Vertical HIV-1 transmission in the setting of maternal broad and potent antibody responses

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22

23 **Abstract**

24 Despite the worldwide availability of antiretroviral therapy (ART), approximately 150,000
 25 pediatric HIV infections continue to occur annually. ART can dramatically reduce HIV mother-to-
 26 child transmission (MTCT), but inconsistent drug access and adherence, as well as primary
 27 maternal HIV infection during pregnancy and lactation are major barriers to eliminating vertical
 28 HIV transmission. Thus, immunologic strategies to prevent MTCT, such as an HIV vaccine, will
 29 be required to attain an HIV-free generation. A primary goal of HIV vaccine research has been
 30 to elicit broadly neutralizing antibodies (bnAbs) given the ability of passive bnAb immunization to
 31 protect against sensitive strains, yet we previously observed that HIV-transmitting mothers have
 32 more plasma neutralization breadth than non-transmitting mothers. Additionally, we have
 33 identified infant transmitted/founder (T/F) viruses that escape maternal bnAb responses. In this
 34 study, we examine a cohort of postpartum HIV-transmitting women with neutralization breadth
 35 to determine if certain maternal bnAb specificities drive the selection of infant T/F viruses. Using
 36 HIV pseudoviruses that are resistant to neutralizing antibodies targeting common bnAb
 37 epitopes, we mapped the plasma bnAb specificities of this cohort. Significantly more
 38 transmitting women with plasma bnAb activity had a mappable plasma bnAb specificity (six of
 39 seven, or 85.7%), compared to that of non-transmitting women with plasma bnAb activity (seven
 40 of twenty-one, or 33.3%, $p=0.029$ by 2-sided Fisher exact test). Our study suggests that having
 41 multispecific broad activity and/or uncommon epitope-specific bnAbs in plasma may be
 42 associated with protection against the vertical HIV transmission in the setting of maternal bnAb
 43 responses.

44 **Importance**

As mother to child transmission (MTCT) of HIV plays a major part in the persistence of the HIV/AIDS epidemic and bnAb-based passive and active vaccines are a primary strategy for HIV prevention, research in this field is of great importance. While previous MTCT research has investigated the neutralizing antibody activity of HIV-infected women, this is to our knowledge the largest study identifying differences in bnAb specificity of maternal plasma between transmitting and non-transmitting women. Here, we show that among HIV-infected women with broad and potent neutralization activity, more postpartum-transmitting women had a mappable plasma broadly neutralizing antibody (bnAb) specificity, compared to that of non-transmitting women, suggesting that the non-transmitting women more often have multi-specific bnAb responses or bnAb responses that target uncommon epitopes. Such responses may be required for protection against vertical HIV transmission in the setting of maternal bnAb responses.

Introduction

Antiretroviral therapy (ART) can dramatically reduce mother-to-child transmission (MTCT) of HIV-1, but challenges remain in ART access and adherence during pregnancy and throughout breastfeeding. Despite the worldwide availability of ART, around 150,000 new pediatric HIV infections occurred in 2020 (1). Further, with interrupted ART supply and access during the Severe Acute Respiratory Syndrome-Coronavirus-2 (SARS-CoV-2) pandemic that began in 2019, this number is likely to have increased significantly (2). Moreover, acute maternal infections during pregnancy and breastfeeding are not addressed in the current ART-based strategies to reduce or eliminate MTCT. Thus, additional strategies to prevent MTCT will be required to attain an HIV-free generation. Interestingly, in the absence of ART, only 15-30% of HIV-infected mothers transmit the virus to their child with additional risk associated with breastfeeding, suggesting that there are immunologic factors that modify the risk of MTCT of

HIV-1 (3, 4). Maternal vaccination strategies seek to enhance immune factors that are partially protective against vertical HIV transmission to further reduce HIV MTCT.

To design immune-based strategies that can synergize with ART to further reduce MTCT, it is important to understand the role of circulating and placentally transferred maternal antibodies in vertical transmission. Many studies have shown that despite having a diverse array of HIV-1 variants circulating in the mother (5-10), only one or a few maternal variants get transmitted to the infant, known as the infant transmitted/founder (T/F) variant(s). This genetic bottleneck suggests that the maternal immune system selects for viruses that are transmitted to the infants. Yet, while broadly neutralizing antibodies (bnAbs) have been shown to protect against susceptible virus strains in animal studies (11-16), there is no consensus about the role of neutralizing antibodies (nAbs) in driving the genetic bottleneck of MTCT (17-19). We previously studied the role of maternal antibodies in the selection of infant T/F viruses with a cohort of 16 clade B infected, peripartum transmitting mother-infant pairs from the United States (17) and demonstrated that infant T/F viruses are more neutralization-resistant to paired maternal plasma compared to non-transmitted maternal variants. Our findings were in line with a previous study investigating autologous virus neutralization activity in 12 transmission pairs from Nairobi, Kenya (19), yet resistance to maternal plasma neutralization was not a defining feature of infant T/Fs in another study of 10 *in utero* and 9 intrapartum, clade C-infected pairs from Malawi (18). Differences in the results of these studies could be due to sample size, HIV-1 subtype, and mode of transmission. Hence, it remains unclear if maternal plasma neutralizing activity drives infant T/F selection.

Conflicting findings have also been observed in studies comparing the plasma neutralization activity of transmitting and non-transmitting mothers against circulating, non-transmitted maternal variants. Some studies have shown that autologous virus neutralizing activity is higher in non-transmitting women than transmitting women (8, 20), while more recent

studies observed no difference at all ([18](#), [21](#)). In addition to autologous virus neutralization, some studies have tested heterologous virus neutralization activity of maternal and infant plasma as a risk factor for MTCT. For example, in a study of postpartum transmission, breadth and potency of infant plasma were tested against a global panel of eight heterologous viruses ([22](#)). No correlation of heterologous virus breadth and potency of placentally acquired neutralizing antibodies with transmission was observed. In contrast, we previously reported that HIV-transmitting mothers had significantly higher heterologous virus neutralization breadth and potency than that of non-transmitting mothers against a different global panel of 11 viruses in a cohort of 63 mother-infant pairs from the Breastfeeding, Antiretrovirals, and Nutrition (BAN) study of breast milk transmission ([23](#), [24](#)). High breadth and potency of maternal plasma suggests that the development of maternal bnAb activity in plasma may be associated with risk of MTCT, a mechanism of which is yet unknown.

BnAb activity in plasma has been identified in about 10-25% of chronically HIV-infected adults and targets highly conserved epitopes on the HIV envelope, allowing them to neutralize a majority of HIV variants found globally with high potency ([25-30](#)). Additionally, most chronically infected individuals display low-to-moderate plasma neutralization breadth ([31](#)). Since MTCT occurs in the presence of maternal antibodies, it is important to understand the role of maternal bnAbs in the prevention of MTCT, as well as the impact of future bnAb-based prophylaxis and therapy approaches on MTCT. Viral evolution and escape from naturally occurring bnAbs in HIV-infected individuals has been well characterized ([32-36](#)), yet while bnAb-resistant infant T/Fs have been identified ([37-40](#)), the selection and escape of such viruses has not previously been studied in relationship to maternal bnAb activity. Importantly, we recently identified an infant T/F virus that was distinguished from non-transmitted maternal variants by resistance to V3 glycan-specific plasma bnAb responses in paired maternal plasma ([41](#)), indicating that maternal bnAb activity can drive the selection of escape variants leading to MTCT.

Taken together, broad and potent neutralization activity of maternal plasma is clearly not sufficient for protection against MTCT. Yet, with passive immunization and induction of such plasma neutralization activity as a current dominant approach to preventing HIV acquisition, it is imperative to identify whether there are specific characteristics of broad and potent neutralization responses that select for vertically transmitted variants. In this study, we characterized and mapped the bnAb specificity of maternal plasma from transmitting and non-transmitting mothers using samples selected from two HIV-infected maternal-infant cohorts prior to the era of universal ART in pregnancy (Breastfeeding, Antiretroviral, and Nutrition Study and the Center for HIV/AIDS Vaccine Immunology 009, both in Malawi) using the criteria of broad and potent neutralization activity of plasma against a global virus panel (23). We found that transmitting mothers more often have mappable single epitope-specific bnAb activity in plasma than non-transmitting mothers, suggesting that polyclonal or uncommon neutralization responses occur more frequently in non-transmitting women. Overall, these findings will help define the characteristics of protective neutralizing antibody responses against MTCT, bringing insight to bnAb-based HIV vaccine development and the elimination of pediatric HIV.

Methods

Study Design

Patient samples were selected from the Breastfeeding, Antiretroviral, and Nutrition Study (BAN) and the Center for HIV/AIDS Vaccine Immunology 009 (CHAVI009) cohorts, both enrolled in Malawi in 2004-2010 and 2007-2009, respectively. Both cohorts were infected primarily with clade C HIV, and their enrollment criteria have been previously described (24, 37, 42, 43)(ClinicalTrials.gov no. NCT00164736). BAN cohort samples were selected from the control arm and received a combination ART for a duration of 7 days after delivery (44). CHAVI009

cohort patients received one dose of the antiviral Nevirapine at the time of delivery. Transmission events were identified by HIV DNA PCR testing of the infants at birth, at 4-6 weeks, and every 3 months until weaning. The CHAVI009 study was approved by the College of Medicine Research and Ethics Committee in Malawi and by all participating institutions' institutional review boards (IRBs). The BAN Study was approved by the Malawi National Health Science Research Committee and by all participating institutions' IRBs. All women provided written informed consent for themselves as well as on behalf of their infants. Maternal and infant samples from the CHAVI009 and BAN cohorts were received as deidentified material and were deemed "not human subject research" by the Duke University School of Medicine IRB (BAN: Duke IRB Pro00030437, CHAVI009: Duke IRB Pro00016627) and Boston University School of Medicine IRB.

Neutralization Assays

Maternal plasma and monoclonal antibodies were tested against the 11 virus global panel and infant T/Fs pseudoviruses for neutralization activity using a TZM-bl neutralization assay as described previously (23). Inhibitory dilution 50% (ID₅₀s) and inhibitory concentration 50% (IC₅₀s) were measured for maternal plasma or monoclonal antibodies using 3-fold dilutions ranging from 1:40 to 1:87480 and 25 µg/mL to 0.011 µg/mL respectively. To identify HIV-1 Env epitopes targeted by the neutralizing antibodies present in the maternal plasma, we assessed neutralization against HIV Env pseudoviruses with unique mutations in established bnAb epitopes, including: CD4 binding site (TRO.11.N279A, TRO.11.G458Y, TRO.11.N276Q, X1632.D279N, X1632.G458Y, and X1632.N276Q), V2 glycan (TRO.11.N160K and BJOX2000.N160K), V3 glycan (TRO.11.N332A and BJOX2000.N332A), and MPER (TRO.11.W672A and BJOX2000.W672A). Neutralization epitope-specificity was identified as a 2 fold reduction or greater in ID₅₀ of a mutant pseudovirus compared to wild type.

Screening Maternal Plasma for Broad and Potent Neutralizing Activity

Maternal plasma samples collected both prior and closest to the first positive infant HIV test (27-66 days) from the BAN cohort were previously tested for breadth, and the plasma that neutralized TRO11 and BJOX2000 (2 viruses from the global panel) at an $ID_{50} \geq 120$ were selected from the available samples ($n = 6$) (24). To match the transmitting mother sample timepoints, 67 plasma samples collected 4-6 weeks after delivery from mothers from the CHAVI009 cohort who only received peripartum ART were screened for neutralization against 11 global panel HIV Env pseudovirus (23, 37, 42, 43). Broad neutralization activity was defined as plasma neutralization at an $ID_{50} \geq 50$ for six of the eleven viruses tested. For samples not tested at a dilution of 50 in Figure 2A, we calculated the % neutralization at a dilution of 50 using the mathematical formulation for a single antibody neutralization curve(45). The breadth and potency (BP) score was defined according to equation 1 in Ghulam-Smith et al. (24).

Pseudovirus Preparation

HIV-1 Env pseudoviruses were produced as previously described (46) in 293T cells through co-transfection using 4 μ g of envelope expression plasmid and 8 μ g of backbone plasmid pSG3 Δ Env or pQ23 Δ Env. VSV-G and SVA.MuLV viruses were prepared by co-transfection in 293T cells with backbone plasmid pNL-LucR-E and pSG3 Δ Env respectively. Virus was harvested after 48 hours post transfection, filtered and stored at -80°C at 20% FBS concentration. Pseudoviruses were tittered by infecting Tzm-bl cells at 5-fold dilutions from 1:10 to 1:97,656,250 and the dilution that achieves 50,000 relative luminescence units (RLU) during infection was used in all assays. If the pseudovirus infection did not reach 50,000 RLU, then a dilution that provided an RLU ten times higher than the uninfected cell control was chosen for assays. Maternal amplicons for pseudovirus preparation were selected using a previously used algorithm to represent viral diversity (37). 2315 infant amplicons for pseudovirus preparation were visually selected based on the unique mutations in the known bnAb epitopes (Fig. 5C).

Monoclonal antibody production

Monoclonal antibodies PGT121, PG9, VRC01, and 10E8 were produced with plasmids obtained through the NIH HIV Reagent Program, Division of AIDS, NIAID, NIH. Heavy and light chain plasmids were transfected into 293i cells using the Expifectamine kit (ThermoFisher A14524) as per manufacturer guidelines. Transfected cells were incubated for 5 days before harvesting the cell supernatant. Antibodies were purified from culture supernatants using affinity chromatography with protein A beads as previously described (47). After purification, all antibodies were then buffer exchanged into a citric acid buffer (pH 6) before use.

VRC01-blocking ELISA

VRC01-blocking activity of maternal plasma was detected by competition ELISA method as previously described (41). Briefly, 384 well plates were coated with 45 ng/mL of 1086CK160N gp120 overnight at 4°C. 10ul of plasma diluted at 1:30 were added in duplicate and incubated at RT for one hour. Plates were washed twice and 16ng of biotinylated VRC01 was added and incubated at RT for one hour. Plates were washed twice, and HRP-conjugated streptavidin was used at 1:10000 dilution and incubated at RT for one hour. Plates were washed four times and developed using 20µl of SureBlue substrate per well and incubated for 3 minutes and 30 secs. The reaction was stopped using stop solution and the plate was read using a spectrophotometer at 450 nm. Percent blocking was determined by the percent difference in OD (max OD=2.23) between uninhibited bnAb binding and the sample. 50% blocking was defined as the positivity cutoff.

Single Genome Amplification (SGA)

SGA sequences were obtained as described previously (37). In brief, RNA was extracted from breast milk and plasma samples and reverse transcribed. The obtained cDNA was used to PCR amplify full length *env* genes. PCR reactions were performed in 96 well plates at a dilution of

cDNA to yield no more than 20% PCR positive wells to fit a Poisson distribution of one template per reaction. Autologous *env* sequences were aligned using Seaview (48) and compared using LANL Gene Cutter (https://www.hiv.lanl.gov/content/sequence/GENE_CUTTER/cutter.html) and SeqPublish (<https://www.hiv.lanl.gov/content/sequence/SeqPublish/seqpublish.html>). Key broadly neutralizing antibody binding sites were found using LANL Neutralizing Antibody Contacts and Features database (https://www.hiv.lanl.gov/components/sequence/HIV/featuredb/search/env_ab_search_pub.com) and identified in the autologous virus envelope sequences using LANL HIV Sequence Locator (<https://www.hiv.lanl.gov/content/sequence/LOCATE/locate.html>) to compare them to HXB2. Total envelope glycosylation count was calculated using LANL N-Glycosite (<https://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html>) and variable region length and glycosylation were calculated using LANL Variable Region Characteristics (https://www.hiv.lanl.gov/content/sequence/VAR_REG_CHAR/index.html).

Statistical Analysis

Counts of mapped versus non-mapped bNAb specificities were compared between transmitting and non-transmitting mothers using a 2-sided Fisher exact test. Breadth and breadth-potency scores between transmitting and non-transmitting mothers were compared using 2-sided Wilcoxon tests. All tests were run on the R platform (<https://www.R-project.org>). Heatmaps were created using the Heatmap tool provided on the LANL database (https://www.hiv.lanl.gov/content/sequence/HEATMAP/heatmap_mainpage.html). Phylogenetic trees were created with MEGA-X using a Kimura 2 Parameters (K2P) model (49). Env variable loop lengths, number of glycosylation sites, and net charge were obtained using the LANL tool Variable Region Characteristics (https://www.hiv.lanl.gov/content/sequence/VAR_REG_CHAR/index.html) and compared between mother and infant for ID 2315 using 2-sided Wilcoxon test.

Results

Identifying HIV-infected transmitting and non-transmitting mothers with plasma HIV-neutralization breadth

All transmission events occurred postnatally, with the infants testing HIV DNA PCR positive after 4-6 weeks of age. BAN cohort maternal plasma samples were previously assessed for breadth (24). Of 19 transmitting mothers from this cohort, 16 neutralized 6 or more viruses from the 11 virus global panel. Of the 44 non-transmitting mothers in the BAN cohort, 24 mothers neutralized 6 or more of the 11 viruses from the global panel. From the CHAVI009 cohort, we initially screened 3 transmitting and 62 non-transmitting mothers for neutralization breadth. Of the transmitting mothers, only one (subject 1209) demonstrated neutralization breadth. Of the 62 non-transmitting mothers in the CHAVI cohort, 18 demonstrated neutralization breadth. Combining the cohorts, we screened a total of 22 transmitting and 106 non-transmitting mothers, for a total size of 128 samples (Fig. 1). Consistent with previous findings that BAN cohort transmitting mothers had higher neutralizing breadth than non-transmitting mothers ($p < 0.001$) (24), the combined cohort had more transmitting mothers (17/22, or 77%) than non-transmitting mothers (42/106, or 40%) that displayed neutralization breadth.

In order to explore the specificity of the plasma neutralizing activity in HIV-transmitting and non-transmitting women, we selected a subcohort of mothers with comparable breadth and neutralization potency. To this end, we selected plasma samples that had sufficient volume ($>140 \mu\text{l}$) and neutralized at least one virus with an $\text{ID}_{50} > 120$ to facilitate neutralization epitope-specificity mapping (Fig. 1). This ID_{50} cut off was chosen based on the ability to observe a three-fold reduction in neutralization activity against a bnAb epitope mutant virus measured at a starting plasma dilution of 1:40. Six transmitting and four non-transmitting women from the BAN

cohort and one transmitting and 17 non-transmitting women from the CHAVI009 cohort met criteria for the neutralization epitope mapping, yielding a study sample size of seven transmitting and 21 non-transmitting women (Fig 2A, Table 1). By design, given how we selected the women, the Breadth Potency Score (BP score, as defined by Ghulam-Smith et al.(24) and the number of viruses neutralized was not different ($p=0.12$ by Wilcoxon test) among the transmitting and non-transmitting women selected for inclusion in our analysis based on the selection criteria (Fig. 2B).

Neutralization epitope specificity mapping

To identify potential differences in the predominant neutralization epitope specificity in transmitting and non-transmitting women with broad and potent neutralizing plasma responses, we further investigated the specificities of broad neutralization activity in both HIV-transmitting and non-transmitting mothers. Using HIV pseudoviruses that have mutations in epitopes targeted by bnAbs, we sought to map the bnAb specificity of maternal plasma with broad neutralization activity (Fig 3). The panel included viruses with mutations to the CD4 binding site (N279A or D279N, G458Y, and N276Q), the V2 glycan (N160K), the V3 glycan (N332A), and the membrane-proximal external region (W672A) (see Methods section). We were able to map 6 of 7 (85.7%) transmitting women's plasma broad neutralizing activity, two to the N276 CD4 binding site (CD4bs), one to the N160 V2 glycan, and two to the N332 V3 glycan. One transmitting mother's broad neutralizing plasma activity was mapped to both the N276 CD4bs and the V3 glycan residue. Of the non-transmitting mothers' plasma, we were able to map 7 of 21 (33.3%) that had neutralizing activity. The plasma broad neutralizing activity of four of the non-transmitting mothers was mapped to the N276 CD4bs, while V2 glycan, and V3 glycan regions were mapped for one non-transmitting maternal plasma each. One non-transmitting mother's plasma broad neutralizing activity was also mapped to both the N276 CD4bs and V3 glycan epitopes (Fig 3C). Yet, for the majority of non-transmitting mothers ($n = 14$, 66.6%), we

were not able to map the plasma broad neutralizing activity to a bnAb epitope, indicating that their plasma neutralizing responses are either multispecific, or alternatively, target an epitope not included in the mutant virus panel. Transmitting mothers in our cohort more frequently had epitope-mappable plasma bnAb specificity that targets at least one of the 4 most common bnAb epitopes (OR=10.9, $p=0.029$, 2-sided Fisher exact test) compared to non-transmitting women (Fig. 3C). These results suggest that the plasma HIV-neutralizing breadth among transmitting versus non-transmitting mothers with broad and potent neutralizing activity may be mediated via different antibody specificities.

Maternal HIV variant neutralization sensitivity in paired plasma and breast milk

All transmission events studied in this cohort occurred postpartum. As breast milk is known to have lower neutralizing activity than plasma (37) and to be populated by locally-replicating variants (42), thus we sought to determine if bnAb resistance profiles were distinct between breast milk and plasma HIV variants in women with plasma bnAb activity. Autologous viruses from four CHAVI009 mothers, one transmitting and three non-transmitting, with plasma broad neutralizing activity were previously sequenced via single genome amplification (SGA) from plasma and breast milk (37). In transmitting mother 1209, *env* 1209bmH5 was identified as most similar to the infant 1209 virus population through phylogenetic analysis (Fig. 4) We tested these autologous Env pseudoviruses for bnAb sensitivity against monoclonal antibodies PGT121, PG9, VRC01, and 10E8 (Fig 5A). No notable differences in bnAb sensitivity of the milk and plasma viruses were found in the mothers tested. Pseudoviruses prepared using SGAs from both breast milk and plasma of mothers 1209 and 4707 were uniformly resistant to VRC01. Autologous viruses from mother 3305 were uniformly resistant to neutralization against bnAbs PGT121 and PG9. In summary, the neutralization sensitivity profiles of breast milk and plasma-isolated Envs appear to be comparable in this cohort.

VRC01-blocking activity of maternal plasma

To explore whether the bnAb resistance of circulating maternal HIV variants could be explained by bnAb activity that was unmappable in the mutant HIV Env pseudovirus assay, we measured VRC01-blocking activity in maternal plasma (Fig. 6). All HIV Env pseudoviruses from mothers 1209 and 4707 had uniform resistance to VRC01 (Fig. 5). To determine if VRC01-like antibody binding was present in plasma, we measured VRC01-blocking activity in maternal plasma using a competition ELISA, where percent blocking over 50 percent was considered positive (Fig. 6). Interestingly, some mothers (129, 1306, 0205, 3507, 2709, 4707) with unmappable plasma bnAb activity by neutralization assay had detectable levels of VRC01 blocking, while plasma of 1209, which was mapped to V2 glycan-specific neutralization activity and who postnatally-transmitted a VRC01-resistant virus, too had positive VRC01 blocking (68.6% blocking). As expected, autologous HIV Env pseudoviruses from mother 3305 were all sensitive to VRC01 and little VRC01 blocking activity was observed in plasma (14.1% blocking).

Infant T/F variant sensitivity to maternal plasma neutralizing activity and mapped bnAb specificities

We sought to determine whether viruses transmitted to infants were escape variants of maternal plasma bnAb activity. We analyzed 3 mothers who transmitted virus to their infant, had plasma activity mapped to a defined epitope, and had successful identification of infant virus *env* sequences (1844i, 2315i, and 1209i). For infant 1209i, the viral population was too diverse to identify an infant T/F virus, so the phylogenetically closest maternal breast milk virus (1209bmH5) was used to represent the infant virus population(37). Phylogenetic tree analysis revealed varying levels of genetic similarity between maternal and infant virus envelopes (Fig. 4). A T/F could not be identified for 1844 and 2315 because of too few infant Envs and extensive diversity among the isolated Envs respectively. Since 1844i and 2315i Envs were

genetically different from their maternal counterparts, we chose to focus on these infant sequences for neutralization sensitivity analysis.

We addressed whether infant viruses acquired mutations that would lead to neutralization escape from paired maternal plasma bnAbs. While subject 1209's maternal plasma bnAb activity was mapped to the V2 glycan specificity, most of the autologous viruses from this sample, including 1209bmH5, were sensitive to V2 glycan-targeting bnAb PG9 and paired maternal plasma (Fig. 5A), indicating that this transmission was not related to escape from the dominant maternal bnAb response. 1844g3i was sensitive to paired maternal plasma neutralization and MPER-targeting bnAb 10E8 (Fig. 5A) yet neutralization resistant to all other bnAbs tested, including CD4bs-targeting VRC01. Interestingly, 1844 maternal plasma was mapped to the N276 CD4 binding site. No mutations in infant virus 1844g3i *env* were identified in the N276 region, yet it was phenotypically resistant to VRC01. Both maternal and infant viruses did have an isoleucine insertion at position 460 which could affect the glycosylation of the G458 glycan, which is necessary for the binding of VRC01 and other bnAbs to the CD4bs (50-52).

Infant HIV Env pseudovirus 2315g7i was potently neutralized by paired maternal plasma, PG9, and 10E8 but was neutralization resistant to VRC01 and PGT121 (Fig. 5A). Interestingly, maternal plasma corresponding to infant 2315 was mapped to the N332 V3 glycan specificity and 2315g7i was resistant to corresponding bnAb PGT121 (Fig. 3, Fig. 5A). We explored this V3 glycan-targeting bnAb resistance of 2315g7i by creating Env pseudovirus mutants 2315g7i.N135K and 2315g7i.T334R. We looked at differences in the V1 and V2 regions of 2315 maternal and infant virus *envs*, as mutations in these regions of the envelope can affect V3-specific bnAb activity (53, 54). These regions in the infant virus *envs* vary greatly from the maternal variants (Fig. 5C). Notably, 5 of 8 infant viruses had a K135N mutation which led to the insertion of a putative glycosylation site. Reverting this mutation in 2315g7i.N135K

was hypothesized to have increased neutralization resistance to V2 glycan and possibly V3 glycan-targeting bnAbs, but the mutant pseudovirus had a similar neutralization profile to the wild-type virus (Fig. 6A). For the V3 glycan region, all infant *envs* included the N332 V3 glycan while all maternal *env* sequences contained mutation T334R which deglycosylates the N332 V3 glycan region (Fig. 5C). We hypothesized that inducing mutation T334R in pseudovirus 2315g7i would increase the Env's neutralization resistance to a larger number of V3 glycan-targeting bnAbs. 2315g7i.T334R still retained neutralization resistance to PGT121 and PGT128 and acquired neutralization resistance to DH427. (Fig. 5B). However, this mutation also slightly increased neutralization sensitivity to 10-1074 compared to the wild-type pseudovirus (1.96 fold).

Finally, we compared the length and N-linked glycosylation in the V1V2 and V5 regions as well as the total glycosylation in maternal and corresponding infant envelopes (Fig. 7), as lengthening and glycosylation in these regions can affect bnAb neutralization sensitivity (53-55). Interestingly, infant 2315i *env* sequences had significantly higher glycosylation sites in the hypervariable V1V2 regions compared to maternal *env* sequences ($p=0.00032$ by 2-sided Wilcoxon test) and significantly longer V1V2 hypervariable loops compared to maternal sequences ($p=0.0003$ by 2-sided Wilcoxon test). Compared to maternal Envs, all 8 infant sequences have insertions 3-11 amino acid long at HXB2 position 188 (Fig. 5C). While previous studies have found that T/F viruses tend to have shorter V1V2 lengths as it favors transmission fitness(56), longer V1 V2 loops have been associated with resistance to V3 and CD4bs bnAbs (54, 55), consistent with the fact that these viruses are all neutralization resistant to PGT121 and VRC01 (Fig 5A). This suggests that different transmission fitness pressures could be working on infant T/F viruses in the context of MTCT vertical transmissions. Finally, the net charge of 2315i V1V2 regions were also significantly different than maternal variants. 2315i V1V2 regions tended to be negatively charged while maternal variants were predominantly positively charged

($p=0.00044$). It has been previously shown that a net positive charge in the V2 strand is correlated with sensitivity to PG9 and PG16 (57), however 2315g7i is phenotypically sensitive to PG9 (Fig. 5A). In summary, our study is suggestive, yet not definitive on whether infant *env* mutations are frequently driven by maternal bnAb selection pressure.

Discussion

To achieve an HIV-free generation, supplemental strategies in conjunction with ART will be required to prevent MTCT. In this study, we investigated vertical HIV transmission in pregnant women with plasma neutralizing breadth and potency to understand the characteristics of epitope-specific responses that may be protective against virus transmission and/or contribute to selection of transmitted variants during postpartum transmission. Importantly, due to the limited success of the single bnAb prevention approach studied in the Antibody Mediated Prophylaxis (AMP) trial in adults (58), it is clear that understanding transmitted virus escape of bnAb activity is critical to advancing bnAb-based prophylaxis and therapy. The gap in knowledge of protective bnAb combinations for mothers and infants urgently needs addressing, as it will define vaccine strategies and antibody therapies that will be effective in preventing MTCT.

We aimed to define protective bnAb activity in postpartum transmitting and non-transmitting mothers from the BAN and CHAVI cohorts by mapping their epitope-specificity using a panel of mutant Env pseudoviruses. We found that more transmitting women had bnAb responses that mapped to 1 or 2 known bnAb specificities compared to non-transmitting women, where the majority were unable to be mapped to common bnAb epitopes, indicating multiple specificities of the neutralization response, responses against undefined epitopes, or responses that were too low to be detected in the pseudovirus neutralization epitope mapping

assay. This pattern suggests that multiple bnAb specificities are more common in non-transmitting than transmitting women with broad neutralizing activity and may be required to prevent MTCT. Alternatively, this data could also suggest that a currently undefined and infrequent specificity of bnAbs may play a role in prevention of MTCT.

We previously demonstrated that autologous HIV viruses isolated from transmitting mothers and their infected infants were enriched with genetic motifs that were associated with resistance to bnAbs (59), suggesting that acquired plasma neutralization-resistance is a common feature of vertically transmitted variants. We also demonstrated that an in utero-transmitted virus from a mother with plasma V3 glycan-specific bnAb activity was selected for its ability to escape the mother's dominant bnAb specificity (41). In this study we described a mother-infant transmission pair where the infant envs exhibited V1V2 variable loop characteristics associated with V3 and/or CD4bs bnAb resistance: higher number of glycosylation sites compared to maternal envs, longer V1V2 insertions, and negative net charge. Thus, it is possible that mothers with a single dominant plasma bnAb specificity could drive the selection of resistant viral variants that are in turn, more likely to be transmitted to their infants. An *in vitro* study using HIV strain BG505 showed that plasma neutralization breadth is moderately correlated to ease of escape via a single mutation (60). Our work demonstrating the potential for lower vertical transmission risk in women with broad, unmappable neutralization activity suggests that transmission may be averted by maternal bnAbs that target uncommon epitopes with a lower capacity for antibody escape via single mutations or with polyclonal bnAb activity. Viruses circulating in women with polyclonal and broad neutralization activity may be less likely to escape bnAbs compared to maternal plasma with a single, dominant specificity, impeding transmission by preventing escape variants.

We also tested breast milk and plasma autologous viruses from four mothers for neutralization resistance against bnAbs targeting common bnAb epitopes. We observed no

differences in neutralization sensitivities between breast milk and plasma viruses within each mother. This is consistent with a study of 13 subjects from Zambia, which did not find a restriction in viral genotype in breast milk (61) and a previous CHAVI009 study which observed limited compartmentalization of breast milk viruses (62). In this study, several mothers had plasma virus populations with uniform neutralization resistance to some bnAbs. Mothers 1209 and 4707 had viruses that were all neutralization resistant to CD4bs-specific bnAb VRC01. Interestingly, mothers with VRC01-resistant plasma viruses had detectable plasma VRC01-blocking activity not identified in the plasma bnAb mapping assay. This could be because binding assays are generally more sensitive than neutralization assays, but as a result we could not confirm these antibodies' function or contribution to the selection of VRC01-resistant viruses.

We were also able to generate infant HIV Env pseudoviruses for transmitting mother-infant pairs with epitope-mapped bnAb responses (1209, 2315, 1844). We hypothesized that infant HIV Env variants would be resistant to bnAbs targeting the same epitopes as found in the paired maternal plasma. In fact, infant HIV Env variants generated from mother-infant pairs 2315 and 1844 were resistant to the maternal mapped specificities, V3 glycan and CD4bs respectively. Despite this, the paired maternal plasma was able to neutralize both infant viruses potentially, likely due to other neutralizing antibodies targeting unique specificities present in the plasma. These results suggest that bnAb selection pressure could be driving the transmission of bnAb-resistant viruses, but neutralization resistance may not be required for MTCT.

Our study is not without limitations. Our cohort is small due to the sample availability and neutralization activity selection criteria, as bnAb activity is detected in a minority of HIV-infected adults. Additionally, we isolated only a few maternal and infant *env* variants from the transmitting mother-infant pairs from timepoints late after transmission due to lack of sample availability. Thus, we were not always able to determine if infant-transmitted variants were

resistant to paired maternal antibody specificities, as the sequenced virus populations could experience reversions when there is less maternal antibody selection pressure. Another limitation of this study is that we only focused on postpartum transmissions, so observations in this study may not be extrapolated to other modes of MTCT. Future studies should expand our understanding of protective bnAbs by investigating all modes of MTCT, increasing sample size, increasing autologous viruses isolated, and characterizing maternal monoclonal antibodies. Despite our study limitations, this is to our knowledge the largest study identifying differences in bnAb specificity of maternal plasma between transmitting and non-transmitting women.

Overall, our results demonstrate that transmitting women with plasma broad neutralizing activity often have antibody activity directed against dominant bnAb epitopes, compared to non-transmitting women whose broad neutralizing activity was unable to be mapped to known specificities. Thus, possessing only a single, common bnAb specificity may be a risk factor for vertical HIV transmission. These results have important implications regarding the use of passive bnAb administration or bnAb-targeting vaccines in HIV-infected pregnant women, who may require the administration or induction of two or more bnAb specificities to prevent maternal virus variants from acquiring antibody escape mutations that would allow for vertical transmission in the setting of pre-existing antibodies. This work underscores supplemental strategies that administer bnAbs to mothers and infants to prevent MTCT must be developed as combinations to effectively contain vertical transmission of HIV and make further gains in the elimination of pediatric HIV.

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Conflict of Interest

Sallie Permar provides individual consulting services to Moderna, Merck, Dynavax, Pfizer, and HOOKIPA Biotech GmbH. Merck Vaccines and Moderna have provided grants for her Institutional sponsored programs.

Figure 1. Diagram of the screening criteria used to identify mothers with bnAb activity.

Transmitting and non-transmitting mothers were screened for plasma neutralization breadth and potency to determine if they had plasma broad neutralizing activity. Mothers with at least 140 μ l of available plasma and ID₅₀ >120 against at least 1 virus met criteria for this study.

Figure 2. Breadth-potency comparison of transmitting and non-transmitting HIV-infected women with plasma neutralization breadth.

A) Maternal plasma was tested against an 11-virus global panel. The % neutralization at a dilution of 1:50 is displayed in the heat map. Samples not tested at an 1:50 dilution have their % neutralization calculated for said dilution using the mathematical formulation for a single antibody neutralization curve. Colors for % neutralization are described on the color key/histogram. B) Plot of the % of viruses neutralized (x-axis) vs. breadth/potency score (y-axis) for transmitting (red) and non-transmitting (blue)

women. The breadth/potency score was not significantly different in between transmitting and non-transmitting women in this cohort.

Figure 3. Plasma bnAb epitope-specificity was more often identified in HIV-infected

transmitting women compared to non-transmitting women. A) Mothers with plasma broad neutralizing activity were tested for bnAb specificities using mutant envelope (Env) pseudoviruses (pseudovirus). Activity was measured by fold change compared to the wild type Env pseudovirus. Colors for fold decrease are described on the color key/histogram. A >2 fold decrease in neutralization compared to the wild type pseudovirus was our positivity threshold. B) ID₅₀s for wild type and mutant pseudoviruses are shown for women with mapped specificities. C) Mapping data is summarized as a pie chart. Significantly more transmitting women were able to be mapped to a bnAb epitope (p=0.029, 2-sided Fisher exact test).

Figure 4. Phylogenetic tree analysis of env SGA from transmitting mothers and their

infants. Phylogenetic trees were prepared using the Kimura parameter 2 method. The 1209 tree is rooted at maternal milk sequence 1209bmH5, the env most closely related to the infant virus population. Maternal SGA timepoints are indicated as weeks postpartum while infant SGA timepoints are indicated as weeks post infection.

Figure 5. BnAb neutralization sensitivity of maternal and infant HIV Env pseudoviruses

isolated from breast milk and plasma. (A) Neutralization sensitivity to autologous maternal plasma, PGT121 V3 glycan-specific IgG bnAb, PG9 V2 glycan-specific IgG bnAb, VRC01 CD4bs-specific IgG bnAb, and 10E8 MPER-specific IgG bnAb. Neutralization was measured using ID₅₀ for plasma and IC₅₀ for bnAbs as described in the color keys/histograms. Blood plasma isolated variants are denoted in normal font and Breast milk isolated variants are denoted with “BM” in the name and are in bold. (B) Neutralization sensitivity of 2315g7i autologous pseudovirus and mutants to V3 glycan-specific IgG bnAbs 10-1074, DH427, PGT128, and PGT121. (C) Transmitting mother-infant pair 2315 isolated HIV env sequences

had mutations in the V1, V2, and V3 glycan regions that could affect V3 glycan-specific binding. Infant sequences end in “i” and maternal sequences end in “m”. The mutations observed were K135N or T, V2 loop extensions at 188, and T334R mutations, respectively as denoted with red arrows and text.

Figure 6. VRC01 bnAb-blocking activity of plasma of mothers with broad neutralizing activity. Percent VRC01 blocking was found by calculating the percent change in OD binding compared to uninhibited VRC01 binding to 1086Cgp120K160N. The dotted lines indicate the positivity cutoff of 50% blocking.

Figure 7. V1V2 variable characteristics comparing pair 2351 maternal and infant Envs. Frequency plots of the V1V2 variable region lengths (top), number of glycosylation sites (middle), and net charge (bottom) for 2351 maternal sequences (left) compared to 2351 infant sequences. All p-values were obtained using a 2-sided Wilcoxon test. Red bars indicate the medians of each distribution.

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Figure 5 **A**

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Figure 7

Table 1

Table 1. Maternal clinical characteristics, timepoints of infant HIV diagnosis, and timepoints of maternal plasma samples of Malawian HIV-infected transmitting and non-transmitting women.

Maternal ID	Days PP Sample Collection	Maternal CD4 ⁺ cells/mm ³	Maternal log ₁₀ plasma VL	Days PP Infant 1st HIV ⁺	# Viruses Neutralized Over 50%	Maternal ID	Days PP Sample Collection	Maternal CD4 ⁺ cells/mm ³	Maternal log ₁₀ plasma VL	# Viruses Neutralized Over 50%
129	174	309	4.39	240	10	1306	14	360	4.43	11
878	13	248	5.02	56	7	1666	13	258	4.91	10
1402	14	293	5.64	56	9	1782	171	293	4.68	11
1785	168	336	4.29	196	10	399	84	396	4.39	6
1844	15	426	4.59	42	11	704	86	293	3.99	11
2315	1	312	4.78	43	11	205	32	156	2.6	11
1209	25	94	5.24	200	7	3305	118	284	5.43	11
						6905	28	123	4.45	11
						5309	33	434	NA	11
						3507	84	385	3.9	9
						3706	87	571	3.73	11
						2709	29	166	2.6	11
						9701	31	153	4.8	8
						301	28	406	4.88	11
						4707	32	247	4.9	7
						1804	48	833	2.68	8
						9606	31	558	3.71	6
						6302	33	115	NA	8
						5506	25	420	NA	6
						8908	31	178	3.97	6
						0608	60	275	2.6	6

Red: Transmitting

Black: Nontransmitting

PP: Postpartum

NA: Not Available