086C gp140 antigens were measured at a 1:12,500 sample dilution. All breast milk IgA samples were measured at a 1:5 dilution, and all plasma IgA samples were measured at a 1:10 dilution. These dilutions were predetermined to be within the linear range of the assay based on testing serial dilutions of a small subset of breast milk/plasma samples.

**ELISA.** Enzyme-linked immunosorbent assay (ELISA) plates (384 wells; Corning Life Sciences, Corning, NY) were coated overnight with 30 µg/well of consensus clade B gp140 (B.con env03 gp140) in 0.1 M sodium bicarbonate. The plates were then washed with washing buffer (phosphate-buffered saline [PBS]–0.1% Tween 20) and blocked with Superblock (PBS containing 4% whey protein, 15% normal goat serum, and 0.5% Tween 20). Breast milk was incubated for 1 h in eight 3-fold serial dilutions, beginning at 1:5; then the plates were washed two times. For total IgA detection, 10 µl/well of 0.08 µg/ml peroxidase-conjugated goat anti-human IgA (Southern Biotech) was added to the wells. After 1 h of incubation and four washes, the substrate (SureBlue Reserve Microwell Substrate; VWR, Radnor, PA) was added, followed by addition of TMB (3,3',5,5'-tetramethylbenzidine) Stop Solution (VWR). For SIgA detection, 10 µl/well of mouse anti-human secretory component IgA monoclonal antibody (Sigma-Aldrich, St. Louis, MO) at a concentration of 0.87 µg/ml was added after the sample incubation. After 1 h of incubation and two washes, this was followed by incubation with 10 µl/well of a 1:4,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Southern Biotech). After 1 h of incubation and four washes, the substrate and then the stop solution were added. Absorbance of the wells was read at 450 nm immediately after addition of the stop solution.

**Statistical methods.** Logistic regression was used to calculate the association between plasma and breast milk HIV-1 Env-specific IgG and IgA with the outcome of postnatal HIV-1 transmission or nontransmission. Separate models were run for each antigen, and a false discovery rate (FDR) (33) was used to account for multiple comparisons within a specific set of plasma or breast milk samples by isotype. Logistic regression was also applied to the assessments of ADCC activity and TZM-bl HIV-1 neutralization. No adjustments to the alpha level were made for these comparisons. All logistic models controlled for CD4<sup>+</sup> T cell count, which was also used for matching, and plasma viral load at an alpha level of 0.05.

Wilcoxon two-sample tests (also known as Mann-Whitney U tests) were performed at the alpha 0.05 level to compare results in uninfected controls with those in HIV-1-infected transmitters and nontransmitters, as well as for cohort comparisons. No adjustments to the alpha level were



made for these comparisons. This research is a laboratory substudy using samples from the control arm of the Malawi-based BAN randomized clinical trial with a fixed number of transmitters ( $n = 22$ ). Thus, the number of available transmitter samples limited the size of the cohort, and the research study was not powered to a specific outcome based on an assumed difference between transmitters and nontransmitters.

## RESULTS

**Breast milk neutralization responses are not associated with postnatal HIV-1 transmission risk.** To investigate if maternal neutralization responses were associated with postnatal transmission in our study cohort, we assessed the HIV-1 neutralization activity of breast milk from transmitting and nontransmitting mothers using the TZM-bl HIV-1 neutralization assay. Milk from HIV-1-uninfected women was included to define the low, innate background levels of neutralization commonly observed with breast milk and other mucosal samples (8, 34, 35). We found that the milk from transmitting and nontransmitting women had higher neutralization activities against the tier 1 clade-matched isolate C.MW965 than those observed in uninfected control women (Fig. 1A). However, we found no significant difference in the frequencies of milk samples with detectable neutralization responses among transmitting and nontransmitting women (95.5% and 86.2%, respectively;  $P = 0.441$ , Fisher's exact test). Moreover, the milk neutralization potency (measured as the 50% inhibitory dose [ $ID_{50}$ ]) against C.MW965 was not associated with transmission risk (odds ratio [OR], 1.40;  $P = 0.661$ ) (see Table S1 in the supplemental material). We also measured neutralization potency of available plasma samples from 10 transmitting and 32 nontransmitting mothers collected in parallel with milk samples to determine if the maternal peripheral neutralizing antibody response was associated with postnatal transmission. The plasma neutralization  $ID_{50}$  titers against the tier 1 isolate C.MW965 were similar among transmitters and nontransmitters and did not indicate any association with transmission status (OR = 4.05,  $P = 0.210$ ).

We next evaluated the neutralization potency of breast milk against a non-clade-matched tier 1 HIV-1 isolate, B.MN, and a clade-matched tier 2 transmitted founder virus isolated from a postnatally infected Malawian infant, C.1209BMH5 (Fig. 1B and C) (23). The  $ID_{50}$  neutralization titers against these isolates did not significantly differ between HIV-infected nontransmitting women and uninfected controls (B.MN,  $P = 0.201$ ; C.1209BMH5,  $P = 0.092$ ; Wilcoxon test).

**Breast milk ADCC activity is not associated with postnatal transmission risk.** ADCC activities of breast milk of transmitting and nontransmitting women were first assessed against target cells coated with recombinant gp120 representing the clade C Malawian breast milk-transmitted virus 4403BMC5 using the ADCC-GTL assay (23, 24). Milk of the HIV-1-infected transmitting and nontransmitting women displayed specific ADCC activity which was significantly higher ( $P \leq 0.001$ , Wilcoxon test) than that of noninfected control mothers (Fig. 2A and B). However, neither the milk ADCC potency (OR = 1.00,  $P = 0.891$ ) nor milk ADCC Ab titers (OR = 0.82,  $P = 0.638$ ) were associated with reduced risk of postnatal HIV-1 transmission (see Table S1 in the supplemental material). Concordant results were obtained in assays conducted with available maternal plasma samples (potency, OR = 1.01 and  $P = 0.902$ ; titer, OR = 2.57 and  $P = 0.591$ ).

To further characterize the ADCC response of transmitting

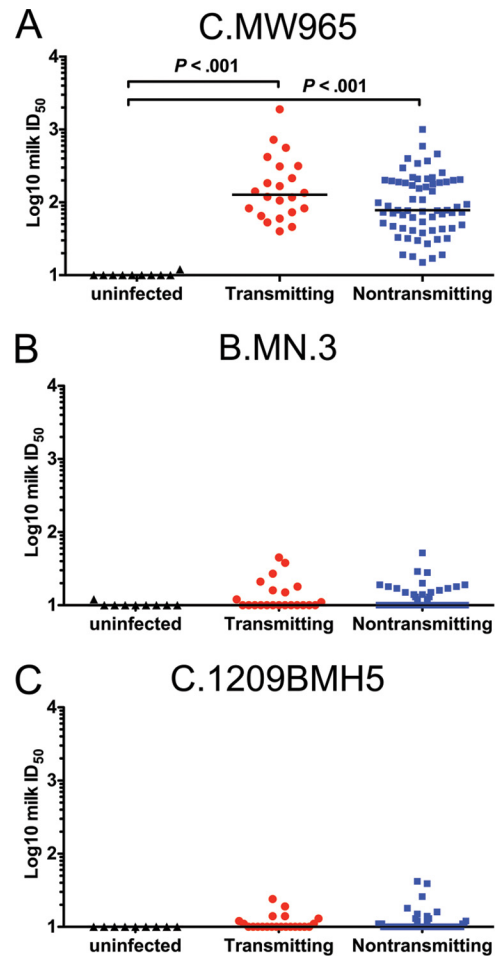
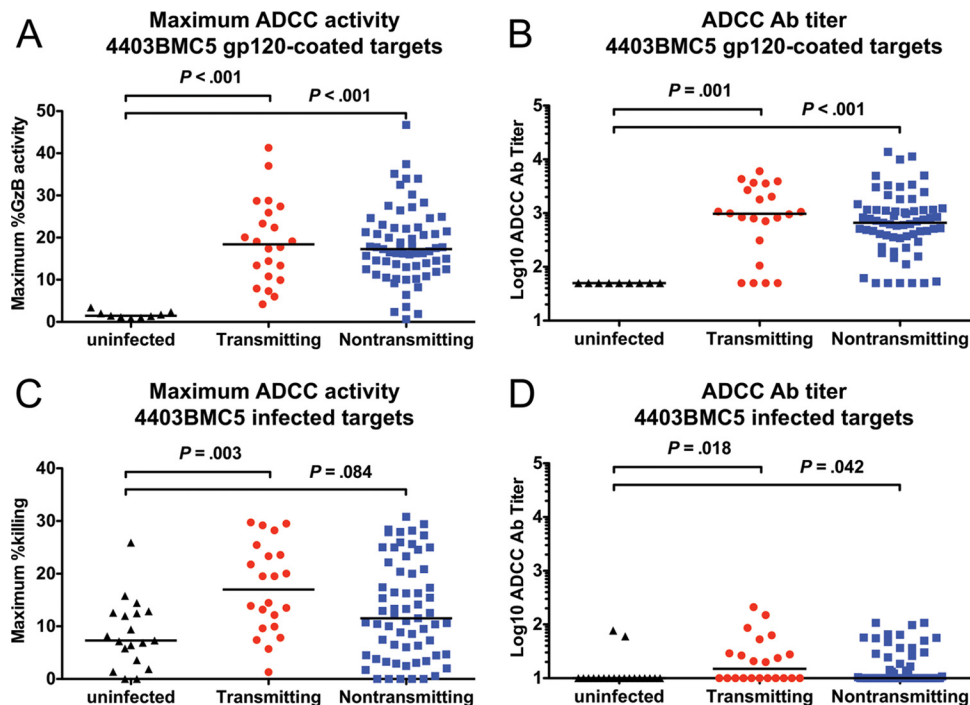


FIG 1 Magnitude of breast milk neutralization in uninfected and postnatal transmitting and nontransmitting mothers. The breast milk neutralization 50% inhibitory dose ( $ID_{50}$ ) was measured by TZM-bl pseudovirus assay against tier 1 C.MW965 (A), tier 1 B.MN.3 (B), and tier 2 C.1209BMH5 (C). Samples from nine uninfected controls were included to represent nonspecific background activity. Tier 1 C.MW965 neutralization responses among transmitting and nontransmitting women were significantly above background levels observed in uninfected control women (Wilcoxon test). Each dot represents milk from one donor, and medians are indicated with black horizontal lines.

and nontransmitting women, we next evaluated the milk ADCC activity directed against target cells infected with HIV-1 4403BMC5 IMC virus (27) using the ADCC-Luc assay (Fig. 2C and D). HIV-1-infected target cells express the diversity of native forms of cell surface HIV-1 Env and likely better mimic the type of target cells present *in vivo* (36). However, neither breast milk ADCC potency (OR = 1.05,  $P = 0.133$ ) nor ADCC Ab titers (OR = 1.82,  $P = 0.269$ ) against HIV-infected target cells were associated with risk of postnatal transmission of HIV-1 (see Table S1 in the supplemental material).

**Breast milk Env-binding IgG responses are not associated with postnatal transmission risk.** In the absence of identifying functional antibody responses associated with reduced risk of postnatal HIV-1 transmission, we next evaluated the magnitude and specificities of HIV-1 Env-binding antibodies present in breast milk of transmitting and nontransmitting mothers. We measured the magnitude of breast milk IgG binding to a multi-



**FIG 2** Antibody-dependent cell-mediated cytotoxicity (ADCC) activity and ADCC antibody (Ab) titer against gp120-coated and HIV-1-infected target cells in breast milk from uninfected and postnatal transmitting and nontransmitting mothers. (A and B) Maximum observed ADCC activity measured as percent granzyme B (GzB)-positive target cells (A), and ADCC Ab titers of breast milk samples against 4403BMC5 gp120-coated target cells in the ADCC GranToxiLux (ADCC-GTL) assay (B). (C and D) Breast milk maximum percent specific ADCC killing activity (C), and ADCC Ab titers against 4403BMC5 HIV-1-infected target cells in the ADCC-Luc assay (D). Samples from nine uninfected controls were included to represent nonspecific background activity and were tested in two independent experiments, for a total  $n = 18$  in the ADCC-Luc assay. Maximum ADCC activities and Ab titers among transmitting and nontransmitting women were above background levels observed in uninfected control subjects as indicated (Wilcoxon test). Each point represents an individual donor, except in panels C and D, where duplicate testing of the uninfected controls is included, and all 18 data points are shown. Medians are indicated with black horizontal lines.

clade panel of Env antigens that included epitope regions found to correlate with reduced infection risk in the RV144 clinical trial (clade B and C V1/V2 and clade C V3 [14–19]). The panel also included a clade C Env gp140 (1086C) (32), clade C breast milk-transmitted founder gp120s (4403 BMC5 and 1209 BMH5) (23), consensus gp120 (Con6), consensus gp140 protein (ConS), and B.MN gp41. Using logistic regression modeling, we found that the magnitudes of total milk IgG binding to the interrogated Env antigens were not associated with postnatal HIV-1 transmission risk (see Table S2 in the supplemental material). The magnitude of maternal plasma IgG Env-binding responses also did not associate with postnatal transmission risk (see Table S3 in the supplemental material).

**Breast milk Env-specific IgA responses and postnatal transmission risk.** We next assessed the magnitude of the Env-binding IgA response against a multiclade panel of HIV-1 Env antigens, including gp140 and gp120 Env proteins, as well as gp41, V3, C1, and V1V2 Env antigens.

We found no significant difference in the magnitude of total milk IgA (Table 2) or plasma IgA (see Table S4 in the supplemental material) binding to any of the interrogated Env antigens between transmitting and nontransmitting women in the logistic regression model. Although the antibody binding magnitudes were not significantly different after correction for multiple comparisons, the magnitude of breast milk total IgA binding to consensus gp140 antigens A1.con env03 and B.con env03 gp140 indicated a trend toward association with reduced transmission

**TABLE 2** Odds ratios for the association between breast milk IgA binding to HIV-1 antigens by BAMA and HIV-1 transmission to the infant via breast milk in the BAN Study, Malawi

| Antigen <sup>a</sup>       | Logistic regression model for transmitters |                             |                  |
|----------------------------|--|-----------------------------|------------------|
|                            | OR (95% CI) <sup>b</sup>                   | <i>P</i> value <sup>c</sup> | FDR <sup>d</sup> |
| <b>A1.con env03 gp140</b>  | <b>0.40 (0.17–0.93)</b>                    | <b>0.034</b>                | <b>0.240</b>     |
| A244 gD <sup>+</sup> gp120 | 0.82 (0.52–1.32)                           | 0.419                       | 0.488            |
| <b>B.con env03 gp140</b>   | <b>0.57 (0.38–0.86)</b>                    | <b>0.007</b>                | <b>0.101</b>     |
| V3.C                       | 1.17 (0.72–1.88)                           | 0.530                       | 0.571            |
| Bio-C1_104.BC              | 0.76 (0.47–1.21)                           | 0.242                       | 0.408            |
| 4403 BMC5 gp120            | 0.80 (0.53–1.21)                           | 0.292                       | 0.408            |
| C1                         | 0.67 (0.42–1.08)                           | 0.098                       | 0.314            |
| Con6 gp120/B               | 0.75 (0.52–1.09)                           | 0.135                       | 0.314            |
| ConS gp140                 | 0.74 (0.51–1.09)                           | 0.129                       | 0.314            |
| MN gp41                    | 0.73 (0.50–1.06)                           | 0.096                       | 0.314            |
| gp70 B.CaseA2_V1V2/169K    | 1.24 (0.77–2.00)                           | 0.381                       | 0.485            |
| gp70 B.CaseA_V1V2          | 1.32 (0.81–2.15)                           | 0.269                       | 0.408            |
| gp70 C.1086C_V1V2          | 1.02 (0.64–1.64)                           | 0.932                       | 0.932            |
| 1086C gp140                | 0.78 (0.55–1.11)                           | 0.163                       | 0.326            |

<sup>a</sup> Detailed descriptions of antigens can be found in Materials and Methods.

<sup>b</sup> Odds ratios (ORs) and 95% confidence intervals (CI) for binding antibody multiplex assay (BAMA) mean fluorescence intensities were determined by logistic regression modeling controlling for  $\log_{10}$  plasma viral load and peripheral CD4<sup>+</sup> T cell count. Significant odds ratios less than 1 indicate that nontransmitters have higher responses than transmitters.

<sup>c</sup> Immune variables with unadjusted *P* values of  $<0.05$  are in boldface.

<sup>d</sup> FDR, false discovery rate.

(A1.con env03 gp140,  $P = 0.034$  and FDR = 0.240; B.con env03 gp140,  $P = 0.007$  and FDR = 0.101) (Table 2). These observations prompted additional exploration into the potential role of breast milk IgA in protection against postnatal HIV-1 transmission.

We first compared the magnitude of breast milk IgA binding to A1.con and B.con env03 gp140s of transmitting and nontransmitting women to uninfected women. Interestingly, the magnitudes of IgA binding responses to the A1.con env03 and B.con env03 gp140 antigens in breast milk of transmitting women were similar to those of 30 uninfected women, yet milk of nontransmitting women bound with significantly higher magnitude than milk of uninfected women (A1.con env03 gp140,  $P = 0.018$ ; B.con env03 gp140,  $P < 0.001$ ) (Fig. 3A and B). These observations support the trend observed in the logistic regression model and suggest that the breast milk IgA responses of nontransmitting mothers are different from those of transmitting mothers.

**Breast milk IgA binding to B.con env03 gp140 is associated with reduced postnatal HIV-1 transmission risk.** To further probe these differences, we performed exploratory analysis to determine if breast milk IgA binding responses directed against the A1.con and B.con env03 gp140 antigens were associated with reduced risk of transmission. For these exploratory analyses, no adjustments for multiple comparisons were made to the  $P$  values of the logistic regression models. We used a standard single-antigen plate-based ELISA to confirm that the binding of total breast milk IgA to the antigen that most closely associated with transmission risk in the primary analysis, B.con env03 gp140, was in fact associated with postnatal mother-to-child transmission risk. The magnitudes of the Env-specific IgA binding responses (measured as  $\log_{10}$  area under the curve [AUC] above positivity threshold) of both transmitting and nontransmitting women measured by ELISA were significantly higher than those observed in uninfected controls (Fig. 3C). Moreover, this response was associated with transmission risk in the exploratory analysis using logistic regression modeling (OR = 0.51,  $P = 0.020$ ) (Table 3). Thus, the magnitude of breast milk IgA binding to B.con env03 gp140 is a possible correlate of postnatal HIV-1 transmission risk in this study cohort.

**Breast milk SIgA binding to B.con env03 gp140 is associated with reduced postnatal transmission risk.** We next used SIgA BAMA to assess the relationship between the magnitude of breast milk SIgA binding to consensus Env gp140 antigens and postnatal transmission risk. The magnitudes of SIgA binding to consensus A, B, and C gp140s were significantly higher among transmitting and nontransmitting women than among uninfected controls (see Fig. S1A to C in the supplemental material). Moreover, using exploratory logistic regression analysis (no adjustment of  $P$  value for multiple comparisons), the magnitude of breast milk SIgA binding to B.con env03 gp140 was significantly associated with postnatal transmission risk (OR = 0.54,  $P = 0.032$ ) (Table 3), concordant with the results obtained for total IgA binding with this antigen.

To confirm the association between the magnitude of the Env-specific SIgA response and transmission risk, we also utilized a standard single-antigen ELISA to determine the relationship between breast milk SIgA binding to B.con env03 gp140 and postnatal transmission risk (see Fig. S1D in the supplemental material). While the SIgA responses in transmitting and nontransmitting women measured by ELISA were not significantly associated with postnatal transmission risk in the multivariate logistic

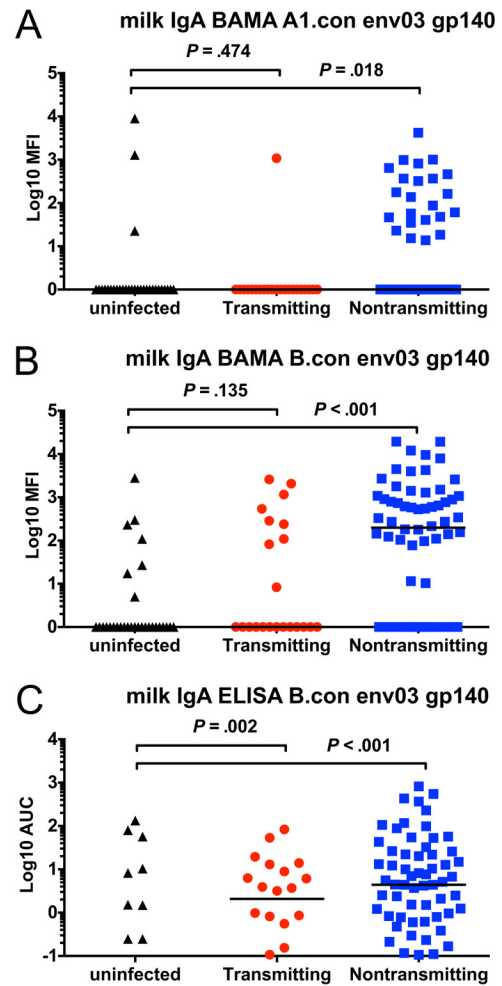


FIG 3 Breast milk total IgA binding to select HIV-1 envelope (Env) antigens in uninfected and postnatal transmitting and nontransmitting mothers. The magnitude of the milk IgA binding responses measured by binding antibody multiplex assay (BAMA) against A1.con env03 gp140 (A) and B.con env03 gp140 (B) in transmitting women were similar to responses of 30 uninfected women, whereas the nontransmitting women had significantly higher IgA responses against these Env antigens than uninfected women, as indicated (Wilcoxon test). Binding magnitude is reported as mean fluorescent intensity (MFI). (C) Breast milk total IgA binding to B.con env03 gp140 measured by enzyme-linked immunosorbent assay (ELISA). The magnitude of binding, measured as the area under the curve among transmitting and nontransmitting women, was significantly above background levels observed in uninfected control subjects (Wilcoxon test) when measured by ELISA. Each dot represents milk from one donor, and medians are indicated with black horizontal lines.

regression model, the point estimate was in the direction of higher responses among nontransmitting women and reduced risk of HIV-1 transmission (OR = 0.51,  $P = 0.053$ ) (Table 3).

**No significant interactions between breast milk Env-binding IgA and ADCC responses associated with postnatal transmission risk.** In the secondary *post hoc* analysis of the immune correlates of infection risk in the RV144 clinical trial, it was reported that ADCC activity was associated with reduced infection risk in vaccine recipients with low levels of Env-specific plasma IgA (15). Accordingly, we probed for similar interactions within our study cohort. Logistic models with breast milk IgA binding to A1.con env03 gp140 and B.con env03 gp140 were assessed for interaction



**TABLE 3** Odds ratios for the association between breast milk total IgA and secretory IgA binding to consensus HIV-1 gp140 antigens by ELISA and BAMA with HIV-1 transmission to the infant via breast milk in the BAN Study, Malawi

| Antigen <sup>c</sup>                   | Logistic regression model for transmitters |                             |
|--|--|-----------------------------|
|  | OR (95% CI) <sup>a</sup>                   | <i>P</i> value <sup>b</sup> |
| <b>IgA B.con env03 gp140 ELISA AUC</b> | <b>0.51 (0.29–0.90)</b>                    | <b>0.020</b>                |
| SIgA A1.con env03 gp140 BAMA MFI       | 0.66 (0.42–1.03)                           | 0.066                       |
| <b>SIgA B.con env03 gp140 BAMA MFI</b> | <b>0.54 (0.30–0.95)</b>                    | <b>0.032</b>                |
| SIgA C.con env03 gp140 BAMA MFI        | 0.69 (0.41–1.16)                           | 0.161                       |
| SIgA B.con env03 gp140 ELISA AUC       | 0.51 (0.26–1.01)                           | 0.053                       |

<sup>a</sup> Odds ratios (ORs) and 95% confidence intervals (CI) for were determined by logistic regression modeling controlling for log<sub>10</sub> plasma viral load and peripheral CD4<sup>+</sup> T cell count.

<sup>b</sup> Immune variables with *P* values of <0.05 are in boldface.

<sup>c</sup> ELISA AUC, enzyme-linked immunosorbent assay area under the curve; SIgA, secretory IgA; BAMA MFI, binding antibody multiplex assay mean fluorescence intensity.

with ADCC potency and ADCC titers as determined for both HIV-1 Env gp120-coated target cells and HIV-1-infected target cells. These two antigens were selected for the interaction analysis because the IgA responses to these antigens were most strongly associated with reduced risk of postnatal HIV-1 transmission in our cohort. However, no significant interactions between breast milk ADCC responses and milk IgA binding levels to these Env gp140 antigens were identified (see Table S5 in the supplemental material).

## DISCUSSION

In this study, we compared the HIV-1 Env-specific breast milk and plasma antibody responses of clade C HIV-1-infected postnatal transmitting and nontransmitting mothers in the control arm of the Malawian BAN randomized clinical trial. This study provided a unique opportunity to investigate humoral correlates of reduced postnatal mother-to-child transmission risk in the absence of postnatal antiretroviral therapy (20). Although no statistically significant associations were identified in the primary logistic regression models after correction for multiple comparisons, in subsequent exploratory analysis, the breast milk IgA responses against B.con env03 gp140 Env were associated with reduced HIV-1 postnatal transmission risk by two independent but related measures: total IgA binding by single-antigen ELISA and SIgA binding by BAMA. In addition, the SIgA ELISA data suggested an association with reduced risk of transmission (*P* = 0.053), further supporting the potential role of breast milk consensus gp140-specific IgA responses in protection against HIV-1 postnatal transmission.

In the RV144 trial, high levels of Env-specific plasma IgA responses directly correlated with HIV-1 infection risk, generating the hypothesis that plasma IgA may interfere with beneficial IgG-mediated antibody responses such as ADCC activity (15, 37). Unfortunately, no mucosal samples were collected during the RV144 clinical trial, and infant plasma samples were not included from the BAN study cohort, thereby impeding direct comparisons regarding the role of plasma and mucosal IgA in risk of infection/transmission across these two studies. However, these two opposing risk correlates for circulating plasma IgA and breast milk total and SIgA suggest that systemic and mucosal IgA may play differ-

ential roles in protection from HIV-1 infection, likely depending on the site and mode of infection and on the characteristics of the IgA responses, including fine specificity. In the RV144 trial, some vaccine-induced IgA antibodies and potent ADCC-mediating IgG antibodies competed for binding to conformational epitopes within the first constant (C1) region of Env (15, 38). The epitope specificities of the mucosal IgA antibodies correlating with reduced transmission risk in our study have not yet been identified. However, we found no interaction between the magnitude of IgA binding responses and breast milk ADCC activity, suggesting that the mucosal IgA response in nontransmitting mothers targets epitopes that do not interfere with potentially beneficial IgG responses. Moreover, we observed no association of breast milk and plasma IgA or IgG binding to V1/V2 or V3 with postnatal transmission risk, suggesting that potentially protective mucosal IgA responses of nontransmitting mothers target epitopes not previously identified as potentially protective in the RV144 clinical trial (14–19).

In our study, neither the neutralization activity nor the ADCC activity of breast milk or plasma was associated with postnatal HIV-1 transmission risk. Neutralization responses have inconsistently been associated with reduced prenatal, perinatal, or postnatal mother-to-child HIV-1 transmission risk, and prior studies differ in study design, cohort size, prevalent virus clade, use of autologous or heterologous HIV-1 virus strains, mode of HIV-1 transmission, and timing of sample collection (39, 40).

A recent study conducted by Mabuka et al. demonstrated higher Env gp120-specific ADCC activity in milk of nontransmitting women than in that of postnatally transmitting women in a small (*n* = 19), clade A-infected cohort (11). We did not identify any association between gp120-specific ADCC activity and risk of postnatal transmission in our larger (*n* = 87) clade C-infected cohort. ADCC activity in both studies was measured against target cells coated with a recombinant gp120 representing a clade-matched infant-transmitted Env. Our study also assessed ADCC activity against CD4<sup>+</sup> T cells infected with clade-matched infant-transmitted founder virus and found that neither ADCC activity nor ADCC antibody titer against the HIV-infected targets was associated with postnatal transmission risk. Importantly, there are additional differences between the study cohorts beyond size and prevalent virus clade. In the Mabuka study, milk was collected from women with high plasma viral load (above the cohort median), and inclusion was further restricted to only women exhibiting robust plasma neutralizing antibody responses (11). In contrast, our cohort was restricted only by peripheral CD4<sup>+</sup> T cell count (>200 cells/μl). Plasma viral load, a known transmission risk factor, was significantly higher among transmitting women in our cohort, and thus, we controlled for log<sub>10</sub> plasma viral load in our logistic regression model. These differences may account for the seemingly contradictory study outcomes. It is, however, notable that in both studies the frequency of breast milk samples with detectable gp120-specific ADCC activity was high (>90%), suggesting that ADCC activity is prevalent in breast milk and may contribute to the overall low rate of HIV-1 transmission observed for breastfed infants. A recent study described by Milligan and colleagues also found no association between maternal plasma ADCC and infant transmission risk (41). However, their study also demonstrated that plasma samples collected from infants born to HIV-1-infected mothers within the first postpartum week were able to mediate ADCC. Interestingly, infant ADCC levels

were associated with reduced risk of infant mortality, suggesting a role for passively transferred maternal ADCC antibodies in the control of infant HIV-1 disease progression (41). The degree to which ADCC and/or virus neutralization contributes to reducing postnatal transmission rates is likely affected by whether each individual transmission event involved cell-free or cell-associated virus, an area of study of paramount importance to better understand the mechanisms of mother-to-child transmission and the types of antibody responses best suited for protection (42).

The identification of total and secretory IgA binding to a consensus B Env protein as being associated with reduced transmission risk was unexpected for our clade-C-infected Malawian cohort. However, as previously discussed, the epitope specificities of the breast milk IgA responses have not been mapped in detail, nor have the sequences of the transmitted virus isolates been determined for this maternal/infant cohort. It is possible that the B.con env03 gp140 better matched crucial epitopes of the circulating clade C viruses prevalent in the cohort than the clade C antigens included in our binding panel. In addition, it is important to note that the B.con env03 gp140 is a recombinantly produced monomeric protein, not a native envelope trimer. It is conceivable that certain monomeric envelope proteins may more accurately represent particular structural aspects of the native envelope than others, regardless of envelope clade. The identification of a mucosal Env-specific IgA response as a potential correlate of reduced transmission risk is unexpected as our group (8) and others (10, 11) have demonstrated that the Env-specific IgG responses are of higher magnitude than those of IgA in breast milk and are responsible for the majority of breast milk virus neutralization and ADCC. Yet mucosal IgA may function through alternative antiviral mechanisms, such as virus aggregation, mucus binding, or inhibition of epithelial cell transcytosis (43, 44). Interestingly, mucosal Env-specific IgA responses have also been observed among highly exposed seronegative individuals, including female sex workers and discordant couples (45). In contrast, previous studies have not found breast milk Env-specific IgA to be associated with reduced risk of postnatal HIV-1 transmission (9, 11–13). A significant difference between our study and previous breast milk studies is that we assessed the magnitude of Env-binding IgA responses, not frequency of responders, as correlates of transmission risk. Moreover, we used the highly sensitive technique of BAMA, which may be able to better discriminate low-level IgA responses than the techniques previously employed.

Importantly, the results of this study suggest a protective role for mucosal HIV-1 Env-specific IgA in the context of postnatal mother-to-child transmission. We have recently demonstrated that combined systemic and mucosal vaccination strategies can induce robust Env-specific IgA responses in the breast milk of lactating rhesus monkeys (30). Thus, our results support further investigations to gain a better understanding of the antiviral functions of mucosal IgA and the potential mechanisms by which it reduces the risk of HIV-1 transmission via breastfeeding. Moreover, methods to enhance milk Env-specific IgA responses should be further developed and tested in preclinical nonhuman primate models as a potential strategy to increase the safety of breastfeeding for all infants in areas of HIV-1 prevalence.

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