Defining neutralizing antibody specificities that target diverse HIV-1 transmitted strains

Leslie Goo

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Reading Committee:
Julie Overbaugh, Chair
Raymond Scott McClelland
Leonidas Stamatatos

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Abstract

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Leslie Goo

Chair of the Supervisory Committee:
Affiliate Professor Julie Overbaugh
Full Member, Division of Human Biology, Fred Hutchinson Cancer Research Center

Studies in animal models have shown that pre-existing neutralizing antibodies (NAbs), which target the human immunodeficiency virus type 1 (HIV-1) envelope (Env) to prevent entry into host cells, can block infection. A limitation of these models is that typically only one challenge virus is used. Given the diversity of HIV-1 in real world settings, a NAb-based vaccine must have broad and potent activity against multiple strains. This thesis describes NAb specificities that target diverse HIV-1 transmitted variants in natural infection within a high-risk population of exposed infants and women.

First, we investigated how transmitted viruses 'escape' NAbs by comparing neutralization-resistant viruses in infants to neutralization-sensitive maternal variants near the time of transmission in 2 mother-infant pairs. Although the molecular determinants of escape were distinct, NAb escape involved conformational masking of distal epitopes in both pairs. This strategy may allow HIV-1 Env to utilize limited changes to simultaneously evade multiple NAb specificities while preserving the ability to infect a new host.

Next, we determined the activity of 7 broadly neutralizing antibodies (bNAbs) against 45 heterosexually transmitted viruses obtained from Kenyan women. NIH45-46W, which targets

the CD4 binding site, was most broad, neutralizing 91% of viruses. Viruses resistant to NIH45-46W were neutralized by PGT128, which targets variable loop 3 of Env, suggesting that combining bNAbs with distinct specificities would provide optimal coverage of HIV-1 variants.

Finally, we explored the ability of the early immune system to develop bNAbs by screening 28 infant plasma samples against heterologous viruses. Remarkably, as early as 2.5 years of life and of HIV infection, some infants generated NAbs of similar breadth and potency to those found in adults identified as having very broad responses after approximately 5 years of infection. This finding implies that there is sufficient B-cell functionality in early life to generate broad and potent responses against HIV-1.

Overall, these studies suggest that for a preventative vaccine, bNAbs targeting distinct conserved epitopes may be required to overcome HIV-1 diversity, and that characterizing the early development of bNAbs in infants may provide insight into how to elicit such NAb responses.

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AIDS: acquired immunodeficiency syndrome

bNAb: broadly neutralizing antibody

CD4bs: CD4 binding site

CDR3: complementarity determining region

Env: envelope

HCDR3: heavy chain complementarity determining region 3

HIV: human immunodeficiency virus

IgG: immunoglobulin G

mAb: monoclonal antibody

MPER: membrane proximal external region of HIV-1 Env

MTCT: mother-to-child transmission

NAb: neutralizing antibody

V1/V2: variable regions 1 and 2 of HIV-1 Env

V3: variable region 3 of HIV-1 Env

VH: variable heavy chain

VL: variable light chain

SVL: set-point viral load

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Dedication

To my grandma, Anna Maria Setiawati (Njoo Siep Nio),

who eagerly anticipated the day I would become a ‘doctor’

R.I.P. October 5, 1921 – June 29, 2009

Chapter I

Introduction

Despite over 3 decades ago since its discovery as the pathogen that causes acquired immunodeficiency syndrome (AIDS) (13), the human immunodeficiency virus (HIV) remains a major global health problem. UNAIDS estimates that in 2012, there were 1.6 million deaths and 2.3 million new infections from HIV, while an estimated 35.3 million were living with HIV (226). The burden of HIV infection is disproportionately distributed, with 70% of all HIV infections occurring in sub-Saharan Africa, and approximately 50% of infections occurring in women. Mother-to-child transmission (MTCT) is also common, with 260,000 of children newly infected in 2012, 88% of whom live in sub-saharan Africa (226). Although there have been tremendous advances in treatment options, access and adherence to antiretroviral therapy in the regions most affected by HIV remain a challenge, highlighting the need for interventions to prevent HIV infection in the first place, such as the development of a vaccine.

HIV-1 origin and diversity

Developing an effective vaccine for HIV has been challenging due to the genetic diversity of the virus, which results from an error-prone viral polymerase, reverse transcriptase, (135), and from high rates of viral replication (81). HIV originated from multiple cross-species transmissions of simian immunodeficiency virus (SIV), leading to 2 different lineages of HIV: HIV-1 originated from SIV in chimpanzees (73), while HIV-2 originated from SIV in sooty mangabeys (116). Each lineage is further divided into groups, with HIV-1 group M being

responsible for the pandemic. Within HIV-1 group M, there is yet considerable diversity, resulting in 9 subtypes designated by A-D, F-H, and J- K, and a number of circulating recombinant forms. These subtypes differ in their geographical distribution (77), and can be distinguished by variation in the sequence of the envelope (Env) protein, which can differ by 8-17% at the amino acid level within subtypes, and by 17-35% between subtypes (186).

The enormous genetic diversity of HIV-1, particularly in Env, presents a significant challenge for a vaccine designed to target this protein. This may be best appreciated by comparing HIV-1 to influenza, another virus with extensive diversity for which a seasonal antibody-based vaccine exists. It is estimated that the diversity of worldwide influenza sequences within a year is comparable to the diversity of HIV found within an infected individual at one time point (107)

HIV-1 proteins

HIV-1 is a member of the family *Retroviridae* in the genus *Lentivirus*. The HIV-1 genome contains 2 copies of a single-stranded RNA genome that encodes for 9 proteins, 3 of which are structural and enzymatic proteins common to all retroviruses: Gag (group specific antigen), which mediates assembly and release of virus particles and encapsidates the viral RNA genome; Pol (polymerase) enzymes, including protease, reverse transcriptase, and integrase, which cleaves viral polyprotein precursors, converts the viral RNA genome to double-stranded DNA, and facilitates integration of viral DNA into the host chromosome, respectively; and Env, which mediates entry into host cells. Additionally, HIV-1 encodes regulatory proteins such as Tat (trans-activator of transcription), which potently stimulates transcription from the HIV-1 long terminal repeat, and Rev (regulator of virion), which mediates nuclear export of partially

spliced viral mRNAs, as well as accessory proteins such as Vif (viral infectivity factor), Vpr (viral protein R), Vpu (viral protein U), and Nef (negative factor). These accessory proteins are so called because they are dispensable for virus replication, at least *in vitro*, but contribute to efficient virus spread and disease induction (63). Of these proteins, Env is the main focus of this thesis and will be discussed in more detail.

HIV-1 Env: Genetic organization, structure, and function

Env is translated in the endoplasmic reticulum as a gp160 polyprotein precursor, which is then processed by cellular proteases in the Golgi, resulting in the surface gp120 subunit, and the gp41 transmembrane subunit (reviewed in (64)) (Figure 1.1A). Following cleavage, gp120 and gp41 remain non-covalently associated, and on the virion surface, Env exists as trimeric spikes of gp120 and gp41 heterodimers. As shown in Figure 1.1B, gp120 is organized into five conserved regions (C1-C5) and highly variable domains (V1-V5), while gp41 contains 6 major domains: the fusion peptide, heptad repeat regions 1 and 2, the membrane proximal external region (MPER), the transmembrane anchor, and the cytoplasmic tail. The conserved regions of gp120 are shielded by the variable loops (251), which are structurally defined by disulfide-linked loops, and are heavily glycosylated (16, 117)

In vivo, HIV-1 primarily infects CD4+ T-cells and cells of the monocyte/macrophage lineage. Viral entry into host cells is initiated by binding of gp120 to CD4, which triggers a conformational change in gp120 that enhances its affinity for its coreceptor (64). The most physiologically relevant HIV-1 coreceptors are CCR5 and CXCR4, members of the G protein-coupled receptor superfamily of seven-transmembrane domain proteins. Binding to the coreceptor triggers further conformational changes in both gp120 and gp41 that promote fusion of viral and host cell membranes, which is primarily mediated by the gp41 ectodomain (35, 237).

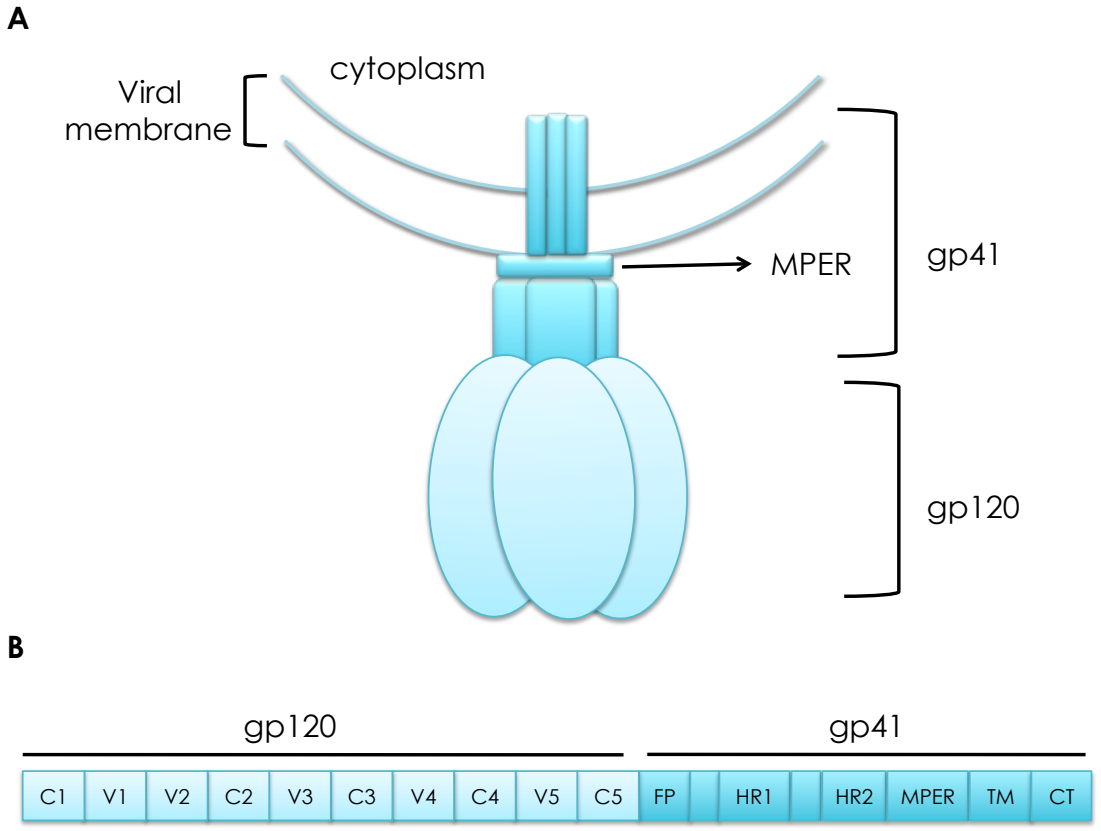

Figure 1.1. Structural and genetic organization of HIV-1 Env.
(A) Schematic of Env spike on the virion surface. Env is composed of 3 gp120 surface subunits (light blue) non-covalently linked to 3 gp41 transmembrane subunits (dark blue). The membrane proximal external region (MPER) of gp41 accessible to neutralizing antibodies is highlighted. **(B)** gp120 is organized into 5 constant (C1-C5) and 5 variable regions (V1-V5), while gp41 consists of 6 major domains: fusion peptide (FP), heptad repeat 1 (HR1), heptad repeat 2 (HR2), the membrane proximal external region (MPER), the transmembrane domain (TM), and the cytoplasmic tail (CT).

Development of antibodies against Env over the course of HIV-1 infection

Figure 1.2 depicts the typical course of HIV-1 infection and the kinetics of antibody development. After breaching the mucosal barrier, HIV-1 is thought to infect CD4+ T cells and to rapidly disseminate, initiating systemic infection (72). During acute infection, high replication rates result in a peak plasma viral load, usually around 21 days post-infection (122), and depletion of CD4+ T cells. After this initial burst in viral replication, there is a rapid decline of viral loads by several orders of magnitude to a steady state viral load set-point (70), which is an important predictor of disease progression to AIDS (144). This decline in viral load during acute infection is accompanied by a recovery of CD4+ T-cell numbers, which has been attributed primarily to the activity of cytotoxic T lymphocytes (21, 90, 108, 210). In adults, viral replication is typically maintained at this steady state for a period of approximately 10 years into chronic infection before there is widespread destruction of CD4+ T-cells resulting in immune suppression and the onset of AIDS (172).

HIV-1 infection results in the generation of antibodies to most viral proteins, but only antibodies to Env, the sole viral protein expressed on the surface, can mediate virus neutralization to block initial infection of target cells. Although binding antibodies to gp41 and variable regions of gp120 can be detected as early as 8 days after infection (151, 223), neutralizing antibodies (NAbs), which bind Env to abrogate entry, are detected only after 12-16 weeks of infection (45, 119, 150, 153). Although this initial wave of NAbs can reach high titers, they are usually highly strain-specific as they target exposed variable regions and drive escape mutations that allow HIV-1 to rapidly evade NAbs (4, 44, 183, 188, 236). However, in chronic infection, a subset of individuals develops broadly neutralizing antibodies (bNAbs), which are capable of recognizing diverse variants from multiple clades (54, 68, 145, 174, 201, 213). The

delay in the development of HIV-1 NAbs is partly attributed to HIV-induced B-cell dysfunction and loss of germinal centers in blood and gut-associated lymphoid tissue B-cells soon after HIV-1 infection (118). Additionally, bNAbs identified so far have unique characteristics (discussed later in this chapter) that may explain why they develop late and only in some individuals.

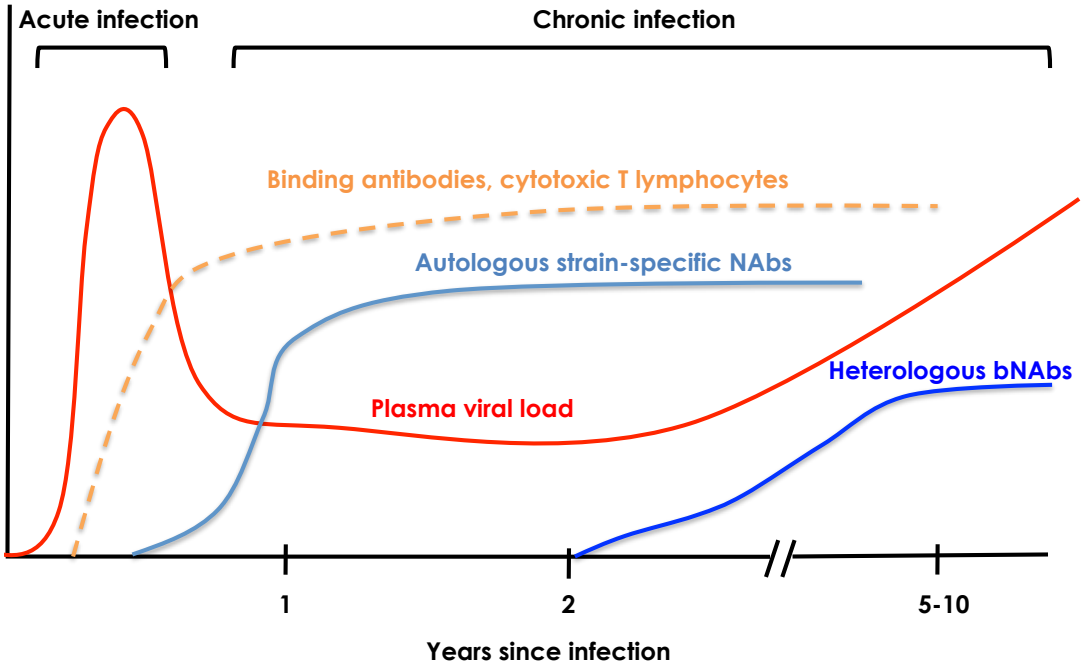

Figure 1.2. Development of adaptive immune responses during a typical course of HIV-1 infection in the absence of treatment.
Figure is adapted from (140). Acute infection is highlighted by high rates of viral replication (solid red line) leading to peak viremia. This is followed by a decrease in replication rates and stabilization to a viral set-point that is maintained at a steady state throughout chronic infection before rebounding to high levels at approximately 10 years after infection, resulting in drastic immune suppression leading to AIDS. Binding antibodies and cytotoxic T lymphocytes (dotted orange line) appear within a few days of acute infection. Almost all infected patients develop strain-specific autologous neutralizing antibodies (solid light blue line) appear after approximately 3 months of infection, while heterologous broadly neutralizing antibodies (bNAbs, solid dark blue line) develop much later, at approximately 2-4 years of infection in a subset of individuals.

Mechanisms of HIV-1 evasion from neutralizing antibodies

In general, the term 'neutralization' refers to the ability of antibodies to reduce the infectivity of a virus particle at one of several different steps in the virus life cycle (100). However, for studies described in this thesis, neutralization refers specifically to antibody-mediated inhibition of virus entry into target cells *in vitro*. Most neutralizing activity in HIV-infected individuals can be attributed to IgG antibodies (207, 223), which are Y-shaped proteins composed of 2 heavy and 2 light chains (Fig. 1.3a). Each heavy and light chain consists of a variable (V) region, which together form the antigen binding site (Fab), and a constant region (Fc), which provides structural support and mediates antibody effector functions. The light chains are bound to the heavy chains by non-covalent interactions and by disulfide bonds, and V regions of the heavy (VH) and light (VL) chains pair in each arm of the Y to generate 2 antigen binding sites.

In addition to extensive genetic heterogeneity, HIV-1 Env has evolved multiple strategies to evade NAb responses, as described below.

Env spike structure

As mentioned, the conserved regions of HIV-1 are effectively shielded from antibodies by highly variable loops (251). As a result, early NAbs are typically targeted to these variable regions, which accounts for the strain-specific activity of these NAbs, and for the ability to HIV-1 to readily escape these responses (44, 65, 153). Molecular mechanisms of NAb escape include amino acid substitutions, insertions, and deletions, either in the regions directly targeted by NAbs or in distal regions that mediate escape via conformational masking (156, 188, 219). The first and second variable loops (V1/V2), in particular, have been shown to be a major regulator of

neutralization sensitivity by shielding epitopes (34, 176, 187, 192, 217). More conserved regions on Env such as the CD4 binding site is occluded by conformational masking and steric hindrance. For example, analysis of the structure of CD4-bound gp120 reveals that the CD4 binding pocket is deeply recessed and flanked by glycosylated and highly disordered variable loops (112), imposing a conformational and energetic barrier on antibodies that bind this region (109).

Glycan shield and heterogeneity

Approximately 50% of the molecular weight of gp120 is composed of glycans (117). Because they are host-derived, these glycans provide HIV-1 with another mode of antibody evasion as they are generally non-immunogenic, although a number of bNAbs that interact with glycans have been identified (32, 225, 232, 233). Additionally, as a result of glycan processing in the Golgi, Env can be decorated with various glycoforms, which affect sensitivity to NAbs even within a given virus strain (52, 158). Finally, amino acid changes that insert, delete, or shift potential N-linked glycosylation sites in Env provide an 'evolving glycan shield' that confers NAb escape (198, 236).

Non-functional Env proteins

Because binding to functional Env trimer, but not to non-functional Env, correlates with neutralization (152), the ability of non-neutralizing antibodies to specifically capture HIV-1 *in vitro* (28, 177) suggests that not all Env proteins on the virion surface exist as functional trimers. Indeed, it has been shown that HIV-1 particles bear non-functional gp120/gp41 monomers and gp120-depleted gp41 stumps (152), implying that non-covalent interactions among Env protomers are unstable. Because as few as 4 functional Env trimers are sufficient to mediate

entry (215), the prevalence of non-functional Env proteins may serve to divert the antibody response, resulting in mostly non-neutralizing antibodies.

Low density of surface Env spikes

The above immune escape mechanisms are not unique to HIV-1, as they are employed by other viruses such as influenza (111). An additional immune evasion mechanism that may be unique to HIV is the low density of Env spikes, which could affect antibody avidity, defined as the ability of the antibody antigen binding sites to simultaneously bind two epitopes tethered on the same surface. Antibody avidity has been shown to enhance neutralizing potency in cases where cross-linking the surface of a virus is efficient (239, 245). However, HIV-1 has an average of only 14 spikes per virus particle (123, 261), in contrast to influenza type A virus, which incorporates ~450 spikes per virion (253). The low Env density on the HIV-1 surface may limit the ability of antibodies to bind Env proteins with high avidity and to achieve potent neutralization, as the majority of nearest neighboring distances of Env spikes are greater than the distance between the 2 IgG antigen binding sites (Fig. 1.3) (262). Interestingly, some bNAbs have been shown to apparently compensate for the lack of neighboring Env spikes by binding to an epitope on Env with one binding site, and to a non-viral specific epitope on the virus membrane with the other binding site to increase avidity (3, 159). However, such antibodies tend to be polyreactive, and are typically selected against by tolerance mechanisms (36, 230).

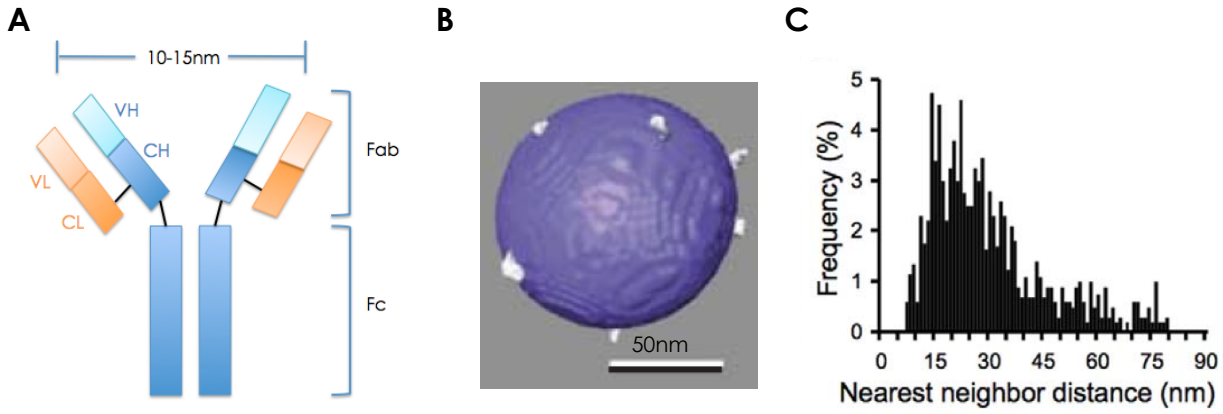

Figure 1.3. Antibody avidity against HIV-1 Env
(A) IgG antibody architecture. An IgG antibody is composed of 2 identical heavy (blue) and light (orange) chains. Each heavy and light chain consists of a variable (VH or VL) and constant (CH or CL) region. The antigen binding site is formed by VH and VL at the tip of the antigen binding fragment (Fab), while the constant fragment (Fc) mediates antibody effector functions. The 2 Fabs are linked to the Fc region by a flexible hinge, which typically allows a 15nm distance between the centers of the antigen binding sites. The 2 heavy chains are linked to each other and to a light chain by disulfide bonds (black lines). **(B)** HIV-1 Env spike density. Cryoelectron microscopy tomography (cryoET) of HIV-1 virion is shown. Image is taken from (262). **(C)** Distribution of nearest neighbor distances of virion surface Env spikes. Data were obtained from (262) and were based on cryoET analyses of 40 HIV-1 virions.

Broadly neutralizing antibodies against HIV-1

Despite the various antibody evasion mechanisms employed by HIV-1, a number of antibodies that can potently neutralize diverse HIV-1 strains have been identified during natural infection in some patients.

Factors associated with the development of bNAbs

Beginning at 2-4 years after HIV-1 infection, approximately 10-30% of individuals develop neutralizing antibodies that can neutralize heterologous viruses from multiple subtypes (54, 68, 145, 201, 213). The broad and potent neutralizing activity of these antibodies is mediated by either a single monoclonal response to a conserved epitope in some cases (209, 233, 247), or by a polyclonal response targeted against multiple epitopes in others (102, 157, 207). The development of these bNAbs has been positively associated with a number of factors, including viral load set-point (68, 174, 228), early envelope diversity (174), early CD4+ T cell decline (68, 228), time since infection (145, 201), and most recently, a highly functional subset of T-follicular helper cells(124). As most of the factors associated with the development of bNAbs are those that are usually also associated with disease progression (144, 197), it is unsurprising that bNAbs do not appear to provide clinical benefit for patients who develop them (68, 174, 228), perhaps because they develop too late in chronic infection (65). Rather, these observations suggest that high antigenic stimulation drives NAb breadth. In support of this, studies have shown that HIV-1 superinfection, defined as sequential infection with diverse strains from different source partners, is associated with the development of bNAbs (41, 154, 178).

Mechanisms of neutralization and epitope specificities of bNAbs

During the first 20 years of HIV-1 research, only a handful of bNAbs capable of neutralizing diverse HIV-1 strains were identified. These antibodies include b12, which targets the CD4 binding site (CD4bs) (30, 185); 2F5 (40, 160), 4E10 (218), and Z13 (264), which target the MPER; and 2G12, which target a glycan epitope on gp120 (32, 225). However, the recent development of high throughput single-cell methods that allow culturing of B-cells and amplification of B-cell receptors (208, 222, 233, 235, 247) has revolutionized our ability to isolate and characterize bNAbs. Indeed, since 2009, more than 20 additional monoclonal antibodies with broad and potent neutralizing activity have been isolated and characterized from HIV-1 infected individuals (42, 87, 209, 232, 233, 247, 249). These 'second generation' bNAbs are in some cases 10-100 fold more potent than the original prototype bNAbs (232). Moreover, as shown in Figure 1.4, these bNAbs define 4 main 'sites of vulnerability' on Env: the CD4bs on gp120, targeted by bNAbs such as VRC01 (247) and NIH45-46W (51); glycan-dependent epitopes on V1/V2 (PG9/16-like bNAbs) (233) or V3 (PGT-like bNAbs) (232) on gp120; and the MPER on gp41 (4E10/10E8-like bNAbs) (87).

A number of mechanisms by which these bNAbs block viral entry have been proposed. For example, monoclonal antibodies (mAbs) 2F5, 4E10, and b12 mediate irreversible neutralization by inducing gp120 shedding from gp41 (191), rendering Env non-functional. In contrast, the broad and potent neutralizing activity of VRC01, which targets the CD4bs, is due to partial mimicry of the interaction of CD4 with gp120, and precise recognition of the initial CD4 attachment site (120). Additionally, although members of the PGT family of NAbs target glycan-dependent epitopes on V3 that are distal to the CD4bs, they prevent conformational rearrangements in gp120 necessary for optimal CD4 binding by allosteric mechanisms (95).

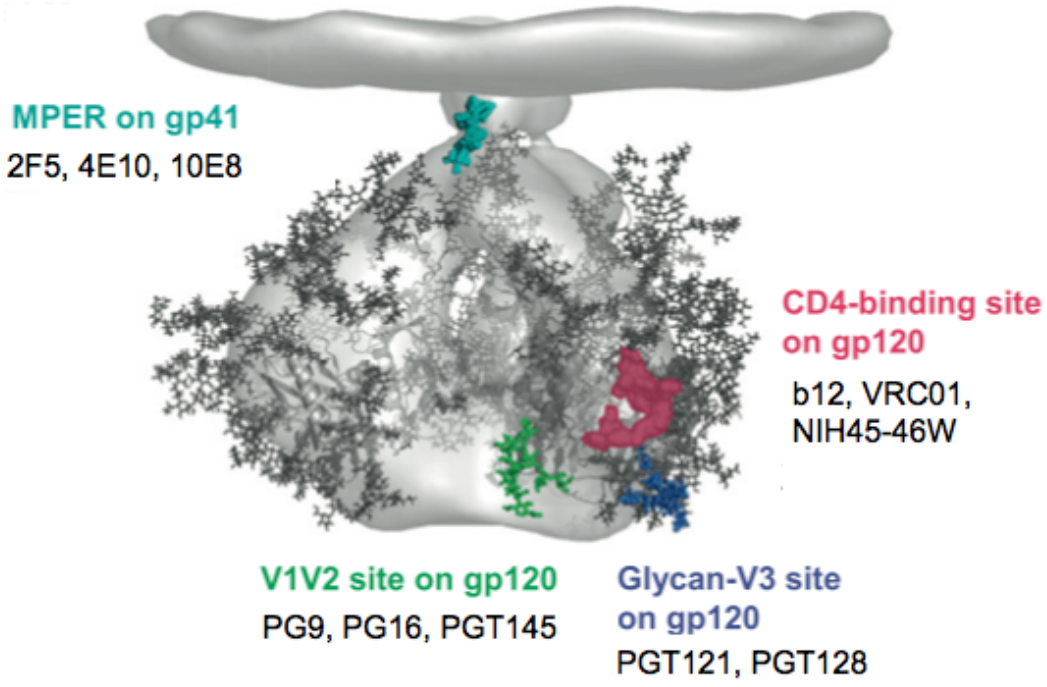

Figure 1.4 Epitope specificities of bNAbs.
Image is adapted from (110). A cryoelectron tomogram of the HIV-1 BaL isolate viral spike (123)is shown in gray, with 3 copies of gp120 core in the CD4-bound conformation (166). Glycans and epitopes of bNAbs are modeled, including CD4bs (red), N160 glycan in V1/V2 (green), N332 glycan in V3 (blue), and MPER of gp41 (cyan). bNAbs targeting each site of vulnerability that are relevant for this thesis are shown.

Antibody development and unusual properties of HIV-1 bNAbs

Unfortunately, almost all of the bNAbs identified to date have one or more unusual properties. In order to portray the atypical characteristics of HIV-1 bNAbs, it is useful to first describe the development of typical antibodies. Each B-cell has a unique receptor that is secreted to give rise to antibodies. As mentioned above, neutralization is primarily mediated by IgG antibodies, which consist of 2 heavy and 2 light chains (Fig. 1.3a). Multiples copies of three (V, D, J) or two gene segments (V, J) encode the variable (V) regions of heavy (VH) or light (VL) chains of IgG, respectively. Rearrangement and joining of one copy of each gene segment results in the generation of a complete germline V region. The antigen binding site of an

antibody is formed by the joining of the heavy and light V regions, and consists of six hypervariable loops or complementarity determining regions (CDR), 3 from each heavy and light chain, of which CDR3 of the heavy chain (HCDR3) makes the most contact with antigen and therefore tends to be most variable. The regions between the CDRs make up the rest of the V region are less variable and are termed framework regions. When a B-cell encounters an antigen, it is stimulated to undergo somatic hypermutation in germinal centers, and only B-cells whose receptors have mutations that increase the affinity for antigen are selected for proliferation.

Typically, high affinity binding to antigen is achieved after the accumulation of 10-15 mutations at the nucleotide level in the CDRs (149, 244). However, some HIV-1 bNAbs contain 40-100 somatic mutations that span not only the CDRs (209, 233, 247), but also the relatively constant framework regions (101). These extensive mutations appear to be necessary to improve from the weak gp120 binding of unmutated germline B-cell receptors to the high affinity recognition by mature antibodies (101, 159, 209, 258). The need for an accumulation of a large number of mutations to achieve affinity maturation may partly explain why bNAbs are delayed in development.

Additionally, some HIV-1 bNAbs have exceptionally long HCDR3 of 24-32 amino acid residues versus 12-13 residues for most human antibodies (39). For bNAbs that target V1/V2, this long HCDR3 may be required for penetrating the glycan shield to contact the underlying protein antigen (143, 171). Another example is b12, which has a long HCDR3 of 18 residues that may allow access to the recessed CD4 binding site on Env (168, 200, 265). In contrast to b12, VRC01, a bNAb that also targets the CD4bs has a more typical HCDR3 length of 12 residues. However, in addition to extensive somatic hypermutation, VRC01-like antibodies

originate from a restricted set of VH genes (209, 247, 249), perhaps due to the unique nature of contact sites encoded by these genes (238, 249, 258). Table 1.1 summarizes the unusual characteristics of bNAbs that are relevant for studies described in this thesis.

mAb	Specificity	Extensive somatic hypermutation	Long HCDR3	Auto/polyreactive	Reference
b12	CD4bs	No	Yes	Yes	(30, 185)
VRC01	CD4bs	Yes	No	No	(209, 247)
NIH45-46W	CD4bs	Yes	No	Yes	(51, 209)
PG9	V1/V2	No	Yes	No	(143, 233)
PG16	V1/V2	No	Yes	No	(233)
PGT145	V1/V2	No	Yes	No	(232)
PGT121	V3	Yes	Yes	No	(232)
PGT128	V3	Yes	No	No	(171, 232)
2F5	MPER	No	Yes	Yes	(40, 160)
4E10	MPER	No	Yes	Yes	(218)
10E8	MPER	Yes	Yes	No	(87)

Table 1. Characteristics of bNAbs relevant for this thesis

Challenges of eliciting bNAbs

Although the isolation of bNAbs provide proof of concept for the ability of the human immune system to generate potent NAbs against HIV-1, the observation that all known bNAbs have one or more unusual features suggest that elicitation of these bNAbs by vaccination may be challenging. For example, extensive somatic hypermutation, which may partly explain the delayed appearance of bNAbs, suggests that prolonged antigenic stimulation is likely required to achieve sufficient affinity maturation, thus presenting a hurdle for immunization strategies. Additionally, inducing antibodies with long HCDR3s may be challenging as these antibodies appear to originate from a rare set of germline gene segments (23). Moreover, because antibodies with long HCDR3 have been associated with self- and polyreactivity (89), tolerance mechanisms have been shown to select against these antibodies, further limiting the development of bNAbs (36, 230).

In addition to understanding how to stimulate B-cells to generate unusual antibodies, another challenge in HIV-1 vaccine design is the absence of an atomic level resolution structure of the native Env trimeric spike that would inform the design of an effective immunogen (86, 112, 123, 136, 137, 166, 240, 259). Notably, various recombinant forms of Env have been tested as immunogens in human clinical trials, but none has been successful at eliciting bNAbs, even when the epitopes of bNAbs are presented (140). One possible interpretation is that current immunogens differ substantially in epitope presentation relative to functional Env trimers.

To date, the RV144 trial conducted in 2009 is the only that has demonstrated a modest degree of short-lived protection in humans (181). The prime-boost regimen consisted of a recombinant canary pox vector encoding Gag, Pol, Nef and gp120 as the prime, and bivalent gp120 as the boost. Interestingly, this regimen had a 31% vaccine efficacy without inducing high NAb titers. Instead, non-neutralizing antibodies to V1/V2 of Env were found to be a correlate of protection (75), suggesting that non-neutralizing antibodies may be an important component of a protective vaccine. However, although non-neutralizing Fc-dependent effector functions have been shown to play a role in antibody protection against HIV (79), a recent passive immunization study comparing b12, a neutralizing antibody, and b6, a non-neutralizing antibody, both targeting the CD4bs, demonstrated that only the former was effective at mediating protection against vaginal infection (29). Moreover, studies in macaques evaluating the protective potential of non-neutralizing antibodies capable of mediating antibody-dependent cytolytic and non-cytolytic functions have provided inconsistent results (61, 62). These studies suggest that a protective HIV-1 vaccine will likely need to mediate broad and potent neutralization. The recent identification and isolation of numerous bNAbs has rejuvenated

optimism for the ability of these bNAbs to afford protection when present at the time of virus exposure.

The role of NAbs in preventing HIV-1 infection in experimental infection models

As mentioned above, HIV-1 bNAbs usually arise late in the course of infection and do not appear to provide clinical benefit. However, it is thought that they can afford protection from infection when present prior to viral exposure, as in the case of prophylactic vaccination. This hypothesis is supported by numerous passive immunization studies in non-human primate models, in which pre-existing NAbs protect against intravenous, vaginal, rectal, and oral transmission (7, 80, 83, 139, 141, 170). Importantly, a study by Hessell, *et al*. in macaques using repeated exposures to low doses of virus similar to those seen in human infection showed that relatively low amounts of mAb b12, which targets CD4bs, were sufficient to provide protection. Average b12 serum concentrations for challenges not resulting in infection were about 40μg/ml, corresponding to an average serum IC50 titer of 1:200 in *in vitro* neutralization assays (80), which is within the range of NAb levels elicited during natural infection. Given that the more recently isolated bNAbs are much more potent than b12, it is predicted that even lower concentrations of bNAbs may be sufficient for protection. Indeed, the average serum concentration at the time of challenge necessary for protection of 50% of macaques is 100-fold lower for PGT121 (1.8μg/ml), a more recently isolated bNAb, compared to b12 (200μg/ml) (148).

Although important in providing proof of concept for the ability of NAbs to protect against infection, experimental infection models in non-human primates are limited due to the fact that only one challenge virus that is typically highly neutralization sensitive (perhaps with one exception (11)) is used (7, 80, 83, 139, 141, 170). In contrast, human populations are

exposed to multiple diverse HIV-1 strains that exhibit a spectrum of neutralization sensitivities (17, 19, 211). In order to study the role of NAbs in limiting HIV-1 transmission in real world settings, natural infection models in humans, in which there is regular sampling to allow accurate estimation of the timing of infection, are ideal. The following section describes 2 such cohorts in Kenya.

The role of NAbs in limiting HIV-1 infection in natural infection models

Vertical versus heterosexual transmission of HIV-1

The cohorts described below represent 2 different settings of HIV-1 transmission: vertical and heterosexual. For both modes of transmission, only a subset of viruses from the diverse population present during chronic infection is transmitted (195). These heterosexually and vertically transmitted viruses often have unique properties compared to non-transmitted viruses, including shorter variable loops and fewer potential N-linked glycosylation sites (37, 47, 193, 246). Additionally, some studies have found that vertically transmitted viruses tend to be poorly recognized by maternal NAbs compared to non-transmitted viruses (48, 105, 114, 204, 246), suggesting that maternal NAbs select for 'escape' variants to be transmitted. In contrast, heterosexually transmitted viruses appear to be those that are more neutralization-sensitive to antibodies in the transmitting donor compared to non-transmitted viruses (47). One possible explanation for this difference is that unlike the setting of MTCT, in which passive antibodies are present in infants, heterosexual transmission usually occurs in the absence of pre-existing NAbs in the exposed person, except in cases of superinfection. Thus, it is possible that additional selective pressures other than NAbs may be important in the context of heterosexual transmission. The availability of cohorts at risk for heterosexual and vertical transmission of

HIV-1 described below provides a unique opportunity to explore antigenic properties of viruses from different settings.

The Nairobi Breastfeeding Trial: Mother-to-child transmission (MTCT) of HIV-1

In the absence of interventions such as antiretroviral prophylaxis, 30-40% of HIV-1 infected mothers transmit the virus to their infants either in-utero, intra-partum, or via breastfeeding (43, 57, 161). MTCT has been associated with maternal virologic, clinical, and genetic factors such as high maternal plasma (49, 216), genital (38, 91), and breastmilk (189, 190) viral loads; low CD4+ T cell counts (146); co-infections (25); maternal-infant human leukocyte antigen (HLA) concordance (134); and certain HLA alleles (133). However, the role of immune factors in MTCT has been less defined. HIV-1 MTCT provides a unique setting in which to study the role of NAbs in limiting transmission or a number of reasons. First, the index and exposed individuals are known, allowing the comparison of transmitted and non-transmitted viruses. Second, antibodies are passively transferred from mother to infant, mimicking the scenario of immunization, in which antibodies are present at the time of virus exposure. Finally, with regular follow-up and sampling, the timing of transmission can be accurately estimated, enabling the identification of potential correlates of risk or protection by studying antibodies and viruses near the time of transmission.

The Nairobi Breastfeeding Trial (161), which was conducted in 1992-1998, provides a valuable resource to study whether NAbs reduce MTCT risk. This trial was conducted to determine the timing and risk of HIV-1 breastmilk transmission, and involved longitudinal follow-up of 425 HIV-1 infected mothers and their infants randomized to either a breastfeeding or formula feeding arm starting at the mother's third trimester of pregnancy until the infant's first

2 years of life. Maternal blood and breastmilk samples and infant blood samples were collected within the first week post-delivery, at 6 weeks, 14 weeks, 6 months, and quarterly thereafter up to 2 years. None of the mothers received antiretroviral therapy, which was not the standard of care for prevention of MTCT at the time the trial was conducted (1992-1998). Subtype A (70.3%) was most common among mothers in this cohort, followed by subtype D (20.5%), subtype C (6.9%), intersubtype recombinants (2.2%), and subtype G (0.3%) (162). As determined by HIV-1 specific PCR, a total of 92 infants were infected, 11 of whom were infected at birth, 39 by week 6, 62 by month 6, 74 by month 12, and 92 by month 24. In this study, breastfeeding nearly doubled the risk of HIV-1 MTCT, with 20.5% of infants infected in the formula feeding group, versus 36.7% in the breastfeeding group (161). Seventy five percent of HIV-1 breastfeeding transmission occurred by 6 months of life.

By studying viruses and antibodies near the time of transmission from 12 mother-infant pairs in this cohort, Wu, *et al*. demonstrated that viruses transmitted to infants were significantly less sensitive to neutralization by maternal antibodies compared to non-transmitted viruses in the mothers (246). This suggests that maternal NAbs may contribute to the genetic bottleneck observed during MTCT (1, 113, 205, 229, 243) by limiting the transmission of neutralization-sensitive variants and selecting for NAb escape variants for transmission. However, it is unclear what epitopes are targeted in the neutralization-sensitive variants to limit their transmission. Conversely, the molecular determinants of NAb escape during MTCT are unknown.

The Mombasa Cohort: Heterosexual transmission of HIV-1

The Mombasa Cohort is an open longitudinal cohort that was established in 1993 to investigate the virological and immunological characteristics of early heterosexual infection

among commercial female sex workers who attend the Ganjoni Municipal Clinic in Mombasa, Kenya (138). Women who visit the clinic are offered confidential HIV counseling and testing, and are screened for HIV infection. HIV seronegative women are invited to enroll in the cohort after written informed consent and are asked to visit the clinic monthly for HIV serological testing. Between February 1993 and December 2008, 7943 women were screened for HIV, of whom 3818 (48%) were seronegative. Of these seronegative women, approximately 2000 joined the cohort and over 300 have seroconverted. Following seroconversion, women visit the clinic monthly, with blood samples collected every 3 months. The median follow-up time as of 2008, when these thesis studies were initiated, was 4.4 years post-infection. HIV infected women in the clinic are offered a routine package of HIV care at no cost. Plasma viral load is determined by Gen-Probe (58), and as of 1998, CD4 counts are measured. Beginning in 2004, HIV positive women receive antiretroviral therapy if they qualify based on the Kenyan National Guidelines for ART (currently CD4<350 or and AIDS-defining illness).

As with the Nairobi Breastfeeding Trial, the Mombasa cohort is a valuable resource for studying immune correlates of protection from HIV-1 infection because of regular sampling to facilitate accurate estimation of HIV-1 infection and detailed analysis of the dynamics of viral and host immune factors throughout infection. Several studies investigating the association between NAbs and risk of heterosexual infection have been performed using this cohort. For example, to test the hypothesis that deficits in the NAb response predisposes to HIV-1 infection, Blish, *et al*. compared NAb breadth against heterologous viruses among women who became superinfected (those who became re-infected by a second HIV-1 strain from a different source partner) to a control group of women who were singly infected with HIV-1. Superinfected cases and controls were matched according to the initial HIV subtype, the timing of plasma samples

according to initial infection, and viral load. Surprisingly, there was no difference in NAb breadth or potency between superinfected cases and non-superinfected controls (18) prior to the timing of superinfection, again suggesting that the breadth and potency of NAb responses elicited during natural infection may not be sufficient to prevent infection. Interestingly, a recent study by Cortez, *et al*. compared NAb responses of superinfected cases and controls *after* superinfection, and found that superinfection broadened and strengthened the HIV-specific NAb response (41), supporting the hypothesis that high antigenic stimulation drives NAb breadth.

The accurate estimation of the timing of infection for most women in this cohort also enables the isolation and characterization of transmitted viruses. Approximately 45 functional full-length envelope sequences representing subtypes A, C, and D have been isolated and characterized from 22 women in the cohort early in their infection. These transmitted viruses display a range of neutralization sensitivities to monoclonal antibodies and pooled plasma from HIV infected individuals (17, 19), highlighting the need to assess NAb responses against more than just one virus to determine their potential for inclusion in a protective vaccine. As current bNAbs target distinct specificities, investigating their neutralization profiles against these recently transmitted viruses will allow the identification of NAb specificities that are most effective against the strains of HIV-1 that are circulating in the most affected populations.

Goals of this thesis

Using the longitudinal cohorts described above, my overall goal was to investigate NAb specificities that impact heterosexual and vertical transmission of HIV-1. First, I describe the molecular determinants of NAb escape in viruses transmitted to infants to identify potential maternal NAb specificities that may limit MTCT (Chapter II). Next, in Chapter III, I investigate which bNAb specificities provide the best coverage of a multi-subtype panel of heterosexually transmitted viruses from the Mombasa cohort. In Chapter IV, I explore the ability of infants to develop broad and potent NAb responses, which have so far only been described in adults. Finally, in Chapter V, I will discuss the implications of this work for NAb-based HIV-1 vaccine design.

Chapter II

Neutralizing antibody escape during HIV-1 mother-to-child transmission involves conformational masking of distal epitopes

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Introduction

As mentioned in Chapter I, 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 a HIV-1 transmission bottleneck (243); despite a heterogeneous population in the chronically infected mother, only one variant is typically transmitted to the infant (1, 113, 193, 205, 229, 246, 256). These studies suggest that variants with certain properties may be selected during transmission, and similar findings have been observed in cases of heterosexual transmission (195). In support of this, variants that are transmitted from mother to child have been found to possess fewer potential N-linked

glycosylation sites compared to variants found in the index case in some studies (193, 246). Vertically transmitted viruses also have been reported to have enhanced replication kinetics (105) and fitness (106) compared to non-transmitted 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 NAb titers against autologous viruses compared to non-transmitting mothers (48, 105, 114, 204), although not all studies have shown this association (71, 78, 88). Some of the differences in these findings could reflect inconsistencies in sampling viruses and antibodies near the window of transmission (Omenda, Overbaugh, manuscript in preparation). 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 (31).

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 compared to matched variants found in the infecting mother (48, 246, 257). This has not been observed in all studies (97, 193, 221) and it is unclear if these different findings represent methodological differences of the type 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 (246). 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 2-20 weeks of infection and these NAb responses can ultimately reach high titers, exerting selective pressure on Env and resulting in neutralization escape (4, 69, 119, 183, 236). NAb escape within an infected individual has been shown to involve multiple pathways, including an evolving "glycan shield" (236), insertions and deletions, and amino acid substitutions in Env (156, 188, 219). NAb escape during intrapatient evolution of the virus has also been shown to involve different domains of Env, including V1/V2, C3-V4, and V5 of the gp120 surface subunit, as well as the ectodomain of the gp41 transmembrane subunit (156, 188, 219). It is not known whether these similar domains and pathways are involved during NAb escape in the context of transmission, where 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 (69, 119, 153). NAb responses often broaden over time (145, 154, 201), and recognize new epitopes (156, 188), 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.

Here, 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.

Materials and methods

Study population

Mother-infant pairs were participants of the Nairobi Breastfeeding Trial conducted in Nairobi, Kenya in 1992-1998 (161). Infants' HIV infection status was monitored by PCR on PBMC or dried blood spots (167). An infant was defined as HIV infected if there were two consecutive HIV-1 DNA positive PCR results. The date of infection was estimated to be the time of first PCR positive test. If 2 consecutive PCR positive results were unavailable, an infant was considered HIV-1 positive if a single blood sample gave a positive result and there were no follow-up samples, or if there was a HIV-1 positive serum antibody result at the last visit.

Amplification and cloning of HIV-1 *env* genes

HIV-1 *env* clones were obtained from 2 mother-infant pairs, as previously described (179, 246). In Figure 2.1, BS208m6bmc.B1 corresponds to S208 B^{res}; MS208w6bmc.B1 (GenBank: DQ187009) to S208 M^{sens1}; MS208w6bmc.C1 (GenBank: DQ187014) to S208 M^{sens2}; BF535.w0m.A1 (GenBank: DQ208431) to F535 B^{res}; and F535 MF535.w0m.B1 (GenBank: DQ208425) to F535 M^{sens}. Maternal and infant envelope variants shown in Figure 2.1 were representative of the diversity of the virus population found in mothers and infants, as determined by phylogenetic analysis (179). Mother-infant chimeric and mutant *env* genes shown in Figure 3 were created by overlap PCR on 10ng starting template using TaqPlus Precision PCR system (Stratagene, La Jolla, CA). Overlapping PCR fragments were digested with Dpn1 for 1

hr at 37°C and then mixed (0.5ul 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 30s, 55°C for 30s, 68°C for 4min, and 1 cycle of 68°C for 10min. S208 full-length *env* second round PCR products were amplified by vpr11 + nef30 primers, and were cloned into pCI-Neo. Full-length *env* second round PCR products for F535 were amplified by either vpr11 + nef30 or vpr11 + nef24 primers, and were cloned into pCI-Neo or pCDNA3.1/V5-His-TOPO, respectively. Primers used for amplification are listed in Tables 2.1-2.3. Full-length envelope chimeras were verified by sequencing the entire region amplified (Big Dye; Applied Biosystems) to ensure 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. Sworfford, 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 co-transfected with an *env*-deficient subtype A proviral plasmid (Q23Δenv; (125)) at a 1:2 mass ratio into 2 x

10^6 293T cells plated in a T-75 flask 24h prior to transfection. For each transfection, 4ug total DNA was mixed with 12ul Fugene6 (Roche). To screen envelope chimeras for biological function, transfection supernatant from 48 hours post-transfection was sterile-filtered through a 0.2μm filter and used to infect TZM-bl cells in Dulbecco's modified Eagle medium 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 hours post-infection after staining fixed cells for ß-galactosidase activity (246).

Neutralization assay

Approximately 500 infectious pseudovirus particles as determined by infecting TZM-bl cells were diluted to a volume of 25μl 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 ($1x10^4$ in 100μl DMEM) were then added to each well. At 48h post-infection, 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 (IC50) was determined from a dose-response curve after log-transformation of plasma dilution or monoclonal antibody/sCD4 concentration (246). Neutralization profiles of all pseudoviruses with chimeric envelopes were assessed in at least 2 independent experiments. IC50 values presented represent the average of these experiments.

Monoclonal antibodies used were b12, 2F5, 4E10 (Polymun), PG9, PGT121, PGT 128, PGT 145 (kindly provided by the IAVI Neutralizing Antibody Consortium), and VRC01 (kindly provided by X. Wu and J. Mascola, NIH 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 (Cat # 4983; NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH) was assessed by adding serial dilutions of the inhibitor at a starting concentration of 1μM to TZM-bl cells for 1hr at 37°C prior to the addition of pseudovirus.

For peptide competition neutralization experiments, 25 μl peptides (GenScript) were 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. (145). The following peptides were tested: S208 M^{sens1} V4 (FSSTQESSDPITLP), S208 M^{sens2} HR2 (EISKYSDTIYNLLEDTQNQ), F535 M^{sens} V1 (V1_1: VTLNCTEASINNATV, V1_2: NNATVNGTSDQNVTV, and V1_3: QNVTVTTTSMEMK 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 (EQDLLALDKWANLWN). Competition of plasma antibodies by peptides was determined by calculating the area under the curve (AUC) in the presence or absence of peptide using GraphPad Prism 5.0 (San Francisco, CA). The percentage 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. (247), with slight modifications. Briefly, a final concentration of 25 μg/ml of wildtype

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 percentage 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 (Cat # 12042) and RSC3Δ371I (Cat # 12043) were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Drs. Zhi-Yong Yang, Peter Kwong, and Gary Nabel.

Western blotting of pseudoviral envelopes

Western blotting was performed on cell-free virus supernatants as described (126), using rabbit polyclonal antisera to HIV-1 envelope (55) and mouse-anti-p24 as primary antibodies (Cat # 4121; NIH ARRP), 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.

S208 B^{res} and M^{sens1} chimeras			
gp120			
First round	F	S208_C5cons_F	caaggagaagagtggtggagag
First round	R	S208_C5cons_R	ctctccaccactcttctccttg
Second round	F	vpr11	atactaagacgcgtgaagcacccgggaagtcagcct
Second round	R	nef30	atattcttgcggccgcgtctcgagatactgctcc
V1			
First round	F	S208usV1_F	cctctctgcgttactttagattgtag
First round	R	S208usV1_R	ctacaatctaaagtaacgcagagagg
First round	F	S208C2_F	ggagttcagtggaacagggcc
First round	R	S208C2_R	ggccctgttccactgaactcc
Second round	F	vpr11	atactaagacgcgtgaagcacccgggaagtcagcct
Second round	R	nef30	atattcttgcggccgcgtctcgagatactgctcc
V4			
First round	F	MB1V4_F	cctataactctcccatgcagaataaagc
First round	R	MB1V4_R	gctttattctgcatgggagagttatagg
Second round	F	vpr11	atactaagacgcgtgaagcacccgggaagtcagcct
Second round	R	nef30	atattcttgcggccgcgtctcgagatactgctcc
S208 B^{res} and M^{sens2} chimeras			
gp120		**Primer name**	**5' to 3' sequence**
First round	F	S208_C5_F	tgaaccactaggagtagc
First round	R	S208_C5_R	gctactcctagtggttca
Second round	F	vpr11	atactaagacgcgtgaagcacccgggaagtcagcct
Second round	R	nef30	atattcttgcggccgcgtctcgagatactgctcc
C4-CT			
First round	F	S208_V4_F	gaggagaatttttctattgtaa
First round	R	S208_V4_R	ttacaatagaaaaattctcctc
Second round	F	vpr11	atactaagacgcgtgaagcacccgggaagtcagcct
Second round	R	nef30	atattcttgcggccgcgtctcgagatactgctcc
C4-HR2		Template: C4V5HR2	
First round	F	S208_dsC4_F	ggaataatattaacaagagatggtgg
First round	R	S208_dsC4_R	ccaccatctcttgttaatattattcc
First round	F	S208_dsHR1_F	ccactaatgtgccttggaactccag
First round	R	S208_dsHR1_R	ctggagttccaaggcacattagtgg
Second round	F	vpr11	atactaagacgcgtgaagcacccgggaagtcagcct
Second round	R	nef30	atattcttgcggccgcgtctcgagatactgctcc
HR2		Template: C4V5HR2	
First round	F	S208_dsHR1_F	ccactaatgtgccttggaactccag
First round	R	S208_dsHR1_R	ctggagttccaaggcacattagtgg
Second round	F	vpr11	atactaagacgcgtgaagcacccgggaagtcagcct
Second round	R	nef30	atattcttgcggccgcgtctcgagatactgctcc

Table 2.1. Primers used to generate S208 envelope chimeras.
All first round products were amplified with vpr11 and nef30 at the flanking 5' and 3' ends, respectively.

S208 M^{sens1} and M^{sens2} N160K			
First round	F	MS208_N160K_F1	ctgctctttcaagatgaccacacagaac
First round	R	MS208_N160K_R1	gttctgtgtggtcatcttgaaagagcag
Second round	F	vpr11	atactaagacgcgtgaagcacccgggaagtcagcct
Second round	R	nef30	atattcttgcggccgcgtctcgagatactgctcc
S208 M^{sens1} N332A			
First round	F	MS208B1_N332A_F1	gcacattgtgctgtcagtgg
First round	R	MS208B1_N332A_R1	ccactgacagcacaatgtgc
Second round	F	vpr11	atactaagacgcgtgaagcacccgggaagtcagcct
Second round	R	nef30	atattcttgcggccgcgtctcgagatactgctcc
S208 M^{sens2} N332A			
First round	F	MS208C1_N332A_F1	gcacattgtgctgtcagtag
First round	R	MS208C1_N332A_R1	ctactgacagcacaatgtgc
Second round	F	vpr11	atactaagacgcgtgaagcacccgggaagtcagcct
Second round	R	nef30	atattcttgcggccgcgtctcgagatactgctcc

Table 2.2. Primers used to generate N160K and N332A mutants in S208 M^{sens1} and M^{sens2}
All first round products were amplified with vpr11 and nef30 at the flanking 5' and 3' ends, respectively.

F535 V1-V3 chimeras			
First round	F	F535usV1_F	gtaaccccactctgtgtcac
First round	R	F535usV1_R	gtgacacagagtggggttac
First round	F	MF535B1C3part1_F1	gtcttatatctcctattatgtctgctg
First round	R	MF535B1C3part_R1	gtcttatatctcctattatgtctgctg
Second round	F	vpr11	atactaagacgcgtgaagcacccgggaagtcagcct
Second round	R	nef30 for M^{sens}	atattcttgcggccgcgtctcgagatactgctcc
Second round	R	nef24 for B^{res}	tacttgtgattgctccatgt
F535 V1/V2 chimeras			
First round	F	F535_C2_F	ccagctggatttgcaattc
First round	R	F535_C2_R	gaattgcaaatccagctgg
Second round	F	vpr11	atactaagacgcgtgaagcacccgggaagtcagcct
Second round	R	nef30 for M^{sens}	atattcttgcggccgcgtctcgagatactgctcc
Second round	R	nef24 for B^{res}	tacttgtgattgctccatgt
F535 V3 chimeras			
First round	F	F535V3us_F	gcaaaaaccataatagtacagcttaatgag
First round	R	F535V3us_R	ctcattaagctgtactattatggtttttgc
Second round	F	vpr11	atactaagacgcgtgaagcacccgggaagtcagcct
Second round	R	nef30 for M^{sens}	atattcttgcggccgcgtctcgagatactgctcc
Second round	R	nef24 for B^{res}	tacttgtgattgctccatgt

Table 2.3. Primers used to generate F535 envelope chimeras.
All first round products were amplified with vpr11 and nef30 for M^{sens}, or vpr11 and nef24 for B^{res} at the flanking 5' and 3' ends, 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 (161), we showed that variants transmitted to infants were overall less sensitive to neutralization by maternal plasma compared to variants found in the mothers near the time of transmission (246). 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, (246)). Infants from both pairs were HIV-1 negative at birth and breastfed. In the case of S208, the infant tested negative at 3 months post delivery, but positive at the next time point tested (6 months post delivery), strongly suggesting breastmilk transmission. The infant from pair F535 was positive at 6 weeks post delivery, and was thus likely infected either via breastfeeding 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 Figure 2.1A. 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 potency (average IC50 of 470 and 493 for S208 and F535, respectively; data not shown).

A

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

B

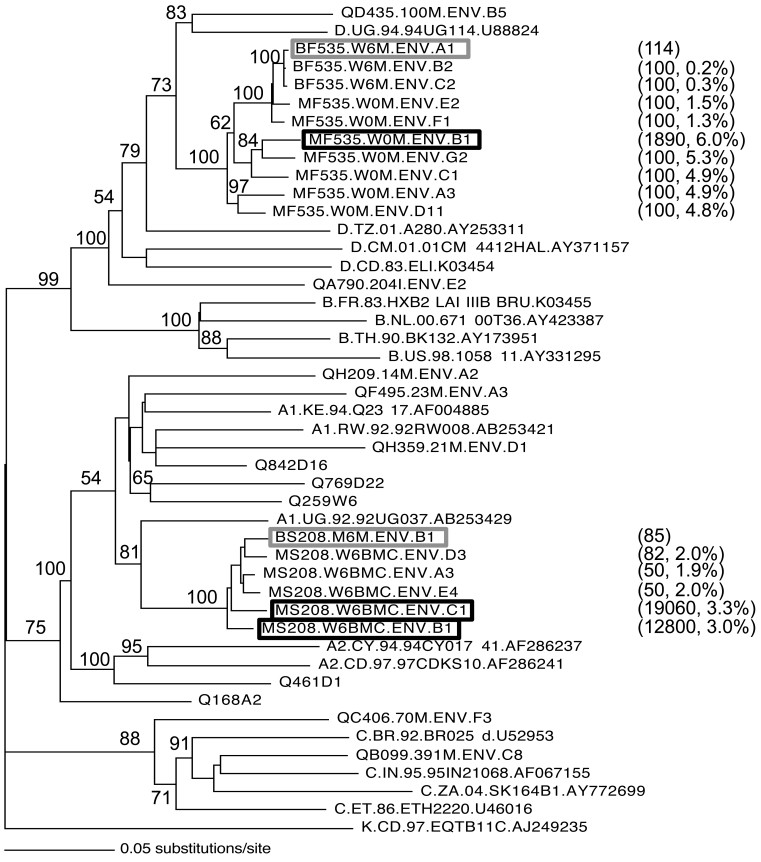


Figure 2.1. Mother-infant pairs selected for NAb epitope mapping.
(A) Summary of number and timing of isolation of envelope clones for mother-infant pairs. [a]Time since delivery. N/A, not applicable. **(B)** Neighbor-joining tree based on pairwise distance of full-length envelope sequences from S208 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 grey boxes, respectively. IC50 values are indicated in parentheses to the right of sequence names. Pairwise distances in envelope of all maternal and baby variants compared to baby variants from the same mother-infant pair analyzed in this study are indicated next to IC50 values.

Figure 2.1B shows a neighbor-joining tree based on full-length *env* sequences of maternal and infant variants, and a summary of their IC50 values against maternal plasma. As previously described, S208 and F535 were infected with subtypes A and A/D, respectively (246). For S208, 2 of the 5 maternal variants isolated were over 100-fold more sensitive to neutralization by maternal plasma compared to 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 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 (185), and 2F5 and 4E10, which target linear regions in the membrane proximal external region (MPER) of the gp41 ectodomain (160, 214, 263, 264). S208 and F535 baby variants were resistant or only moderately sensitive to neutralization by b12, 2F5, and 4E10, with IC50 values ranging from 2 - >25 μg/ml (Fig. 2.2A). Maternal variants were overall more sensitive to neutralization by these mAbs, with IC50 values

less than 2ug/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 similar neutralization profile to S208 B^{res} against these mAbs. mAb 2G12, which targets a cluster of glycans (203, 225), failed to neutralize all maternal and infant variants (data not shown; (246)).

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 (247), and mAbs PG9, PGT121, PGT128, and PGT145, all of which target glycan-dependent epitopes (143, 171, 232, 233). We found that these mAbs displayed varying potencies and breadth against S208 and F535 maternal and infant variants (Fig. 2.2A). VRC01, PG9, and PGT145 were able to potently neutralize both S208 maternal and infant variants (IC50 0.03 - 0.5ug/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 (1μg/ml). Interestingly, B^{res}, but not M^{sens}, was sensitive to VRC01, PG9, and PGT128 (IC50 0.001 – 0.1ug/ml). The neutralization profiles of F535 variants against VRC01, PG9, and PGT128 were opposite to 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 (6) (Fig. 2.2A). 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 (IC50 0.1μg/ml vs. 0.005μg/ml and 0.01μg/ml). However, for F535, the infant variant was >50-fold more sensitive to inhibition by TAK-779 than was the maternal variant (IC50 0.001μg/ml vs. 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 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 residue N332 required for PGT121 and PGT128 recognition (171, 232) was shifted to position 334 (Fig. 2.2B). 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 (143, 233), 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.

A

		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 PGT145			PGT128 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

Figure 2.2. Neutralization profiles of S208 and F535 variants against a panel of mAbs and entry inhibitors.
(A) IC50 values 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 25μg/ml. mAbs VRC01, PG9, PGT121, PGT128, and PGT145 were tested at a starting concentration of 1μg/ml. TAK-779 was tested at a starting concentration of 1μM. Grey 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; shift from position 332 to 334; dot, conserved amino acid; *, K168 is present in variants shown.

NAb escape in mother-infant pair S208 involves V4 and HR2

Figure 2.3A 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 differences that were only observed 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 in 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} (IC50 85, Fig. 2.3B) relative to M^{sens1}, which was highly sensitive to neutralization by maternal plasma (IC50 12800). Figure 2.3B summarizes the IC50 values of chimeras used to fine map the region that conferred NAb resistance, while Figure 2.3C shows neutralization curves of representative viruses from Figure 2.3B. 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 (IC50 100), while the chimera containing gp41 of B^{res} remained sensitive (IC50 7212). This suggested that the determinants of resistance mapped to gp120 of B^{res}. Because transmitted variants tend to have shorter variable loops (37, 47, 193, 256), and since V1/V2 has been shown to regulate sensitivity to neutralization (156, 176, 187, 188), 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 (IC50 480), suggesting V1 may be one determinant of

resistance, but it was not the only region contributing to the resistant phenotype of B^{res}. Introducing V1 of M^{sens1} into B^{res} resulted in a modest increase of neutralization sensitivity (IC50 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. 2.3A), and found that this insertion, when introduced into the maternal envelope, significantly decreased neutralization sensitivity and resulted in a virus with a similar neutralization profile to B^{res} (IC50 92, Fig. 2.3B-C). The reciprocal chimera, in which V4 of B^{res} was replaced with that of M^{sens1} displayed an intermediate neutralization profile (IC50 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} (IC50 19060, Fig. 2.3D). 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 IC50 values 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 Figure 2.3E. 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 (IC50 51200), 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 (IC50 17360), while the reciprocal chimera remained neutralization resistant (IC50 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 (IC50 100), whereas the reciprocal chimera partially restored neutralization sensitivity (IC50 1550). 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 2.3A), were sufficient to confer neutralization resistance to maternal plasma (IC50 58, Fig 2.3D-E). Replacing HR2 of B^{res} with that of M^{sens2} did not restore neutralization sensitivity (IC50 50, Fig 2.3D-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 resistant phenotype of B^{res} (data not shown).

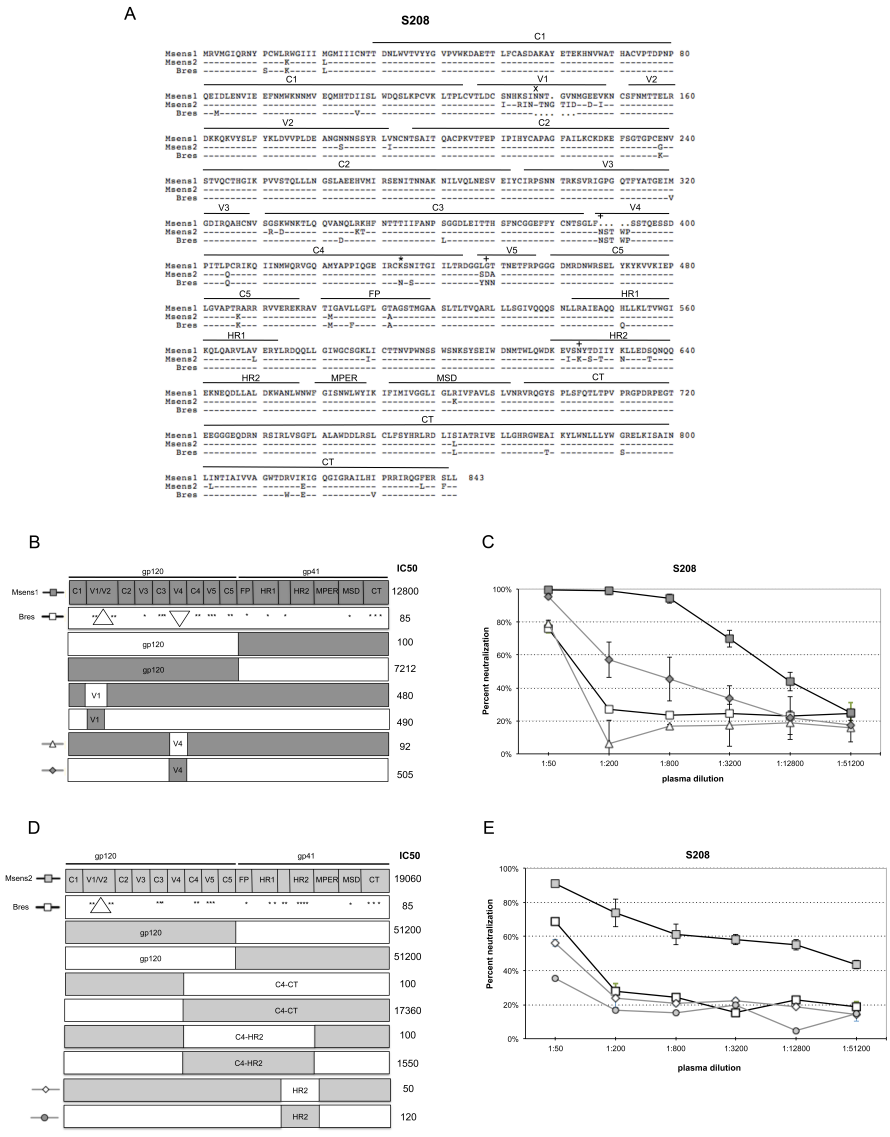


Figure 2.3. Determinants of NAb escape for S208.
(A) Amino acid alignment of envelope region of S208 variants. The sequence for S208 M^{sens1} is shown. Symbols: Dashes, amino acids similar to S208 M^{sens1}; dots, deletions; x, loss of PNGS in Bres; +, gain of PNGS in B^{res}. Modifications in PNGS highlighted are relative to one or both of sensitive maternal variants. Constant (C1-C5) and variable regions (V1-V5) of the surface gp120 subunit of the envelope are indicated. For gp41, FP=fusion peptide, HR1=heptad region 1, HR2=heptad region 2, MPER = membrane proximal external region, MSD = membrane spanning domain, CT = cytoplasmic tail. Neutralization profiles of S208 pseudoviruses bearing chimeric envelopes from **(B)** B^{res} and M^{sens1} or **(D)** Bres and M^{sens2} against maternal plasma. Average IC50 values from at least 2 independent experiments are shown next to bars representing chimeric envelopes. Grey bars represent envelope sequence of Msens1 or Msens2. White bars represent envelope sequence of B^{res}. Asterisks denote regions in B^{res} with amino acid differences relative to maternal envelope. Right side up triangle represents deletion in Bres. Inverted triangle represents insertion in B^{res}. Neutralization curves of chimeric viruses bearing regions found to confer resistance to maternal plasma (V4 in **(C)** and HR2 in **(E)** are shown relative to native maternal and infant viruses. Percent neutralization vs. plasma dilution is shown.

V1-V3 mediates NAb escape in mother-infant pair F535

Figure 2.4A shows the Env amino acid differences between sensitive maternal and resistant infant variants for F535. The resistant baby variant, B^{res} (IC50 114, Fig 2.4B) contained deletions in V1/V2 relative to the sensitive maternal variant, M^{sens} (IC50 1890), 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 that of native B^{res} and M^{sens} (IC50 700-800), indicating that while V1/V2 was a determinant of differences in neutralization sensitivity, other regions were required for NAb escape. We next introduced V1-V3 of B^{res} into the M^{sens} envelope and found that this chimera was resistant to neutralization by maternal plasma (IC50 121, Fig. 2.4B-C). Furthermore, we found that V3 of B^{res} alone (IC50 430, Fig. 2.4B) 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-V3 of B^{res} was required to drive escape from F535 maternal NAbs. Replacing V1-V3 of B^{res} with those of M^{sens} restored complete neutralization sensitivity to maternal plasma (IC50 1510, Fig. 2.4B-C), while V1/V2 or V3 alone of M^{sens} only partially restored neutralization sensitivity (Fig. 2.4B), suggesting that V1-V3 in combination may form epitopes that together account for the major target of maternal NAbs.

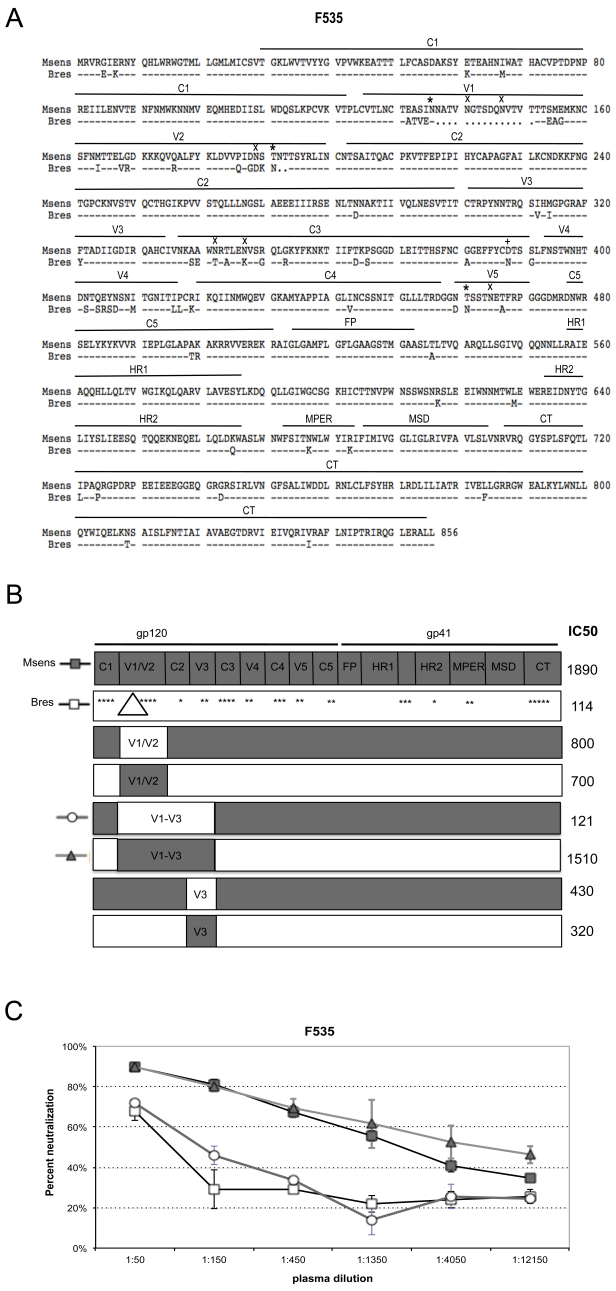


Figure 2.4. Determinants of NAb escape for F535.
(A) Amino acid alignment of envelope region of F535 variants. The sequence of F535 M^{sens} is shown. Symbols: Dashes, regions in B^{res} that are identical to the maternal sequence shown; dots, deletions in B^{res}; x, loss of PNGS in B^{res}; +, gain of PNGS in B^{res}; *, shift of PNGS in B^{res}. Abbreviations are described in Figure 2.3. **(B)** Neutralization profiles of F535 pseudoviruses bearing chimeric envelopes from B^{res} and M^{sens} against maternal plasma. Grey bars represent envelope sequence of Msens. White bars represent envelope sequence of B^{res}. Average IC50 values 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 vs. plasma dilution is shown.

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. 2.5A). 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 (241).

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% - 1%, Fig. 2.5B) 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.

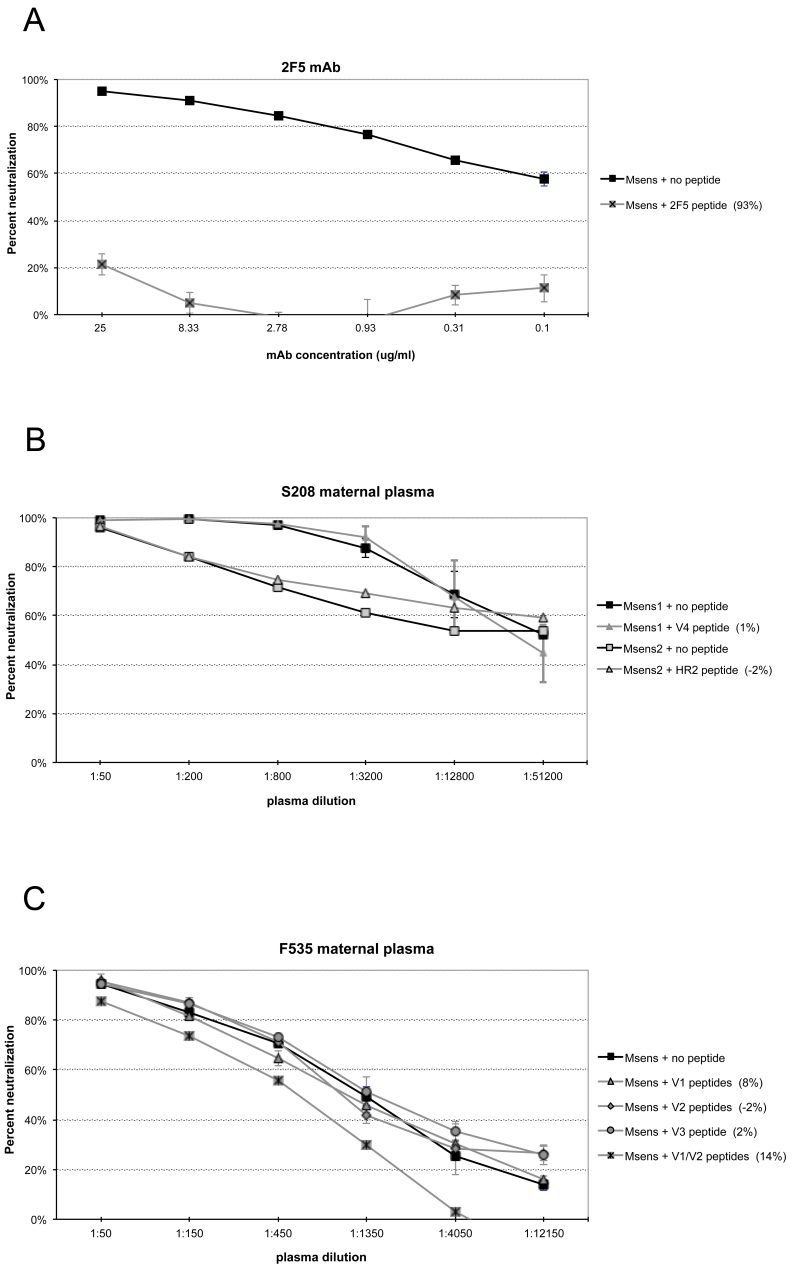


Figure 2.5. Peptide competition neutralization assays.
Percent neutralization vs. mAb concentration or plasma dilution is shown. **(A)** Serially diluted mAb 2F5 was pre-incubated with 2F5 peptides prior to addition of F535 M^{sens} in the neutralization assay. **(B)** S208 maternal plasma was pre-incubated with either V4 or HR2 peptides before addition of S208 M^{sens1} or M^{sens2}, respectively. **(C)** F535 maternal plasma was pre-incubated 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 percentage 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 legends.

For F535, we found that V1-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 V1 and V2 overlapping 15mers (data not shown). However, overall, V1, V2, and V3 peptides appeared to contribute to only a small fraction of maternal plasma neutralizing activity, as pre-incubation of maternal plasma with these peptides resulted only in a subtle shift in neutralization curves (AUC reduction of -2% – 8%, Fig. 2.5C). 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. 2.5B), and because replacing these regions with corresponding maternal sequences resulted in at most only partial restoration of sensitivity (Fig. 2.3B-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 neutralization profiles of native S208 B^{res} and M^{sens2} against these mAbs reflected those against maternal plasma (i.e. neutralization sensitivity of $M^{sens2} >> B^{res}$, Fig 2.2A). Interestingly, we found that regions of B^{res} that conferred resistance to maternal plasma simultaneously conferred resistance to the mAbs tested (Fig. 2.6A). For example, reciprocal gp120 chimeras, as well as chimeras containing C4-CT or C4-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-CT, C4-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 to not only 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 (185), introducing HR2 of B^{res} into M^{sens2} markedly reduced sensitivity to b12 relative to 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 neutralization profiles against these mAbs (Fig 2.2A).

For F535, we similarly found that regions of B^{res} (V1-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 2.2B), introducing V1-V3 of B^{res} into M^{sens}, resulted in a virus that was less sensitive to these mAbs relative to M^{sens} (Fig. 2.6B). We also found that the reciprocal chimera, in which V1-V3 of M^{sens} was introduced into B^{res}, restored sensitivity to not only maternal plasma but also to mAb 4E10. V1-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 2.2B). These results support the hypothesis that V1-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 (248). In fact, in some cases, the neutralization profile against b12 appeared to be opposite to that against maternal plasma. For example, V1-V3 of B^{res} was sufficient for resistance to maternal plasma,

but had only a modest effect on b12 sensitivity relative to the maternal variant. Similarly, the reciprocal chimera containing V1-V3 of M^{sens} conferred full sensitivity to maternal plasma but did not increase sensitivity to b12 relative to B^{res}.

As mentioned above, the neutralization profile of F535 maternal and infant variants against PG9 and VRC01 were opposite to that seen against maternal plasma: F535 B^{res}, but not M^{sens} was neutralized by PG9 and VRC01. When we tested F535 chimeras 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 2.6B). 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 (143) for PG9 (Fig. 2.2B). 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.

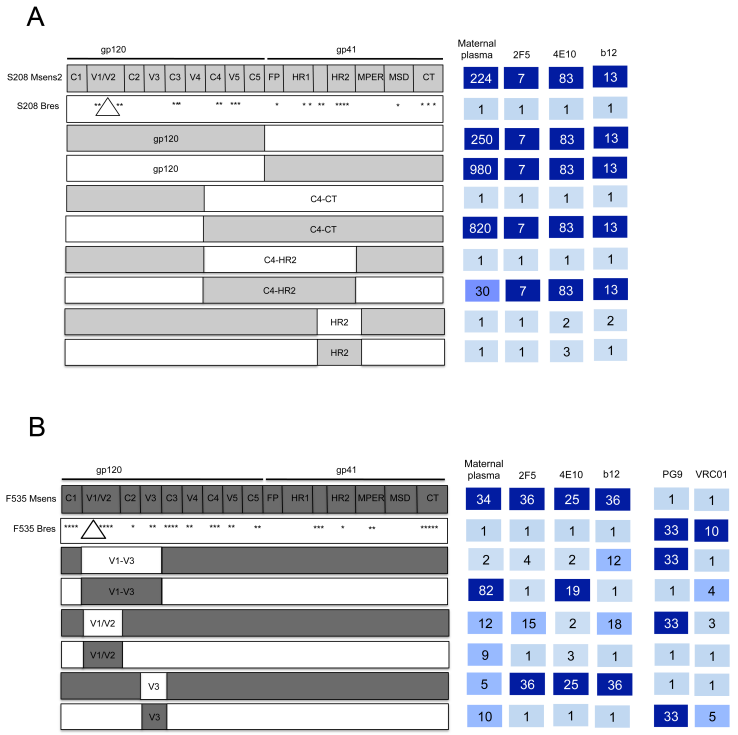


Figure 2.6. Neutralization profiles of chimeras against maternal plasma and mAbs.
(A) S208 Bres and Msens2 chimeras. **(B)** F535 B^{res} and M^{sens} chimeras. Numbers in colored boxes denote fold neutralization sensitivity based on IC50 values relative to B^{res}, which was assigned a 1 for neutralization by maternal plasma, b12, 2F5, and 4E10. For PG9 and VRC01, numbers in colored boxes denote fold neutralization sensitivity based on IC50 values relative to M^{sens}, which was assigned a 1. Darker shading denotes increasing sensitivity. Symbols and abbreviations for envelope regions are described in Figure 2.3.

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 (233) and PGT mAbs (232), and VRC01 (247), 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) (143, 232, 233). Therefore, to screen maternal plasma for PG9- and PGT145-like NAbs, we created a N160K mutation in both sensitive S208 maternal variants, M^{sens1} and M^{sens2}, and compared the neutralization phenotypes of these mutants to those of wildtype maternal variants. As a positive control, we showed that both 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% - 94% AUC reduction, Fig. 2.7A). However, both S208 M^{sens1} and M^{sens2} N160K mutants were still as potently neutralized by maternal plasma as were the wildtype maternal variants (-10% - 5% AUC reduction, Fig. 2.7B).

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 (171, 232). Again, we found that S208 M^{sens1} and M^{sens2} N332A mutants were still sensitive to neutralization by maternal plasma (Fig. 2.7B). In fact, for M^{sens1}, we saw a markedly enhanced neutralization

by maternal plasma when the N332A mutation was introduced (-48% AUC reduction, Fig. 2.7B).

As mentioned above, F535 M^{sens} was not recognized by PG9, despite the presence of N160 (Fig 2.2B). 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 2.2B). 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. Wildtype, N160K, and N332A S208 M^{sens2} variants were all neutralized to a similar extent by F535 maternal plasma (Fig. 2.7C), with N160K and N332A mutations resulting in a reduction 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.

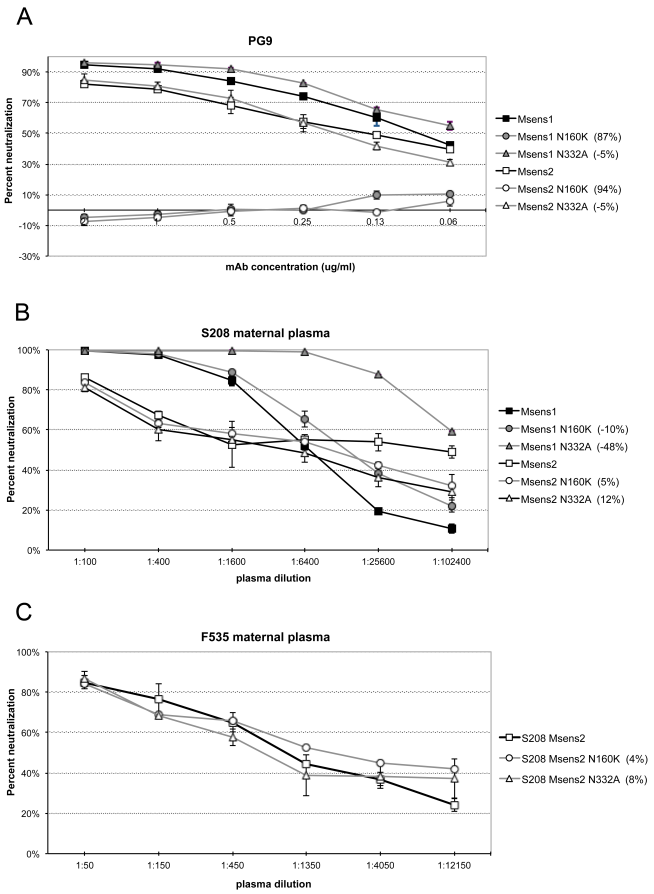


Figure 2.7. Neutralization profiles of wildtype, N160K, and N332A variants of S208 Msens variants.
(A) PG9, **(B)** S208 maternal plasma, and **(C)** F535 maternal plasma. Percent neutralization vs. mAb concentration or plasma dilution is shown in each panel. Contribution of epitopes dependent on N160 or N332 to PG9 or maternal plasma neutralizing activity was calculated as percentage reduction in AUC relative to PG9 or plasma neutralization of wildtype maternal variants. Values for average percent reduction in AUC from at least 2 independent experiments are shown in parentheses in legends.

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 (247). We used either wildtype 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 neutralizing activity of VRC01 was competed by RSC3, but not by RSC3Δ371I (74% vs. 1% AUC reduction, Fig. 2.8A). 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 lack of a substantial shift in neutralization curves, and by the negligible reduction in AUC (Fig. 2.8B-C), suggesting that the major NAb responses in maternal plasma were not against epitopes overlapping the CD4 binding site.

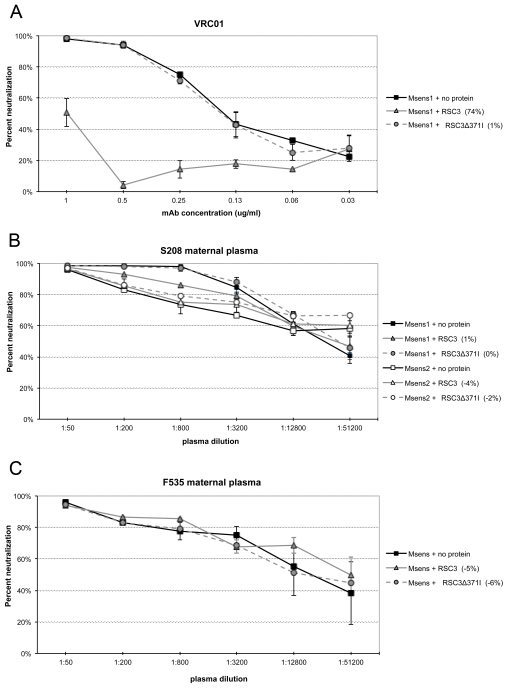


Figure 2.8. RSC3 protein competition neutralization experiments.
Serially diluted mAb VRC01 or maternal plasma was pre-incubated with RSC3 or RSC3Δ371I before use in neutralization assays against **(A)** S208 M^{sens1}, **(B)** S208 M^{sens1} and S208 M^{sens2}, or **(C)** F535 M^{sens}. Percent neutralization vs. mAb concentration or plasma dilution is shown in each panel. Contribution of antibodies against the CD4 binding site to overall neutralizing activity of VRC01 or maternal plasma was calculated as percentage 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 legends.

Maternal and infant variants have similar envelope content

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. 2.9A). 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} relative to B^{res} (Fig. 2.9B). 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.

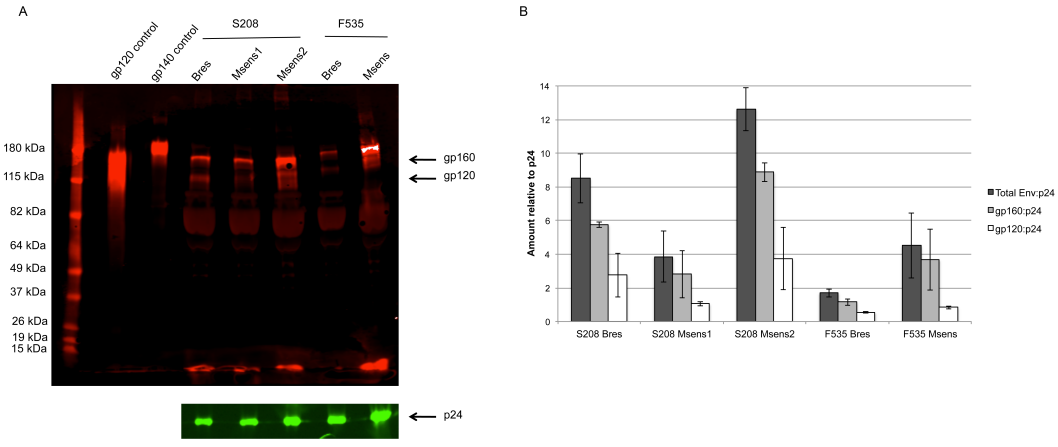


Figure 2.9. Envelope content of S208 and F535 maternal and infant variants.
(A) SDS-PAGE Western-blotting of viral supernatants using rabbit polyclonal rabbit polyclonal antisera to HIV-1 and mouse-anti-p24 as primary antibodies. An equal number of infectious particles based on titers in TZM-bl cells was loaded for each virus. The first lane shows the molecular weight marker with sizes included to the left of the image. Approximate molecular weights 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.

Discussion

We have previously described that HIV-1 variants that were resistant or only weakly sensitive to maternal NAbs present near the time of transmission were commonly transmitted from mother to infants (246), suggesting that maternal NAbs may apply selection pressure during transmission. Here, 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 common mechanism to evade maternal NAbs through conformational masking of distal epitopes. Three observations supported this hypothesis: 1) replacing regions of the infant Env that mediated neutralization resistance with those of maternal Env did not always confer sensitivity, 2) peptide competition experiments showed that regions involved in escape were not major linear targets, and 3) 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 (188, 219), 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-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 accounting 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-V3 of 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 (74, 123, 240, 250). Additionally, it has been shown that V1/V2 modulate exposure of epitopes in V3 (192). 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 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 intra-patient evolution (156,

188, 219, 236). Although the gp41 ectodomain as a whole has been found to play a role in mediating NAb escape (188), 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, suggesting that some mutations in gp41 alter overall Env conformation to affect distal epitopes, as has been shown previously (8, 20, 99, 164, 169, 180, 220, 242). 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 in NAb escape during intra-patient evolution (156, 188). Studies have shown that glycans play an important role in modulating epitopes of NAbs both by serving as direct targets (143, 171, 203) and by shielding epitopes (236). Glycans at positions N160 and N332, which have been shown to form epitopes targeted by recently identified broad mAbs (171, 233) 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 maternal envelope conferred sensitivity to PG9. This region of

both the maternal and infant variant contained the N160 residue, which is required for neutralization by PG9 (233), as well as other residues such as N156 and K168 that contribute to sensitivity against PG9 (143). 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 (184). Similarly, it is likely that there are other determinants for recognition by mAbs PGT121 and 128 besides N332 (171), 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 PGT121/128 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 to 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 PG9/16 compared to variants found in the infecting mother (221). 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 (255). Alternatively, increased Env content may enhance neutralization through increased avidity (104, 159). Moreover, recent studies have reported that increased Env content may be a signature of transmitted variants (5, 66). 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 (246). In the case of infant S208, there was a longer period between the last HIV-1 negative to the first positive test (negative at 3 months, 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

[(24); 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 for 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.

Chapter III

A combination of broadly neutralizing HIV-1 monoclonal antibodies targeting distinct epitopes effectively neutralizes variants found in early infection

Introduction

NAb protection against HIV-1 may require broad and potent antibodies targeting multiple epitopes. Recently identified HIV-1 monoclonal antibodies (mAbs) capable of potently neutralizing diverse variants have spurred optimism for a NAb-based vaccine, as these mAbs may define key targets for protective NAb responses, and may also be candidates for gene delivery (9, 92), and potentially for passive immunization to prevent or modify the course of infection (194). However, it is unclear how effective these mAbs are specifically against transmitted variants, which may comprise a unique subset of HIV variants (195) that have distinct characteristics compared to variants in chronic infection, such as shorter variable loop lengths and fewer potential N-linked glycosylation sites (PNGS) (37, 47, 196, 246), and in some cases, different neutralization profiles compared to non-transmitted variants (47, 48, 246, 257).

We analyzed the neutralization profile of 45 HIV-1 envelope variants of diverse subtypes (A, C, D), which were obtained soon after heterosexually acquired infection [median = 59 days post infection (17, 19, 125)] against 7 recently identified broadly neutralizing mAbs targeting several distinct epitopes. These included VRC01, which targets the CD4 binding site (CD4bs) (247); NIH45-46W (51), which also targets the CD4bs but is an engineered mutant that improves

the neutralization breadth and potency of mAb NIH45-46, a clonal variant of VRC01(209); PG9, PG16, and PGT145, which recognize a glycan-dependent quaternary epitope in V1/V2 and V3 (231, 233); and PGT mAbs 121 and 128 (231), which form another class of antibodies targeted to glycan-dependent epitopes in V3.

Materials and methods

Pseudovirus panel

Envelope variants used in the pseudovirus panel were isolated from early heterosexual infection of women in the Mombasa cohort by limiting dilution single copy PCR from PBMC DNA or plasma RNA (17, 19, 125). Banked blood samples prior to seroconversion were tested for HIV RNA to determine the timing of infection, defined as 17 days prior to the first HIV RNA positive visit (115). For women who had no plasma viremia recorded prior to seroconversion, the timing of infection was estimated to be the mid-point between the last seronegative and first HIV seropositive visits (115). Pseudoviruses were created by co-transfecting 293T cells with each of the cloned viral envelopes and a full-length subtype A proviral clone defective in the envelope gene as described in Chapter II.

Monoclonal antibodies

PG9, PG16, PGT121, PGT128, and PGT145 were provided by the IAVI Neutralizing Antibody Consortium; VRC01 by Xueling Wu and John Mascola; and NIH45-46W by Ron Diskin, Paola Marcovecchio, and Pamela Bjorkman. Serial dilutions of all mAbs were tested at a starting concentration of 1μg/ml against envelope pseudoviruses in the TZM-bl assay, as described in Chapter II. This starting mAb concentration was chosen due to both limited reagent

availability as well as the reported breadth of the mAbs even at low concentrations (51, 231, 233, 247).

Heat map and hierarchical clustering analysis

IC50 heatmaps were generated using "heatmap.2" and "RColor Brewer" version 1.0-5 packages in R (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria, 2008). An IC50 of 100 was set as the threshold value.

Results

mAbs have variable breadth and potency against heterosexually transmitted viruses

The mAbs tested had variable neutralizing activities against the panel viruses, with IC50 values ranging by more than 3 orders of magnitude from 0.0003 - >1 μg/ml (Figure 3.1). The CD4bs mAb NIH45-46W neutralized 91% of variants with a geometric mean IC50 of 0.09μg/ml, while VRC01, another CD4bs mAb neutralized 71% of variants with a geometric mean IC50 of 0.36μg/ml (Figure 3.2). The glycan-dependent PG and PGT mAbs were less broad and potent than the CD4bs mAbs, neutralizing only 16%-49% variants with a geometric mean IC50 of 0.24-0.78μg/ml.

Because the PG and PGT mAbs failed to neutralize a majority of variants, we investigated whether these variants lacked the PNGS required for neutralization by these mAbs (Figure 3.1). In some cases, resistance to these mAbs could be explained by the absence of a key PNGS. For example, variants isolated from a number of patients, including Q769, QG984, QH209, and QH359 that were resistant to PGT121 and PGT128 lacked the N332 residue required for neutralization (171, 231). Two of the four PG9/16-resistant variants isolated from subject QF495, did not have the full glycosylation sequon that is a target for these mAbs, despite

having the N160 residue (143, 233). Similarly, one of the QD435 variants resistant to these mAbs did not have the N156 residue required for recognition by PG9 (143). However, for all other variants resistant to PG9/16, the absence of known PNGS targets could not account for resistance as these variants possessed key residues required for neutralization (N156, N160) (143, 233). Moreover, the presence of positively charged residues in positions 168, 169, 171, which have been reported to be important for recognition by PG9/16 (53), did not always predict sensitivity to these mAbs (data not shown).

Some viruses, such as those from QF495, QH343, and QA465 had key PNGS for PGT121 and 128 recognition (N301 and N332) (20) yet were resistant to one or both of these mAbs. For other viruses, such as those isolated from Q259, Q168, and QD435, the shift of PNGS at position 332 to 334 may account for resistance to PGT121 and PGT128. However, this shift in PNGS did not always predict resistance to PGT121 and PGT128, as exemplified by Q842 variants, which had a shift of PNGS to position 334, yet were sensitive to these mAbs. Thus, the presence of known residues important for neutralization by the PG and PGT mAbs did not fully explain differing neutralization profiles among these early variants, suggesting that there may be other determinants of sensitivity to these mAbs. Indeed, the fact that some viruses such as those from QF495 and QA465 had the expected epitope targets yet were resistant to most mAbs suggests that these viruses may have altered conformations that result in global neutralization resistance, as was observed for another early subtype A virus from heterosexual transmission (20), and for subtypes A and A/D vertically transmitted variants (67). Of note, variants that possessed the canonical epitopes for PG and PGT mAbs (QA013, QB857, QF495 QH343, and QH359, variants) were still not neutralized even when these mAbs were tested at a higher starting concentration of 10μg/ml (data not shown). An alignment of V1-V3 sequences of all

variants did not readily reveal signature sequences that would predict sensitivity to these mAbs (Fig. 3.3), although QF495 variants had a large insertion in V2, including the addition of multiple PNGSs, that could explain their resistance to most mAbs tested here.

														PG9, PG16		PGT128	
														PGT145		PGT121	
Subject ID	Subtype	Year of infection	Days PI*	Virus	NIH45-46W	VRC01	PG9	PG16	PGT121	PGT128	PGT145	NIH45-46W + PGT128	N156	N160	N301	N332
Q769	A	1996	56	Q769b9	0.03	0.17	0.05	0.01	>1	>1	>1	0.04	.	.	.	x
		1996		Q769d22	0.16	0.23	0.14	>1	>1	>1	>1	0.20	.	.	.	x
		1996		Q769h5	0.31	0.41	0.03	0.10	>1	>1	>1	0.31	.	.	.	x
Q842	A	1994	49	Q842d12	0.03	0.13	0.11	0.03	0.004	0.007	>1	0.004	.	.	.	shift
		1994		Q842d14	0.09	0.37	0.01	0.003	0.004	0.02	>1	0.001	.	.	.	shift
		1994		Q842d16	0.08	0.52	0.46	0.07	0.06	0.11	>1	0.24	.	.	.	shift
Q259	A	1994	76	Q259d2.17	0.12	0.28	0.41	>1	>1	>1	>1	0.12	.	.	.	shift
		1994		Q259d2.26	0.04	0.43	>1	>1	>1	>1	>1	0.03	.	.	.	shift
		1994		Q259.w6	0.04	0.34	>1	>1	0.002	>1	>1	0.03	.	.	.	shift
Q461	A	1995	26	Q461c2	0.08	0.45	>1	>1	>1	>1	>1	0.04	.	.	.	shift
		1995		Q461d1	0.04	0.29	0.74	0.20	>1	>1	>1	0.02	.	.	.	shift
		1995		Q461e2	0.10	0.58	>1	>1	>1	>1	>1	0.23	.	.	.	shift
Q168	A	1995	23	Q168a2	0.05	0.42	0.31	0.05	>1	>1	>1	0.14	.	.	.	shift
		1995		Q168b23	0.04	0.17	0.04	0.004	>1	>1	>1	0.06	.	.	.	shift
Q23	A	1995	485	Q23ENV.17	0.37	0.36	0.004	0.0003	0.04	0.03	1.00	0.05	.	.	.	.
QB726	A	1996	70	QB726.70M.ENV.B3	0.09	0.72	0.30	0.57	0.77	0.08	0.35	0.04	.	.	.	.
QF495	A	2005	23	QF495.23M.ENV.A1	0.23	>1	>1	>1	>1	>1	>1	0.60	.	x	.	.
		2005		QF495.23M.ENV.A3	0.17	>1	>1	>1	>1	>1	>1	0.26	.	x	.	.
		2005		QF495.23M.ENV.B2	0.16	>1	>1	>1	>1	>1	>1	0.28	.	.	.	.
		2005		QF495.23M.ENV.D1	0.13	>1	>1	>1	>1	>1	>1	0.23	.	.	.	.
QG984	A	2004	21	QG984.21M.ENV.A3	0.03	0.10	0.20	0.04	>1	>1	>1	0.03	.	.	.	shift
QH209	A	2005	13	QH209.14M.ENV.A2	0.02	0.17	>1	>1	>1	>1	0.07	0.04	.	.	.	shift
QH343	A	2005	21	QH343.21M.ENV.A10	>1	>1	>1	>1	>1	0.11	0.07	0.06	.	.	.	.
		2005		QH343.21M.ENV.B5	>1	>1	>1	>1	>1	0.10	0.06	0.06	.	.	.	.
QH359	A	2005	21	QH359.21M.ENV.C1	0.37	>1	>1	>1	>1	>1	0.80	0.80	.	.	.	shift
		2005		QH359.21M.ENV.D1	0.25	>1	>1	>1	>1	>1	>1	0.60	.	.	.	shift
QA255	A	1998	21	QA255.21P.ENV.A15	0.20	0.82	0.07	0.01	>1	0.04	>1	0.03	.	.	.	.
QA790	A/D	1996	204	QA790.204I.ENV.A4	0.03	0.06	>1	0.20	>1	>1	>1	0.05	.	.	.	T
		1996		QA790.204I.ENV.C1	0.001	0.05	0.21	0.01	>1	>1	>1	0.002	.	.	.	T
		1996		QA790.204I.ENV.C8	0.22	0.12	0.68	0.45	>1	>1	>1	0.07	.	.	.	T
		1996		QA790.204I.ENV.E2	0.01	0.11	>1	0.49	>1	>1	>1	0.02	.	.	.	T
QG393	A2/D	2004	60	QG393.60M.ENV.A1	0.01	0.04	0.16	>1	>1	>1	>1	0.01	.	.	.	shift
		2004		QG393.60M.ENV.B7	0.02	0.06	0.06	0.08	>1	>1	>1	0.02	.	.	.	shift
		2004		QG393.60M.ENV.B8	0.01	0.03	0.13	>1	>1	>1	>1	0.004	.	.	.	shift
QB099	C	1995	391	QB099.391M.ENV.B1	0.06	0.43	0.44	0.02	0.03	0.05	>1	0.02	.	.	.	.
		1995		QB099.391M.ENV.C8	0.20	0.15	0.63	0.06	0.03	0.06	>1	0.04	.	.	.	.
QC406	C	1997	70	QC406.70M.ENV.F3	0.13	>1	0.20	>1	0.01	0.007	>1	0.01	.	.	.	.
QA013	D	1995	70	QA013.70I.ENV.H1	0.11	0.81	>1	>1	>1	>1	>1	0.47	.	.	.	S
		1995		QA013.70I.ENV.M12	0.22	>1	>1	>1	>1	>1	>1	0.45	.	.	.	S
QA465	D	1993	59	QA465.59M.ENV.A1	>1	>1	>1	>1	0.03	>1	>1	>1	.	.	.	.
		1993		QA465.59M.ENV.D1	>1	>1	>1	>1	0.03	>1	>1	>1	.	.	.	.
QB857	D	1997	110	QB857.110I.ENV.B3	>1	>1	>1	0.20	>1	0.07	0.65	0.12	.	.	.	.
QD435	D	1994	100	QD435.100M.ENV.A4	0.17	0.35	>1	>1	>1	>1	0.20	0.13	.	.	.	shift
		1994		QD435.100M.ENV.B5	0.08	0.51	>1	>1	>1	>1	>1	0.08	D	.	.	shift
		1994		QD435.100M.ENV.E1	0.08	0.59	>1	>1	>1	>1	>1	0.26	.	.	.	shift

IC50 µg/ml
<0.03
0.03-0.08
0.09-0.23
0.24-1
>1

Figure 3.1. Neutralization profiles of viruses from early heterosexual infection against mAbs. Subject ID, virus subtype based on V1-V5 envelope sequence, and calendar year of infection are shown in the first 3 columns. Each row shows the virus name, IC50 for mAbs tested, and known core residues based on HXB2 numbering required for neutralization by mAbs shown. For some patients, multiple viruses were obtained from the same timepoint to represent the diversity of the virus population at that timepoint, as determined by phylogenetic analysis (19). Symbols: asterisk, estimated days post infection at which envelope clone was obtained; dot; amino acid is present; x, amino acid is present but not in a glycosylation sequon; shift, present but in position 334; D, T, S, amino acid substitutions. Darker shading indicates increasing mAb potency, as indicated by the key at the bottom, grouped by quartiles of IC50 values for all virus-mAb combinations. Grey color indicates that 50% neutralization was not achieved at the highest concentration of mAb tested (1µg/ml). The combination of NIH45-46W + PGT128 was tested at a starting concentration of 1ug/ml of each mAb. IC50 values shown are averages from at least 2 independent experiments performed in duplicate.

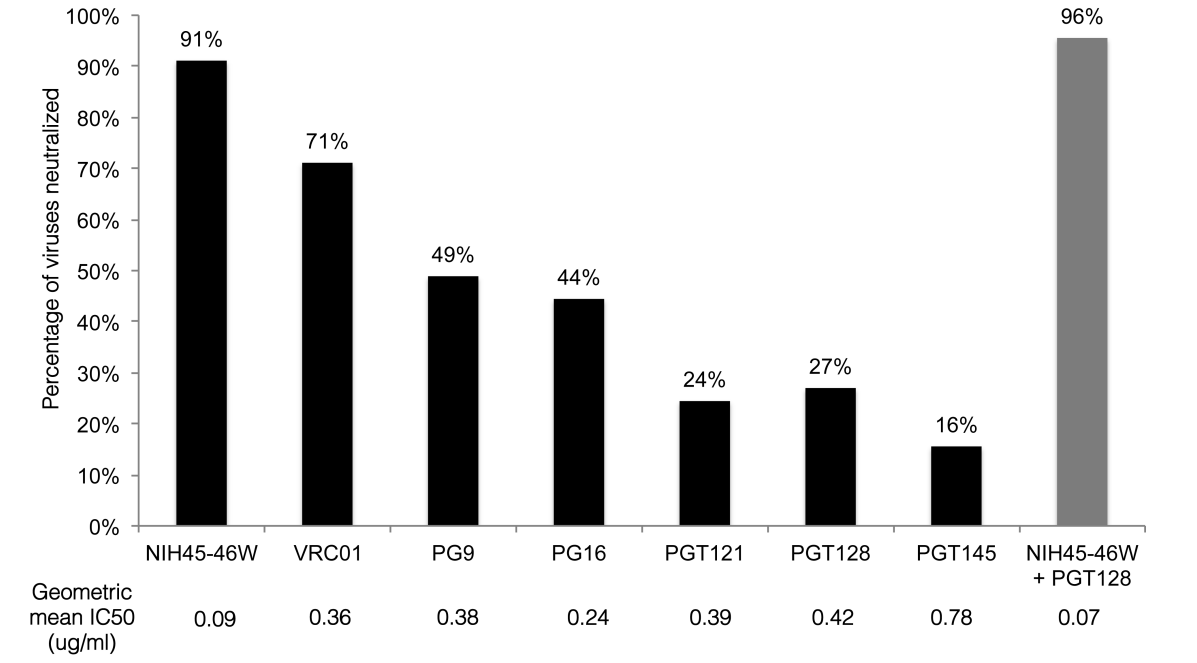


Figure 3.2. Neutralization breadth and potency of mAbs against 45 viruses from early heterosexual infection.
Percentage of viruses neutralized for each mAb indicated at the bottom of the graph is shown. Black bars represent neutralization breadth of individual mAbs. Gray bar represents neutralization breadth of a 1:1 combination of NIH45-46W and PGT128. Geometric mean IC50 value for each mAb is indicated below mAb names. IC50 values greater than the highest mAb concentration tested (1μg/ml) were assigned a value of 1 in the geometric mean IC50 calculations.

CD4bs and V3-specific mAbs display complementary neutralizing activity

NIH45-46W neutralized all but 5 viruses in the panel, including some viruses that were not neutralized by any other mAb (QF495, QH359, and QA013; Figure 3.1). Interestingly, although mAbs PGT121, 128, and 145 displayed limited breadth at the highest concentration tested in these experiments (1μg/ml), they potently neutralized variants that were resistant to all other mAbs, including to NIH45-46W. (Figures 3.1 and 3.4). Specifically, QA465 and QH343 variants were only neutralized by PGT121, PGT128, and/or PGT145 (IC50 $\leq$ 0.11μg/ml). Hierarchical clustering analyses suggested that combining NIH45-46W and PGT128 would neutralize all but 2 variants, which were recognized by PGT121 (Figure 3.4).

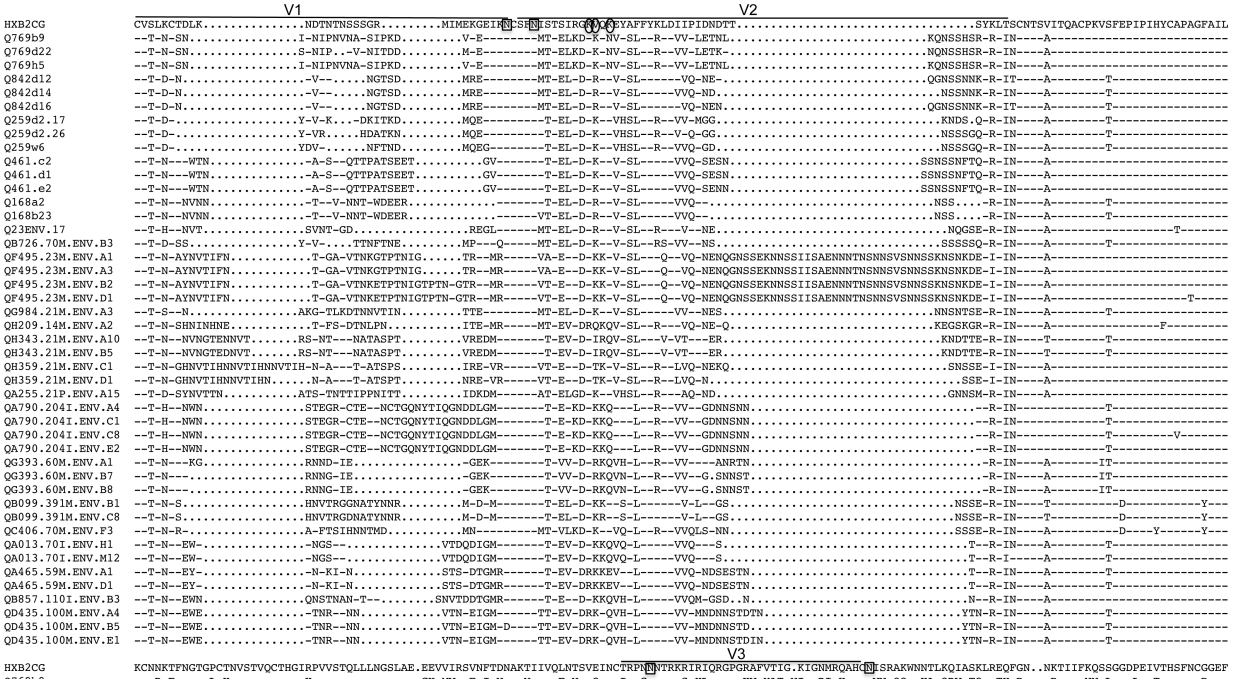


Figure 3.3. Amino acid alignment of the V1-V3 envelope region of 45 viruses from early heterosexual infection.

HXB2 sequence is shown at the top as reference. Symbols: dashes, consensus amino acid sequences; dots, deletions; boxes, PNGS at residues 156 and 160 in V1/V2, and at residues 301 and 332 in V3 important for PG9/16 and PGT121/128 neutralization, respectively; circles, positively charged residues in positions 168, 169, and 171 of V2 important for PG9/16 and PGT145 neutralization.

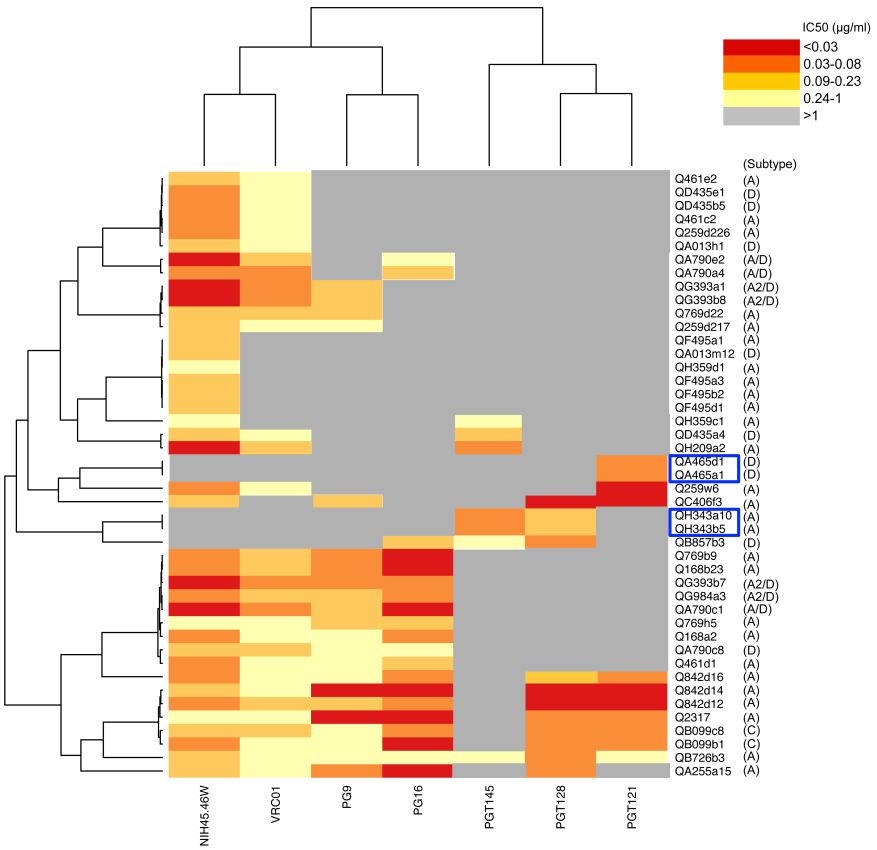


Figure 3.4. Hierarchical clustering of mAbs and panel viruses.
A heatmap of IC50 values for each virus-mAb combination is shown, with darker shading indicating increasing potency, as indicated by the key. The grey color indicates that 50% neutralization was not achieved at the highest concentration of mAb tested (1µg/ml). Blue boxes indicate viruses that are only neutralized by PGT121, PGT128, and/or PGT145.

To test the hypothesis that NIH45-46W and PGT128 would complement rather than interfere with each other's neutralizing ability, we investigated the neutralization profiles of a subset of viruses against NIH45-46W and PGT128 alone or in a 1:1 combination. We chose viruses that were either 1) neutralized by one, but not the other mAb (Q168.A2, QH343.21M.ENV.A10), or 2) neutralized by both mAbs (QB099.391M.ENV.B1 and QC406.70M.ENV.F3). The presence of one mAb did not interfere with the activity of the other mAb regardless of whether the virus tested was sensitive to one (Figure 3.5A) or both (Figure 3.5B) mAbs, with IC50 values for mAb tested alone or in a 1:1 combination within 2-fold. Because NIH45-46W is an engineered antibody, we also confirmed that a naturally occurring broad and potent CD4bs mAb, VRC01, would not interfere with PGT128 neutralization (Figure 3.5). These results demonstrate that broad and potent mAbs targeting the CD4bs and V3 do not compete for neutralization. Overall, the combination of NIH45-46W and PGT128 neutralized 96% of variants at a geometric mean IC50 of 0.07μg/ml (Figures 3.1 and 3.2). The remaining 2 variants not neutralized by NIH45-46W and PGT128 alone or in combination were potently neutralized by PGT121 (Figure 3.3). Because PGT121 and PGT128 have previously been shown to compete for binding (231), we investigated whether these mAbs would interfere with each other's neutralizing capacity against these 2 viruses. The combination of PGT121 and PGT128, with or without NIH45-46W present, neutralized both viruses, which were sensitive to PGT121 but resistant to PGT128, to a similar extent as PGT121 alone (Figure 3.6). These results demonstrate that the presence of PGT128 does not interfere with PGT121 neutralization against these variants. It is possible that these observations reflect an absence of binding of PGT128 to the viruses tested, rather than lack of competition between the mAbs.

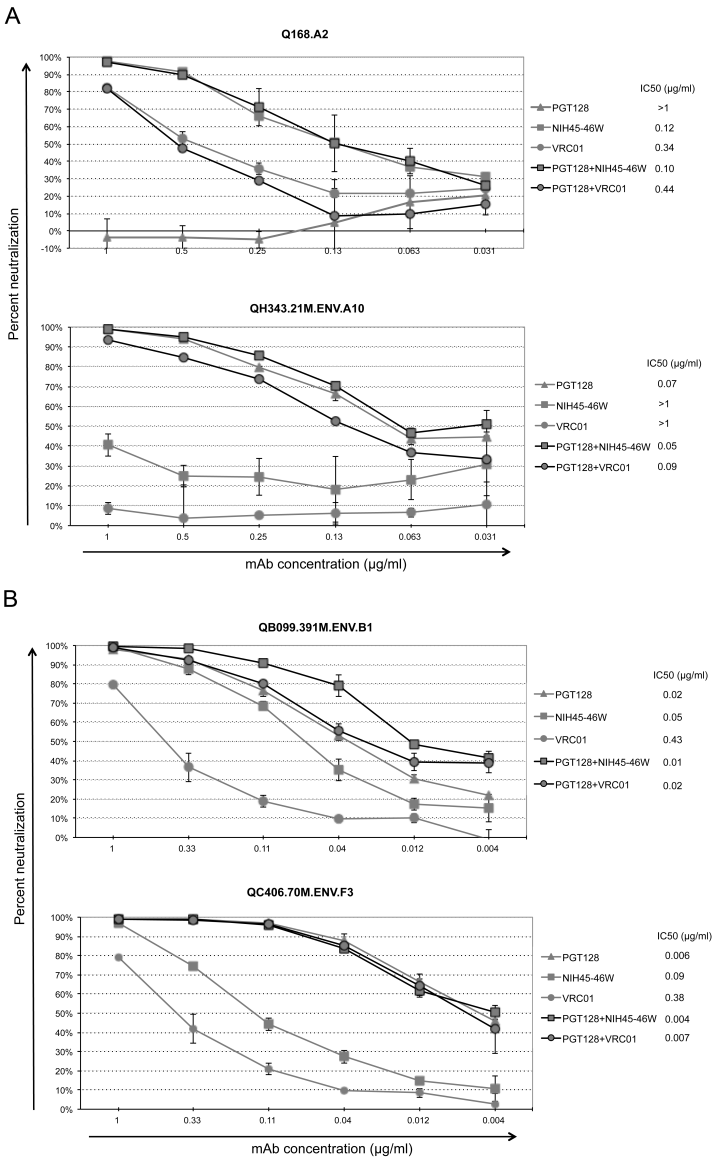


Figure 3.5. Neutralization of viruses by PGT128 alone, NIH45-46W alone, VRC01 alone, or a 1:1 combination of PGT128, NIH45-46W, and VRC01.

mAbs were tested at a starting concentration of 1µg/ml. Percent neutralization against mAb concentration is plotted. IC50 values are shown in the key and are based on averages from 2 independent experiments, only one of which is shown. **(A)** Neutralization curves of viruses sensitive to NIH45-46W and VRC01, but not PGT128 (top panel), or vice versa (bottom panel). **(B)** Neutralization curves of viruses sensitive to NIH45-46W, VRC01, and PGT128.

Subject ID	Subtype	Year of infection	Days PI*	Virus	NIH45-46W + PGT128	PGT 121	PGT121 + PGT128	PGT121 + PGT128 + NIH45-46W
QA465	D	1993	59	QA465.59M.ENV.A1	>1	0.03	0.04	0.02
		1993		QA465.59M.ENV.D1	>1	0.03	0.05	0.05

IC50 µg/ml
<0.03
0.03-0.08
0.09-0.23
0.24-1
>1

Figure 3.6. Neutralization of viruses not neutralized by a combination of NIH45-46W and PGT128 against PGT121 alone and in combination with either PGT128 or PGT128 and NIH45-46W.
Asterisk, estimated days post infection at which envelope clone was obtained. Darker shading indicates increasing mAb potency, as indicated by the key. Grey color indicates that 50% neutralization was not achieved at the highest concentration of mAb tested (1µg/ml). IC50 values shown are averages from at least 2 independent experiments performed in duplicate.

Discussion

In this study, we found that recently isolated HIV-1 mAbs display varying breadth and potency against variants from early heterosexual infection. The CD4 binding site mAb, NIH45-46W, was most broad and potent, neutralizing 91% of variants with a geometric mean IC50 of 0.09μg/ml. Combining NIH45-46W and a V3-specific mAb, PGT128, neutralized 96% of viruses, while PGT121, another V3-specific mAb, neutralized the remainder. These results suggest that, 2-3 antibody specificities may be required to provide optimal coverage of most circulating HIV-1 variants. Indeed, the same combination of CD4bs- and V3-specific antibodies was found to provide 100% neutralization coverage of 111 variants of diverse subtypes obtained from mothers and infants in the Nairobi Breastfeeding Trial (131).

We observed generally similar neutralization breadth for the individual mAbs NIH45-46W and VRC01, and only slightly lower breadth for PG9 and PG16 compared to previous reports (51, 233, 247). However, the neutralization breadth of PGT121, 128, and 145 (16%-24%) was 2-3 fold lower than observed previously (231). In the prior study by Walker et al. (231), these mAbs were tested against a panel of viruses weighted towards variants from chronic infection, potentially suggesting differences in efficacy of the mAbs against variants in early versus chronic infection. However, differences in assays used and the subtypes of viruses examined could also be relevant and studies that directly compare these variables will be needed to understand differences in efficacy against different virus panels.

Prior studies of a subset of mAbs tested here, including CD4bs and V1/V2 mAbs, suggested that the combination of PG9 and VRC01 provided almost universal coverage of the viruses tested, which included subtype B viruses (59) as well as viruses from diverse subtypes (56). When PG9 and VRC01 were tested at 1μg/ml, 26% of viruses in our panel were resistant

to both mAbs (Figure 3.1), which is more than what was reported in the prior cross-clade study [~10%; (56)]. This difference could again reflect differences in the virus panel, which included viruses from both acute and chronic infection in the study by Doria-Rose (56). Additionally, differences in the calendar period from which viruses were isolated could also influence sensitivity to these mAbs (13), but our sample size and distribution of sampling period (Figure 1) were not adequate to rigorously address this. Finally, the study by Doria-Rose used higher mAb concentrations (50μg/ml) and a larger dilution range, and we have observed a tendency to see higher IC50 values compared to the lower starting concentration (1μg/ml) and tighter dilution range used here for viruses that were potently neutralized (IC50 < 0.1μg/ml). However, this would not have altered our overall results, which focused on whether mAbs could neutralize variants at1μg/ml, although it could lead to small differences in the geometric mean IC50. Notably, at the lower mAb concentration used here, only 4% of viruses in our panel were resistant to NIH45-46W and PGT128, two mAbs that were not included in previous studies, implying that these mAbs may be among the most effective against variants found early in infection. Although 7/45 viruses were obtained later in infection (Q23, QA790, and QB099) and thus may not be representative of recently transmitted variants, removing these viruses from the analysis did not significantly affect the results of our study. For example, even by excluding these viruses, NIH45-46W was still the most broad and potent mAb, while PGT121, PGT128, and PGT145 the least broad and potent mAbs against our panel.

In summary, we demonstrated that broadly neutralizing HIV-1 mAbs have variable activity against variants found early in infection. NIH45-46W, an engineered mutant of NIH45-46 that targets the hydrophobic CD4 binding cavity in gp120 (51), displayed remarkable breadth and potency against these viruses. However, this mAb was unable to neutralize ~10% of viruses

in the panel, which were potently neutralized by glycan-dependent mAbs, PGT121, PGT128, and/or PGT145. PGT128 and NIH45-46W displayed no competition for neutralization and a combination of these mAbs improved neutralization coverage of variants. Our results suggest that optimal neutralization coverage of transmitted variants may be achieved by combining a potent CD4bs NAb with one or more NAbs directed to glycan-dependent epitopes in V3, and provide motivation to focus on the these epitopes for vaccine design, given that the antibody combination against them can neutralize viruses representing recently transmitted variants.

Chapter IV

Early development of broad and potent neutralizing antibodies in HIV-1 infected infants

Introduction

Although the early neutralizing antibody response against HIV-1 is typically strain-specific, after 2-3 years of infection, approximately 25% of adults can develop cross-clade broadly neutralizing antibodies (bNAbs) (54, 68, 145, 201, 213, 228). Moreover, among adults who generate bNAbs, approximately 1% exhibit 'elite' neutralizing activity, defined as the ability to neutralize more than one virus across 4 different clades at plasma dilutions greater than 1:300(213). The development of bNAbs in a subset of adults suggests that the mature immune system can produce broad and potent antibodies against HIV-1, and provides optimism for the potential of an effective antibody-based HIV-1 vaccine. However, it is not known whether bNAbs can also arise in HIV-infected infants, who typically progress to disease faster, presumably in part due to an immature immune system (182, 212).

Compared to adults, peak and set-point viral loads are approximately one log higher in HIV-1 infected infants (182). Because high viral loads have been associated with NAb breadth (68, 145, 174, 201), it is possible that infants develop bNAbs more commonly than adults do. Alternatively, high viral loads in infants may lead to faster B-cell dysfunction that may impede the generation of bNAbs (118, 147). Additionally, although, infants can develop *de novo* NAbs against transmitted viruses that have escaped maternal NAbs (246), it is unclear whether these NAbs can evolve in breadth and potency, as seen in adults. As some NAb escape mutations have been shown to induce bNAbs (155), one hypothesis is that infection with escape variants may lead to more rapid appearance of bNAbs in infants. Moreover, the presence of HIV-specific

passive antibodies has been shown to accelerate the development of *de novo* NAbs in newborn macaques, presumably by reducing viremia to preserve B-cell function (163). Thus, it is unclear whether bNAbs can develop in infants, and if so, whether they arise with similar frequency and kinetics as in adults.

In this Chapter, we assessed the presence of bNAbs in 28 infants who acquired HIV-1 *in-utero*, during delivery, or via breastfeeding in the Nairobi Breastfeeding Trial (161), and who had plasma samples available at least after 12 months of life. This late timepoint was chosen to increase our chances of detecting *de novo* NAbs, which usually develop after 6 months (127, 246). We show that bNAbs develop at least as commonly in infants as in adults. Cross-clade NAb responses were detected in 11/28 infected infants, in some cases, within 1 year of infection. Moreover, among infants with the top quartile of responses, neutralization of Tier 2-3 variants from multiple clades was detected at 20 months post-infection, which is earlier than reported in adults (68, 145, 201, 228). These findings suggest that, even in early life, there is sufficient B-cell functionality to mount bNAbs against HIV. Additionally, the earlier appearance of bNAbs in some infants compared to adults may provide a unique setting for understanding the pathways of B-cell maturation leading to bNAbs.

Materials and methods

Study population

Plasma samples were obtained from antiretroviral-naïve mother-infant pairs enrolled in the Nairobi Breastfeeding Trial (161). Infants were screened for bNAbs in this study if they had plasma samples available at least after 12 months of birth. Twenty-eight infants met this criterion, of which 17 were breast-fed and 11 formula-fed. Infants were defined as HIV-1 infected by PCR using PBMC or dried blood spot DNA, and by HIV-1 RNA testing using the

Gen-Probe HIV-1 Viral Load assay (161). Time post-infection is defined as the time of a plasma sample in relation to the infant's first HIV-1 positive nucleic acid test.

For each of the 28 infants, the presence of bNAbs was measured using the last available plasma sample. To determine kinetics of NAb breadth in 7 infants, earlier available samples were tested, beginning at either the first week of life (3 infants), week 14 (3 infants), or month 6 (1 infant). Maternal plasma samples were obtained from the last HIV negative timepoint of their corresponding infants. HIV-1 clade was determined by a combination of heteroduplex mobility assay of V1-C3 (21/28 subjects), and sequence analysis of V1-V5 (7/28 subjects) of maternal virus envelopes, as described previously (162). Viral loads were quantified by the Gen-Probe HIV-1 viral load assay (58). Plasma samples from 2 HIV-1 infected adult women, QB850 and QA255, who were participants of a heterosexual transmission cohort in Mombasa, Kenya, we also screened for HIV-1 specific NAbs using samples from ~5 years PI. QB850 is a top broad neutralizer identified in a screen of 12 superinfected and 36 singly infected women (41), while QA255 is a top broad neutralizer in a similar screen of 70 singly infected women (174).

Pseudovirus Panels and neutralization assay

The 4-virus panel (Panel 1) used in the initial screen for bNAbs consists of 2 clade A (Q461.D1, Q842.d16), 1 clade D (QD435.A4), and 1 clade A/D recombinant (BF535.A1). Infant plasma samples that could neutralize 2 additional viruses other than Q461.D1 (n=7), an easy-to-neutralize virus (20, 211) at an IC50 > 100 were classified as having bNAbs (213), and were further screened against Panel 2 viruses, which included 19 viruses in addition to Panel 1 viruses. Panel 2 includes 8 clade A (Q461.D1, Q23, Q842.d16, Q769.B9, Q259d2.26, BJ613.E1, Q168.a2, Q842.d12), 1 clade A/D (BF535.A1), 6 clade B (SF162, TRO.11, THRO4156.18,

CAAN.A2, THRO4156.18, PVO.4), 6 clade C (ZM109F.PB4, QC406.F3, DU156.12, DU422.1, DU172.17, CAP210.E8), and 2 clade D (QD435.A4, QB857.B3) viruses. Panel 3 viruses (Q461.D1, Q769.B9, SF162, TRO.11, QC406.F3, DU156.12, QD435.A4, QB857.B3) were tested against longitudinal infant samples and maternal plasma samples. A negative control pseudovirus from the envelope of simian immunodeficiency virus (SIV), SIVMneCl8 (175), was also tested against all plasma samples to confirm that neutralizing activity was HIV-specific. Plasma samples from BG505 and BB391, the top 2 broad neutralizers, were also tested against an additional 6-virus panel (Panel 4) that was previously used to identify elite neutralizers (213). This panel consists of 1 clade A (94UG103), 2 clade B (JRCSF, 92BR020), 2 clade C (IAVI C22, 93IN905), and 1 clade CRF01_A3 (92TH021) virus.

Procedures for pseudovirus production and neutralization assays were described in Chapter II. Heat-inactivated plasma samples (56°C, 1hr) were tested using six 2-fold serial dilutions, starting at 1:100. IC50 values represent the reciprocal plasma dilution resulting in a 50% reduction of virus infectivity. For viruses not neutralized at the lowest dilution tested (1:100), IC50 values of 100 were assigned for analyses. For viruses that did not reach 50% neutralization at the highest dilution tested (1:3200), IC50 values of 3200 were assigned for analyses. IC50 values represent the average of at least 2 independent experiments.

Breadth and potency scores

A composite breadth score was calculated for each infant plasma sample, as previously described (18). Briefly, the breadth score for a given sample is the number of viruses neutralized at an IC50 higher than the median IC50 for that virus across all samples. For example, for 7 infant samples that were tested against Panel 2 (23 viruses), the minimum and maximum possible breadth scores were 0 and 23, respectively. We also calculated potency scores by

dividing the IC50 for a given plasma-virus combination by the median virus IC50 value and summing these values for all viruses tested (41). For BG505 and BB391, the top 2 broad infant samples, and QB850 and QA255, the top 2 broad adult samples in our Mombasa cohort, an additional 'neutralization score' was calculated using a scoring procedure that was deemed most suitable for identifying samples with bNAbs ('Score 1'), as described in Simek et al. (213). Briefly, an individual's neutralization score is the average of log-transformed titers across virus Panel 4. Log-transformed titers were obtained by dividing IC50 values by 100 followed by a log-base 2 transformation and adding 1: (Y = log2 (IC50/100)+1). All IC50 values below the limit of detection of 100 were given a log-transformed value of 0 for calculating neutralization scores.

Immunoglobulin G (IgG) purification and quantification

Total IgG was purified from 50μl of plasma using the Melon Gel IgG purification kit (Thermo Scientific) in a final volume of 500ul (1:10 dilution in purification buffer). Purified IgG was quantified and was tested in neutralization assays at a starting concentration of 2mg/ml for BG505 or 1mg/ml for BB391, which had more limited sample, against Q461.D1, Q842.d16, TRO.11, QC406.F3, QD435.A4, and SIV.

Total and HIV-1-specific ELISA

Total IgG ELISA in plasma samples from mother-infant pairs were quantified using a human IgG ELISA kit (Immunology Consultants Laboratory) according to manufacturer's instructions. HIV-1 Env-specific IgG ELISAs was performed as previously described (132) using 25ng/well of purified recombinant gp120 (Immune Technology Corp, New York, NY) from Q461.D1, a Tier 1 virus in Panel 1. Plasma samples were serially diluted 2-fold starting at

a dilution of either 1:1,000 or 1:10,000 to obtain an end-point titer (EPT), defined as the reciprocal dilution at which the average optical density (OD) value was greater than or equal to twice the average OD value of background. Total IgG levels and EPT values reported represent the average of at least 2 independent experiments.

Expression and purification of RSC3 proteins

Proteins were expressed and purified as described by Wu, et al. (247), with slight modifications. Briefly, proteins were produced by transient transfection of 293F cells (1.2 x 10^6 in 400 ml) using 293fectin (Invitrogen). Culture supernatants were harvested 5 days after transfection, filtered through a 0.45mm filter, and concentrated by centrifugation in 100kDa Centricon-Plus 70 filter tubes (Millipore) with buffer exchange into PBS. Proteins were first purified by DEAE sepharose (GE) ion exchange chromatography, followed by His-Select Nickel (Sigma) affinity chromatography.

Epitope mapping

To screen for PG9/16- and PGT128-like NAbs(232, 233), plasma neutralization of a commonly used clade A wildtype virus, Q23.17 (68, 155) was compared to that of N160K or N332A mutants, respectively for most infant samples. Mutants were generated by overlap PCR and verified by sequencing. BG376 and BN469 could not neutralize Q23.17 at the lowest dilution tested (1:100) and were instead tested against JRCSF, a subtype B variant, and corresponding mutants, which have been used to detect PG- and PGT-like antibodies in previous studies (233, 234). In all experiments, PG9 and PGT128 were included as positive controls for N160K and N332A, respectively, while VRC01, which targets the CD4 binding site, served as a

negative control for non-specific effects of both mutants. A sample was considered positive for PG- or PGT-like antibodies if the reduction in area under the curve comparing mutant to wildtype viruses for that sample was at least 3-fold higher than that observed for the VRC01 negative control against Q23.17 wildtype and mutant viruses. The presence of CD4bs (VRC01-like) antibodies was assessed by ELISA as described above for Q461.D1 gp120, except a resurfaced stabilized core (RSC3) gp120 protein and its mutant (RSC3D371I) were used (247), and plasma samples were tested at a starting dilution of 1:100. EPT was calculated as described above, and an EPT >100 for RSC3, and a corresponding EPT reduction of greater than 3-fold for RSC3D371I was considered to be indicative of the presence of CD4bs binding antibodies (128). To map MPER specificities, we compared neutralization of a HIV-2 full-length construct (7312A) to that of a HIV-2/HIV-1 MPER chimera (7312-C1) (87). An IC50 > 3-fold higher for 7312-C1 versus 7312-A was considered positive.

Statistical analyses

All analyses were performed in R 2.10.1 and GraphPad Prism 9.0. For all analyses, maternal IgG levels and neutralization profiles were based on plasma obtained from the last HIV negative timepoint of their corresponding infants or the earliest timepoint available if the infant was predicted to be infected *in-utero*, while infant *de novo* IgG and neutralization profiles were assessed at the latest available timepoint. To compare maternal and infant total IgG levels and $\log_2$ transformed Q461.d1 EPT, we used Wilcoxon's signed rank test and paired t-test, respectively. To investigate the relationship between maternal Nabs and infant *de novo* NAbs, we tested samples from mothers of the 7 infants who developed bNAbs against Panel 3 viruses. Paired t-test was then used to determine the relationship between maternal and infant *de novo* average $\log_2$(IC50) values against these viruses.

For a subset of 22 infants who had plasma availability within the first week of life, we also determined the association between passive and *de novo* antibodies. IC50 values for passive neutralization for 13/22 infants were already available from a previous study investigating the breadth of passive NAb responses starting at a plasma dilution of 1:25 (127), while neutralization data for the remaining 9/22 infants were generated in this study at a starting plasma dilution of 1:100 to preserve sample. For all analyses, viruses not neutralized at the lowest plasma dilution tested were assigned IC50 values of 25 or 100 (the lowest dilution tested in the prior or current study, respectively). We used Pearson's correlation coefficient to compare the average $\log_2$(IC50) values of passive and *de novo* antibodies against 3 viruses: Q461.D1 (clade A), DU156.12 (clade C), and QD435.A4 (clade D), which represented the overlap between the virus Panel from this prior study and Panel 3 viruses. Pearson's correlation coefficient to compare the average $\log_2$(IC50) values of all 28 infants in this study and their mothers was also based on neutralization of these 3 viruses.

Viral load measurements were available for mothers of 27/28 infants, excluding BM378, and were obtained between 32-39 weeks of pregnancy (9/27), at delivery (14/27), or within 6 weeks of delivery (4/27). Infant set-point viral load measurements, defined as the first available measurement within 4-12 months of infection, were available for all infants except BB391. Pearson's correlation coefficient was used to investigate the association between maternal and infant passive NAbs (n = 22 pairs) against Q461.D1, DU156.12, and QD435.A4 (average $\log_2$(IC50)); and maternal (pre-transmission) and infant (set-point) viral loads (n = 26 pairs, excluding B391 and M378). To compare set-point viral loads between infants who developed bNAbs (n=6, excluding BB391) and those who did not (n=21), as determined in Figure 1, we performed an unpaired t-test with Welch's correction.

Univariate linear regression models were used to identify factors associated with *de novo* NAb breadth in all 28 infants, defined as the average $\log_2$(IC50) value against 3 viruses representing clades A, C, and D (Q461.D1, DU156.12, and QD435.A4, respectively). In these models, logarithmic transformations were performed for passive NAb IC50 values against these viruses, set-point viral load, and Q461.D1 gp120-specific EPT. Duration of HIV-1 infection was calculated by subtracting the timepoint of the first HIV-positive result from the plasma timepoint used for neutralization assays. For infants who were predicted to be infected *in-utero* based on an HIV-positive cord blood sample, or at delivery, the timepoint of first HIV positive result was set to 0. Covariates significantly associated with *de novo* NAb breadth in univariate analyses ($p < 0.05$) were included in multivariate models. Analyses involving passive NAbs and set-point viral load were performed for 22/28 and 27/28 infants, respectively.

Results

Identifying infants with cross-clade HIV-1 bNAbs

As most infants of the 28 infants included in this study were infected with clade A or D viruses, samples at the last timepoint after birth (median = 24 months, range = 12-30 months) were first screened against 4 viruses (Panel 1, Fig. 4.1), including Q461.D1 and Q842.d16 (both clade A), QD435.A4 (clade D), and BF535.A1 (clade A/D). In some cases, the timepoint after birth was similar to that after HIV-1 infection as most infants were first detected HIV positive within 2 months of life (median = 1.5 months, range = 0-9.1 months). Overall, 27/28 samples (96%) neutralized at least one virus, and 11/28 samples (39%) showed evidence of cross-clade NAbs against a Tier 2 virus at a median time of 20 months post-infection (PI) (range = 11.4-28.2). In comparison, approximately 13%-43% of adults developed similar cross-clade NAbs against a Tier 2 virus after 2-4 years PI (68, 145, 201, 228).

Based on this initial screen, 7/28 samples that could neutralize at least 2 viruses in addition to Q461.D1, an easy-to-neutralize virus (20, 211), were tested against 19 other viruses representing various clades and neutralization sensitivities (17, 19, 211) (Panel 2, Fig. 4.2A). All 7 samples displayed broad responses(213), neutralizing at least one virus across 4 clades with IC50 > 100 at a median time of 20.3 months PI (range = 12 – 28.2). BG505 and BB391 had the most impressive responses, neutralizing 91%-96% of viruses with the top 2 breadth and potency scores (18). Purified IgG from these 2 infants neutralized HIV variants but not SIV (Fig. 4.2B), demonstrating that IgG antibodies mediated HIV-specific NAb breadth.

	Age in months						Clade A		Clade D	Clade A/D	
							Tier 1B	Tier 2	Tier 2	Tier 2	Tier-2
ID	Last HIV-	First HIV+	Plasma	Time PI	Breastfed	Clade	Q461.D1	Q842.d16	QD435.A4	BF535.A1	Cross-clade NAbs
BG505	0.0	1.7	27.0	25.3	Yes	A	>3200	482	548	129	+
BB391	0.1	1.8	30.0	28.2	No	A	455	232	333	189	+
BG376	3.0	6.0	24.0	18.0	No	C	799	142	176	<100	+
BT326	-1.0	0.0	12.0	12.0	No	A	1944	147	128	<100	+
BF520	0.3	3.8	27.0	23.2	Yes	A	2063	151	112	<100	+
BN469	1.4	3.7	24.0	20.3	No	A	551	126	106	<100	+
BH217	0.0	4.0	24.0	20.0	No	A	360	125	106	<100	+
BB539	6.0	9.1	24.0	14.9	Yes	A	2102	<100	235	<100	+
BJ412	0.0	1.5	24.0	22.5	Yes	C	558	151	<100	<100	+
BE012	-1.0	1.6	13.0	11.4	No	A	362	<100	147	<100	+
BL846	-1.0	1.4	15.0	13.6	Yes	A	160	<100	103	<100	+
BJ349	-1.0	0.3	25.0	24.7	No	A	278	219	<100	<100	-
BF013	-1.0	0.2	25.0	24.8	Yes	A	838	102	<100	<100	-
BF055	3.5	6.1	24.0	17.9	Yes	D	1588	<100	<100	<100	-
BF403	-1.0	0.0	27.0	27.0	Yes	A	667	<100	<100	<100	-
BM378	-1.0	0.0	22.0	22.0	No	A	612	<100	<100	<100	-
BH026	0.0	1.5	15.0	13.5	No	A	592	<100	<100	<100	-
BK202	-1.0	0.0	21.0	21.0	Yes	A	582	<100	<100	<100	-
BI759	-1.0	0.1	24.0	23.9	No	A	573	<100	<100	<100	-
BF264	-1.0	0.0	24.0	24.0	Yes	D	527	<100	<100	<100	-
BI352	6.0	9.0	24.0	15.0	Yes	A	375	<100	<100	<100	-
BM827	-1.0	1.6	24.0	22.4	Yes	A	293	<100	<100	<100	-
BF089	0.0	1.9	25.0	23.1	No	A	259	<100	<100	<100	-
BB988	3.6	7.0	22.0	15.0	Yes	D	217	<100	<100	<100	-
BH285	-1.0	0.0	15.0	15.0	Yes	C/?	177	<100	<100	<100	-
BI507	-1.0	0.0	25.0	25.0	Yes	D	148	<100	<100	<100	-
BI102	0.0	1.4	28.0	26.6	Yes	D	109	<100	<100	<100	-
BF535	0.4	1.4	24.0	22.6	Yes	A/D	<100	<100	<100	<100	-
						Median IC50	539	<100	<100	<100	

IC50
<100
100-300
301-1000
>1000

Figure 4.1. Neutralization profiles of 28 infant samples against Panel 1 viruses.
Each row shows data for an individual infant, whose ID is shown in the first column, followed by the last HIV-1 negative timepoint, where '-1.0' indicates testing of cord blood sample, and '0.0' indicates delivery timepoint. A combination of '-1.0' for the last HIV-1 negative timepoint and '0.0' for the first HIV-1 positive timepoint suggests *in-utero* transmission. The plasma timepoint used for neutralization assay is followed by the estimated time post-HIV-1 infection (PI) of that sample, calculated as time from first detection of HIV-1. The column labeled 'Clade' indicates the infecting virus clade based on V1-C3 or V1-V5 envelope sequence (162). BH285 was infected with a clade C recombinant virus but portions of the envelope gene could not be readily assigned to any known subtype. The clades of the 4 virus panel tested here and tiered categorization of neutralization sensitivity (41, 211) are shown above virus names. In the last column, '+' and '-' indicate the presence or absence of cross-clade NAbs against a Tier-2 virus, respectively. IC50 values, shown as reciprocal plasma dilutions from at least 2 independent experiments, are color coded with darker shading representing greater neutralization potency, as shown in the key. Gray boxes indicate that 50% neutralization was not achieved at the lowest plasma dilution tested (1:100). Median IC50 values against all samples are shown at the bottom. The 7 samples selected for further testing are shown above the horizontal line.

A

			ID (months post-infection)							
Clade	**Tier**	**Virus**	**BG505 (25.3)**	**BB391 (28.2)**	**BT326 (12.0)**	**BF520 (23.2)**	**BN469 (20.3)**	**BH217 (20.0)**	**BG376 (18.0)**	**Median IC50**
A	1B	Q461.D1	>3200	455	1944	2063	551	360	799	799
A	1B	Q23	295	163	123	123	<100	108	<100	123
A	2	Q842.d16	482	232	147	151	126	125	142	147
A	2	Q769.B9	170	535	189	118	<100	129	<100	129
A	2	Q259d2.26	245	148	<100	<100	<100	<100	<100	<100
A	2	BJ613.E1	<100	268	173	156	138	134	152	152
A	2	Q168.a2	164	259	114	<100	<100	<100	<100	<100
A	2	Q842.d12	253	197	166	<100	135	133	<100	135
A/D	2	BF535.A1	129	189	<100	<100	<100	<100	<100	<100
B	1A	SF162	>3200	404	>3200	1127	1691	1230	895	1230
B	2	TRO.11	262	202	384	106	120	120	132	132
B	2	THRO4156.18	<100	<100	<100	<100	<100	<100	<100	<100
B	2	CAAN.A2	179	120	130	<100	<100	<100	<100	<100
B	3	TRJO4551.58	171	148	222	<100	199	112	128	148
B	3	PVO.4	169	178	149	<100	<100	<100	<100	<100
C	1B	ZM109F.PB4	149	187	212	191	165	168	152	168
C	2	QC406.F3	971	303	166	2028	306	251	193	303
C	2	DU156.12	248	181	<100	<100	<100	113	102	102
C	2	DU422.1	282	300	469	<100	145	145	165	165
C	2	DU172.17	286	197	197	<100	139	<100	<100	139
C	2	CAP210.E8	590	191	192	<100	128	115	137	137
D	2	QB857.B3	185	225	118	114	122	129	124	124
D	2	QD435.A4	548	333	128	112	106	106	176	112
		Breadth	20	18	15	5	3	2	1	
		Potency	50.1	36.9	32.8	28.5	22.4	21.1	21.7	
		% Neutralized	91%	96%	83%	48%	61%	70%	56%	
		SIV	114	<100	<100	<100	<100	<100	<100	<100

IC50
<100
100-300
301-1000
>1000

B

		BG505 IC50		BB391 IC50	
Subtype	Virus	Plasma (dilution)	Purified IgG (mg/ml)	Plasma (dilution)	Purified IgG (mg/ml)
A	Q461.D1	>3200	0.04	455	0.51
A	Q842.d16	420	0.32	232	nd
B	TRO.11	262	1.23	202	0.27
C	QC406.F3	971	0.22	166	0.83
D	QD435.A4	465	0.07	333	0.24
	SIV	114	>2	<100	>1

Figure 4.2. Neutralization profiles of 7 infant plasma samples with bNAbs.
(A) Neutralization of Panel 2 viruses, which are indicated in the first three columns along with clade and Tier designations. Infant IDs are shown in top rows, followed in parentheses by the months post-infection at which plasma samples were tested. Corresponding IC50 values are shown below each infant ID and are color coded as in Figure 4.1. The last column shows median IC50 for each virus against all samples. Breadth and potency scores were calculated by normalizing the IC50 for each virus-plasma pair to the median IC50 for that virus (41), and are shown in the box below IC50 data, along with percentage of viruses neutralized. Neutralization of SIV, a negative control, is shown at the bottom. **(B)** Neutralization profile of plasma and purified IgG from BG505 and BB391 against a subset of HIV-1 variants from clades A, B, C, and D, and SIV, a negative control virus. IC50 values are shown as reciprocal dilution for plasma, and as concentration in mg/ml for purified IgG. nd = not determined due to limited sample availability.

These results suggest that, as seen in adults, approximately 25% of infants can develop bNAbs (54, 68, 145, 201, 213). However, as cross-clade neutralization of Tier 2 viruses with IC50 >100 is typically detected starting at 2-4 years PI in adults (68, 145, 201, 228), and samples at or after 24 months PI were only available for 9/28 infants (Fig. 4.1), we are probably underestimating the prevalence of bNAbs in infants. Three infants in the initial screen had some evidence of cross-clade responses by 12-15 months PI (BE012, BB539, BL846, Fig. 4.1) but we could not assess whether they ultimately developed more broad and potent NAbs as later samples were unavailable. Notably, some infants such as BT326 and BG376 displayed cross-clade breadth against Tier 2 and 3 viruses as early as 12-18 months PI (Fig. 4.2A).

Kinetics of development of *de novo* bNAbs

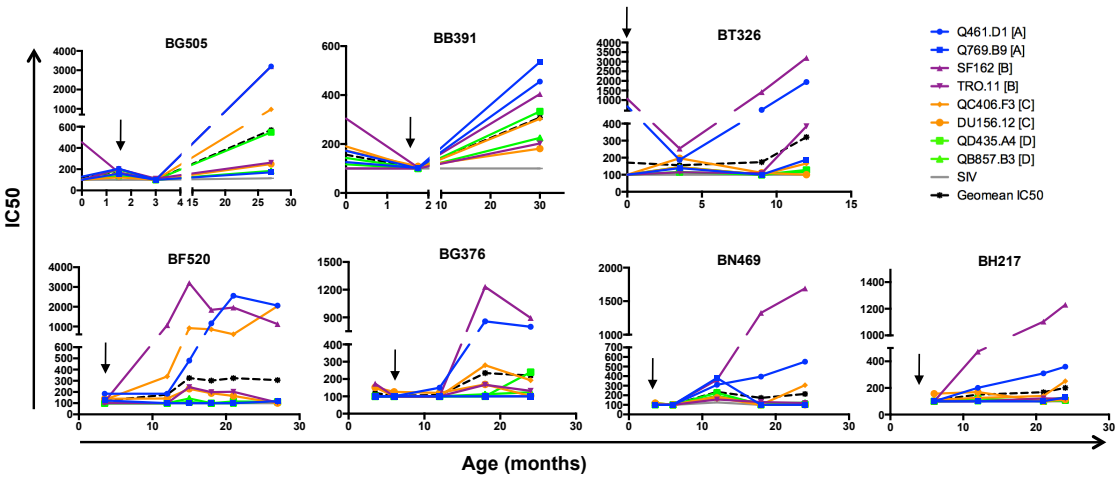
Because passively transferred HIV-1 NAbs typically decay by 6 months of life (127, 246), the observed NAb breadth at ~20 months in infants likely reflects *de novo* responses. To confirm this, and to determine the kinetics of NAb breadth, we tested longitudinal samples, beginning at the earliest timepoint available after birth (median = week 14, range = week 1 to month 6) against 8 viruses representing clades A, B, C, and D (Panel 3, Fig. 4.3A and Table 4.1). At the earliest timepoints, BG505, BB391 and BT326 had high neutralizing titers against Tier 1 viruses (Q461.d1 and SF162). These titers likely reflect those of passive NAbs as they waned by ~3 months before rebounding and peaking at the last timepoint (Fig. 4.3A). For infants infected somewhat later (BF520, BG376, BN469, BH217), there was only modest early neutralizing activity, possibly because the first sample tested was after passive antibodies had decayed. Beginning at month 12, *de novo* responses that increased in potency over time were observed for most samples (geometric mean IC50 = 201-570 at the last timepoint). By 12 months of life (~8-

12 months PI), some infants such as BN469 and BT326 had already developed broad *de novo* responses, neutralizing at least one virus across 4 different clades with IC50 >100 (213), while BF520 and BG376 developed similar breadth by 15 and 18 months of life, corresponding to 11 and 12 months PI, respectively (Table 4.1). These results confirm that NAb breadth was due to *de novo* responses, and suggest that some infants can develop bNAbs within the first year of life and of HIV-1 infection.

Comparison of NAb breadth in infants and adults

To compare NAb breadth of BG505 and BB391, the top 2 infants with bNAbs, to that of adults, we tested these infant samples against 6 viruses (Panel 4, Fig. 4.3B) used to screen ~1800 infected adults to identify 'elite neutralizers' (the top 1%) (213). A neutralization score based on average log-transformed IC50 values against these viruses was calculated. BG505 and BB391 had scores of 2.1, which fall short of the rare subset of adults with elite neutralizing activity, defined as a score $\geq$ 2.5 (213). Nevertheless, after less than 2.5 years of HIV-1 infection, these infants had scores similar to those of the top 22 of 1800 (1.2%) adult samples initially screened for bNAbs against a larger panel of viruses after at least 3 years PI in the prior study (213), and to those of QB850 and QA255 (neutralization scores of 2.3 and 1.6, respectively), 2 adult women identified as having bNAbs at ~5 years PI in our previous screens of 48 and 70 women, respectively (41, 174). Thus, NAb responses in these 2 infants at ~2.5 years PI are approaching those found in the top 1% of adults at later times in their infection.

A



B

				Clade A	Clade B		Clade C		CRF01_A3	
ID	Subject	Age (years)	Plasma PI (years)	94UG103	92BR020	JRCSF	IAVI22	93IN905	92TH021	Score
BG505	Infant	2.3	2.1	467	106	143	223	309	176	2.1
BB391	Infant	2.5	2.4	399	119	252	245	222	163	2.1
QB850	Adult	40.8	4.8	138	436	170	330	1582	<100	2.3
QA255	Adult	45.1	5.1	209	<100	209	206	194	121	1.6

IC50
<100
100-300
301-1000
>1000

Figure 4.3. Kinetics of infant NAb breadth.
(**A**) Graphs show IC50 values against Panel 3 (shown in the key in the upper right corner) viruses over time (age in months). Viruses are color-coded by clade, as shown in the key. The geometric mean IC50 for each sample against all viruses is shown as a dotted black line. Black arrow denotes when HIV-1 was first detected. **(B)** Neutralization profile of 2 infants with greatest NAb breadth (BG505, BB391) and 2 adult samples (QB850, QA255) identified as having bNAbs in previous screens (41, 174) against Panel 4 viruses to determine 'elite neutralizing' activity. Plasma timepoint tested in shown in years post-infection (PI). The subject's age at the plasma timepoint tested is also shown. IC50 values are color coded as in Figure 1. Average IC50 values from 2 independent experiments were used to calculate neutralization scores (213).

			Clade A		Clade B		Clade C		Clade D			
ID[a]	Plasma[b]	Time PI[c]	Q461.D1	Q769.B9	SF162	TRO.11	QC406.F3	DU156.12	QD435.A4	QB857.B3	Geomean IC50	SIV
MG505	W0	N/A	1022	241	>3200	370	153	132	333	646	434	<100
BG505	WC	-	130	<100	454	<100	<100	<100	<100	<100	125	<100
BG505	W6	+	205	158	169	202	123	162	138	196	166	<100
BG505	W14	1.3	<100	<100	<100	112	<100	<100	<100	<100	101	<100
BG505	M27	25.3	>3200	170	>3200	262	971	248	548	185	570	114
MB391	P38	N/A	396	<100	283	<100	<100	<100	<100	<100	135	<100
BB391	W0	-	172	126	304	<100	190	173	117	143	156	<100
BB391	W7	+	<100	102	104	<100	<100	109	<100	<100	102	<100
BB391	M30	28.2	455	535	404	202	303	181	333	225	308	<100
MT326	W0	N/A	>3200	144	>3200	161	254	261	109	<100	338	<100
BT326	WC	+	676	<100	1070	<100	<100	<100	<100	<100	171	<100
BT326	W14	3	187	139	254	114	145	197	116	144	156	<100
BT326	M9	9	491	<100	1420	109	<100	111	<100	<100	174	<100
BT326	M12	12	1944	189	>3200	384	166	<100	128	118	321	<100
MF520	W1	N/A	190	190	224	<100	<100	<100	<100	<100	130	<100
BF520	W14	+	184	124	113	<100	130	140	<100	<100	121	<100
BF520	M12	8.2	184	<100	1069	<100	339	142	<100	<100	177	<100
BF520	M15	11.2	480	<100	>3200	244	922	228	110	142	325	<100
BF520	M18	14.2	1162	<100	1842	199	859	189	<100	<100	302	<100
BF520	M21	17.2	2556	<100	1962	202	612	165	115	<100	323	<100
BF520	M27	23.2	2063	118	1127	106	2028	100	112	118	306	<100
MG376	W0	N/A	144	<100	456	481	799	235	<100	<100	222	<100
BG376	W14	-	<100	<100	173	<100	152	152	<100	<100	119	<100
BG376	M6	+	<100	<100	<100	<100	<100	126	<100	<100	103	<100
BG376	M12	6	150	<100	<100	<100	<100	120	<100	<100	108	<100
BG376	M18	12	858	<100	1231	167	280	169	<100	112	235	<100
BG376	M24	18	799	<100	895	132	193	102	176	124	212	<100
MH217	P39	N/A	1299	<100	>3200	133	218	179	108	<100	264	<100
BH217	M6	*	<100	<100	<100	<100	112	157	100	<100	107	<100
BH217	M12	8	201	<100	473	<100	125	169	121	104	150	<100
BH217	M21	17	310	<100	1104	122	140	<100	<100	108	168	<100
BH217	M24	20	360	129	1230	120	251	113	106	129	201	<100
MN469	P32	N/A	1282	140	1377	225	561	326	182	<100	342	<100
BN469	W14	+	<100	<100	<100	<100	<100	119	<100	119	104	<100
BN469	M6	2.3	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100
BN469	M12	8.3	310	381	362	157	187	174	227	194	236	126
BN469	M18	14.3	395	<100	1325	130	101	<100	<100	117	173	<100
BN469	M24	20.3	551	<100	1691	120	306	<100	106	122	214	<100

IC50
<100
100-300
301-1000
>1000

Table 4.1. Neutralization profile of maternal and longitudinal infant plasma against Panel 3 viruses.
[a]Subject ID. M, maternal sample; B, infant sample
[b]Plasma timepoint tested. P, weeks after pregnancy; W, weeks after birth; M, months after birth.
[c]Time post HIV-1 infection in months.
'N/A', not available; '-', timepoint prior to HIV infection; '+', first HIV positive timepoint;
'*', timepoint immediately after first HIV positive test for BH217

Association between maternal and infant NAbs and viral loads

To determine whether infant NAb responses were correlated to those of their mothers, we tested maternal samples from the last pre-transmission timepoint against Panel 4 viruses. Overall, 4/7 maternal samples neutralized at least one virus from each clade with IC50 > 100 (Table 4.1), but there was a trend for lower NAb titers in mothers compared to paired infants (p=0.098, Fig. 4.4A), although there was no difference between maternal and infant total (p = 0.227, Fig. 4.4B) or Env-specific IgG levels (p = 0.337, Fig. 4.4C). There was also no correlation between maternal and infant *de novo* responses among this subset of 7 pairs (Pearson's r = 0.59, p = 0.138, Fig. 4.4D) or among all 28 pairs (Pearson's r = 0.02, p = 0.913, Fig. 4.4E). These findings suggest that unique antigenic features of the viral population shared by mother and infant were not the major factor driving infant NAb breadth.

For 22/28 infants with plasma available within the first week of life, we also determined the association between passive and *de novo* antibodies. Passive neutralization data for 13/22 infants were available from a prior study investigating passive NAb breadth (127). We compared passive and *de novo* NAbs against Q461.D1 (clade A), DU156.12 (clade C), and QD435.A4 (clade D), which represent the overlap between Panel 3 and the virus panel from this prior study, and found no correlation between passive and *de novo* NAb titers (Pearson's r = 0.25, p = 0.258, Fig. 4.4F). Interestingly, excluding BG505, an outlier in this analysis, resulted in a significant correlation between passive and *de novo* NAbs (Pearson's r = 0.50, p = 0.022, Fig. 4.4G), suggesting that passive antibodies may influence *de novo* responses overall, but there may be unique factors contributing to *de novo* NAb breadth in BG505.

Surprisingly, we found a trend for a positive correlation between passive NAbs and set-point viral load (SVL), defined as the first viral load measurement between 4-12 months PI (182)

(Pearson's r = 0.40, p = 0.069, Fig. 4.4H). Because maternal viral load has previously been shown to predict infant SVL (165), and because viral load is associated with NAb breadth (68, 174, 201, 228), we hypothesized that the association between infant passive NAbs and SVL was driven by maternal viral load. However, maternal viral load obtained around the time of delivery was neither associated with infant SVL (Pearson's r = 0.28, p =0.161, Fig. 4.4I), nor passive NAbs (Pearson's r = 0.17, p = 0.486, Fig. 4.4J), suggesting that the association between infant passive NAbs and SVL was not explained by maternal viral load.

Factors associated with NAb breadth in infants

We analyzed neutralization of 3 clade A, C, and D variants (Q461.D1, DU156.12, and QD435.A4, respectively) by infant plasma to investigate factors that might be associated with *de novo* NAb breadth, including infant SVL, duration of HIV-1 infection, and total, and Env-specific IgG levels at the latest timepoint, and passive NAbs. In univariate and multivariate linear regression models, SVL and Env-specific IgG levels were significantly associated with NAb breadth (Fig. 4.5A). Moreover, there was a trend for higher Env-specific IgG levels among the 7 infants with bNAbs identified in Figure 4.1 compared to 21 infants without bNAbs (mean $\log_2$ end-point titer of 17.8 vs. 15.8, p = 0.052, Fig. 4.5B), and SVL was significantly higher in the former compared to the latter group (mean SVL of 6.62 vs. 5.85 $\log_{10}$copies/ml, p = 0.0004, Fig. 4.5C).

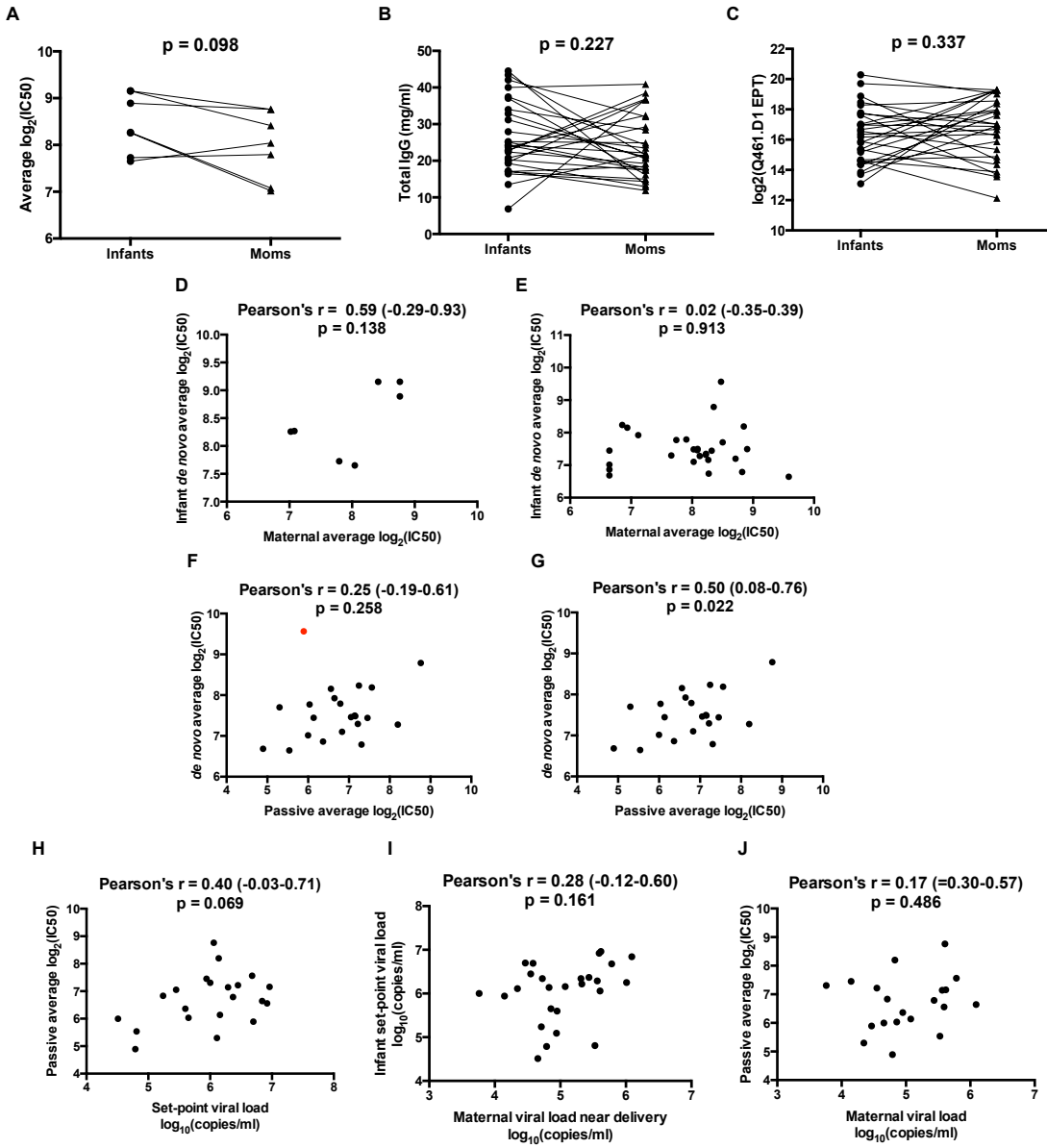


Figure 4.4. Association between maternal and infant antibodies and viral loads.
(**A)** Paired t-test comparing average log_2(IC50) values of 7 infants with bNAbs and their corresponding mothers against Panel 3 viruses. **(B)** Wilcoxon's signed rank test comparing maternal and infant total IgG levels from 28 pairs. **(C)** Paired t-test with Welch's correction comparing maternal and infant Env-specific IgG end-point titers (EPT) from 28 pairs. **(D)** Correlation between average log_2(IC50) values of plasma from 7 infants with bNAbs and their mothers against Panel 3. **(E)** Correlation of average log_2(IC50) values of 28 mother-infant pairs against Q461.D1, DU156.12, and QD435.A4. Correlation between passive and *de novo* infant average log_2(IC50) values against Q461.D1, DU156.12, and QD435.A4 **(F)** including (n=22) or **G)** excluding (n=21) BG505 (red dot in F). **(H)** Correlation between infant passive average log_2(IC50) values against Q461.D1, DU156.12, and QD435.A4 and set-point viral load (n=21). **(I)** Correlation between maternal viral load and infant set-point viral load (n=26). **(J)** Correlation between maternal viral load obtained near delivery and infant passive average log_2(IC50) values against Q461.D1, DU156.12, and QD435.A4 (n=20).

A

Univariate linear regression

Covariate	n	Estimate	95% CI	p-value
Set-point viral load	27	0.606	0.256-0.957	0.003*
Duration of infection	28	0.001	-0.061-0.064	0.965
Total IgG	28	0.009	-0.030-0.047	0.667
Env-specific IgG	28	0.191	0.060-0.323	0.01*
Passive NAbs	22	0.192	-0.131-0514	0.258

Multivariate linear regression

Covariate	n	Estimate	95% CI	p-value
Set-point viral load	22	0.392	0.036-0.748	0.045*
Env-specific IgG	22	0.164	0.032-0.296	0.025*

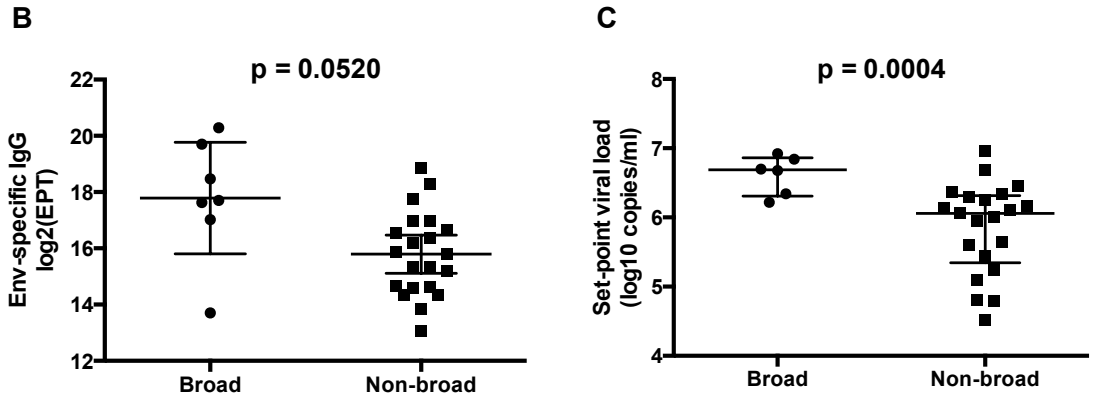
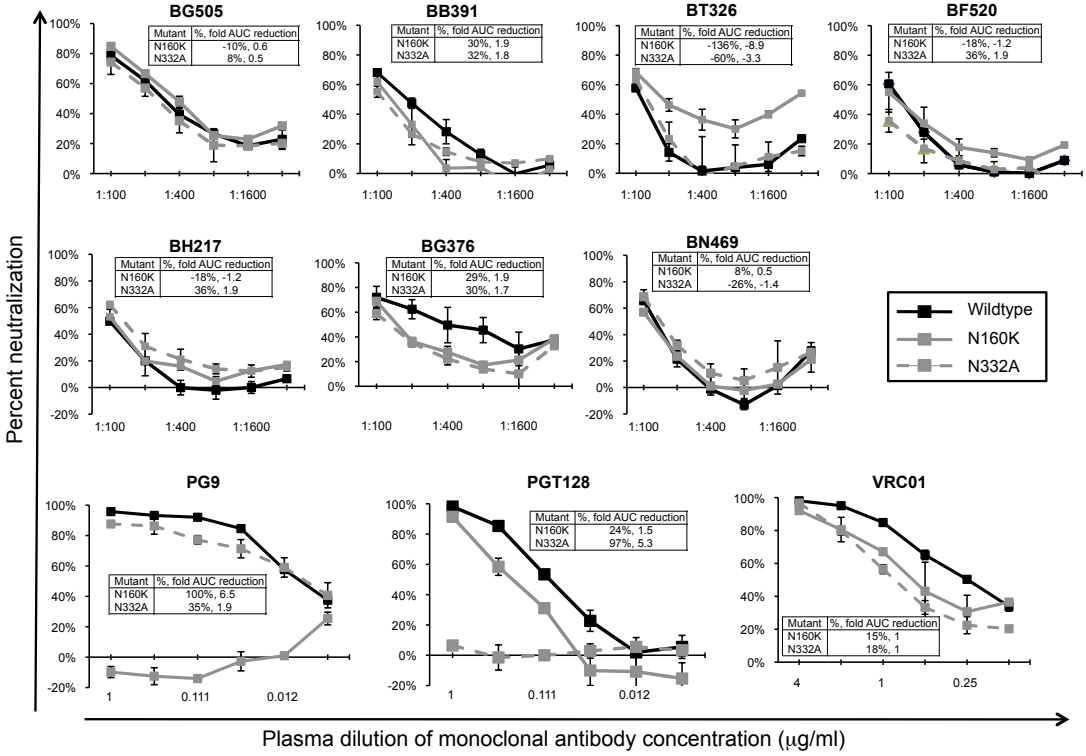


Figure 4.5. Analysis of factors associated with bNAbs.
(A) Univariate and multivariate linear regression analyses of infant set-point viral load, duration of HIV-1 infection, total IgG levels, Env-specific (Q461.D1 gp120) levels, passive NAb titers, and *de novo* NAb breadth, defined as the average $\log_2$(IC50) against Q461.D1 (clade A), DU156.12 (clade C), and QD435.A4 (clade D). Passive NAb data and set-point viral load measurements were available for 22/28 and 27/28 infants, respectively. '*', p-value < 0.05. 'n', number of infants included in analysis. **(B)** Unpaired t-test with Welch's correction comparing Env-specific (Q461.d1 gp120) log-transformed end-point titers (EPT) of plasma from infants with (n=7) and without (n=21) bNAbs. **C)** Unpaired t-test with Welch's correction comparing set-point viral load of infants with (n=6, excluding BB391, who had no available set-point viral load data) and without (n=21) bNAbs identified in Figure 4.1. Horizontal lines in **(B)** and **(C)** represent mean and 95% confidence intervals.

bNAbs in infants do not target known epitopes

Currently identified bNAbs target 4 main epitopes on Env: on gp120, glycan-dependent epitopes in V1/V2 (PG9-like) (233) or V3 (PGT128-like) (232), and the CD4 binding site (CD4bs) (VRC01-like) (247); and on gp41, the membrane proximal external region (MPER) (4E10/10E8-like) (87). By performing mapping experiments that are currently standard for detecting these bNAbs (68, 87, 128, 145, 155), we found that the predominant NAbs in infants did not target these known epitopes. For all samples, there were no appreciable differences in the reduction in area under neutralization curves comparing wildtype Q23 or JRCSF to the corresponding N160K and N332A mutants relative to those observed with VRC01, a monoclonal antibody that served as a control for non-specific effects of these mutants, suggesting that NAb breadth was not due to PG- or PGT-like NAbs (Fig. 4.6A). Similarly, by performing ELISA at a starting plasma dilution of 1:100, only 2 samples bound to a resurfaced stabilized core protein (RSC3) designed to optimally display the CD4 binding site (247), indicating that the majority of antibodies were not VRC01-like (Fig. 4.6B). Additionally, only BG505 neutralized the HIV-2/HIV-1 MPER chimera with a ~3-fold increase in titer compared to HIV-2 (Fig. 4.6C), suggesting limited MPER NAbs. Altogether, these results suggest that there is no dominant response against known bNAb epitopes in infant plasma, and that breadth may be due to either a bNAb with novel specificity and/or to a polyclonal response.

A



B

ID	End-point titer RSC3	RSC3d371	Fold difference
BG505	<100	<100	1
BB391	<100	<100	1
BT326	<100	<100	1
BF520	<100	<100	1
BG376	<100	<100	1
BN469	133	<100	1.3
BH217	100	<100	1
VRC01	<0.04	1.25	31.3
2G12	2.5	2.5	1

C

ID	IC 50 7312A	7312A-C1	Fold difference
BG505	224	703	3.1
BB391	156	<100	0.6
BT326	<100	<100	1.0
BF520	<100	<100	1.0
BG376	<100	133	1.3
BN469	<100	<100	1.0
BH217	<100	<100	1.0
4E10	>4	0.17	23.5

Figure 4.6. Epitope mapping of bNAbs in infants.
(**A)** The presence of PG9- and PGT128-like antibodies was determined by comparing neutralization of a clade A wildtype virus, Q23.17 relative to N160K and N332A mutants, respectively(68, 155). Two samples (BG376 and BN469) that could not neutralize Q23.17 at the lowest dilution tested (1:100) were instead tested against JRCSF (clade B) and corresponding mutants (232, 233). Monoclonal antibodies PG9 and PGT128 were included as positive controls, while VRC01 served as a control for non-specific effects for both mutants. For each sample, the percentage reduction in area under the curve (AUC) comparing mutant to wildtype virus was normalized to that seen for VRC01 (% sample AUC reduction / % VRC01 AUC reduction), and is expressed as fold AUC reduction, as shown in boxes. **(B)** Infant antibodies against CD4bs were assessed by binding to RSC3 or RSC3d371I in ELISAs (128). End-point titers are shown. Monoclonal antibodies VRC01 and 2G12 were included as positive and negative controls, respectively. **(C)** Infant plasma samples were screened against the 7312A HIV-2 full-length construct, or the 7312A-C1 HIV-2/HIV-1 MPER chimera (87) to detect the presence of MPER-specific NAbs. IC50 values are shown. Monoclonal antibody 4E10 was included as a positive control.

Discussion

In this study, we have shown that approximately 25% of HIV-infected infants can develop bNAbs. This may actually be an underestimate because for some infants with modest cross-clade responses at 12 months PI, later samples were not available to determine if they ultimately developed more broad and potent NAbs. Notably, after only 2.5 years of infection, BG505 and BB391 developed NAbs that were comparable in breadth and potency to those observed in adults identified as having the broadest responses after ~5 years of infection in larger studies (41, 174, 213). Thus, despite having higher viral loads and faster disease progression compared to adults, infants maintain sufficient B-cell function to mount bNAbs. In fact, as seen in adults, higher SVL in infants was associated with NAb breadth, supporting the hypothesis that high early antigenic load drives the development of bNAbs (68, 174, 228).

These results suggest that HIV-1 bNAbs can develop early in life. HIV-1 bNAbs in adults display extensive somatic hypermutation (87, 232, 233, 247). Infants can achieve adult-like IgG diversity and concentration by 8-12 and 12-24 months, respectively (212), raising the possibility that rapid somatic hypermutation early in life may contribute to the development of infant bNAbs. Although infant IgG diversity was not explored here, we did find that at ~24 months, infants did not differ from their mothers in total or Env-specific IgG titers. Additionally, the reported predominance of IgG1 and IgG3 over IgG2 in infants (212) may contribute to enhanced HIV-1 neutralizing activity (7, 33, 206).

Although HIV-specific passive IgG has been shown to reduce viremia and accelerate the appearance of *de novo* NAbs in newborn macaques (163), presumably by mitigating HIV-induced B-cell dysfunction, which is thought to delay NAb responses (118), we found a trend for a positive correlation between higher passive NAbs and infant SVL, which in turn was

associated with *de novo* breadth. Because only passive IgG matched to the infecting virus, and not mismatched IgG, successfully reduced viremia in the prior macaque study (163), the failure of passive NAbs to control viremia in infants may be explained by the selection NAb escape variants for mother-to-child transmission (48, 105, 246). It is possible that these transmitted NAb escape variants must also have high replication fitness (105, 106) when there is efficient transfer of passive NAbs, which might explain the observed trend for a positive correlation between passive NAb levels and SVL.

We observed a positive correlation between passive and *de novo* NAbs only when an outlier, BG505, the infant with greatest NAb breadth in this screen, was excluded from the analysis, suggesting that potentially unique factors may contribute to the development of *de novo* responses in BG505. Interestingly, HIV-1 envelope isolated from the first HIV positive timepoint in BG505 was recently found to be remarkably stable as soluble trimers (93, 129) that preferentially express epitopes for bNAbs over non-NAbs (94, 199), and to elicit NAbs against Tier 2 viruses as a monomeric immunogen (82). We have previously shown that, as in most cases of vertical transmission, viruses transmitted to BG505 appeared to have escaped maternal NAbs (246). This process of NAb escape from maternal NAbs, and perhaps from early *de novo* responses, could have indirectly exposed conserved epitopes that led to bNAbs, as has been shown recently in heterosexual infection (155). Thus, it is interesting to speculate that the transmission of NAb escape variants plays a role in the development of bNAbs.

As infants are monitored frequently for infection as part of prevention of mother-to-child transmission efforts, pre-infection and longitudinal samples are available in many infant cohort studies. Given the growing interest in the field to delineate pathways to HIV-specific bNAbs, these samples may be especially valuable for characterizing pathways to bNAbs, as some infants

appear to develop bNAbs relatively rapidly, and at their early age, may have been exposed to fewer non-HIV-specific antigens than their adult counterparts.

Chapter V

Implications for NAb-based HIV-1 vaccine design

The studies described in this thesis provide insight into NAb specificities that are relevant for both heterosexual and vertical transmission of HIV-1. In the context of MTCT, transmitted viruses escape maternal NAbs by conformational changes in Env that mediate evasion from multiple NAb specificities, suggesting that NAbs targeting conserved epitopes may be required to block MTCT. Indeed, even though these escape variants are resistant to neutralization by maternal NAbs, they are potently neutralized by a number of bNAbs targeting distinct, conserved epitopes. In particular, a combination of bNAbs that target the CD4bs and a conserved glycan-dependent epitope in V3 provides optimal coverage of both vertically and heterosexually transmitted viruses. Additionally, this work demonstrates the ability of the human immune system to generate broad and potent responses against HIV-1, even in early life. These results highlight additional areas for future studies relevant for HIV-1 vaccine design that I will discuss in this chapter.

Identifying bNAb specificities that are effective against transmitted variants

Based on the neutralization profile of bNAbs identified to date against a panel of diverse heterosexually transmitted variants in Chapter III, it appears that no single bNAb can provide 100% neutralization coverage of viruses, but bNAbs targeting CD4bs and V3 displayed complementary activity, and combining these bNAbs improves coverage. This same combination of bNAbs has been shown to provide optimal coverage of viruses in some studies (22, 131), while others have shown that different combinations of bNAb specificities, including

V1/V2 and V3 (131), and CD4bs and V1/V2 (56, 59) can improve neutralization coverage of various HIV-1 strains. Overall, these studies suggest that a protective vaccine may need to elicit more than one bNAb specificity to effectively protect against diverse circulating viruses.

A recent study has shown that complementary monoclonal antibodies targeting distinct epitopes (CD4bs and V3) that mediate broad and potent NAb responses can in fact develop within a single HIV-1 infected individual (102), but it is unclear how commonly these responses are generated. Indeed, although a few studies have estimated the prevalence of NAb responses targeting conserved epitopes of bNAbs using a variety of mapping strategies, some of which are described in Chapters II and IV, these studies have focused on individuals known to be broad neutralizers (145, 207, 234), and not on a more general HIV-1 infected population. Studying the prevalence of bNAb specificities in the latter population would provide insight into which antibody specificities are feasible to elicit via vaccination as a particular NAb specificity that is generated frequently during natural infection may be less challenging to induce than one that is rarely observed.

In order to evaluate which bNAbs would be most effective in preventing transmission, it would also be valuable to regularly screen them against viruses circulating in the current epidemic. An updated panel of viruses representing those presently circulating is especially relevant given that HIV-1 Env appears to be adapting towards higher resistance to bNAbs at a population level over the course of the epidemic (22, 26, 59). Although the combination of NIH45-46W, which targets CD4bs, and PGT128, which targets V3 appears to be effective against viruses as recent as 2010 (22), it is conceivable that Env may gradually evolve resistance to these mAbs. Thus, ongoing longitudinal cohorts at risk of vertical and heterosexual transmission provide a valuable resource for obtaining diverse transmitted viruses circulating in

the ongoing epidemic that can be used to monitor HIV-1 Env evolution and NAb escape at the population level.

The collection of diverse variants from early infection may also be useful for studying structural and antigenic properties of transmitted viruses that may be distinct from those found in chronic infection. As previously described, compared to viruses found in chronic infection, transmitted viruses tend to have unique properties such as shorter variable loops and fewer PNGS, which modify neutralization sensitivity (37, 47, 193, 198, 236, 246). Despite this observation, with a few exceptions (50, 82, 94), most Env constructs used for structural and immunogenicity studies are based on lab-adapted or chronic infection strains (14, 15, 46, 86, 96, 98, 112, 123, 136, 137, 166, 240, 259). Interestingly, a recent study found that an envelope variant isolated from the first HIV positive timepoint from infant BG505, who was found to have the most broad and potent NAbs among infants screened in Chapter IV, displayed remarkable stability as a trimer, which is a rare property among soluble Env constructs (94). Additionally, the BG505 envelope variant elicited NAbs against some relatively difficult to neutralize viruses (Tier 2) as a monomeric immunogen (82). It is unclear whether enhanced stability and immunogenicity are properties shared by most envelopes of transmitted viruses, or whether these are unique to vertically transmitted viruses, or even just to BG505. Regardless, the structural and antigenic properties of Env from transmitted viruses are largely unexplored and warrant further study, as these are the viruses that a protective NAb response must target. Moreover, detailed analyses of these viruses may provide clues into the structural basis for the observed complementary activity of certain combinations of bNAbs targeting distinct epitopes.

Env mutational tolerance to mediate NAb escape

Studies characterizing the dynamics of NAb escape within acute heterosexually infected individuals have shown that continuous waves of escape variants arise, beginning at approximately 100 days post-infection in most cases (10, 156), but as early as 2 weeks post-infection in others (4). It is likely that regular sampling of viruses and antibodies from individuals enrolled in longitudinal cohorts will improve estimations of the kinetics of NAb escape. Interestingly, even very low NAb titers (IC50 of 1:20-1:50) appear to select for escape variants in acute heterosexual infection (10).

In the context of MTCT, transmitted escape variants can elicit potent *de novo* autologous NAb titers in infants by 6 months of life in most cases (Chapter IV and (246)), but it is unclear whether further escape from *de novo* infant NAbs occurs with similar kinetics and mechanisms to those observed in acute heterosexual infection. The availability of longitudinal samples from HIV-1 infected infants provides a unique opportunity to characterize the kinetics of mechanisms of further NAb escape from de novo responses, and into the mutational tolerance of Env to mediate immune evasion. As described in Chapter II, the molecular determinants of NAb escape in vertically transmitted variants are complex, with distinct regions in Env mediating escape through conformational modifications that affect multiple distal epitopes, which may reflect the requirement for escape from polyclonal maternal NAbs throughout chronic infection. Because vertically transmitted variants have already undergone multiple rounds of NAb escape, one hypothesis is that further mutations needed to evade infant *de novo* NAbs are more likely to exert a fitness cost. If this were true, then the detection of escape variants from infant *de novo* NAbs may be rare or delayed compared to those in acute heterosexual infection. Although there have been conflicting findings on the effect of NAb escape mutations on viral fitness during

heterosexual infection (27, 202, 227), there are some studies suggesting that HIV Env may have a limited capacity to evolve continuously to evade NAbs (45, 202). It is possible that epitope specificity of NAbs affects the ability of Env to evolve to mediate escape. For example, Sather, *et al*. found that escape from broad and potent NAbs targeting the conserved CD4bs occurs with a fitness cost on the virus (202). Thus, identifying sites on Env that are accessible to NAbs and that have low mutational tolerance will be important for understanding how to restrict pathways of NAb escape.

Escape of vertically transmitted viruses from *de novo* NAbs may occur via distinct mechanisms from those observed during acute heterosexual infection. For example, preliminary findings suggest that the PNGS at residue 332 (N332), which is associated with NAb escape (155), is more prevalent among vertically transmitted than among heterosexually transmitted viruses ((155) and Goo, Mabuka, Overbaugh, unpublished). The retention of this residue in vertically transmitted viruses may facilitate NAb escape during transmission in the presence of pre-existing passive antibodies in infants. In heterosexual transmission, the N332 residue evolves during chronic infection among variants that have escaped from autologous NAbs. Interestingly, as described in previous chapters, N332 is the target of PGT-like bNAbs (232), and the presence of this residue coincides with the development of bNAbs in some heterosexually infected individuals (155). Because most vertically transmitted viruses already possess N332, they likely evolve additional mechanisms of escape from de novo NAbs, possibly exposing additional sites of vulnerability on Env. Thus, continuous cycles of Env evolution in vertically transmitted viruses to mediate NAb escape from de novo responses may indirectly expose conserved epitopes, and may explain the early development of bNAbs in some infants as described in Chapter IV.

Early development of bNAbs in infants

Chapter IV demonstrates that HIV-1 infected infants develop bNAbs at least as commonly as adults do. Moreover, cross-clade NAb responses develop earlier in some infants than in adults. As antigenic stimulation is important for the development of bNAbs (68, 174, 201, 228), one explanation for the early appearance of NAb breadth in infants may be that set-point viral loads reach higher levels in infants than they do in adults (182). Additionally, as has been shown in newborn macaques, the presence of passive antibodies may accelerate the development of de novo NAbs (163), although the exact mechanism by which this occurs is unknown. It is also possible that, as described above, infection with variants that have already undergone multiple rounds of NAb escape may lead to the exposure of conserved epitopes that induce broad and potent NAb responses sooner in infants. Isolation and characterization of viruses and monoclonal antibodies will be necessary to understand viral and immunological factors that contribute to these responses.

As mentioned, most bNAbs identified to date have undergone extensive somatic hypermutation that is necessary for high affinity binding to Env. The sequences of germline unmutated ancestors of such bNAbs have been indirectly inferred by a combination of deep sequencing, computational and phylogenetic methods (76, 260). Surprisingly, in all but one case (121), unmutated germline ancestors of B-cell receptors display either weak or undetectable binding to HIV-1 Env (2, 84, 130, 142, 252), suggesting that 1) only certain Envs can engage germline B-cell receptors, or 2) computational predictions of unmutated ancestors are often inaccurate, or 3) a non-HIV antigen engaged the initial B-cell receptor that eventually led to HIV-1 bNAbs. The identification of B-cell maturation pathways to HIV-1 specific bNAbs is additionally complicated by the fact that the prototype bNAbs were usually isolated from adults

during very late chronic infection (209, 247, 249), when there is likely massive B-cell hyperactivation and dysfunction (147). Because some infants develop early bNAbs, and because they are likely to have been less frequently exposed to multiple non-HIV antigens compared to adults, they may provide a unique model to more directly trace the evolution of HIV-1 specific bNAbs, particularly in cases where there are pre-infection and longitudinal samples available to characterize pre-cursor and evolved HIV-specific B-cells, respectively.

The development of bNAbs during chronic infection does not appear to provide clinical benefit (68, 174, 228), perhaps because NAbs can at best control only a subset of variants found in a diverse quasispecies present during chronic infection (65, 227). However, it is possible that if broad and potent NAbs were present soon after infection, when envelope diversity is lower (173), they may effectively control early viral replication to impact subsequent disease progression. In indirect support of this, a study to determine the *in vivo* efficacy of neutralizing mAbs found that lower NAb titers were required to control viremia in acute compared to chronic infection in humans (224). Additionally, passive immunization studies in macaque models have demonstrated that even if NAbs cannot protect against infection, they can still reduce early plasma viremia (60, 141, 163, 170, 254). This early control of viral replication could potentially reduce viral set-point, which is an important predictor of disease progression (144). Moreover, one study found that the early development of de novo NAbs in newborn macaques by 12 weeks was associated with reduced plasma viral load and maintenance of high CD4+ T-cell counts for the six months of follow-up (163). Although we found that some infants develop very broad and potent NAbs by 2.5 years of infection, which is earlier than most adults, we could not investigate the association between bNAbs and disease progression due to small sample size. Although potentially challenging, an important future study would be to utilize samples from a

longitudinal cohort to investigate the breadth and potency of NAbs prior to the establishment of set-point viral load to determine whether early bNAbs can impact disease progression. This finding would provide rationale for the use of NAbs as a therapeutic vaccine in the absence of a preventative vaccine. Indeed, recent studies showed that a combination of bNAbs administered at approximately 20 days after established infection can effectively control HIV-1 replication and suppress viral load to levels below detection in humanized mice (103), and in macaques, even when bNAbs were administered during chronic infection for the latter (12). Moreover, the longer half-life of some bNAbs compared to antiretroviral drugs resulted in more durable viral suppression after cessation of therapy (103). Thus, a combination of bNAbs and antiretroviral drugs may provide a novel therapeutic option to achieve long-lasting treatment of HIV-1 (85).

Conclusion

Overall, this work has identified the epitope specificities of NAbs that are relevant in limiting heterosexual and vertical transmission of HIV-1. Due to the extensive diversity of HIV-1 and the various mechanisms by which Env can evade NAb responses, an effective NAb-based vaccine will likely require a combination of epitope specificities to provide maximum coverage of circulating variants. Although bNAbs that are effective against most transmitted variants have been identified from HIV-1 infected adults and infants, understanding how to elicit such antibodies by vaccination remains a challenge, mainly due to a lack of understanding of the B-cell maturation pathways that lead to bNAbs, and of the structural properties of the native Env trimer, particularly on transmitted viruses, that would guide the rational design of immunogens. Cohorts such as the ones described in this work, in which there are pre-infection and longitudinal samples available to study the properties of transmitted viruses and the dynamics of virus and antibody evolution will be valuable for addressing these issues.

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next-generation sequencing and phylogenetic pairing of heavy/light chains. Proceedings of the National Academy of Sciences of the United States of America 110:6470-6475.

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CURRICULUM VITAE

Leslie Goo

EDUCATION

University of Washington — **Seattle, WA**
Ph.D. Pathobiology — 2013

University of Michigan — **Ann Arbor, MI**
M.P.H. Epidemiology/International Health — 2008

University of Michigan — **Ann Arbor, MI**
B.S. Microbiology — 2004

FELLOWSHIPS AND HONORS

University of Washington Outstanding Doctoral Student Award — 2013
Keystone Symposia Scholarship — 2013
American Society for Virology Student Travel Award — 2012
FHCRC Dual Mentor Interdisciplinary Research Fellowship — 2011
University of Washington Graduate Top Scholar Award — 2008
University of Michigan Dean's Merit Award — 2006-2007
University of Michigan Globalization and Health Fellowship — 2007
University of Michigan Honors — 2001
University of Michigan William J. Branstrom Freshman Prize — 2000

RESEARCH EXPERIENCE

Fred Hutchinson Cancer Research Center — **Seattle, WA**
Graduate Research Assistant, laboratory of Dr. Julie Overbaugh — 2008-2013
Dissertation topic: Defining neutralizing antibody specificities that target diverse HIV-1 transmitted strains

University of Washington Department of Chemistry — **Seattle, WA**
Graduate Research Assistant, laboratory of Dr. Pradip Rathod — Spring 2008
Rotation topic: Defining the therapeutic window of osmotic pump-delivered pyrimethamine

Seattle Biomedical Research Institute — **Seattle, WA**
Graduate Research Assistant, laboratory of Dr. Helen Horton — Winter 2008
Rotation topic: Developing a natural killer cell-based HIV-1 inhibition assay

University of Michigan School of Public Health — **Ann Arbor, MI**
Graduate Research Assistant, Dr. Sioban Harlow — 2007-2008
Master's thesis topic: Effect of intimate partner violence on skilled attendance at most recent delivery among women in Kenya

University of Michigan Medical School — **Ann Arbor, MI**
Part-time Research Assistant, laboratory of Dr. Akira Ono — 2007-2008

Research Topic: The role of phophoinositide in HIV-1 Gag localization in macrophages

National AIDS Research Institute **Pune, India**
Intern, Dr. Sanjay Mehendale and Dr. Seema Sahay Summer 2007
Research topic: Communication of sexual and reproductive health among adolescent students

University of Michigan Medical School **Ann Arbor, MI**
Research Technician Associate, laboratory of Dr. Alice Telesnitsky 2005-2006
Research topic: Mechanisms and outcomes of Murine Leukemia Virus non-homolgous recombination

University of Michigan Medical School **Ann Arbor, MI**
Laboratory Assistant, laboratory of Dr. David Miller 2004-2005
Research topic: Characterizing Flock House Virus assembly in yeast mitochondria

PUBLICATIONS

Goo L, Chohan V, Nduati R, Overbaugh J. "Early development of broad and potent neutralizing antibodies in HIV-1 infected infants." *Submitted October 2013*

Mabuka J, **Goo L**, Omenda MM, Nduati R, Overbaugh J. "HIV-1 maternal and infant variants show similar sensitivity to broadly neutralizing antibodies, but sensitivity varies by subtype." *AIDS* 2013 June;27(10): 1535-44. doi: 10.1097/QAD.0b013e3283faba5.

Goo L, Jalalian-Lechak Z, Richardson BA, Overbaugh J. "A combination of broadly neutralizing HIV-1 monoclonal antibodies targeting distinct epitopes effectively neutralizes variants found in early infection." *J Virol.* 2012 Oct;86(19):10857-61. doi: 10.1128/JVI.01414-12.

Goo L, Milligan C, Simonich CA, Nduati R, Overbaugh J. "Neutralizing antibody escape during HIV-1 mother-to-child transmission involves conformational masking of distal epitopes in envelope." *J Virol.* 2012 Sep;86(18):9566-82. doi: 10.1128/JVI.00953-12.

Goo L, Harlow S. "Intimate partner violence affects skilled attendance at most recent delivery among women in Kenya." *Matern Child Health J.* 2012 Jul;16(5):1131-7.

Duggal NK, **Goo L**, King SR, Telesnitsky A. "Effects of homology minimization on Moloney Murine Leukemia Virus template recognition and frequent tertiary template-directed insertions during non-homologous recombination." *J Virol.* 2007 Nov;81(22): 12156-12168.

PRESENTATIONS

Goo L, Milligan C, Simonich CA, Nduati R, Overbaugh J. "Defining Neutralizing Antibody Specificities that Impact HIV-1 Mother-to-Child Transmission." HIV-1 Vaccines Keystone Symposia, Keystone, CO, February 10-15, 2013. Poster presentation

Goo L, Jalalian-Lechak Z, Richardson BA, Overbaugh J. "A combination of broadly neutralizing antibodies targeting distinct epitopes effectively neutralizes variants found in early infection. University of Nairobi Collaborative Meeting, Nairobi, Kenya, January 23 2013. Oral presentation

Goo L, Milligan C, Simonich CA, Nduati R, Overbaugh J. "Neutralizing Antibody Escape during HIV-1 Mother-to-Child Transmission Involves Conformational Masking of Distal Epitopes in Envelope." American Society for Virology Conference, Madison, WI, July 22, 2012. Oral presentation.

Duggal NK, **Goo, L**, King SR, Telesnitsky A. "Minimal homology required during Moloney Murine Leukemia Virus template recognition and outcomes of forced non-homologous template switch." Cold Spring Harbor Laboratories Retrovirus Conference, May 25, 2007. Oral presentation

Nuga AA, King SR, **Goo L**, Telesnitsky A. "Retroviral packaging and recombination." University of Michigan Global Infectious Disease Symposium, Oct. 17, 2006. Poster presentation.

TEACHING EXPERIENCE

Didactic

PABIO 551 – Biochemistry and Genetics of Pathogens and Their Host.
University of Washington, Seattle, Fall 2009.

Trainees

Bingjie Wang, *Undergraduate Student, laboratory of Dr. Julie Overbaugh*
University of Washington, Seattle, WA, Summer 2012-Fall 2013.
Cassandra Simonich, *Rotating Graduate Student, laboratory of Dr. Julie Overbaugh*
University of Washington, Seattle, WA, Summer 2011.
Caitlin Milligan, *Rotating Graduate Student, laboratory of Dr. Julie Overbaugh*
University of Washington, Seattle, WA, Summer 2010.
Marcy Patrick, *Rotating Graduate Student, laboratory of Dr. Alice Telesnitsky*
University of Michigan, Ann Arbor, Fall 2005.
Vicki Larson, *Research Technician, laboratory of Dr. Alice Telesnitsky*
University of Michigan, Ann Arbor, 2005-2006.

LEADERSHIP and ACTIVITIES

FHCRC Weintraub Award Committee **Seattle, WA**
Reviewed applications and assisted in selection of awardees Spring 2011
University of Washington Dream Project **Seattle, WA**
Assisted high school seniors in writing college application essays Winter 2011
University of Washington Pathobiology Student Activities Committee **Seattle, WA**
Planned and organized program activities Fall 2009