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Neutralizing Antibody Escape during HIV-1 Mother-to-Child Transmission Involves Conformational Masking of Distal Epitopes in Envelope

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HIV-1 variants transmitted to infants are often resistant to maternal neutralizing antibodies (NAbs), suggesting that they have escaped maternal NAb pressure. To define the molecular basis of NAb escape that contributes to selection of transmitted variants, we analyzed 5 viruses from 2 mother-to-child transmission pairs, in which the infant virus, but not the maternal virus, was resistant to neutralization by maternal plasma near transmission. We generated chimeric viruses between maternal and infant envelope clones obtained near transmission and examined neutralization by maternal plasma. The molecular determinants of NAb escape were distinct, even when comparing two maternal variants to the transmitted infant virus within one pair, in which insertions in V4 of gp120 and substitutions in HR2 of gp41 conferred neutralization resistance. In another pair, deletions and substitutions in V1 to V3 conferred resistance, but neither V1/V2 nor V3 alone was sufficient. Although the sequence determinants of escape were distinct, all of them involved modifications of potential N-linked glycosylation sites. None of the regions that mediated escape were major linear targets of maternal NAbs because corresponding peptides failed to compete for neutralization. Instead, these regions disrupted multiple distal epitopes targeted by HIV-1-specific monoclonal antibodies, suggesting that escape from maternal NAbs occurred through conformational masking of distal epitopes. This strategy likely allows HIV-1 to utilize relatively limited changes in the envelope to preserve the ability to infect a new host while simultaneously evading multiple NAb specificities present in maternal plasma.

Neutralizing antibodies (NAbs) target the HIV-1 envelope (Env) to prevent entry into host cells. Passive-immunization studies in nonhuman primate models have provided proof of concept for the ability of preexisting NAbs to protect against infection by HIV-1 (5, 7, 15, 21, 22, 35, 36, 47). However, the majority of these studies represent the ideal setting to detect protection because the host is typically challenged with a single virus that is effectively neutralized by the passively transferred NAbs. An enormous challenge in preventing infection in HIV-1-exposed populations is the requirement to elicit cross-reactive NAbs, which must recognize diverse circulating HIV-1 strains. Mother-to-child transmission (MTCT) of HIV-1 provides a unique setting in which to study the role of NAbs in blocking transmission of a quasispecies of HIV-1 in a natural setting, as well as escape pathways that lead to failure in protection. This setting is relevant because the index case (the mother) is known, allowing the analysis of the ability of her antibodies to impact transmission. Additionally, the timing of infection of the infant can be accurately estimated when there is regular sample collection, allowing the detailed study of variants that are present close to the time of transmission.

MTCT studies were the first to illustrate the concept of an HIV-1 transmission bottleneck (76); despite a heterogeneous population in the chronically infected mother, only one variant is typically transmitted to the infant (1, 29, 58, 63, 69, 77, 82). These studies suggest that variants with certain properties may be selected during transmission, and similar findings have been obtained in cases of heterosexual transmission (59). In support of this, variants that are transmitted from mother to child have been found to possess fewer potential N-linked glycosylation sites than variants found in the index case in some studies (58, 77). Vertically transmitted viruses also have been reported to have enhanced replication kinetics (27) and fitness (28) compared to nontransmitted viruses.

In addition to viral factors, host immune responses could determine which variants are transmitted in the context of MTCT. Indeed, some studies have shown that mothers who transmitted to their infants had lower titers of NAb against autologous viruses than did nontransmitting mothers (13, 27, 30, 62), although not all studies have shown this association (18, 20, 23). Some of the differences in these findings could reflect inconsistencies in sampling viruses and antibodies near the window of transmission. Sampling within this period is critical because of the dynamic nature of the antibody response and the resulting viral evolution in response to antibody pressure (10).

Although there have been inconsistent findings regarding the association between maternal NAbs and infant infection risk, we and others have shown that variants transmitted to infants were less sensitive to neutralization by maternal plasma than matched variants found in the infecting mother (13, 77, 83). This has not been observed in all studies (24, 58, 67), and it is unclear if these different findings represent methodological differences of the type

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noted above or immunological differences that are specific to different populations or routes of MTCT. In our study of 12 breastfeeding infants infected postpartum, regular infant testing and longitudinal sample collection allowed for the analysis of maternal and infant viruses very close to the time of transmission (77). The finding of this previous study, that vertically transmitted viruses tend to be less sensitive to maternal NAbs, suggests that maternal antibodies may limit the transmission of neutralization-sensitive variants and select for transmission of variants that have escaped maternal NAb pressure.

Virtually all patients develop NAbs capable of neutralizing their own virus within 2 to 20 weeks of infection, and these NAb responses can ultimately reach high titers, exerting selective pressure on Env and resulting in neutralization escape (2, 17, 31, 52, 72). NAb escape within an infected individual has been shown to involve multiple pathways, including an evolving "glycan shield" (72), insertions and deletions, and amino acid substitutions in Env (41, 56, 65). NAb escape during intrapatient evolution of the virus has also been shown to involve different domains of Env, including V1/V2, C3 to V4, and V5 of the gp120 surface subunit, as well as the ectodomain of the gp41 transmembrane subunit (41, 56, 65). It is not known whether these similar domains and pathways are involved during NAb escape in the context of transmission, in which there are potentially distinct selective pressures for viruses with unique properties that allow them to establish infection in a new host.

Studies of virus escape within an infected person have focused primarily on the first years of infection, when NAb responses tend to be type specific and directed to variable domains (17, 31, 39). NAb responses often broaden over time (38, 40, 60) and recognize new epitopes (41, 56), and these factors could influence the likely escape pathways. To our knowledge, there is currently no information on the mechanisms of escape from NAbs in the context of transmission during chronic infection, which represents a scenario in which NAbs fail to protect against infection. Thus, understanding NAb escape pathways during MTCT will provide insight into how HIV-1 successfully evades host NAb responses that play a role in limiting transmission.

In this study, we have identified the molecular determinants of NAb escape that contribute to selection for transmitted variants during MTCT in 2 mother-infant transmission pairs. Our results highlight the complexity of conformational interactions among different regions of the envelope and suggest that HIV-1 may need to simultaneously mask multiple epitopes to evade NAb responses in maternal plasma during HIV-1 MTCT.

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ID	Subject	Subtype	Time of first HIV+ result[a]	*env* cloning time[a]	Number of clones	Maternal plasma time[a]
S208	Mother	A	N/A	Week 6	5	Week 14
	Baby	A	Month 6	Month 6	1	
F535	Mother	A/D	N/A	Week 0	7	Week 0
	Baby	A/D	Week 6	Week 6	3	

B

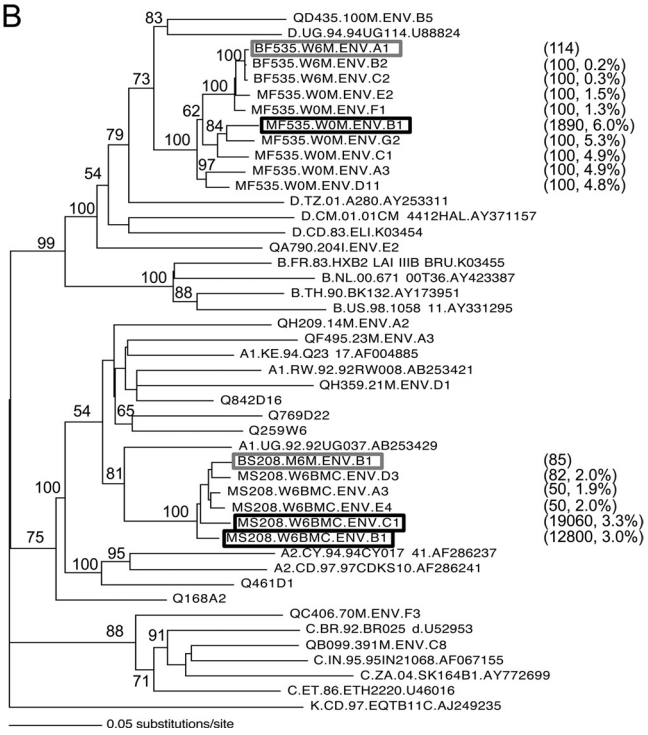

FIG 1 (A) Summary of number and timing of isolation of envelope clones for mother-infant pairs. [a]Time since delivery. N/A, not applicable. (B) Neighbor-joining tree based on pairwise distance of full-length envelope sequences from S208 and F535 maternal (sequence names begin with "M") and infant (sequence names begin with "B") variants. Bootstrap values are indicated to the left of nodes. Maternal and infant variants analyzed for detailed epitope mapping are highlighted in black and gray boxes, respectively. IC_{50}s are indicated in parentheses to the right of sequence names. Pairwise distances in the envelopes of all maternal and baby variants compared to baby variants from the same mother-infant pair analyzed in this study are indicated next to the IC_{50}s.

MATERIALS AND METHODS

Amplification and cloning of HIV-1 *env* genes. HIV-1 *env* clones were obtained from 2 mother-infant pairs, as previously described (50, 77). In Fig. 1, BS208m6bmc.B1 corresponds to S208 B^{res}, MS208w6bmc.B1 (GenBank accession no. DQ187009) to S208 M^{sens1}, MS208w6bmc.C1 (GenBank accession no. DQ187014) to S208 M^{sens2}, BF535.w0m.A1 (GenBank accession no. DQ208431) to B^{res}, and MF535.w0m.B1 (GenBank accession no. DQ208425) to F535 M^{sens}. Maternal and infant envelope variants shown in Fig. 1 were representative of the diversity of the virus population found in mothers and infants, as determined by phylogenetic analysis (50). Mother-infant chimeric *env* genes shown in Fig. 3 were created by overlap PCR on 10 ng starting template using the TaqPlus Precision PCR system (Stratagene, La Jolla, CA). Overlapping PCR fragments were digested with DpnI for 1 h at 37°C and then mixed (0.5 μl each) for amplification of full-length products using primers that bind to *vpr* and *nef*. Cycling parameters were 94°C for 4 min; 15 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 4 min; and 1 cycle of 68°C for 10 min. Full-length *env* PCR products were cloned into either pCDNA3.1/V5-His-TOPO or pCI-Neo (Invitrogen). Primers used for amplification are available upon request. Full-length envelope chimeras were verified by sequencing the entire region amplified (BigDye; Applied Biosystems) to ensure that no additional mutations were present.

Phylogenetic tree analysis. Full-length maternal and infant *env* sequences were aligned and manually edited using MacClade version 4.01 to remove regions that could not be unambiguously aligned. A neighbor-joining tree based on pairwise distance was constructed using the general time reversible model in PAUP* 4.0b10 (D. L. Swofford, Sinauer Associates, Inc., Sunderland, MA). A subtype K unrelated sequence was used as an outgroup. Reference sequences from the Los Alamos National Laboratory HIV database (http://www.hiv.lanl.gov) as well as unrelated sequences from different clades were used to define viral subtype. The reliability of branching orders was assessed by bootstrap analysis with 100 replicates.

Pseudovirus production. To generate pseudoviruses, plasmids containing envelope chimeras were cotransfected with an *env*-deficient sub-

type A proviral plasmid (Q23Δenv [33]) at a 1:2 mass ratio into 2×10^6 293T cells plated in a T-75 flask 24 h prior to transfection. For each transfection, 4 μg total DNA was mixed with 12 μl Fugene6 (Roche). To screen envelope chimeras for biological function, transfection supernatant from 48 h posttransfection was sterile filtered through a 0.2-μm filter and used to infect TZM-bl cells in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and L-glutamine in the presence of DEAE-dextran (10 μg/ml). Viral titer was determined by visually counting blue cells at 48 h postinfection after staining fixed cells for β-galactosidase activity (77).

Neutralization assay. Approximately 500 infectious pseudovirus particles as determined by infecting TZM-bl cells were diluted to a volume of 25 μl and were incubated with an equal volume of serial dilutions of heat-inactivated maternal plasma, monoclonal antibodies (MAbs), or soluble CD4 (sCD4) in duplicate at 37°C for 60 min. TZM-bl cells (1×10^4 in 100 μl DMEM) were then added to each well. At 48 h postinfection, β-galactosidase levels were measured using the Galacto-Lite system (Applied Biosystems, Foster City, CA). Percent neutralization was calculated as the percent reduction in β-galactosidase activity of pseudovirus incubated with a given dilution of plasma or MAb compared to the same virus incubated with only growth medium. The reciprocal dilution of plasma or concentration of MAbs that resulted in 50% inhibition of virus infection (IC_{50}) was determined from a dose-response curve after log transformation of plasma dilution or monoclonal antibody/sCD4 concentration, as described previously (77). Neutralization profiles of all pseudoviruses with chimeric envelopes were assessed in at least 2 independent experiments. IC_{50}s presented represent the averages of these experiments.

Monoclonal antibodies used were b12, 2F5, 4E10 (Polymun), PG9, PGT121, PGT128, PGT145 (kindly provided by the IAVI Neutralizing Antibody Consortium), and VRC01 (kindly provided by X. Wu and J. Mascola, NIH Vaccine Research Center [VRC]). MAbs b12, 2F5, 4E10, and sCD4 (Invitrogen) were used at a starting concentration of 25 μg/ml, while the remaining MAbs were used at a starting concentration of 1 μg/ml. Inhibition by TAK-779 (catalog no. 4983; NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) was assessed by adding serial dilutions of the inihibitor at a starting concentration of 1 μM to TZM-bl cells for 1 h at 37°C prior to the addition of pseudovirus.

For peptide competition neutralization experiments, 25 μl of peptides (GenScript) was added at a final concentration of 10 μg/ml to an equal volume of serially diluted plasma and incubated at 37°C for 60 min before the addition of pseudovirus, as described by Mikell et al. (38). The following peptides were tested: S208 M^{sens1} V4 (FSSTQESSDPITLP), S208 M^{sens2} HR2 (EISKYSDTIYNLLEDTQNQ), F535 M^{sens} V1 (V1_1 [VTLN CTEASINNATV], V1_2 [NNATVNGTSDQNVTV], and V1_3 [QNVTV TTTSMEMK] at 1:1:1 in combination, or tested separately), F535 M^{sens} V2 (V2_1 [SFNMTTELGDKKKQV], V2_2 [KKKQVQALFYKLDVV], and V2_3 [KLDVVPIDNSTNTTS] at 1:1:1 in combination, or tested separately), F535 M^{sens} V3 (QSIHMGPGRAFFTAD), and 2F5 (EQDLLALD KWANLWN). Competition of plasma antibodies by peptides was determined by calculating the area under the curve (AUC) in the presence or absence of peptide using Prism 5.0 (GraphPad, San Francisco, CA). The percent contribution of a given peptide to plasma neutralizing activity was calculated as 100% × (AUC without peptide − AUC with peptide)/(AUC without peptide).

Protein competition neutralization experiments were performed as described by Wu et al. (78), with slight modifications. Briefly, a final concentration of 25 μg/ml of wild-type resurfaced stabilized core (RSC3) HIV-1 envelope core recombinant protein, or the variant containing a CD4 binding site knockout mutation (RSC3Δ371I), was added to serial dilutions of maternal plasma and incubated at 37°C for 30 min before the addition of pseudovirus. The percent contribution of antibodies against the CD4 binding site to overall plasma neutralizing activity was calculated as 100% × (AUC without protein − AUC with protein)/(AUC without protein). RSC3 (catalog no. 12042) and RSC3Δ371I (catalog no. 12043) were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Zhi-Yong Yang, Peter Kwong, and Gary Nabel.

Western blotting of pseudoviral envelopes. Western blotting was performed on cell-free virus supernatants as described previously (34), using rabbit polyclonal antisera to HIV-1 envelope (14) and mouse anti-p24 as primary antibodies (catalog no. 4121; NIH AIDS Research and Reference Reagent Program [AARP]) and 700-DX-conjugated goat-anti-rabbit IgG and 800-DX-conjugated goat-anti-mouse IgG (Rockland Immunochemicals) as secondary antibodies. Protein bands were visualized and quantified using the Odyssey infrared imaging system (LI-COR Biosciences). Purified recombinant subtype C (BL035.W6M.ENV.C1; Immune Technology) and subtype A (Q461.e2 TAIV gp140, kindly provided by L. Stamatatos, Seattle Biomed) Env proteins were used as gp120 and gp140 positive controls, respectively.

RESULTS

Selection of mother-infant variants for epitope mapping. In a previous analysis of 12 mother-infant transmission pairs from the Nairobi Breastfeeding Trial (44), we showed that variants transmitted to infants were overall less sensitive to neutralization by maternal plasma than were variants found in the mothers near the time of transmission (77). Envelope clones were obtained from infants at the first HIV-1 DNA-positive time point and from mothers at a time point just prior to infant diagnosis. To identify the molecular basis of NAb escape of infant variants, we chose to focus on 2 pairs, S208 and F535, with the greatest difference in neutralization sensitivities of maternal and infant variants against maternal plasma (~10 to >100-fold [77]). Infants from both pairs were HIV-1 negative at birth and breast-fed. In the case of S208, the infant tested negative at 3 months postdelivery but positive at the next time point tested (6 months postdelivery), strongly suggesting transmission via breast milk. The infant from pair F535 was positive at 6 weeks postdelivery and was thus likely infected either via breast-feeding or during delivery. Envelope clones were tested against maternal plasma available prior to the first HIV-1-positive time point of the infant, which was the closest time to transmission, as summarized in Fig. 1A. Both S208 and F535 maternal plasma obtained near transmission displayed NAb breadth, neutralizing 6/6 heterologous viruses of subtypes A, C, and D with similar potencies (average IC_{50}s of 470 and 493 for S208 and F535, respectively [data not shown]).

Figure 1B shows a neighbor-joining tree based on full-length *env* sequences of maternal and infant variants, and a summary of their IC_{50}s against maternal plasma. As previously described, S208 and F535 were infected with subtypes A and A/D, respectively (77). For S208, 2 of the 5 maternal variants isolated were over 100-fold more sensitive to neutralization by maternal plasma than was the baby variant. These 2 most sensitive maternal variants (S208 M^{sens1} and S208 M^{sens2}) and the resistant baby variant (S208 B^{res}) were analyzed to identify determinants of NAb escape in S208 B^{res}. The pairwise distances in *env* between B^{res} and M^{sens1} and between B^{res} and M^{sens2} were 3% and 3.3%, respectively. For F535, only 1 out of 7 maternal variants isolated was more than 10-fold more sensitive than the baby variants. To identify regions in the F535 baby variant that conferred escape from maternal plasma, we analyzed the resistant baby variant, F535 B^{res}, and the most sensitive maternal variant, F535 M^{sens} (6% pairwise distance in *env*).

Maternal and infant variants have variable neutralization sensitivity to recently isolated broad monoclonal antibodies and entry inhibitors. To determine whether there were inherent

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		b12	2F5	4E10	2G12	VRC01	PG9	PGT 121	PGT 128	PGT 145	sCD4	TAK-779
S208	Bres	8	2	>25	>25	0.2	0.1	>1	>1	0.03	>25	0.1
	Msens1	>25	2	>25	>25	0.2	0.2	>1	>1	0.5	>25	0.005
	Msens2	0.8	0.3	0.3	>25	0.2	0.1	>1	>1	0.2	0.16	0.01
F535	Bres	>25	>25	>25	>25	0.1	0.03	>1	0.001	>1	>25	0.001
	Msens	0.7	0.7	1	>25	>1	>1	>1	>1	>1	0.14	0.06

Resistant (IC50 >25 μg/ml)
IC50 1-25 μg/ml
IC50 <1 μg/ml
Resistant (IC50 > 1μg/ml)
IC50 0.2-1 μg/ml
IC50 0.03-0.1 μg/ml
IC50 <0.03 μg/ml
IC50 0.02-0.1 μM
IC50 0.006-0.01 μM
IC50 <0.006 μM

B

		2F5					4E10								PG9			PGT128	
															PGT145				PGT121
		E	L	**D**	**K**	**W**	A	N	**W**	**F**	D	**I**	T	K	W	L	**W**	N156	**N160**	K/R168*	N301	**N332**
S208	Bres	A	.	.	.	.	.	.	.	.	G	.	S	N	.	.	.		+	+	+	+	+
	Msens1	A	.	.	.	.	.	.	.	.	G	.	S	N	.	.	.		+	+	+	+	+
	Msens2	A	.	.	.	.	.	.	.	.	G	.	S	N	.	.	.		+	+	+	+	+
F535	Bres	Q	.	.	Q	.	.	.	.	.	S	.	.		.	.	.		+	+	+	+	Shift
	Msens	Q	.	.	.	.	.	.	.	.	S	.	.	N	.	.	.		+	+	+	+	Shift

FIG 2 Summary of neutralization profiles of S208 and F535 variants against a panel of MAbs and entry inhibitors. (A) IC_{50}s for each MAb or entry inhibitor against each virus are shown, with darker shading indicating more potent neutralization, as indicated in the key. MAbs b12, 2F5, and 4E10 and soluble CD4 (sCD4) were tested at a starting concentration of 25 μg/ml. MAbs VRC01, PG9, PGT121, PGT128, and PGT145 were tested at a starting concentration of 1 μg/ml. TAK-779 was tested at a starting concentration of 1 μM. Gray boxes indicate that the virus tested did not reach 50% neutralization at the highest concentration of MAb or inhibitor tested. (B) Comparison of amino acid residues that are known targets of the indicated antibodies for S208 and F535 variants. Boldface characters indicate minimum residues required for neutralization. Symbols: +, present; dot, conserved amino acid; *, presence of K168 in variant. "Shift" indicates a shift from position 332 to 334.

differences in neutralization sensitivity among mother-infant viruses, we tested neutralization of S208 and F535 variants against MAbs of different specificities. These included the extensively studied first-generation HIV-1 broad MAbs, including b12, which targets the CD4 binding site (54), and 2F5 and 4E10, which target linear regions in the membrane-proximal external region (MPER) of the gp41 ectodomain (43, 64, 84, 85). S208 and F535 baby variants were resistant or only moderately sensitive to neutralization by b12, 2F5, and 4E10, with IC_{50}s ranging from 2 to >25 μg/ml (Fig. 2A). Maternal variants were overall more sensitive to neutralization by these MAbs, with IC_{50}s of less than 2 μg/ml for S208 M^{sens2} and F535 M^{sens}. However, sensitivity to these MAbs was not universal among all maternal variants, because S208 M^{sens1} was not neutralized by 4E10 or b12 and overall had a neutralization profile similar to that of S208 B^{res} against these MAbs. MAb 2G12, which targets a cluster of glycans (61, 68), failed to neutralize all maternal and infant variants (data not shown) (77).

We also investigated whether these variants were recognized by more recently isolated broad and potent MAbs. These include VRC01, which targets the initial contact site for CD4 (78), and MAbs PG9, PGT121, PGT128, and PGT145, all of which target glycan-dependent epitopes (37, 48, 70, 71). We found that these MAbs displayed various potencies and breadth against S208 and F535 maternal and infant variants (Fig. 2A). VRC01, PG9, and PGT145 were able to potently neutralize both S208 maternal and infant variants (IC_{50}s, 0.03 to 0.5 μg/ml). In contrast, neither PGT121 nor PGT128 neutralized these variants. Thus, for S208, maternal and infant variants were similar in their neutralization profiles against these recently identified MAbs. For F535, both maternal and infant variants were not neutralized by PGT121 and PGT145 at the highest MAb concentration tested (1 μg/ml). Interestingly, B^{res}, but not M^{sens}, was sensitive to VRC01, PG9, and PGT128 (IC_{50}s, 0.001 to 0.1 μg/ml). The neutralization profiles of F535 variants against VRC01, PG9, and PGT128 were opposite those observed against maternal plasma and b12, 2F5, and 4E10. These results demonstrate that variants transmitted to infants were not inherently resistant to neutralization.

To determine whether transmitted variants show differences in their interactions with CD4 or CCR5 compared to maternal variants, we tested inhibition by sCD4 and the CCR5 antagonist TAK-779 (4) (Fig. 2A). Both S208 and F535 infant variants were resistant to sCD4, but this phenotype was not exclusive to transmitted variants, because S208 M^{sens1} was also resistant to sCD4. All maternal and infant variants were sensitive to TAK-779. For S208, B^{res} was ~20-fold less sensitive to TAK-779 than were S208 maternal variants (IC_{50}, 0.1 μg/ml versus 0.005 μg/ml and 0.01 μg/ml). However, for F535, the infant variant was >50-fold more sensitive to inhibition by TAK-779 than was the maternal variant (IC_{50}, 0.001 μg/ml versus 0.06 μg/ml). Thus, transmitted infant variants did not appear to have unique receptor properties compared to maternal variants.

The core epitopes required for neutralization by the MAbs tested in the experiments described above were often present, with the exceptions of F535 B^{res}, which contained a mutation in the epitope of 2F5, and both F535 M^{sens} and B^{res}, in which the N residue at position 332 required for PGT121 and PGT128 recognition (48, 70) was shifted to position 334 (Fig. 2B). Differences in neutralization profiles against MAbs could not be explained by the presence or absence of known epitope targets. For example, residue N160, which is required for PG9 neutralization (37, 71), was present in both F535 B^{res} and M^{sens}, but only the former was neutralized by this MAb. Similarly, S208 maternal and infant variants were not neutralized by PGT121, even though the N332 residue required for neutralization by this MAb was present.

NAb escape in mother-infant pair S208 involves V4 and HR2. Figure 3A depicts Env amino acid differences between S208 maternal and infant variants that were chosen for detailed epitope mapping. Relative to both M^{sens1} and M^{sens2}, B^{res} contained a deletion in V1, as well as amino acid substitutions in multiple regions of the envelope. Additionally, there were sequence differ-

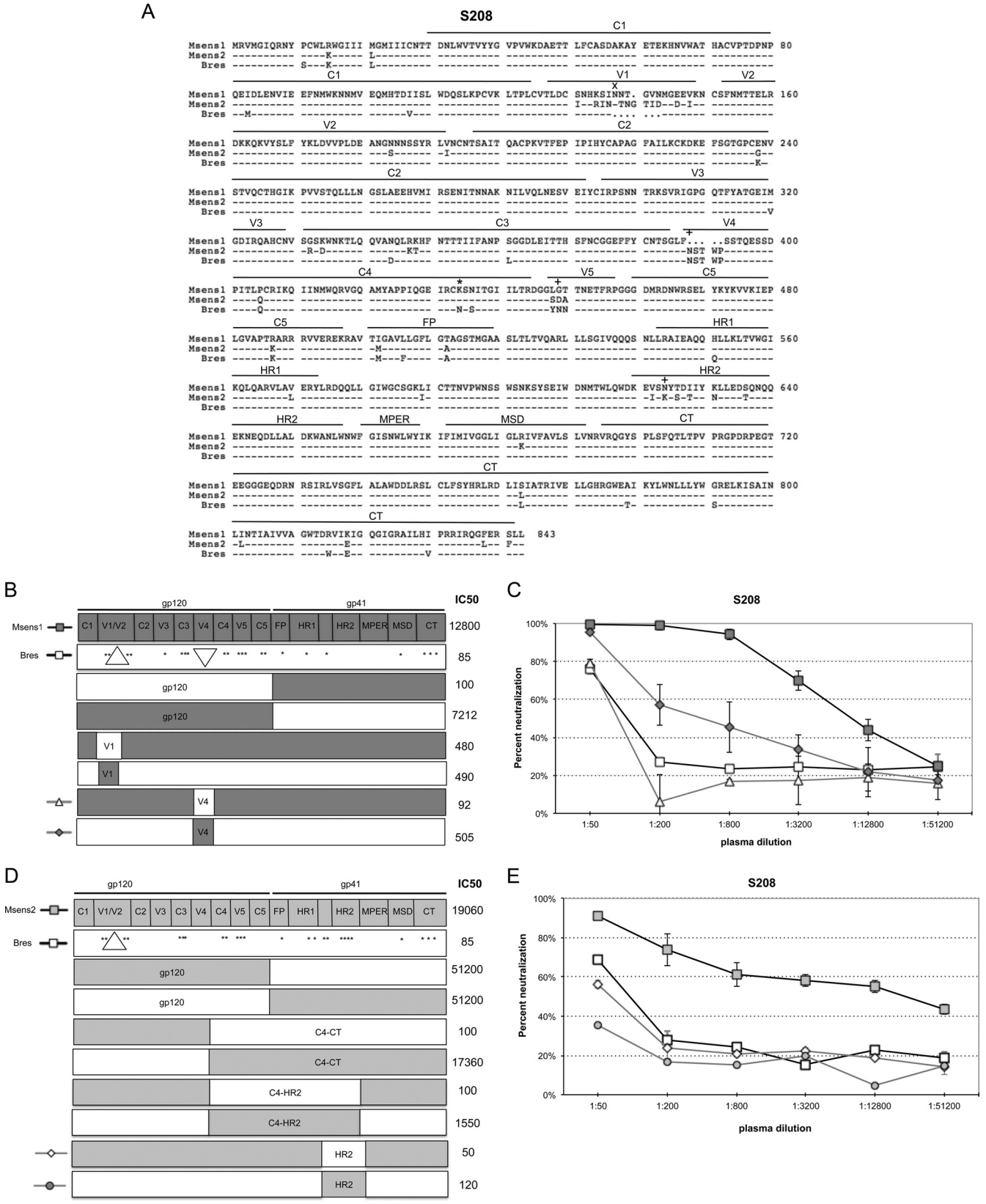


FIG 3 (A) Amino acid alignment of envelope regions of S208 variants. The sequence for S208 M^{sens1} is shown. Symbols: dashes, amino acids similar to those in S208 M^{sens1}; dots, deletions; x, loss of PNGS in B^{res}; +, gain of PNGS in B^{res}. Modifications in PNGS highlighted are relative to one or both of the sensitive maternal variants. Constant (C1 to C5) and variable (V1 to V5) regions of the surface gp120 subunit of the envelope are indicated. For gp41, abbreviations are as follows: FP, fusion peptide; HR1, heptad region 1; HR2, heptad region 2; MPER, membrane-proximal external region; MSD, membrane-spanning domain;

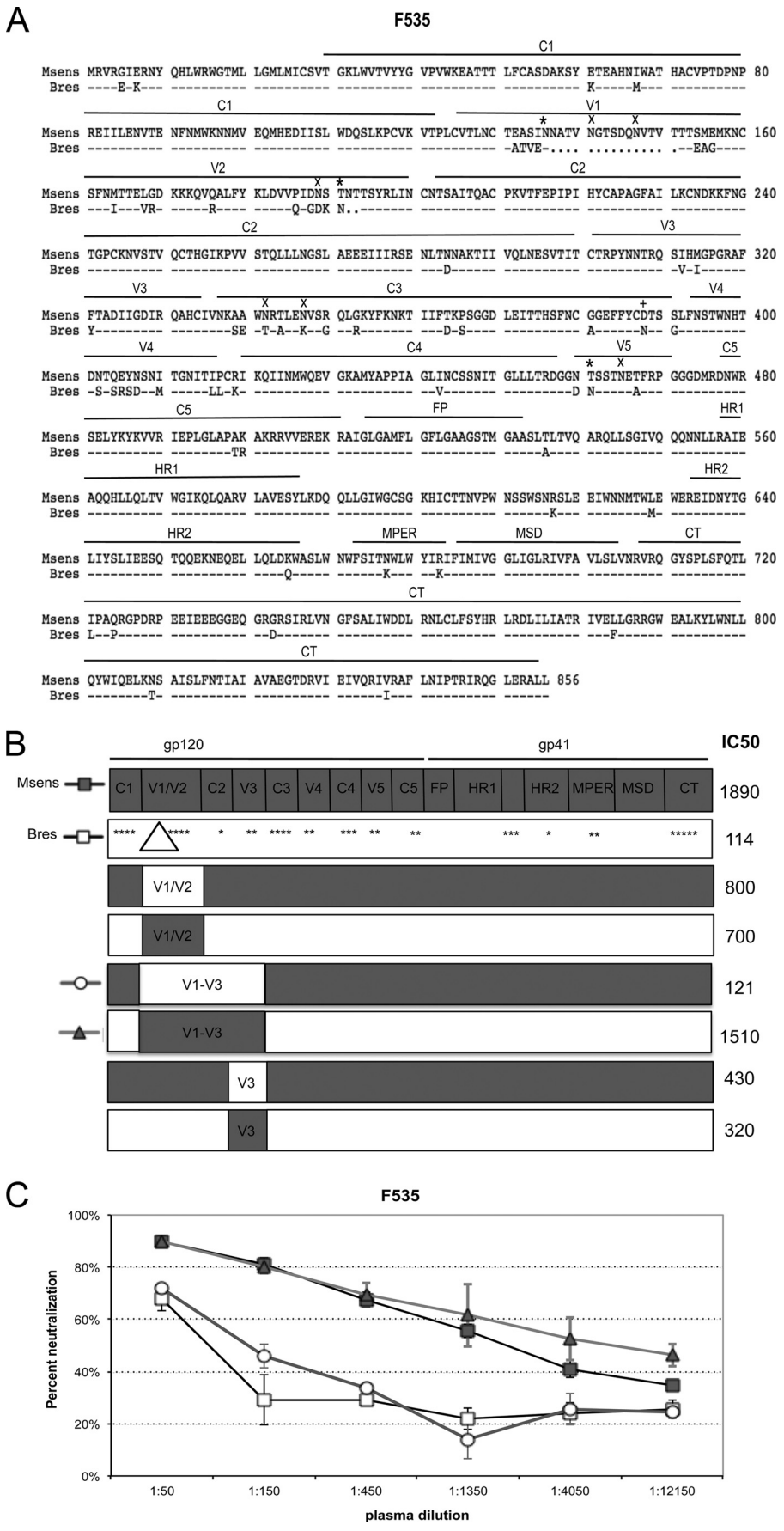
ences that were observed only when B^{res} was compared to either M^{sens1} or M^{sens2}. For example, relative to M^{sens1}, B^{res} contained a 5-amino-acid insertion in V4 and amino acid substitutions in C4, C5, and the fusion peptide, but these regions in B^{res} were identical to those in M^{sens2}. In contrast, compared to M^{sens2}, B^{res} contained amino acid substitutions in V2, C3, and HR2, but these regions were identical between B^{res} and M^{sens1}.

We first investigated the determinants of NAb resistance of B^{res} (IC_{50}, 85 [Fig. 3B]) relative to M^{sens1}, which was highly sensitive to neutralization by maternal plasma (IC_{50}, 12,800). Figure 3B summarizes the IC_{50}s of chimeras used to fine map the region that conferred NAb resistance, while Fig. 3C shows neutralization curves of representative viruses from Fig. 3B. To investigate whether NAb resistance maps to gp120 or gp41 of B^{res}, we created chimeras containing either gp120 or gp41 of B^{res} and found that the chimera containing gp120 of B^{res} was resistant to neutralization by maternal plasma (IC_{50}, 100), while the chimera containing gp41 of B^{res} remained sensitive (IC_{50}, 7,212). This suggested that the determinants of resistance mapped to gp120 of B^{res}. Because transmitted variants tend to have shorter variable loops (11, 12, 58, 82), and since V1/V2 has been shown to regulate sensitivity to neutralization (41, 49, 55, 56), we next tested whether the V1 deletion in S208 B^{res} relative to M^{sens1} contributed to neutralization resistance against maternal plasma. Introducing V1 of B^{res} into M^{sens1} partially reduced sensitivity to maternal plasma (IC_{50}, 480), suggesting that V1 may be one determinant of resistance, but it was not the only region contributing to the resistance phenotype of B^{res}. Introducing V1 of M^{sens1} into B^{res} resulted in a modest increase of neutralization sensitivity (IC_{50}, 490), suggesting that V1 of M^{sens1} may serve as a target of maternal NAbs. Next, we examined a 5-amino-acid insertion in V4 of B^{res}, which included the addition of a potential N-linked glycosylation site (PNGS) relative to M^{sens1} (Fig. 3A), and found that this insertion, when introduced into the maternal envelope, significantly decreased neutralization sensitivity and resulted in a virus with a neutralization profile similar to that of B^{res} (IC_{50}, 92 [Fig. 3B and C]). The reciprocal chimera, in which V4 of B^{res} was replaced with that of M^{sens1}, displayed an intermediate neutralization profile (IC_{50}, 505), suggesting that V4 may serve as a direct target of maternal NAbs. Thus, when we mapped NAb escape relative to M^{sens1}, an insertion of 5 amino acids in V4 of B^{res} was sufficient to confer NAb resistance, while a deletion of 6 amino acids in V1 of B^{res} partially mediated resistance to maternal plasma, independent of the presence of B^{res} V4.

Interestingly, the V4 region of S208 B^{res} was identical in sequence (Fig. 3A) to another maternal sensitive variant, M^{sens2} (IC_{50}, 19,060 [Fig. 3D]). Therefore, we hypothesized that differences in neutralization sensitivity of B^{res} relative to M^{sens2} might be determined by different regions of Env. Figure 3D summarizes the IC_{50}s of chimeras used to fine map the region that conferred NAb escape to B^{res} relative to M^{sens2}, with representative neutralization curves shown in Fig. 3E. To map NAb escape of S208 B^{res} relative to M^{sens2}, we again investigated whether we could map the determinants of resistance to either gp120 or gp41 of B^{res}. Interestingly, the resulting reciprocal chimeras that contained either gp120 or gp41 of B^{res} both displayed a neutralization-sensitive phenotype (IC_{50}, 51,200), suggesting that NAb resistance may require regions in gp120 as well as gp41. Next, we found that the chimera with the 3′ region of B^{res} starting from C4 replaced with M^{sens2} sequences resulted in a neutralization-sensitive phenotype (IC_{50}, 17,360), while the reciprocal chimera remained neutralization resistant (IC_{50}, 100), suggesting that the residues important for neutralization resistance of B^{res} mapped to the region 3′ of V4. In support of this, we found that replacing C4-HR2 of S208 M^{sens2} with corresponding sequences from S208 B^{res} resulted in a neutralization-resistant phenotype (IC_{50}, 100), whereas the reciprocal chimera partially restored neutralization sensitivity (IC_{50}, 1,550). Further fine mapping of this region demonstrated that 6-amino-acid substitutions within HR2 of B^{res}, which included the addition of a PNGS in B^{res} relative to M^{sens2} (Fig. 3A), were sufficient to confer neutralization resistance to maternal plasma (IC_{50}, 58 [Fig. 3D and E]). Replacing HR2 of B^{res} with that of M^{sens2} did not restore neutralization sensitivity (IC_{50}, 50 [Fig. 3D and E]), suggesting that while HR2 was sufficient to confer NAb escape, it may not be directly targeted by maternal NAbs. NAb resistance likely required a combination of the 6 mutations in HR2 of B^{res}, as chimeras that included smaller portions of HR2 of B^{res} in the context of M^{sens2} did not recapitulate the neutralization resistance phenotype of B^{res} (data not shown).

The region containing V1 to V3 mediates NAb escape in mother-infant pair F535. Figure 4A shows the Env amino acid differences between sensitive maternal and resistant infant variants for F535. The resistant baby variant, B^{res} (IC_{50}, 114 [Fig. 4B]), contained deletions in V1/V2 relative to the sensitive maternal variant, M^{sens} (IC_{50}, 1,890), which resulted in shifts and losses of PNGS. Additionally, there were multiple amino acid substitutions throughout the envelope. We first created reciprocal V1/V2 chimeras to determine whether deletions in this region of B^{res} mediated resistance to neutralization by maternal plasma. The reciprocal chimeras displayed a neutralization profile that was intermediate in sensitivity between those of native B^{res} and M^{sens} (IC_{50}, 700 to 800), indicating that while V1/V2 was a determinant of differences in neutralization sensitivity, other regions were required for NAb escape. We next introduced V1 to V3 of B^{res} into the M^{sens} envelope and found that this chimera was resistant to neutralization by maternal plasma (IC_{50}, 121 [Fig. 4B and C]). Furthermore, we found that V3 of B^{res} alone (IC_{50}, 430 [Fig. 4B]) or in combination with C2 (data not shown) conferred only a modest decrease in neutralization sensitivity and was not sufficient to recapitulate the neutralization resistance of B^{res} to maternal plasma. These results demonstrated that a combination of changes in V1 to V3 of B^{res} was required to drive escape from F535 maternal NAbs. Replacing V1 to V3 of B^{res} with those of M^{sens} restored complete neutralization sensitivity to maternal plasma (IC_{50}, 1,510 [Fig. 4B and C]), while V1/V2 or V3 alone of M^{sens} only partially restored

CT, cytoplasmic tail. Neutralization profiles of S208 pseudoviruses bearing chimeric envelopes from B^{res} and M^{sens1} (B) or B^{res} and M^{sens2} (D) against maternal plasma are shown. Average IC_{50}s from at least 2 independent experiments are shown next to bars representing chimeric envelopes. Gray bars represent envelope sequence of M^{sens1} or M^{sens2}. White bars represent envelope sequence of B^{res}. Asterisks denote regions in B^{res} with amino acid differences relative to the maternal envelope. The right-side-up triangle represents deletion in B^{res}. The inverted triangle represents insertion in B^{res}. Neutralization curves of chimeric viruses bearing regions found to confer resistance to maternal plasma (V4 in panel C and HR2 in panel E) are shown relative to native maternal and infant viruses. Percent neutralization versus plasma dilution is shown.


A
F535
C1
Msens MRVRGIERNY QHLWRWGTML LGMLMICSVT GKLWVTVYYG VPVWKEATTT LFCASDAKSY ETEAHNIWAT HACVPTDPNP 80
Bres ----E-K--- ---------- ---------- ---------- ---------- ---------- K-----M--- ----------
C1 V1
Msens REIILENVTE NFNMWKNNMV EQMHEDIISL WDQSLKPCVK VTPLCVTLNC TEASINNATV NGTSDQNVTV TTTSMEMKNC 160
Bres ---------- ---------- ---------- ---------- ---------- -ATVE-.... ....--EAG----
V2 C2
Msens SFNMTTELGD KKKQVQALFY KLDVVPIDNS TNTTSYRLIN CNTSAITQAC PKVTFEPIPI HYCAPAGFAI LKCNDKKFNG 240
Bres ---I---VR- -----R---- -----Q-GDK N..------- ---------- ---------- ---------- ----------
C2 V3
Msens TGPCKNVSTV QCTHGIKPVV STQLLLNGSL AEEEIIIRSE NLTNNAKTII VQLNESVTIT CTRPYNNTRQ SIHMGPGRAF 320
Bres ---------- ---------- ---------- ---------- ---D------ ---------- ---------- -V-I------
V3 C3 V4
Msens FTADIIGDIR QAHCIVNKAA WNRTLENVSR QLGKYFKNKT IIFTKPSGGD LEITTHSFNC GGEFFYCDTS SLFNSTWNHT 400
Bres Y--------- --------SE -T-A--K--G --R------- ---D-S---- ---------- A------N-- G---------
V4 C4 V5 C5
Msens DNTQEYNSNI TGNITIPCRI KQIINMWQEV GKAMYAPPIA GLINCSSNIT GLLLTRDGGN TSSTNETFRP GGGDMRDNWR 480
Bres -S-SRSD--M -----LL-K- ---------- ---------- --V------- ---------D N-----A--- ----------
C5 FP HR1
Msens SELYKYKVVR IEPLGLAPAK AKRRVVEREK RAIGLGAMFL GFLGAAGSTM GAASLTLTVQ ARQLLSGIVQ QQNNLLRAIE 560
Bres ---------- --------TR ---------- ---------- ---------- -----A---- ---------- ----------
HR1 HR2
Msens AQQHLLQLTV WGIKQLQARV LAVESYLKDQ QLLGIWGCSG KHICTTNVPW NSSWSNRSLE EIWNNMTWLE WEREIDNYTG 640
Bres ---------- ---------- ---------- ---------- ---------- ------K--- --------M- ----------
HR2 MPER MSD CT
Msens LIYSLIEESQ TQQEKNEQEL LQLDKWASLW NWFSITNWLW YIRIFIMIVG GLIGLRIVFA VLSLVNRVRQ GYSPLSFQTL 720
Bres ---------- ---------- ----Q----- ------K--- --K------- ---------- ---------- ----------
CT
Msens IPAQRGPDRP EEIEEEGGEQ GRGRSIRLVN GFSALIWDDL RNLCLFSYHR LRDLILIATR IVELLGRRGW EALKYLWNLL 800
Bres L--P------ ---------- --D------- ---------- ---------- ---------- ---F------ ----------
CT
Msens QYWIQELKNS AISLFNTIAI AVAEGTDRVI EIVQRIVRAF LNIPTRIRQG LERALL 856
Bres --------T- ---------- ---------- ------I--- ---------- ------
B
gp120
gp41
IC50
Msens
C1 V1/V2 C2 V3 C3 V4 C4 V5 C5 FP HR1 HR2 MPER MSD CT
1890
Bres
114
V1/V2
800
V1/V2
700
V1-V3
121
V1-V3
1510
V3
430
V3
320
C
F535
Percent neutralization
100%
80%
60%
40%
20%
0%
1:50
1:150
1:450
1:1350
1:4050
1:12150
plasma dilution

neutralization sensitivity (Fig. 4B), suggesting that V1 to V3 in combination may form epitopes that together account for the major target of maternal NAbs.

Regions that confer NAb escape are not direct linear targets of maternal NAbs. Our mapping studies identified specific regions that conferred escape of viruses transmitted to infants, but replacing these regions with those from sensitive maternal variants did not always restore complete sensitivity to neutralization by maternal plasma, suggesting that these regions were not likely to be major linear targets of maternal NAbs. To directly test this hypothesis, we performed competition neutralization assays using peptides corresponding to regions identified to be sufficient for resistance to neutralization by maternal plasma. As a positive control for the competition assay, we incubated MAb 2F5 with the peptide corresponding to its linear epitope and observed that this peptide effectively competed the neutralizing activity of 2F5, as shown by a 93% reduction in AUC (Fig. 5A). Incubation of peptides with virus in the absence of maternal plasma or MAb did not result in inhibition of virus entry, indicating that the peptides did not interfere with virus infectivity (data not shown). In the case of the HR2 peptide, the lack of inhibition may reflect the fact that it includes only the N-terminal portion of HR2, which is not the major region involved in entry inhibition (74).

Our chimera analyses showed that for S208, V4 and HR2 of B^{res} mediated escape from maternal NAbs. In competition assays, peptides corresponding to V4 and HR2 of M^{sens1} and M^{sens2}, respectively, did not compete for neutralizing activity of S208 maternal plasma, as demonstrated by a negligible reduction in AUC (−2% to 1% [Fig. 5B]) in the presence of these peptides. The inability of V4 and HR2 peptides to compete for NAb activity implies that these regions were not direct linear targets of maternal NAbs.

For F535, we found that V1 to V3 of B^{res} conferred escape from maternal plasma. To test whether V1, V2, and V3 were linear targets of maternal NAbs, we performed competition neutralization assays using peptides corresponding to these regions of F535 M^{sens}. We saw some variability in the ability of peptides to compete for maternal NAbs, especially when we tested the overlapping 15-mers of V1 and V2 (data not shown). However, overall, V1, V2, and V3 peptides appeared to contribute to only a small fraction of maternal plasma neutralizing activity, as preincubation of maternal plasma with these peptides resulted only in a subtle shift in neutralization curves (AUC reduction of −2% to 8% [Fig. 5C]). Even when we combined V1/V2 peptides in the competition assay, the average AUC reduction from 3 experiments was at most 14%.

Regions that mediate escape from maternal NAbs alter distal epitopes. Because we found that the regions of S208 B^{res} that conferred escape from maternal NAbs were not linear targets (Fig. 5B), and because replacing these regions with corresponding maternal sequences resulted in at most only partial restoration of sensitivity (Fig. 3B and D), we hypothesized that these regions might instead mediate NAb escape by altering Env conformation to affect exposure of distal epitopes. To test this hypothesis, we determined the neutralization profiles of S208 B^{res} and M^{sens2} chimeras against various MAbs against which there were differences in sensitivity between maternal and infant viruses. MAbs b12, 2F5, and 4E10 were selected because the neutralization profiles of native S208 B^{res} and M^{sens2} against these MAbs reflected those against maternal plasma (i.e., neutralization sensitivity of $M^{sens2} \gg B^{res}$ [Fig. 2A]). Interestingly, we found that regions of B^{res} that conferred resistance to maternal plasma simultaneously conferred resistance to the MAbs tested (Fig. 6A). For example, reciprocal gp120 chimeras, as well as chimeras containing C4 to CT or C4 to HR2 of M^{sens2}, were sensitive to neutralization by maternal plasma and MAbs. Chimeras that were resistant to neutralization by maternal plasma (those bearing C4 to CT, C4 to HR2, or HR2 alone of B^{res}) were also resistant to MAbs. Of note, the chimera containing HR2 of B^{res} in the backbone of M^{sens2} reduced sensitivity not only to maternal plasma but also to the MAbs tested even when the known epitopes of these MAbs were present. For example, although the b12 epitope maps solely to gp120 (54), introducing HR2 of B^{res} into M^{sens2} markedly reduced sensitivity to b12 relative to that of the native M^{sens2}. These results support the hypothesis that HR2 of B^{res} alters multiple distal epitopes to confer neutralization resistance. We could not test neutralization of S208 B^{res}/M^{sens1} chimeras against b12, 2F5, and 4E10 since B^{res} and M^{sens1} had similar profiles of neutralization against these MAbs (Fig. 2A).

For F535, we similarly found that regions of B^{res} (V1 to V3) that mediated escape from maternal plasma appeared to alter distal NAb targets. Specifically, although MAbs 2F5 and 4E10 target linear epitopes in gp41 that were present in M^{sens} (Fig. 2B), introducing V1 to V3 of B^{res} into M^{sens} resulted in a virus that was less sensitive to these MAbs than was M^{sens} (Fig. 6B). We also found that the reciprocal chimera, in which V1 to V3 of M^{sens} was introduced into B^{res}, restored sensitivity not only to maternal plasma but also to MAb 4E10. V1 to V3 of M^{sens} did not confer sensitivity to MAb 2F5, which also targets a linear epitope in gp41. This result is not surprising, given that the 2F5 epitope was mutated in B^{res} (Fig. 2B). These results support the hypothesis that V1 to V3 mutations in F535 B^{res} modify Env conformation to mask distal epitopes. For b12, the neutralization profile for F535 chimeras did not always reflect that seen with maternal plasma, perhaps due to the complex determinants of sensitivity to this MAb (79). In fact, in some cases, the neutralization profile against b12 appeared to be opposite that against maternal plasma. For example, V1 to V3 of B^{res} was sufficient for resistance to maternal plasma but had only a modest effect on b12 sensitivity relative to that of the maternal variant. Similarly, the reciprocal chimera containing V1 to V3 of M^{sens} conferred full sensitivity to maternal plasma but did not increase sensitivity to b12 relative to that of B^{res}.

As mentioned above, the neutralization profiles of F535 maternal and infant variants against PG9 and VRC01 were opposite those seen against maternal plasma: F535 B^{res}, but not M^{sens}, was neutralized by PG9 and VRC01. When we tested F535 chimeras

FIG 4 (A) Amino acid alignment of envelope region of F535 variants. The sequence of F535 M^{sens} is shown. Symbols: dashes, regions in B^{res} that are identical to the maternal sequence shown; dots, deletions in B^{res}; x, loss of PNGS in B^{res}; +, gain of PNGS in B^{res}; *, shift of PNGS in B^{res}. Abbreviations are described in the legend to Fig. 3. (B) Neutralization profiles of F535 pseudoviruses bearing chimeric envelopes from B^{res} and M^{sens} against maternal plasma. Gray bars represent envelope sequence of M^{sens}. White bars represent envelope sequence of B^{res}. Average IC_{50}s from at least 2 independent experiments are shown. (C) Neutralization curves of chimeras bearing regions that confer neutralization resistance to maternal plasma are shown relative to native maternal and infant viruses. Percent neutralization versus plasma dilution is shown.

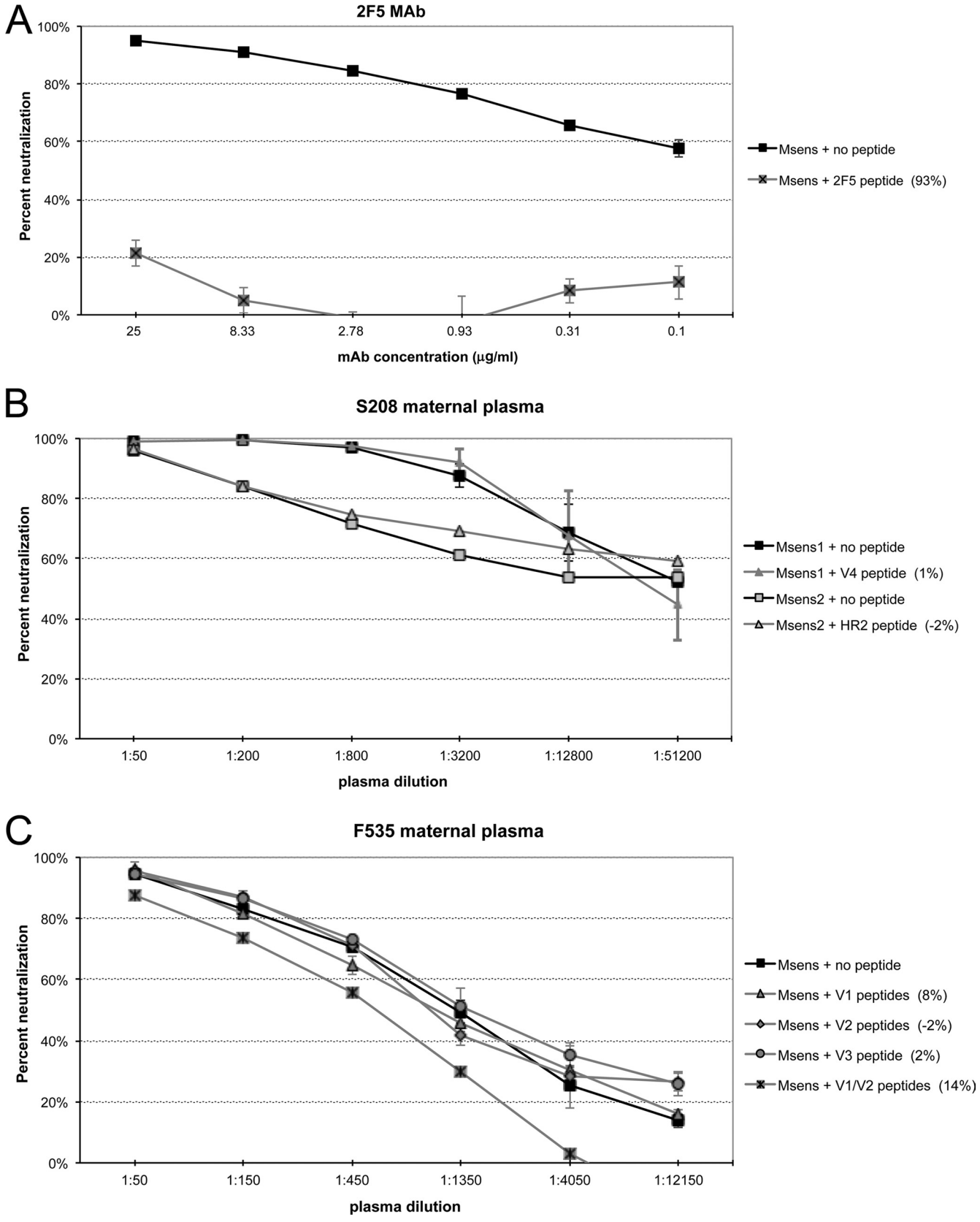


FIG 5 Peptide competition neutralization assays. Percent neutralization versus MAb concentration or plasma dilution is shown. (A) Serially diluted MAb 2F5 was preincubated with 2F5 peptides prior to addition of F535 M^{sens} in the neutralization assay. (B) S208 maternal plasma was preincubated with either V4 or HR2 peptides before addition of S208 M^{sens1} or M^{sens2}, respectively. (C) F535 maternal plasma was preincubated with V1, V2, V3, or V1/V2 peptides before addition of F535 M^{sens}. Contribution of peptides to maternal plasma or 2F5 neutralizing activity was calculated as percent reduction in AUC relative to 2F5 or plasma neutralization in the absence of peptides. Values for average percent reduction in AUC from at least 2 independent experiments are shown in parentheses in the symbol keys.

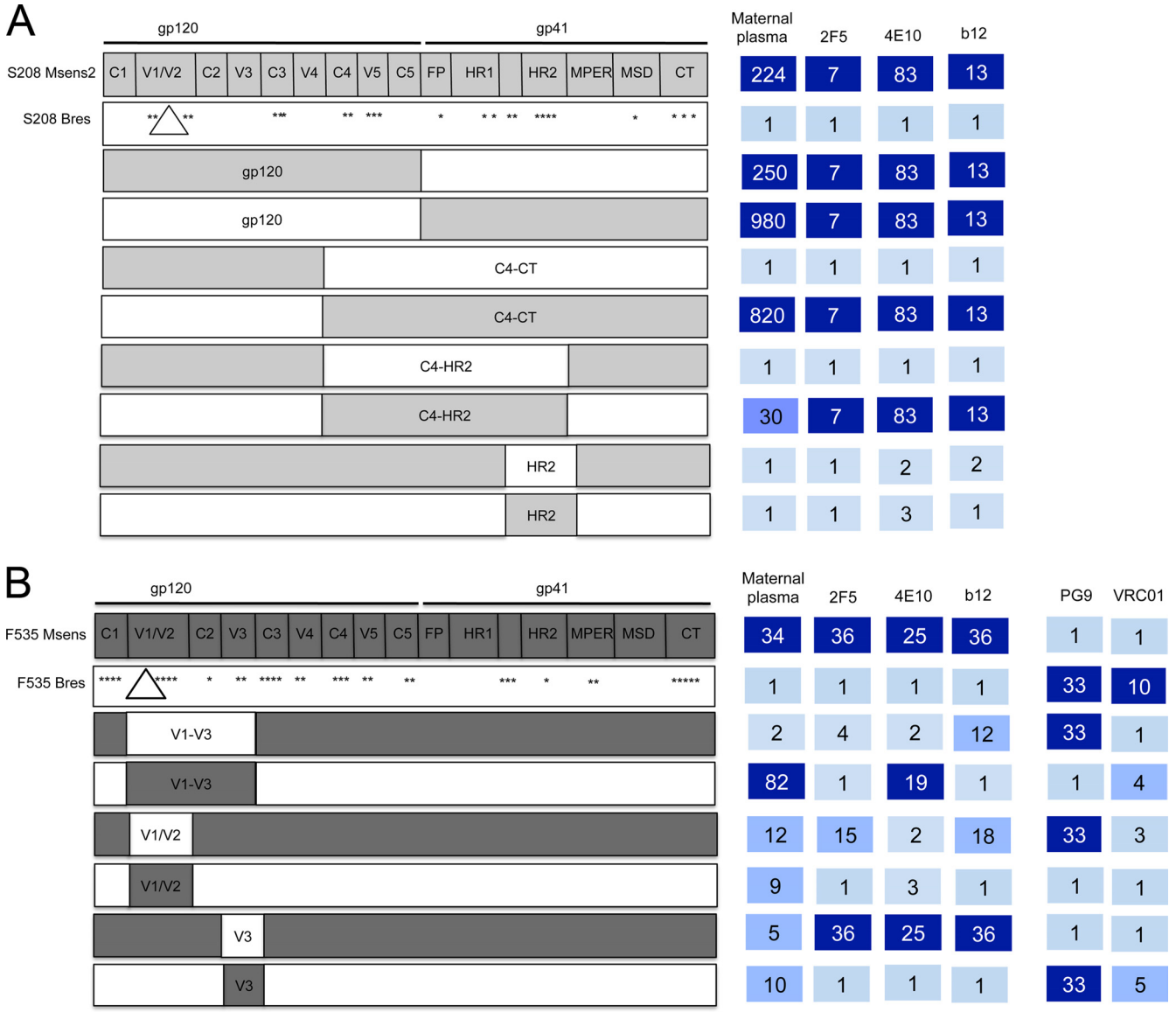


FIG 6 Summary of neutralization profiles of S208 B^{res} and M^{sens2} chimeras (A) and F535 B^{res} and M^{sens} chimeras (B) against maternal plasma and a panel of MAbs. Numbers in colored boxes denote fold neutralization sensitivity based on IC_{50}s relative to B^{res}, which was assigned a 1 for neutralization by maternal plasma, b12, 2F5, and 4E10. For PG9 and VRC01, numbers in colored boxes denote fold neutralization sensitivity based on IC_{50}s relative to that of M^{sens}, which was assigned a value of 1. Darker shading denotes increasing sensitivity. Symbols and abbreviations for envelope regions are described in the legend to Fig. 3.

against these MAbs, we found that the difference in neutralization sensitivity against PG9 mapped to V1/V2. Introducing V1/V2 of B^{res} into M^{sens} conferred sensitivity to PG9, while the reciprocal chimera resulted in resistance to PG9 (Fig. 6B). V1/V2 of both B^{res} and M^{sens} contained the N160 residue required for neutralization, as well as other residues such as N156 and K168 that have been shown to be secondary contact sites (37) for PG9 (Fig. 2B). Thus, differences in sensitivity to PG9 were not explained by the presence or absence of known determinants of sensitivity to this MAb. We were unable to map the determinants of sensitivity to VRC01 with the chimeras tested.

Maternal plasma has limited NAb responses against known conformational epitopes targeted by broad NAbs. Because the regions that conferred NAb escape to maternal plasma appeared to alter Env conformation to mask multiple distal epitopes, we investigated whether we could map maternal NAb responses to known conformational epitopes. Specifically, we determined whether the maternal plasma contained NAb specificities against epitopes that are glycan dependent and those that overlap the CD4 binding site, such as those targeted by the PG (71) and PGT (70) MAbs and VRC01 (78), respectively. The neutralizing activities of PG9 and PGT145 have been shown to be dependent on the presence of a conserved N-linked glycosylation site in V2 (N160) (37, 70, 71). Therefore, to screen maternal plasma for PG9- and PGT145-like NAbs, we created an N160K mutation in both sensitive S208 maternal variants, M^{sens1} and M^{sens2}, and compared the neutralization phenotypes of these mutants to those of wild-type maternal variants. As a positive control, we showed that both

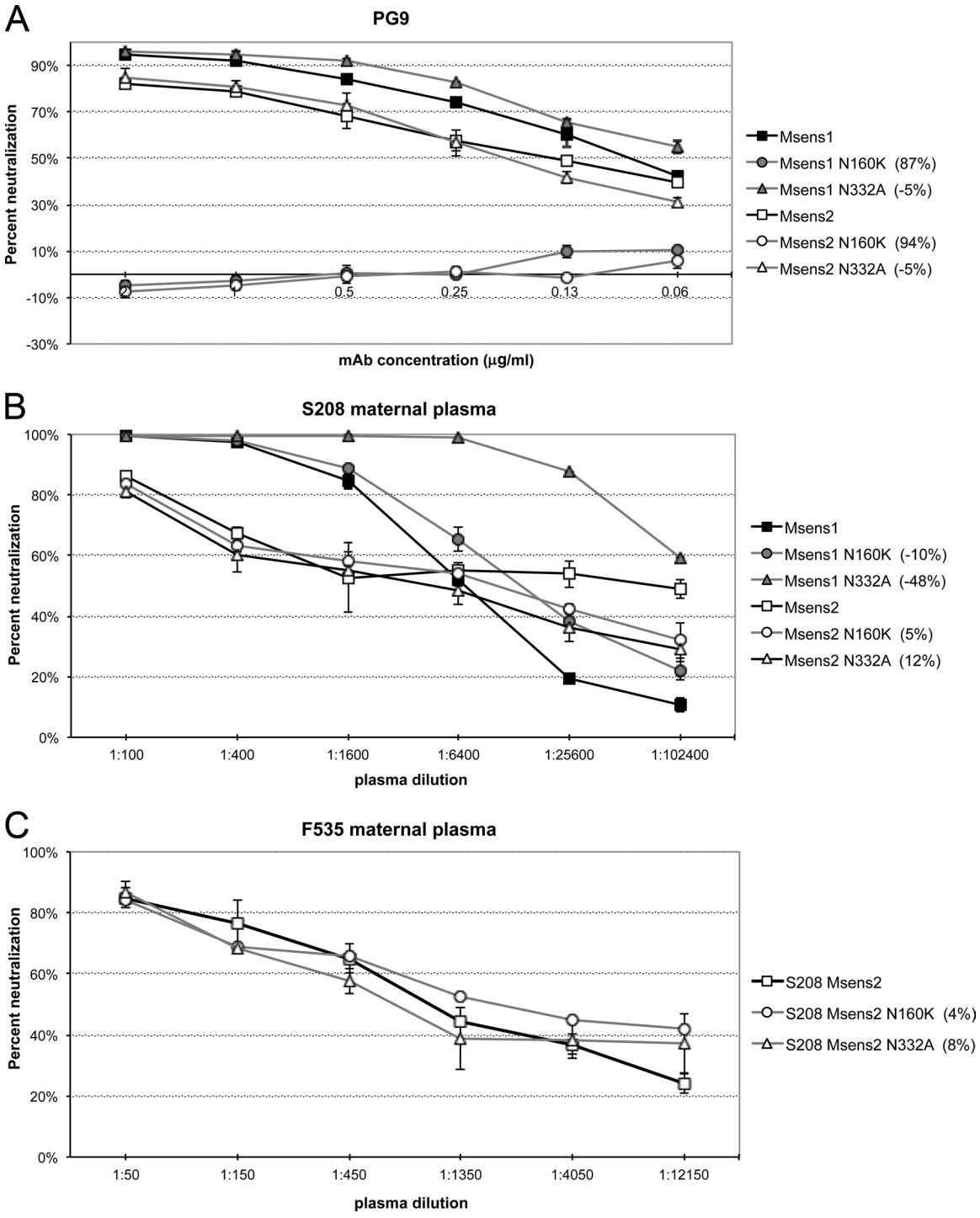


FIG 7 Neutralization profiles of wild-type, N160K, and N332A variants of S208 M^{sens} variants against PG9 (A), S208 maternal plasma (B), and F535 maternal plasma (C). The percent neutralization versus MAb concentration or plasma dilution is shown in each panel. The contribution of epitopes dependent on N160 or N332 to PG9 or maternal plasma neutralizing activity was calculated as the percent reduction in AUC relative to PG9 or plasma neutralization of wild-type maternal variants. Values for average percent reduction in AUC from at least 2 independent experiments are shown in parentheses in the symbol keys.

M^{sens1} and M^{sens2} were potently neutralized by PG9, but as expected, the N160K versions of these variants were resistant to neutralization by PG9 (87% to 94% AUC reduction [Fig. 7A]). However, both S208 M^{sens1} and M^{sens2} N160K mutants were still as potently neutralized by maternal plasma as were the wild-type maternal variants (−10% to 5% AUC reduction [Fig. 7B]).

We also tested the effect of mutating another N-linked glycosylation site in amino acid position 332 since this residue has also been shown to be important for the activity of a number of PGT antibodies, which form another class of glycan-dependent broad NAbs (48, 70). Again, we found that S208 M^{sens1} and M^{sens2} N332A mutants were still sensitive to neutralization by maternal

plasma (Fig. 7B). In fact, for M^{sens1}, we saw a markedly enhanced neutralization by maternal plasma when the N332A mutation was introduced (−48% AUC reduction [Fig. 7B]).

As mentioned above, F535 M^{sens} was not recognized by PG9, despite the presence of N160 (Fig. 2B). Additionally, this variant lacks a PNGS in position 332 of HXB2 but has a PNGS that is shifted two amino acids downstream of this position (N334 [Fig. 2B]). Therefore, we took advantage of the observation that F535 maternal plasma was capable of potently neutralizing the heterologous S208 M^{sens2} to test whether epitopes dependent on N160 or N332 in the S208 backbone had an effect on neutralization by F535 maternal plasma. Wild-type, N160K, and N332A S208 M^{sens2} variants were all neutralized to similar extents by F535 maternal plasma (Fig. 7C), with N160K and N332A mutations resulting in reductions in AUC of no more than 4% and 8%, respectively. These results suggest that the predominant maternal NAb responses in both S208 and F535 were not directed against conformational epitopes that are dependent on N160 or N332.

To determine if either S208 or F535 maternal plasma contains NAbs that target the CD4 binding site, we also screened maternal plasma for neutralizing activity against epitopes overlapping the CD4 binding site that are recognized by b12 and VRC01 by performing competition neutralization assays with resurfaced, stabilized core (RSC3) proteins, as previously described (78). We used either wild-type RSC3 or RSC3 with a mutation that eliminates CD4 binding (RSC3Δ371I) to compete maternal antibodies. As a positive control for the protein competition assay, we showed that the neutralizing activity of VRC01 was competed by RSC3 but not by RSC3Δ371I (74% versus 1% AUC reduction [Fig. 8A]). The neutralizing abilities of both S208 and F535 maternal plasma against sensitive maternal variants were not competed by either RSC3 or RSC3Δ371I, as shown by the lack of a substantial shift in neutralization curves and by the negligible reduction in AUC (Fig. 8B and C), suggesting that the major NAb responses in maternal plasma were not against epitopes overlapping the CD4 binding site.

Maternal and infant variants have similar envelope contents. To examine whether differences in Env content between maternal and infant variants contributed to differing neutralization sensitivities to maternal plasma and MAbs, we performed SDS-PAGE Western blot analyses on pseudoviruses (Fig. 9A). We did not observe a pattern linking neutralization sensitivity and envelope content per particle. For example, total Env, gp160, and gp120 levels of S208 M^{sens1} were lower than those for B^{res}, while these levels were higher for M^{sens2} than for B^{res} (Fig. 9B). The results were generally similar when we performed Western blot analyses under native conditions to determine trimeric Env content (data not shown). Thus, differences in Env glycoprotein levels between these maternal and infant variants did not appear to explain differing neutralization phenotypes.

DISCUSSION

We have previously shown that HIV-1 variants that were resistant or only weakly sensitive to maternal NAbs present near the time of transmission were commonly transmitted from mothers to infants (77), suggesting that maternal NAbs may apply selection pressure during transmission. In this study, we dissected the molecular basis of NAb escape for 2 mother-infant transmission pairs to gain insight into NAb escape mechanisms that select for transmitted variants. We found that although the sequence determinants of NAb escape were different for the infant variants from each pair, these determinants appeared to share a mechanism to evade maternal NAbs through conformational masking of distal epitopes. Three observations supported this hypothesis: (i) replacing regions of infant Env that mediated neutralization resistance with those of maternal Env did not always confer sensitivity, (ii) peptide competition experiments showed that regions involved in escape were not major linear targets, and (iii) regions that conferred escape from maternal NAbs also modulated sensitivity to various MAbs that target distal epitopes.

For S208, an insertion of 5 amino acids in V4, which introduced a PNGS, conferred NAb resistance to the infant variant, relative to one highly neutralization-sensitive variant found in the infecting mother. However, when the same infant variant was compared to another neutralization-sensitive variant from the same mother, a different region of the envelope (6-amino-acid substitutions in HR2, which again introduced a PNGS in the infant variant) conferred resistance to neutralization by maternal plasma. Given that NAbs found in plasma are likely polyclonal in most individuals (56, 65), it is possible that different antibody specificities targeted the 2 sensitive S208 maternal variants. This hypothesis is supported by the observation that these maternal variants had distinct neutralization profiles against MAbs b12, 2F5, and 4E10, suggesting that these variants may have differing epitopes accessible to NAbs. Thus, the observation that different regions of the infant envelope conferred escape from the same maternal plasma may be explained by the need for an escape variant to evade multiple NAb specificities.

For F535, there were also multiple domains (V1 to V3) contributing to NAb escape, in this case within one mother-infant virus pair. In F535 viruses, V1/V2 and V3 may serve as direct targets of maternal NAbs, since reciprocal V1/V2 and V3 chimeras of maternal and infant variants resulted in partial neutralization sensitivity to maternal plasma and V1, V2, and V3 peptides partially competed maternal NAbs in competition neutralization assays, although at most they accounted for 14% or less of total maternal plasma neutralizing activity. However, full sensitivity or resistance to maternal plasma required the combination of residues in V1 to V3 of the maternal or infant variant, respectively. Structural studies of the unliganded envelope trimer using cryoelectron tomography suggest that V1/V2 and V3 may interact at the apex of the trimer (19, 32, 73, 80). Additionally, it has been shown that V1/V2 modulates exposure of epitopes in V3 (57). Our observation that V3 was not a major linear target of F535 maternal NAbs yet was required for full neutralization sensitivity to maternal plasma along with V1/V2 suggests that V3 may also modulate V1/V2 epitopes. Thus, it is possible that V1/V2 and V3 may cooperate to form a conformational epitope(s) recognized by F535 maternal NAbs. The epitope(s) targeted by F535 maternal plasma is likely distinct from that targeted by PG9, given that the maternal variant (M^{sens}), which was sensitive to neutralization by maternal plasma, was not neutralized by PG9. Moreover, PG9-like antibodies were not detected in the maternal plasma.

Most of the regions found to mediate NAb escape in this study—V1/V2, V3, and V4—have previously been shown to be involved in NAb escape during intrapatient evolution (41, 56, 65, 72). Although the gp41 ectodomain as a whole has been found to play a role in mediating NAb escape (56), we demonstrated that NAb escape during MTCT could also occur through changes solely in HR2 of gp41. We observed that even though HR2 mediated NAb escape, it was not a linear target of maternal NAbs,

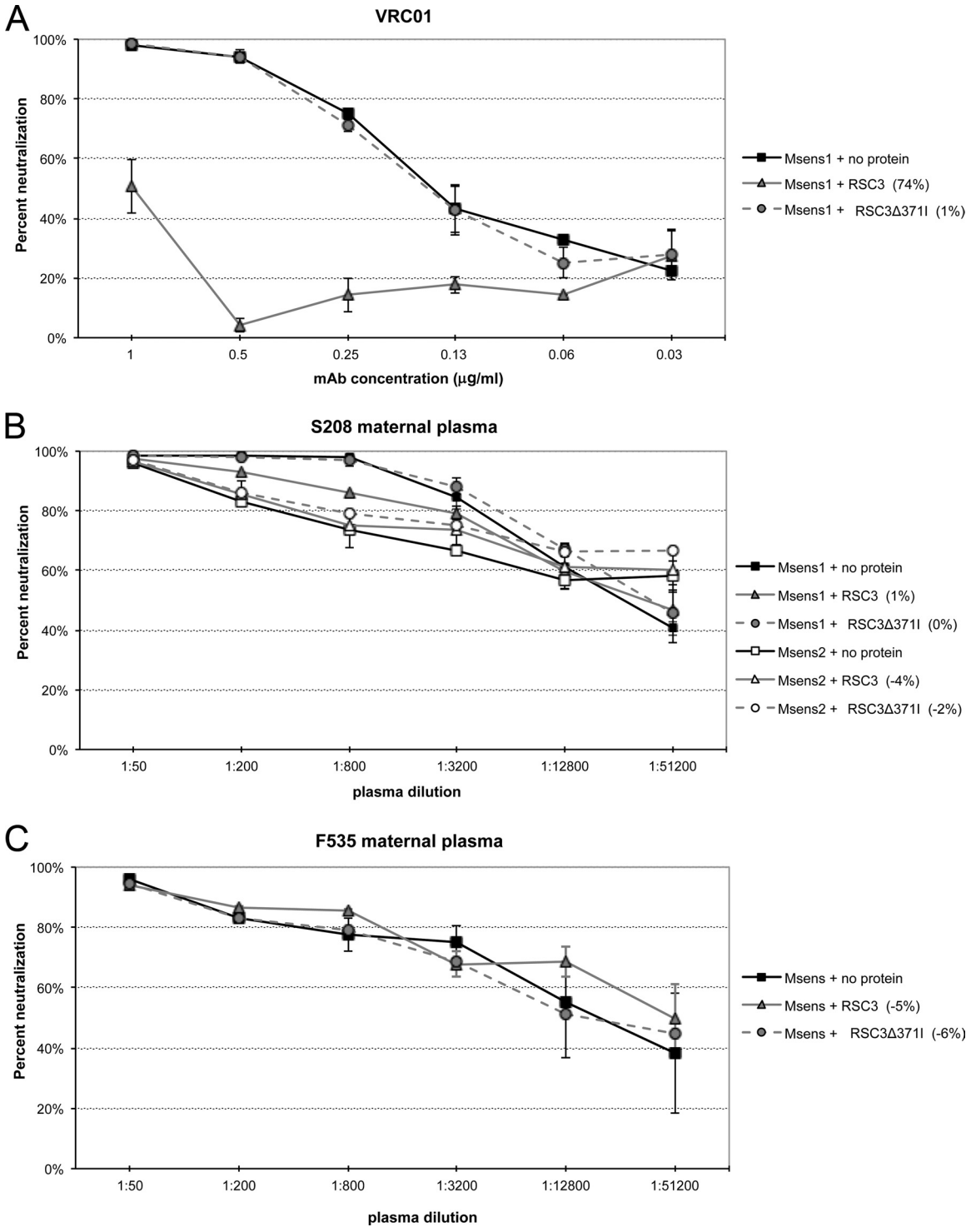


FIG 8 Protein competition neutralization experiments. Serially diluted MAb VRC01 (A) or maternal plasma (B and C) was preincubated with RSC3 or RSC3Δ371I before use in neutralization assays against S208 M^{sens1}, S208 M^{sens1} and S208 M^{sens2}, or F535 M^{sens}. The percent neutralization versus MAb concentration or plasma dilution is shown in each panel. The contribution of antibodies against the CD4 binding site to overall neutralizing activity of VRC01 or maternal plasma was calculated as percent reduction of AUC relative to neutralization by VRC01 or plasma in the absence of protein. Values for average percent reduction in AUC from at least 2 independent experiments are shown in parentheses in the symbol keys.

suggesting that some mutations in gp41 alter overall Env conformation to affect distal epitopes, as has been shown previously (6, 8, 25, 45, 46, 51, 66, 75). In the case described here, changes in gp41 may contribute to the selection of a NAb escape variant during MTCT.

We found that NAb escape during MTCT also involves multiple pathways, including insertions, deletions, and substitutions, which often involve modifications of PNGS, as has been described for NAb escape during intrapatient evolution (41, 56). Studies

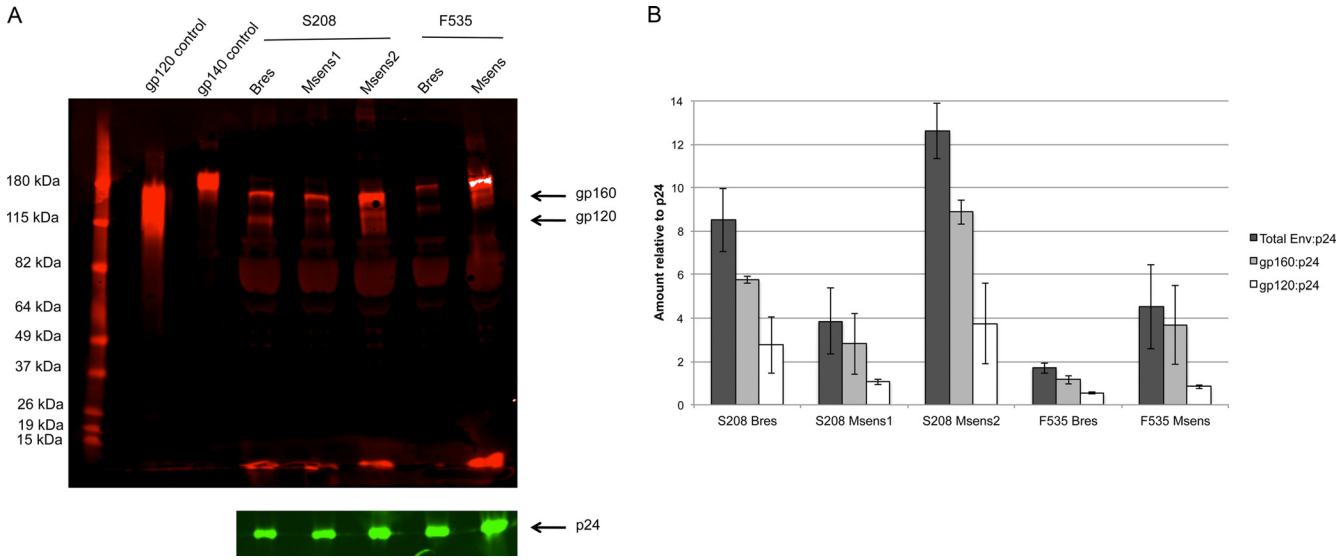


FIG 9 Determination of envelope content of S208 and F535 maternal and infant variants. (A) SDS-PAGE Western blotting of viral supernatants using rabbit polyclonal antisera to HIV-1 and mouse-anti-p24 as primary antibodies. An equal number of infectious particles based on titers in TZM-bl cells was loaded for each virus. The first lane shows the molecular mass markers, with sizes included to the left of the image. Approximate molecular masses of gp160, gp120, and p24 are indicated by arrows to the right of the image. gp120 control, purified recombinant subtype C envelope; gp140 control, purified recombinant subtype A envelope. (B) Levels of total envelope, gp160, or gp120 per particle, calculated as (gp160 + gp120)/p24, gp160/p24, and gp120/p24, respectively. Values shown are averages from 2 independent experiments.

have shown that glycans play an important role in modulating epitopes of NAbs both by serving as direct targets (37, 48, 61) and by shielding epitopes (72). Glycans at positions N160 and N332, which have been shown to form epitopes targeted by recently identified broad MAbs (48, 71), did not appear to contribute to the predominant NAb response in both mothers studied here. In fact, we found that for S208 M^{sens1}, maternal plasma neutralizing activity was enhanced when an N332A mutation was introduced in this variant, perhaps by exposure of additional epitopes masked by a glycan at this position. Thus, it is possible that in the background of certain variants, the glycan at position N332 may serve to shield NAb epitopes.

Although the prototype broad MAbs, b12, 2F5, 4E10, and 2G12, could not potently neutralize variants transmitted to infants, the more recently isolated broad MAbs such as VRC01 and PG9 potently neutralized both S208 and F535 infant variants. Interestingly, for F535, we observed that VRC01 and PG9 neutralized the infant but not maternal variant. Introducing V1/V2 of the infant variant into the maternal envelope conferred sensitivity to PG9. This region of both the maternal and infant variants contained the N160 residue, which is required for neutralization by PG9 (71), as well as other residues such as N156 and K168 that contribute to sensitivity against PG9 (37). Thus, there are likely other determinants of sensitivity to PG9 in V1/V2 other than N156, N160, and K/R168, as has been recently suggested (53). Similarly, it is likely that there are other determinants for recognition by MAbs PGT121 and -128 besides N332 (48), since these MAbs did not neutralize S208 maternal and infant variants, despite the presence of this residue. It is unclear whether these determinants of sensitivity to PG9 and PGT121 and -128 map to specific residues or are the result of conformational effects.

The neutralization profiles of F535 maternal and infant variants against PG9 and VRC01 were opposite those observed with maternal plasma, in which the maternal but not infant variant was sensitive. This suggests that the majority of F535 maternal NAb responses were not targeted against epitopes recognized by VRC01 and PG9, as supported by RSC3 competition and N160K and N332A neutralization experiments. We also found that the predominant S208 maternal NAbs were not PG9- or VRC01-like, implying that the mothers who transmitted in this study did not have NAbs with specificities of these broad MAbs. A recent small study provided indirect evidence for an association between MTCT and lack of NAbs targeting epitopes of broad MAbs. Specifically, that study reported that transmitted infant variants were more sensitive to neutralization by PG9 or -16 than variants found in the infecting mother (67). However, neither full-length *env* sequences nor specificities of maternal NAbs were examined in this study. Thus, a larger screen of full-length mother-infant variants and maternal plasma from MTCT pairs will be required to determine whether epitope specificities of broad MAbs play a role in selecting for variants transmitted from mother to child.

We investigated whether the differing neutralization sensitivities of maternal and infant variants could be explained by differences in Env content. It is possible that increased Env content leads to decreased neutralization sensitivity, since neutralization of HIV-1 appears to require all functional Env trimers to be occupied by at least one antibody (81). Alternatively, increased Env content may enhance neutralization through increased avidity (26, 42). Moreover, recent studies have reported that increased Env content may be a signature of transmitted variants (3, 16). However, for the 2 variants transmitted to the infants examined here, neutralization resistance to maternal plasma did not appear to be explained by differences in Env content relative to maternal variants.

Finally, although we have sampled viruses and antibodies very close to the estimated time of transmission, we acknowledge that if

the infants were infected soon after the last HIV-1-negative test, the transmitted variant could have been subject to selection by the infant's autologous NAbs, in addition to maternal NAbs. In the case of infant F535, this seems unlikely given that the envelope variants were isolated from the first HIV-1-positive sample only 6 weeks after the last negative test (at birth), leaving limited time for development of substantial *de novo* responses. Indeed, autologous NAb responses were not evident until 9 months in this infant (77). In the case of infant S208, there was a longer period between the last HIV-1-negative to the first HIV-1-positive test (negative at 3 months and positive at 6 months after birth), making it harder to rule out a contribution of *de novo* infant responses in driving some of the escape observed. Indeed, this is a potential confounding issue in all studies of NAb escape during transmission when the interval of sampling involves a significant time period where the virus is under immune selection after transmission. However, given the efficiency of passive transfer of HIV NAbs (reference 9 and unpublished data), the levels of passive maternal NAbs are likely to be higher than *de novo* responses in the initial weeks of infection in the infant.

In summary, we have characterized the molecular determinants of NAb escape that are relevant in the context of transmission. We found that NAb escape during HIV-1 MTCT is mediated by distinct domains and pathways that act through a common mechanism of masking distal epitopes. This strategy likely allows HIV-1 to utilize relatively limited changes in envelope to balance the requirement to preserve the ability to infect a new host with the need to simultaneously evade polyclonal NAb responses present in maternal plasma. Although this escape pathway may also permit escape from broad MAbs in some cases, escape from maternal NAbs may be associated with increased sensitivity to these MAbs in other cases. Because the mothers studied here did not have evidence of a monoclonal response of known specificity, such as that of PG9 and VRC01, it remains possible that escape in the face of specific broad MAbs may involve a different pathway.

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