Defining the Role of Maternal Plasma Neutralizing Antibodies in Mother-to-Child Transmission of HIV-1

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Abstract

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Identifying a vaccine that would elicit neutralizing antibodies (Nabs) that prevent HIV-1 infection of host cells is a priority for the HIV-1 research field. Mother-to-child transmission (MTCT) of HIV-1 offers a natural setting in which to evaluate whether Nabs are correlated with protection in exposed individuals. This topic is the focus of this thesis.

Given that Nabs can impact MTCT of HIV-1 by neutralizing viruses within the mother and/or infants, I first evaluated in chapter 2 whether the Env specific Nabs repertoire in the mother correlates with that in their corresponding infants. I compared antibody levels in plasma from 60 mother-infant pairs near the time of birth, including 14 breastfeeding transmission pairs. The Envelope binding titers were strongly correlated ($r=0.91$, $p<0.0001$) and similar (1.4-fold greater in maternal plasma) between a mother and her corresponding infant as were the neutralizing antibody (Nab) levels (*r*

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= 0.80, p<0.0001; 1.3-fold higher), suggesting efficient transfer. However, there was no significant difference in heterologous Nab responses between transmitting (TM) and nontransmitting mothers (NTM), although there was a trend for transmitting mothers to have higher HIV-1-specific Nabs.

Neutralizing antibody response measured against circulating variants (heterologous Nabs) may not be adequate to measure a protective antibody response; rather Nabs responses specific to the maternal own variants (autologous Nabs) are more relevant to protection against MTCT. Indeed, during MTCT, HIV-1 variants relatively insensitive to maternal autologous Nabs (aNabs) are transmitted, suggesting protective effect of such antibodies against sensitive variants. In chapter 3 of this dissertation, I explore the hypothesis that if aNabs are protective against MTCT then NTM, particularly those at high risk of MTCT (high viral load & breastfeeding), may have few neutralization resistant viruses. We tested maternal Env variants that represent each woman's quasispecies against autologous plasma obtained near transmission, and compared the inhibitory concentration (IC50s) of TM to NTM. I found that there was no association between postpartum MTCT risk and IC50 (Odds Ratio = 1.0; p = 0.201). To examine whether sample timing may yield a different conclusion regarding aNab escape, maternal and infant variants obtained near the time of MTCT were tested against contemporaneous maternal plasma (early plasma) versus maternal plasma obtained ~6 months after transmission (later plasma) and compared using GEE (chapter 4). IC50 values against early plasma among maternal variants were 10-fold greater than for infant variants (p<0.0001). However, there was no significant difference in IC50

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values of maternal versus infant variants when tested against later plasma (maternal/infant IC50 ratio =1.3; p=0.69) because the infant variants were more sensitive to later aNabs in the mother (late/early IC50 ratio = 6.3; p<0.0001). Our findings suggest that although maternal aNab induced in natural infection may limit MTCT of sensitive variants, as defined by studies of aNabs near the transmission event, they are not sufficient to explain protection among high-risk NTM.

Overall, maternal Nabs induced in natural HIV-1 infection are present both in the mother and her infant, but the level of these antibodies are insufficient to protect against HIV-1 transmission. However, maternal Nabs present around the time when MTCT, may provide protection against neutralization sensitive variants.

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List of Abbreviations

ADCC: antibody-dependent cell-mediated cytotoxicity

AIDS: acquired immunodeficiency syndrome

ARV: anti-retroviral

BMC: breast milk cells

C1-C5: constant regions 1-5

CCR5: chemokine (C-C motif) receptor type 5

CXCR4: chemokine (C-X-C motif) receptor type 4

dsDNA: double stranded DNA

ELISA: enzyme linked immunosorbent assay

EPT: end point titers

RER: rough endoplastic reticulum

GEE: generalized estimating equations

HIV-1: human immunodeficiency virus type-1

IC50: inhibitory concentration (dilution) at which 50% of the input virus is neutralized

MPER: membrane proximal external region

MTCT: mother-to-child transmission

Nabs: neutralizing antibodies

aNabs: autologous neutralizing antibodies

hNabs: heterologous neutralizing antibodies

PCR: polymerase chain reaction

PNGs: potential N-linked glycosylation site

RT: reverse transcriptase

SIV: simian immunodeficiency virus

SU: surface unit

TM: trans-membrane

UNAIDS: United Nations Programme on HIV/AIDS

V1-V5: variable regions 1-5

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analyzed in this thesis.

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Dedication

To my sons; Ramon & Ryan,

you made life so meaningful to me.

To all my teachers, for the academic torch passed on.

Chapter 1

Introduction

The Human Immunodeficiency Virus (HIV)

HIV is an enveloped virus belonging to the family of *Retroviridae,* genus of complex *Lentiviruses* and is a zoonotic pathogen that seeded infection in humans as a result of cross-species transfer from different primate species. HIV-1, which is the pandemic form of HIV, crossed to humans from Chimpanzees(70) while the less pandemic HIV-2 was passed to human from Sooty mangabeys[(67) and reviewed in (151)]. Since HIV-1 was first diagnosed in gay men in 1981(60) and subsequently isolated as an etiologic agent of acquired immunodeficiency syndrome (AIDS) in 1984(11, 51, 52, 130), it has infected an estimated over 64 million people across the globe(111). However, because of rapid advances in the understanding of the biology and pathogenesis of HIV-1, there has been tremendous improvement in both treatment using antiretrovirals (29, 126) and preventative interventions such as adult male circumcision, vaginal microbicides and ARV-based pre-exposure prophylaxis (1, 7, 9, 56). Given these advances, there has been an overall increase in life expectancy of infected patients as well as reduction in the occurrence of new infections. However, ARV treatment, which is currently the most effective form of intervention, is a complicated option for long term sustainability because of the emergence of drug resistance resulting from poor adherence. Moreover, given the un-proportional

distribution of the number of HIV-1 infected people versus the ones on ARV treatment (14.4 million people eligible for ARV treatment and only 8 million are on treatment) (www.unaids.org, 2012 Global Report), ARV treatment remains a suboptimal and an expensive intervention for complete stoppage of HIV-1 spread among humans. Given these limitations, perhaps only an effective vaccine would be a practical and cost effective strategy to help halt the spread of HIV/AIDS pandemic. Unfortunately the greatest challenge for the development of an effective HIV vaccine is the lack of clear information on which immune correlate of protection should be targeted with a vaccine. The work presented in this dissertation outlines whether neutralizing antibodies induced in natural infection are correlates of protection against HIV-1 infection in the setting of mother-to-child HIV-1 transmission (MTCT)

HIV-1 genome and proteins necessary for pathogenesis

HIV-1 has a 9.8kb genome consisting of two copies of single stranded RNA. The RNA genome specifies three categories of genes including those coding for: 1. Major structural proteins such as *Gag* (group specific antigens), *Pol* (polymerase) & *Env* (envelope), 2. Regulatory proteins such as *tat* (transcriptional transactivator) and *rev* (Regulator of virion); and 3. Accessory proteins including *Vpu* (viral protein U), *Vpr* (viral protein R), *Vif* (viral infectivity factor) and Nef (negative factor). While all these proteins are critical for virus infectivity, the Env participates in the initiation step of infection as it mediates viral binding and fusion with host target cell plasma membranes. Moreover, Env is exposed on the virus surface (Fig1.1) making it a more

attractive target for a vaccine that seeks to induce sterile immunity that would block infection of target cells. Because this thesis focuses on the neutralizing antibody responses to Env, the Env is described in further detail.

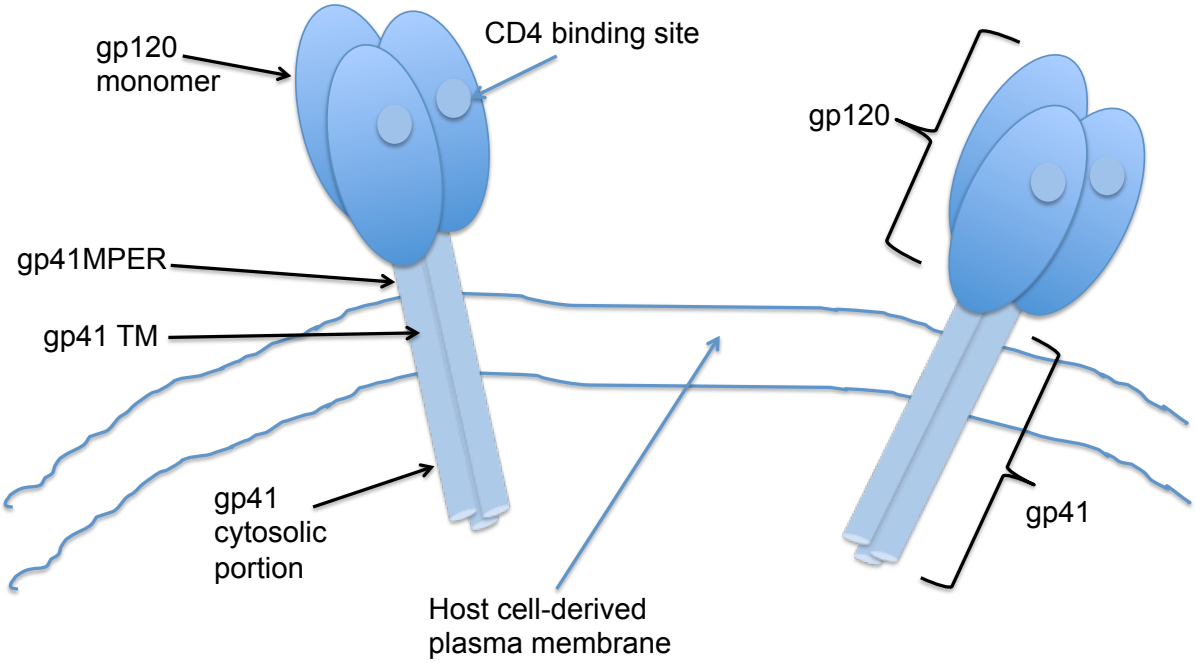


Figure 1.1. A diagrammatic representation of trimeric Envelope spike on the surface of the viral membrane. Three gp120 (blue) and gp41 (light blue) domains linked though non-covalent interaction, form the Env trimer. The CD4 binding site on the gp120 is also shown in light blue. The gp41 TM (transmembrane domain) hooks the Env into the host-derived viral membrane. MPER refers to membrane proximal external region of gp41.

HIV-1 Envelope protein and its role in virus replication

Envelope synthesis: The Env glycoprotein is displayed on the surface of the virion as highly glycosylated spikes, consisting of a trimer of heterodimer of homotrimeric glycoprotein gp120 noncovalently linked to glycoprotein gp41. The Env

glycoprotein is initially translated as a polyprotein precursor (gp160) from a singly spliced mRNA and is co-translationally modified in the rough endoplasmic reticulum (RER) by addition of N- and O-linked side chain oligosaccharides(12, 89). In the RER, gp160 monomers oligomerize into mostly trimers, however dimers and tetramers may also exist. These oligomeric forms traffic through the secretory pathway via the trans-golgi aparatus, where further modification involving trimming of the oligosaccharide side-chains and addition of more complex sugars occurs. In the Golgi, fully processed gp160 precursor protein is cleaved at highly conserved motif (Arg-X-Lys/Arg-Arg), which is recognized by host cell encoded furin-like proteases(49, 63). The cleavage produces two subunits: the surface destined gp120 (SU) and the membrane spanning cytosolic gp41 glycoproteins (TM) subunits (Fig 1.1). These subunits (gp120 and gp41) associate together through noncovalent interaction and on the virus surface, three of these glycoproteins form a functional Env.

The gp120 subunit of Env consists of discontinuous conserved regions named C1-C5, separated by highly variable segments named V1–V5. The variable regions are folded into loops created by cysteine/cysteine intramolecular disulfide bridges(156, 171) (Fig 1.2). In addition, gp120 protein is highly glycosylated (Fig.1.2), with almost half of its molecular weight contributed by both N-linked glycans(4) and O-linked glycans(12). These sugars form a canopy over the Env thereby masking it from host immune recognition, particularly recognition by neutralizing antibodies. In addition to protecting the Env from immune recognition, these sugars also participate in Env folding as well as binding of the virus to the target cells(92, 132).

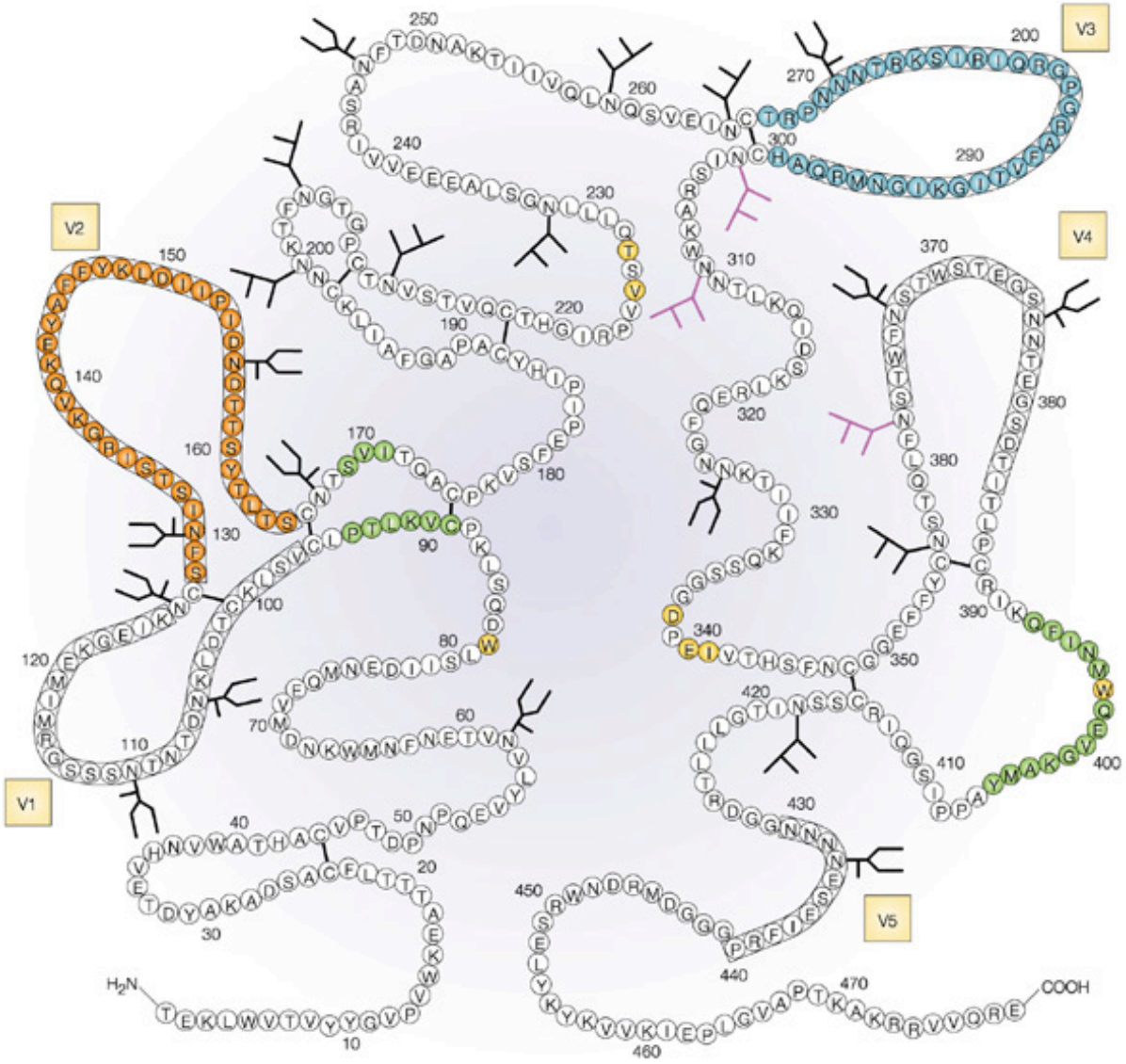


Fig.1.2. Diagramatic representation of HIV-1 Env gp120 glycoprotein. The gp120 variable regions are labeled V1–V5 (yellow boxes). The glycosylation sites containing high mannose-type and/or hybrid-type oligosaccharide structures are indicated by the branched structures, and glycosylation sites containing complex-type oligosaccharide structures are indicated by the U-shaped branches. Some of the key epitopes in gp120 that induce neutralizing antibodies are highlighted in color. These include the highly conformational CD4-binding domain (key epitopes highlighted in yellow), the CD4-induced epitope (green), an epitope composed of α1→2 mannose residues (purple), the V2 loop (orange) and the V3 loop (blue). Adapted with permission from Nat Rev Immunol. 2004 Mar; 4(3):199-210 and reference(89).

The gp120 defines virus tropism because it is the Env subunit that interacts with the virus receptor (CD4) and co-receptor (mostly CCR5 & CXCR4 chemokine receptors) on the surface of the target host cells. The CD4 receptor binding site of gp120 is formed by distal conserved domains including C1, C3, and C4 that are brought in close proximity in the tertiary structure of Env(82, 87). Several seminal studies have suggested that the variable regions of the Env are not directly involved in CD4 binding because deletion of V1, V2, and V3 domains of Env do not abrogate high affinity binding to CD4(176). However, the V3 loop may be critical for mediating fusion between the virus Env and the host plasma membrane(48) as well as co-receptor specificity(121, 152). Therefore, V3 may be critical for virus infectivity. It has also been shown to be targeted by neutralizing antibodies induced during natural infection, and is involved in the virus switch from CCR5 to CXCR4 tropism(108, 178)

The gp41 transmembrane subunit of the Env mediates fusion between the virus and the host cell plasma membrane. It has three domains namely: 1. An extracellular N terminal domain, which consists of a fusogenic peptide region, two α –helical coiled-coil structured regions (HR1 & HR2) and a tryptophan rich membrane-proximal region(34), which is a major target for broadly neutralizing antibodies. 2. A highly conserved transmembrane region, which anchors the Env in the lipid, and 3. C-terminal cytosolic domain (27, 170). The HR1 domain is connected to HR2 through a disulfide bridge and the interaction of the two domains facilitates the fusion process and hence are critical for infectivity. The MPER is also highly conserved across several clades and is one of the first Env regions against which several broadly neutralizing antibodies such 2F5,

4E10 & Z13, have been isolated (181).

HIV-1 infection of target cells: HIV-1 predominantly infects CD4 expressing cells including CD4+ T lymphocytes, macrophages & dendritic cells because CD4 is the primary receptor required by the virus to initiate infection. During infection, the binding of virus to CD4 through gp120 triggers conformational change in this protein that exposes its domains required for recognition of the co-receptor. Although HIV-1 can use other coreceptors, the primary ones include CCR5 and CXCR4. Upon engagement of the co-receptors, additional structural changes occur that extend to gp41. This global conformational change triggers a reaction that culminates in the fusion of the virus Env with host cell plasma membrane [reviewed in(47)]. The fusion reaction is pH-independent and delivers the viral core into the cytoplasm(110). In addition to the pH-independent pathway of viral entry, HIV-1 can also enter target host cells through clathrin-mediated endocytosis(33). However, this alternative pathway requires a subsequential fusion with the endosomal membrane, which is dependent on low pH(33, 112).

Upon entry into the cytosol, the viral RNA genome is first reverse transcribed into complimentary DNA (cDNA) by virus encoded reverse transcriptase enzyme. The reverse transcription process results into RNA/cDNA heterodimer. It is thought that the RNA in the RNA/cDNA complex is concurrently degraded by RNase-H as cDNA synthesis takes place. The generated cDNA is further transcribed into double-stranded DNA by the RT enzyme. The newly synthesized double stranded DNA is translocated as a preintegration complex into the nucleus where it is integrated into the host cell

chromosomal DNA(22, 54). The integration process is mediated by the viral-encoded integrase enzyme while the transcription of viral RNAs from the integrated DNA is catalyzed by host cell DNA polymerase enzymes. The generated viral mRNAs, some of which are fully spliced, are transported into the cytoplasm where they are translated in viral proteins using host cell translation machinery. The viral proteins and two copies of unspliced viral RNA are recruited to the plasma membrane by the Gag protein where assembly into a new generation of viral particles occurs.

The course of HIV-1 infection

The early events resulting in productive HIV-1 infection begin when the virus crosses the epithelial barrier of mucosal surfaces. The potential pathways by which the virus crosses the epithelia include: 1. Direct infection of intraepithelial lymphocyte, 2. Transcytosis either through epithelial or M cells and, 3. Direct transportation of the virus by the dendritic cells (DC) that patrol the mucosa and capture pathogens for example via dendritic cell-specific ICAM-3 grabbing nonintegrin (DC-SIGN), a C-type lectin that is highly expressed on DCs and has high affinity to the envelope protein such as HIV-1 Env (Fig.1.3).

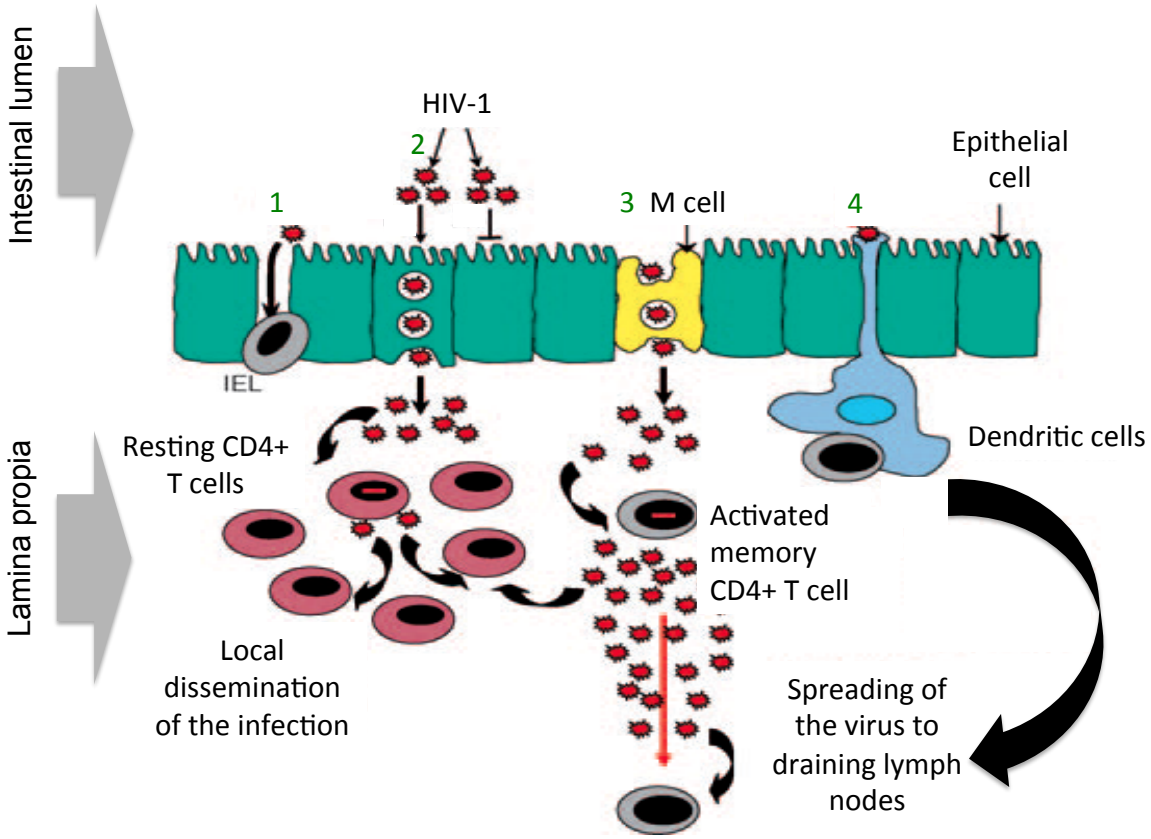


Fig. 1.3. Potential mechanisms by which HIV-1 is thought to establish infection: 1. breach in the epithelium and/or direct infection of intraepithelial lymphocytes (IEL), 2. transcytosis through epithelial cells, 3. transcytosis through M cells, 4. HIV-1 transport by DC. After the virus crosses the mucosal epithelium, low level of virus production occurs within infected resting CD4 memory T cells whereas relatively high levels of virus replication occur within fully activated CD4 memory T cells leading to dissemination of the infection to long distances [Adapted with permission from Centlivre: AIDS, Volume 21(1). January 2, 2007. 1–11].

Once the virus crosses the mucosal epithelium, the virus infects the susceptible cells resident in the lamina propia, such as Langerhans cells, DC, tissue macrophages, and CD4+ T cells. The majority of target CD4+ T cells in the mucosal compartment are poorly receptive to HIV-1 infection because they are mostly in a resting state. Nonetheless, it is thought that infection of these cells, although is characterized by low level of virus replication, creates a threshold pool of viruses that initiate infection, which spread to the draining lymph nodes(24) (Fig.1.3). Because there is high

abundance of activated CCR5+, CD4+ T-cells in the lymph nodes, when HIV-1 reaches these sites there is likely to be a burst in virus production due to infection of the activated CD4+ T cells. The high production of virus causes wanton destruction and depletion of CD4+ T. The exact mechanisms by which the CD4+ T cells get destroyed during primary acute infection is still debatable, however it could be as a result of direct killing due to cytopathic effect of the virus or as a result of bystander effect arising from Fas–Fas ligand mediated T-cell apoptosis. Several studies in nonhuman primates have observed that early in infection (10 days post challenge), majority of CD4+ T cells (over 60%) in the lymph nodes contain proviral DNA and majority (80%) of these infected cells disappear within few days; suggesting that their killing may be due to virus-mediated direct killing rather than spreading killing of bystander uninfected CD4+ T cell(109). Other such studies have reported that only a small percentage (7%) of mucosal CD4 + T cell are infected, thus the massive loss of CD4+ T cells witnessed during acute infection is less likely to be due to direct virus-mediated killing, rather may largely attributable to indirect deaths of bystander uninfected CD+ T cells(93). Nonetheless, the magnitude HIV-1 induced CD4+ T cell death occurring during HIV1 infection; irrespective of the mechanism involved has a direct implication on the success of any effective vaccine against HIV-1 pathogenesis

The burst in HIV-1 replication during acute infection induces adaptive immune responses, particularly those from CD8+ T lymphocytes. These cells are thought to participate in the killing of infected cells thereby causing a reduction in the peak viral load to a steady state usually referred to as viral set point, where a balance is struck

between virus turnover and the immune responses(68, 135). This viral set-point phase, which is characterized by lower viral replication than early stages of infection is maintained for several years (typically 10 years) without any prominent clinical symptoms. After the clinical latency period, apparent disease develops and can progresses to death if no treatment is administered.

The course of HIV-1 infection therefore follows three major phases: 1. Primary infection phase, which is marked by a burst in viral load followed by establishment of a viral load set-point, 2. Asymptomatic chronic phase marked by low/moderate viral replication and chronic decline in CD4 T-cell numbers, and 3. Clinical disease stage (AIDS) associated collapse of immune system and occurrence of death (Fig.1.4). The host immune responses induced in the course of HIV-1 infection are discussed below.

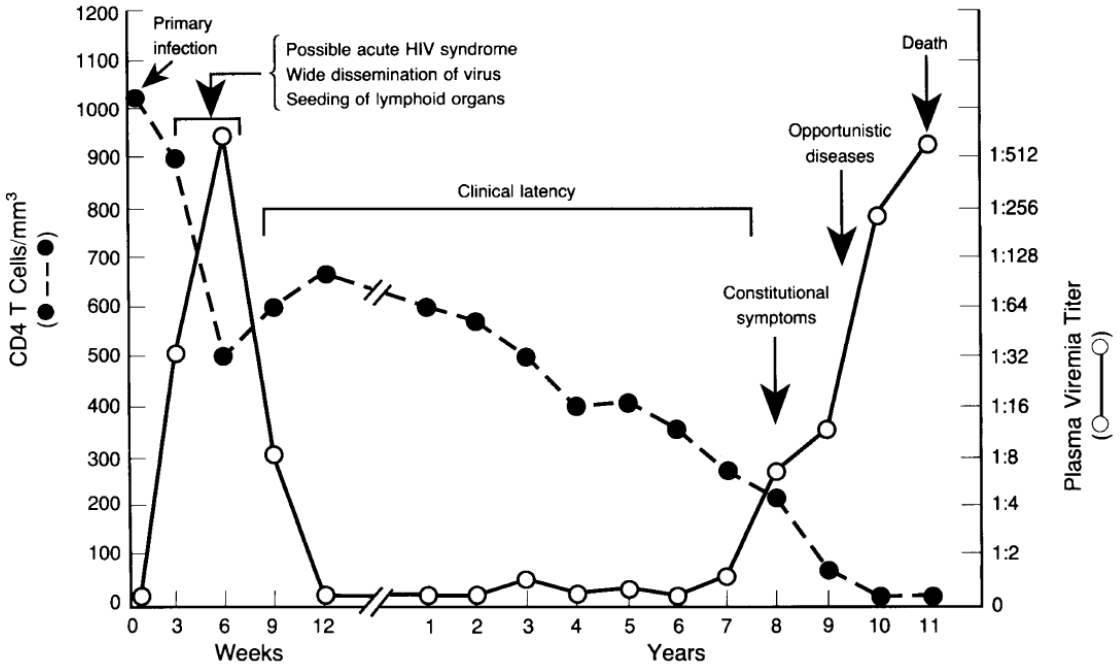


Fig.1.4 Typical course of HIV-1 transmission: After HIV-1 initiates infection at the mucosal sites, there is a widespread dissemination of the virus and concomitant burst in plasma viral load, which often coincides with a drastic drop in CD4+ T cell count in the

circulation. High viral replication induces HIV-1 specific immune responses (mostly CD8+ T cell responses), which coincide with subsequent reduction in plasma viremia. The reduced viremia is sustained for several years, however during this period of clinical latency, the CD4+ T cells are continually destroyed to a critical level beyond which opportunistic infections arise, and death may occur [Adapted from: N Engl J Med 1993; 328:327-335]

Antibody response to HIV-1 infection

The earliest antibody responses following HIV-1 infection typically appear about 1 week after infection and are directed towards non-neutralizing epitopes on Env(158) (Fig.1.5). These antibodies are detectable in plasma as virion/antibody complexes(30) and appear contemporaneously with detectable plasma virus RNA. This early phase of B cell response to HIV-1 infection is followed immediately by freely circulating binding antibodies that first appear to recognize gp41. These anti-gp41 antibody responses are followed in a few weeks by those binding to V3 of gp120(158). Several studies looking at the beneficial effect of these early binding antibodies suggested that they are capable of compliment activation(158) as well as mediating antibody dependent cellular cytoxicity [ADCC] against infected cells(44). The magnitude of the ADCC activity mediated by these binding antibodies was indeed inversely associated with plasma viremia levels(44), suggesting these antibodies may be beneficial. In contrast to these early findings, more recent studies suggest that these early binding antibodies are unlikely to control viral replication (158) and have no detectable selective pressure on the founder/ transmitted virus(72).

Antibodies capable of neutralization function are first elicited several months (2 to 12 months) after infection(58, 134) (Fig.1.5). These early Nabs are only capable of neutralizing the infecting virus strain (autologous virus) and cannot neutralize more

divergent viruses obtained from other people (heterologous viruses)(6, 35, 50, 58, 113, 134, 169). Interestingly, these autologous Nabs are highly potent and exert selective pressure that drives viral escape (105), which is evidenced by the fact that majority of later viruses are less sensitive to earlier plasma(168). Moreover, the potency of these antibodies has been shown to fluctuate most likely due to dynamics in epitope availability and accessibility resulting from appearance of escape variants(116). This is because the majority of aNabs mostly target epitopes resident in the variable regions of the envelope(100, 116, 136). Therefore such epitopes are difficult to be targeted with a universal vaccine that would protect against the highly diverse HIV-1 strains. But, although the arms race between aNabs and HIV-1 mostly involves epitopes occurring in the variable region, continued viral replication as a result of escape from Nabs may result in the exposure of more conserved regions, which may stimulate production of broad and cross-neutralizing antibodies (BCN) in some individuals. Since such antibodies target conserved epitopes on the Env, they are usually able to neutralize viruses across different strains of HIV-1. Antibodies that are able to neutralize viruses from different individuals are usually referred to heterologous Nabs (hNabs).

Unlike aNab, which are induced in almost all HIV-1 infected individuals, hNabs, particularly BCN ones, develop only in a subset of patients and typically after 2 years of infection(41, 57, 165, 166) (Fig.1.5). The factors that restrict the development of these antibodies to only a few individuals is not fully elucidated, though some studies have proposed that it is related to the number of years of infection, plasma viral load(13, 114, 127, 147, 153), disease progression (low levels of CD4+ T cell), and viral diversity(41,

115, 127). Interestingly, the time to development of broadly neutralizing antibodies vary greatly in different individuals suggesting that other host and viral factors may be involved. However the observation that high plasma viral level correlates with elicitation of such antibodies suggests that years of persistent viral stimulation are critical for their generation. Since these antibodies neutralize across subtypes and have been shown to target few similar sites on the envelope including the CD4 binding site, the gp41 MPER, the peptidoglycan epitopes in the V2 & V3 regions(58, 117, 159, 166, 175, 181), their discovery is of immense interest for the development of an effective vaccine. Such a vaccine would elicit Nabs with activity against a diverse panel of primary isolates, hence protecting against the extremely diverse HIV-1 variants circulating in human population. However, a major hindrance to the discovery of a vaccine candidate that could elicit the production of BCN is the apparent lack of understanding of the ontogeny of these types of antibody. It is not clear whether they develop from earlier strain-specific autologous Nabs through bettering of epitope recognition or they just arise due to de novo specificities stimulated by new conserved epitopes arising due to continued virus replication and escape. Because BCN antibodies develop much later (after 2 years) after infection(57, 127), it is logical to think that they arise due to antibody maturation rather than random appearance of de novo specificities elicited by novel epitopes arising from continued viral replication. This hypothesis is further supported by the fact that the antigen binding site of majority of BCN shows atypical high levels of somatic hypermutation, which can only arise due to affinity maturation toward a constant epitope(101, 177). Given this potential of

hierarchy of Nab development, it is critical to understand the role of autologous Nab in HIV-1 transmission. Theoretically, escape from autologous Nab may not be infinite, as the virus may be constrained in its ability to mutate continuously without compromising its fitness(35).

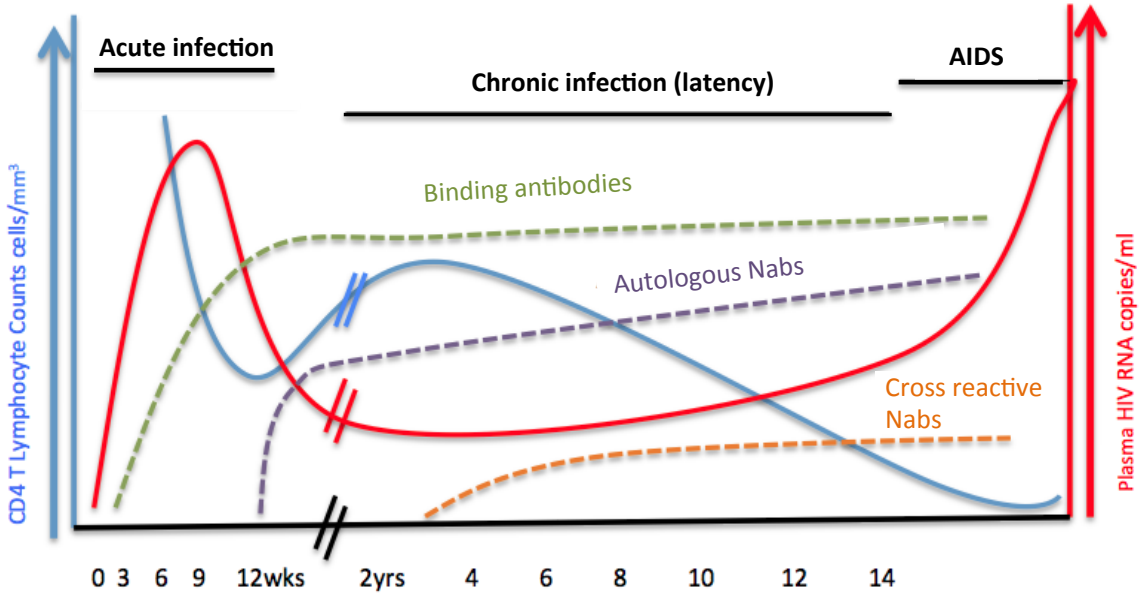


Figure 1.5. Typical B cell response to HIV-1 during the course of infection: Binding antibodies are the earliest to be elicited soon after HIV infection (green). This early immune response is followed by a decline in viral loads (red line) and a rebound in peripheral CD4+ T cells (blue line). A few months later, strain specific autologous antibodies develop (purple line). After ~2 years, heterologous antibodies (orange line) capable of neutralizing a broad range of circulating strains are generated in subset of individuals (modified from Maroa 2012)

Mechanisms of HIV-1 escape from neutralizing antibodies

As outlined previously, Nab antibody responses to HIV-1 are majorly directed against the envelope because this glycoprotein is displayed on the virus surface and is highly immunogenic. Escape from Nabs occurs through several pathways, including amino acid variations created by mutation processes such as deletions, insertions and

substitutions that affect epitopes targeted by Nabs(91, 116, 143). These mutations are generated during the process of reverse transcription because the RT enzyme is highly error prone and lacks proof reading mechanisms. This enzyme is estimated to make about $3.4x10^5$ mutation per site per generation (106). The high replicative error rates coupled with short generation time provide a fertile platform for Nab selection for escape.

HIV-1 Env is highly glycosylated protein. For instance, the gp120 portion of Env contains about 24 potential sites for addition of N-linked glycans (PNGS)(89). Addition of sugars to these sites are not only required for proper folding and processing of Env protein during synthesis(94) but also are necessary for interactions of Env with co-receptors(129, 180) during infection. Moreover, these glycans also mask conserved viral epitopes from antibody recognition thereby providing an escape mechanism for the virus. For this reason, mutational changes in the Env that introduce, remove or shift the PNG sites results in escape from Nabs(25, 133). Moreover, N-linked glycans can also block antibody recognition through steric hindrance, which physically prevent Nab from accessing the antigenic polypeptide surface of the HIV-1 envelope, hence promoting viral escape.

Although all these mechanisms allow the virus to escape from Nabs recognition, there is evidence that the virus might be restrained in its capacity to infinitely mutate because there may be fitness consequences resulting from such endeavors as mutation process may affect virus proteins required for viability.

Dynamics of HIV-1 evolution in response to autologous Nab pressure

Typically, most HIV-1 infected patients generate potent neutralizing antibody responses early after infection. These antibodies, which broaden overtime, are at first directed toward autologous infecting HIV-1 variant and have been shown to exert a selective pressure that drives continuous virus escape(50, 134, 168, 172). The escape phenotype arises from mutations in variable regions of the Env, which affect recognition by antibodies by several pathways including direct alteration of cognate epitope or through global alteration of Env conformation. Irrespective of the pathway of escape, the end result is that the current viruses are less sensitive to contemporaneous plasma (Nabs) while later plasmas potently neutralize earlier variants. In the study by Wei and colleagues, they showed that Nabs activity against the successively emerging virus strains increased overtime, but the early viruses were more potently neutralized by later plasma as compared to contemporaneous viruses(168). Their data hinted that changes in the viral Env are what make successive viruses increasingly resistant to neutralization(168). Similarly, a recent study by Chaillon et al, which focused on a chronic infection case, illustrated that even in chronic infection, there is temporal increases in autologous neutralization potency of subsequent sera toward infectious pseudotyped viruses isolated from previous visits(26). They also showed that a particular isolate at 16 years post infection was resistant to early plasma. These studies suggest that sample timing (plasma & virus) is crucial in the detection of virus escape during HIV-1 infection and may affect our interpretation of the role of Nab in HIV-1 transmission.

Evidence for protective role of Nabs in HIV-1 transmission

The hallmark of many licensed vaccines against viral pathogens is the ability to elicit Nabs(5). The ability of Nabs to provide protection against virus infection can be tested through passive immunization of non-human primates with antibodies followed by subsequent challenge by a virus(124). Indeed for many viruses, this approach has demonstrated that *in vivo* protection correlate with neutralizing activity *in vitro*(164). In the case of HIV-1, it has been shown in passive immunization studies in nonhuman primate model systems, that Nabs originally isolated from HIV-1 infected humans are capable of blocking infection by a simian immunodeficiency virus expressing HIV-1 Env[(8, 65, 66, 69, 107, 125, 138) also reviewed in (95)]. However, because these experiments were done in ideal conditions optimized to detect protection including: 1. The viruses used for challenge was known to be inherently sensitive to the antibodies tested, 2. Extremely high dose of virus inoculum were used to initiate infection, and 3. Only a single strain of the virus was used, their results have failed to fully inform the development of an effect vaccine that can protect against HIV-1 transmission in real world settings in humans, where highly diverse virus variants circulate and little inoculum initiate infection. For example, a combination of monoclonal antibodies (IgG1b12, 2G12, 2F5 and 4E10) originally isolated from individuals infected by HIV-1 subtype B(21, 23), completely protected neonatal rhesus macaques from oral challenge with SHIV 89.6P(43). However, subsequent studies reported that these antibodies poorly neutralized other strains of HIV-1 isolated from people infected with different subtypes(15), suggesting that the initial results from nonhuman primates may not apply

to exposed humans. Moreover, this inconsistency between non-human primate experiments and what happens in humans, is also reflected in the failure of vaccines designed based on the impressive results from non-human primates to induce protective Nabs in humans(73). Therefore, it remains to be defined whether Nabs are a correlate of protection against HIV-1 transmissions in humans.

Mother-to-child transmission of HIV-1 (MTCT)

Stages and mechanisms of MTCT

About 30-40% of infants born to HIV-1 positive mothers become infected in the absence of any interventions. Among those who acquire the virus, an estimated 12% get infected in utero, 29% intrapartum, 20% during delivery, and the remaining 39% are infected postpartally via beastmilk(81). The exact mechanism by which MTCT occurs is not clear. However, for in utero/intrapartum transmissions, it is thought that these transmissions may be caused either by ascending infection from an infected birth canal or through placental microtransfusions by HIV-infected maternal blood cells(84). It is also possible that the infants may be infected during delivery due to exposure to maternal viruses present in the HIV-1 contaminated genital fluids. For postpartum transmissions, infection may occur due to exposure to the maternal viruses secreted in breastmilk. Indeed, several previous studies have reported that both cell-associated and cell-free virions present in breastmilk can contribute to breastmilk MTCT(90, 128, 137). However, the major source of HIV-1 in breast milk as well as the relative contribution of either cell-free or cell-associated in breastmilk MTCT is currently poorly defined. But

the limited available evidence suggests that cell associated viruses are most like to play a significant role as cell free viruses may be readily inactivated by the high acidic environment in the stomach, while cell associated viruses are not(148).

Factors associated with MTCT

Numerous factors of both viral and maternal origin may affect the risk of MTCT. Maternal plasma HIV-1 RNA level(37, 53) as well as lower CD4+ T lymphocyte count, have been shown to be correlated with increased risk of HIV-1 transmission to the infant. In addition, inflammatory conditions of the breast such as mastitis, and chorioamnionitis of the placenta also may increase the risk of MTCT(97, 160) because such conditions promote the recruitment and activation of target cells. Although host immunity may also play a key role in modulating MTCT, its role in MTCT is still poorly defined particularly the role of Nabs, which may play a greater role in reducing the risk MTCT. Typically mothers transfer antibodies to their infants, hence HIV-1 exposed infants may have passive HIV-1 specific antibodies acquired from their mothers, at the time they are exposed to maternal viruses. Such passive antibodies may provide protection against HIV-1 acquisition as has been suggested by studies in animal models(8, 69, 138).

Mother to child transfer of IgG

Transplacentally acquired pathogen specific IgG antibodies form the primary source of early protection against many infectious agents in infants. The transfer of

these maternal antibodies to the infant is facilitated by placental protein hFcRn that binds specifically to the Fc region of IgG antibodies and not other immunoglogulin isotypes(154). Thus, infants acquire mostly IgG antibody isotype from their mothers during pregnancy. Many factors including maternal IgG level, coinfection with multiple pathogens, hypergammaimmunoglobunemia may interfere with transfer of these antibodies to the infants(34). It has been shown that in HIV-1 infected mothers, there is reduced transfer of tetanus, Measles and staphylococcus pneumoniae specific IgG antibodies(34, 39).

It is possible that HIV-1 induced aberrant B cell activation and hyperproduction of immunoglobulin may saturate the hFcRn, thereby abrogating the binding of relevant antibodies hence reducing their transport to the infant. Similarly, infection of placental lymphocytes by HIV-1 virus may result in inflammation-mediated destruction of the placental tissue, which may impair expression of the hFcRn in the placenta, thus reducing the transfer of the antibodies. It is not known whether these scenarios would also interfere with the transfer of HIV-1-specific Nabs to the infant. Information on whether HIV-1 specific passive Nabs in the infant differ from that of the mother is important in understanding whether passive antibodies may be an immune correlate of protection in exposed infants.

MTCT as a natural setting to evaluate the potential of Nabs to protect against HIV-1 transmission in humans

As mentioned above, while studies in nonhuman primates have confirmed that Nabs can block HIV-1 transmission when present at the time of exposure[(8, 69, 107) and reviewed in (95)], they have failed to translate into the development of an effective vaccine that would prevent HIV-1 transmission in humans(73). Typically there are genetically and antigenically diverse variants present in a chronically HIV-1 infected mother. The maternal Nabs raised against these variants may be present in the infants at the time of exposure. Because maternal antibodies may be present in the infant, MTCT provides a unique natural setting, in which to evaluate whether Nabs capable of recognizing an incoming virus can provide protection against HIV-1 transmission. This scenario mimics what would happen in typical vaccination, where a vaccine would induce protective Nabs against future exposures. Moreover, in MTCT the index case (infected mother) and the exposed person (infant) are known and the timing of transmission can be approximated with great precision through close frequent sampling and testing of the infant. These features allow for the virus and antibodies of interest to be isolated and examined within the time frame relevant for transmission.

Indeed, mother-to-child HIV-1 transmission studies were the first to show some protective effect of Nabs(173). For example Kliks *et al.* showed in a small study of 6 mother/baby transmission pairs that viral isolates from infants were generally resistant to neutralization by maternal plasma(104). Moreover, in expanded study by our laboratory involving 12 transmission pairs, where multiple individual envelope

variants (N=64 from the mothers and N=32 from corresponding infants) were examined, Wu *et al* found that the maternal variants transmitted to the infants during intrapartum or via breastfeeding tended to be those in the mother that were poorly neutralized if at al, by maternal plasma near transmission(174). These studies suggested that maternal autologous Nabs could provide some protection against MTCT. However, since in these studies only transmitting mothers (TM) were analyzed, it is difficult to draw conclusions on the overall protective effect of Nabs during MTCT.

One subsequent study that included both nontransmitting mother (NTM) and TM also suggested a protective role for maternal antibodies against vertical transmission of HIV-1(36). In this study, Dickover et al. reported that women who transmitted to their infant during pregnancy (N=14) had infrequently detectable Nabs compared to NTM (N=17). Hence this study, which looked at only primary isolates rather than individual variants, validated and extended the findings of previous studies of only TM. However, this study only illustrated the role for antibody in the in utero setting and not other stages incuding intrapartum and breastmilk transmissions when MTCT is highest(119).

In support of the previous studies that focused on the role of Nabs against autologous viruses, other studies that have used a panel of heterologous viruses have also reported a correlation between maternal antibodies and transmission risk of MTCT(10,143). In the study by Samleerat *et al,* where antibody responses against four standard HIV-1 isolates(146) were analyzed, responses to 2 of the 4 isolates correlated with transmission, with the strongest correlation with one particular viral variant(146).

Together, the results from all these studies suggest that the nature and specificity of the Nabs response in the mothers may be a major determinant of MTCT. But while these studies provide proof-of-principle they failed to examine the role of Nabs in all stages including early breastmilk transmission.

In contrast to the studies that suggest protective role of maternal Nabs against MTCT, other studies have failed to detect any protective role of Nabs(61, 71). This later finding was also confirmed by a more recent study from our lab, which showed that maternal Nabs transferred to the infant are not protective against infant HIV-1 acquisition(98). Although the discrepancy between these studies could be due to many factors such different assay format, viruses used and timing of transmission examined, it suggests that the role maternal Nabs in reducing the risk of vertical HIV-1 transmission is complex and more comprehensive studies are needed.

Goals of this thesis

Several previous studies looking at the role Nabs on MTCT have focused on the mother and few have examined passive antibodies in the infant(10, 76, 146). One study by our laboratory, where the association between infant passive Nabs and the risk of MTCT was examined reported no correlation between infant infection risk and the breadth and potency of passively acquired antibodies in infants exposed to HIV-1 during breastfeeding(99). It is unclear whether the findings by this study reflected poor vertical transfer of HIV-1-specific Nabs or simply that the breadth and potency of maternal Nabs are not correlated with protection against breastmilk HIV-1 transmission. In the second chapter of this thesis, I address this gap by comparing mother Nab levels with that of their corresponding infants.

In contrast to the findings of the above study, other studies where a panel of heterologous envelope variants were analyzed against maternal plasma, suggested that higher maternal antibody levels are protective against intrapartum transmissions(10, 146). Although differences between these studies may be due to many factors, one possible explanation for the difference is that detection of Nabs against heterologous variants is not adequately measuring the presence of a protective Nabs in the mother because these antibodies are not specific to the maternal variants to which the infants are exposed, rather autologous Nabs are more relevant to protecting the infant against autologous variants. The idea that autologous Nabs are more relevant in MTCT is supported by studies examining the effect of maternal aNab. These studies have shown that maternal viruses transmitted to the infant are those that are poorly neutralized by

maternal plasma near transmission, suggesting that they are Nabs escape variants. However, because these autologous studies only focused on TM and did not include NTM, we are unable to draw conclusions as to whether Nabs have protective effect against HIV-1 transmission. In the third chapter of this dissertation, I explore whether Nabs raised against maternal autologous envelope variants that represent virus population in the TM and NTM influence vertical HIV-1 transmission risk.

Interestingly, some recent studies where the time points when the antibodies and virus were analyzed were mismatched with the estimated time of transmission have reported that the transmitted variants have similar neutralization profiles as the non-transmitted variants in the mother. Given the rapid changes in immune response to HIV-1, it is likely that the chance to detect the Nabs escape variants during MTCT could be missed by measuring responses after transmission, which may differ both qualitatively and quantitatively from the earlier responses present near the time of transmission. In chapter four of this dissertation, I address the hypothesis that differences between these studies can most likely be ascribed to differences in timing of sampling in relation to transmission.

Chapter 2

Evidence for Efficient Vertical Transfer of Maternal HIV-1 Envelope-Specific Neutralizing Antibodies but No Association of Such Antibodies with Reduced Infant Infection

Introduction

In the absence of any interventions to reduce transmission, approximately one third of infants born to HIV-infected mothers will become infected. Approximately half of these infections are due to breastfeeding; the remainder occur *in utero* or during delivery.(119) The role of neutralizing antibodies (Nabs) in the setting of mother-to-child transmission (MTCT) has been extensively studied to determine if HIV-specific antibodies present at the time of exposure can protect against HIV infection. Several early studies, each relatively small, showed that non-transmitting mothers tended to have more frequently detected and/or higher Nab responses than transmitting mothers, suggesting a role for Nabs in reducing MTCT(36, 88, 149). However, the results of additional studies have reached divergent conclusions as to whether Nabs are important during all stages of mother-to-child transmission, if at all(36, 71, 146). The association between Nabs and protection has also been somewhat dependent on the virus tested.(146) No association was observed between infant infection risk and the breadth and potency of passively acquired antibodies in infants exposed to HIV-1 during breastfeeding.(99) It is unclear if the results from this study in infants indicate there is simply poor vertical transfer of HIV-1-specific Nabs or that the breadth and potency of maternal Nabs are not a factor in protection against breastmilk HIV-1 transmission.

Placentally acquired antibodies reach their highest level in the infant during the third trimester and have been shown to protect infants from various postpartum exposures to maternal infections. Studies in nonhuman primates have demonstrated efficient transfer of passively infused human monoclonal Nabs(62), but little is known about the transfer of maternal HIV-1-specific Nabs in humans. One study of 35 mother/infants pairs that assessed Nabs against two lab-adapted HIV-1 strains showed a strong correlation between maternal and infants Nabs, suggesting efficient transfer(19). However, responses to more relevant circulating variants of HIV-1 were not examined. Another study that explored the relationship between maternal and infant antibodies showed variation in antibody transfer based on antigen specificities and IgG subclasses, indicating transfer may vary by antibody type(77). Thus, these studies suggest that mother-to-child transfer of HIV-1-specific Nabs may be affected by the nature of the Nabs, including antibody specificity. To address this hypothesis, we examined the transfer of HIV-specific antibodies, including Nabs, to three circulating HIV-1 variants that represent the dominant HIV-1 subtypes in the population under study.

Materials and methods

Samples: Plasmas tested in the current study were from mother/baby pairs who participated in the breastfeeding arm of a randomized clinical trial done in Nairobi, Kenya, from 1992 to 1998 to examine HIV-1 transmission rates between breastfeeding versus formula feeding women(119). Plasmas from the mothers were obtained around the time of delivery and the infant plasmas were obtained within the first week after

birth, a time when passively-transferred antibody levels should be high. Both women and infants were antiretroviral therapy naïve and mother/infant pairs were included in this study based on these criteria: 1) the mother breastfed, 2) the mother had a plasma sample available around delivery, 3) the infant was HIV DNA PCR negative at birth, 4) the infant was either detected as HIV positive after birth or was followed for at least 3 months if HIV negative, and 5) the infant had a plasma sample within the first week after birth(99). Sixty mother/infant pairs met these criteria and were included in the study. The ethical review committees of the University of Nairobi, University of Washington and Fred Hutchinson Cancer Research Center approved this study as well as other studies described in this dissertation. The subjects examined in this thesis provided written consent.

Detection of HIV-1 Envelope-specific IgG: HIV-1 Envelope-specific IgG titers were estimated using an enzyme-linked immunosorbent assay (ELISA) performed as previously outlined using a HIV subtype A gp120 protein (BL035; Immune Technology Corp., New York, NY).(102) Plasmas were diluted starting at 1:4000 followed by 2-fold serial dilutions up to a maximum dilution of 1:512000. In cases where the end point titer (EPT) was outside the range of these dilutions, additional 2-fold dilutions were made either starting with a minimum dilution of 1:1000 or diluting to maximum of 1:2048000. The EPT was defined as the reciprocal plasma dilution at which the average optical density (OD) value was greater than or equal to twice the average OD value of the background. For the purposes of analyses, in cases in which the EPT was less than the lowest dilution tested, the midpoint value (500) between the lowest dilution and

zero was assigned. The EPT results represent the average of two independent duplicate assays.

Generation of pseudoviruses

Three HIV-1 Envs, which were initially cloned from 3 viruses isolated early in infection from 3 Kenyan women, were used to generate pseudoviruses as described(99). Briefly, plasmids containing these Envs were cotransfected into preplated 293T cells together with another plasmid containing a full length subtype A proviral genome with deleted portion of Env (Q23Δenv) using the Fugene-6 transfection reagent (Roche, Indianapolis, IN). In all cases a ration 1:2μg of Env plasmid to Q23Δenv was used in the transfection process, which was done in a T75 tissue culture flask preplated with $2x10^6$ of 293T cells. Forty-eight hours post transfection, supernatants were harvested and sterilized by filtering through a 0.22μm Filter Units (Millipore, Billerica, MA). Transfection supernatants were aliquoted and stored at -80^oC for subsequent use. Titers of the pseudoviruses were determined by serially diluting viruses 10-fold then adding $5x10^4$ TZM-bl cells suspended in growth medium containing a final concentration of 20 μg/mL DEAE dextran and incubating at 37 °C. The TZM-bl cells are HeLa cells derived indicator cell line that are susceptible to infection by HIV-1 because they stably express CD4 and CCR5, the two major proteins required by HIV-1 to infect cells. In addition, these cells also contain integrated reporter genes for firefly luciferase and β-galactosidase, under the influence of HIV-1 long terminal repeat (LTR) promoter(167) and thus, there infection can be readily observed by staining for β-galactosidase and/or

assaying for luciferase activity. For titration, 48 hours post infection with the pseudoviruses, the adhered cells were stained for β-galactosidase activity and cell with blue foci were directly counted.

The three Envs used to generated the pseudoviruses represented two subtype A (Q461.d1 & Q842.d16) and one subtype D [QD435.A4] and were cloned from viruses, which were directly isolated from the blood of the three women in early infection.

Neutralization Assay

Neutralization assays were performed by infecting TZM-bl cells with the pseudoviruses as described elsewhere(99). Briefly, 500 virus infectious particles were incubated in duplicate with media only or 2-fold dilutions of heat-inactivated plasma, starting at a concentration of 1:50, in a final volume of 50μL for 1 hour at 37 °C. Ten thousand TZM-bl cells in 100 μL of complete growth medium were then added to each well. Forty-eight hours after infection, β-galactosidase activity was measured using Galacto-Light Plus reagents (Applied Biosystems, Foster City, CA). The median inhibitory concentration (IC50), which represented the reciprocal plasma dilution at which 50% of infection of the target cells was inhibited, was calculated as described elsewhere(174). The IC50 results represent the average of two independent duplicate neutralization assays.

Statistical Method

Log_2 transformed (log_2 transformed to adjust for the variability in IC50 values) neutralization IC50s between the mother and her infant were compared using Welsh's t-test (with unequal variances). Because maternal plasma viral load is positively associated with increased risk of vertical HIV-1 transmission and Nab levels, we further used linear regression model to adjust for viral load in our sensitive analysis. The association between maternal log_2IC50 values and infant infection outcome was determined using logistic regression, while the correlation between IgG endpoint titers and log2 IC50 levels were examined using paired t-test.

Results

To investigate the association between maternal HIV-1-specific antibody levels and infant passive antibodies, we examined antibody levels in 60 mother-infant pairs from the Nairobi Breastfeeding Trial.(119) All of the infants were HIV-1 DNA negative at birth. Fourteen (23%) infants were HIV positive after birth; one (7%) prior to 6 weeks of life, six (43%) at approximately 6 weeks and the remaining seven (50%) after 6 weeks of life. Although we can't rule out that a few of the seven identified within 6 weeks postpartum were infected late *in utero* or during delivery, prior studies of this cohort indicate that the majority of those detected as HIV DNA positive by 6 weeks were infected through breastfeeding.(119) Thus, the majority of the 14 infants acquired HIV-1 as a result of breastmilk transmission. The rest of the infants (N=46) remained uninfected during the follow-up period (mean = 21 months). In order to assess the presence and correlation of HIV-1-specific antibodies among the mother/baby pairs, we

compared the log_2 transformed EPT values within and between the mothers and corresponding infants. Because HIV-1 subtype A is the predominant subtype in this cohort(120), we measured maternal and infant HIV-1-specific total IgG titers against soluble subtype A gp120 (BL035) Envelope protein isolated from early in infection of an infant from this cohort(174). We found that maternal and infant EPTs were correlated ($r=0.91$, $p<0.0001$, Fig.2.1A) with mothers having 1.4 fold-higher EPTs (95%CI: 1.1,1.6; $p=0.001$), suggesting efficient transfer of HIV-1 specific antibodies.

We examined maternal HIV-1-specific Nab responses against a panel of three viruses including two subtype A (Q461d1 & QD435.A4) and one subtype D (Q842.d16). The corresponding infant Nab responses had been determined as part of a prior study(99). The viruses tested here were among the 8 used in the prior study and included a Tier 1b (Q461.d1) and two Tier 2 (QD435.A4 & Q842.d16) viruses.(99) The majority of maternal plasmas neutralized all three viruses: 55 (90%) neutralized QD435.A4 at greater than 50%, 52 (84%) showed activity against Q461.d1 and 50 (82%) neutralized Q842.d16.

Fig.2.1

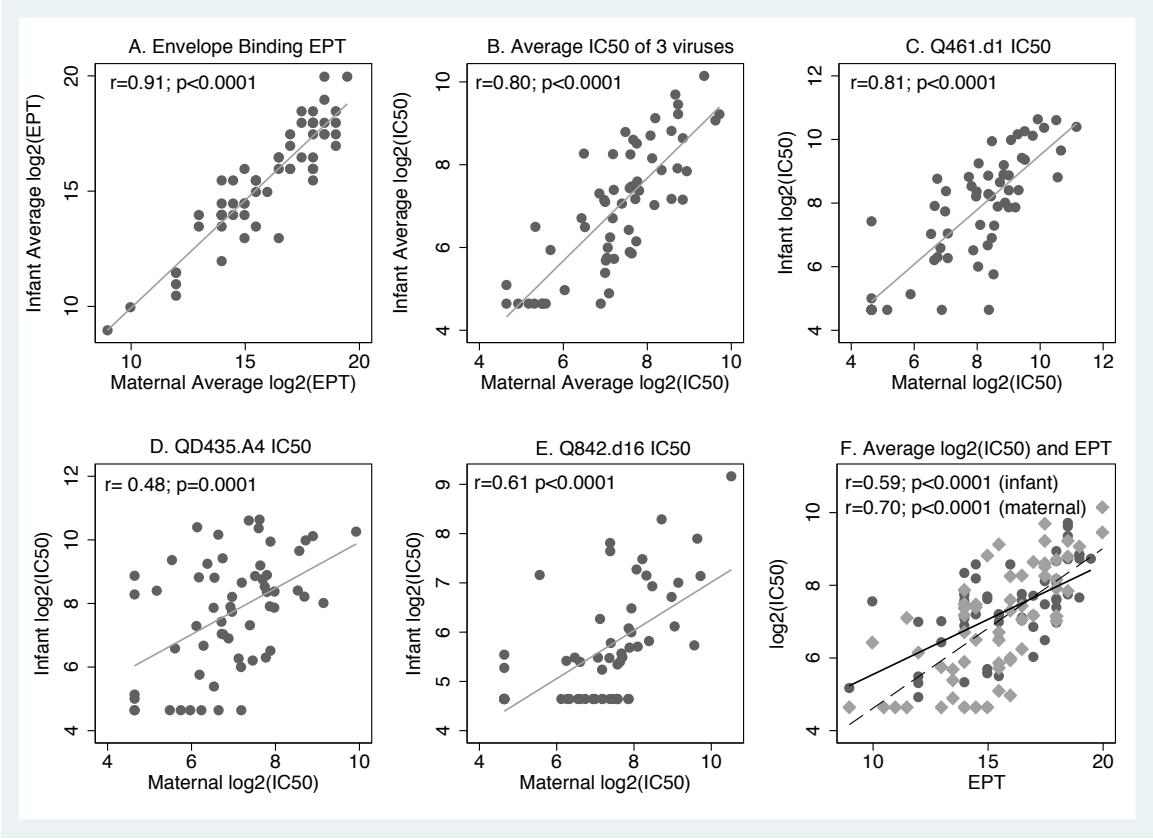


Fig.2.1: Comparison of maternal and infant HIV-1 Envelope-specific antibodies. For panels A-E, maternal and infant antibody levels are shown on the x- and y-axis, respectively. Each dot represents the data from one mother-infant pair. The line represents regression best-fit line and the r is Pearson's product moment correlation coefficient. The p-values shown above each graph are based on the Student's t approximation for the distribution of the corresponding standardized test statistic. The panels include: **A)** Envelope binding EPTs; **B)** average log_2IC50 for the 3 viruses; **C)** Q461.d1 log_2IC50; **D)** QD435.A4 log_2IC50; E) QD842.d16 log_2IC50, and F) the association between EPTs and Nab average log_2IC50 values for both mothers(•−)infants (◆ - - - - - - -).

Next, we assessed the correlation between the capacity of the mothers' and infants' plasma to neutralize the three viruses by comparing the neutralization log_2IC50s of mothers defined in this study to log_2IC50 of infants defined in the prior study(99). using Pearson's correlation coefficient (r) and t-tests (expressed as fold-differences). We found

that, on average, mothers had 1.3-fold (95% CI:1.1,1.5; p=0.0096) greater IC50 values across all viruses as compared to their infants. There was a strong linear correlation for log_2IC50s between mothers and their corresponding infants when the average IC50s of the three viruses tested were considered (r = 0.80, p<0.0001, Fig.2.1B). The magnitude of this correlation varied for each virus with the highest correlation between maternal and infant IC50s observed for the most neutralization sensitive variant Q461.d1 (r= 0.81) and the lowest correlation in log_2IC50 values was for QD435.A4 (r= 0.48) (Fig.2.1 C-E). EPTs were positively correlated with IC50s for both mothers and for infants (r=0.59 and 0.70, respectively, Fig.2.1F).

IC50 values were 1.74 times greater (95% CI: 1.06, 2.83; p=0.030) for transmitting mothers compared to non-transmitting mothers (Fig.2.2). Correspondingly, there was a positive association between IC50 and risk of infant infection (OR=1.90 given a 2-fold greater maternal IC50, 95% CI:1.03,3.47; p=0.039). However, when controlling for log_{10} plasma viral load in linear and logistic regression models respectively, these associations were not statistically significant (fold-difference in IC50 = 1.60, 95% CI: 0.91, 2.83, p=0.10; OR=1.86, 95% CI: 0.88, 3.9; p=0.10). This was true when we examined the average IC50 for the three viruses (Fig. 2.2) or the IC50 for each individual virus in relation to infant outcome (data not shown). Similarly, no association was observed when we analyzed the average IC50 values across the three viruses tested and transmission outcome for the 60 infants considered in this study (data not shown).

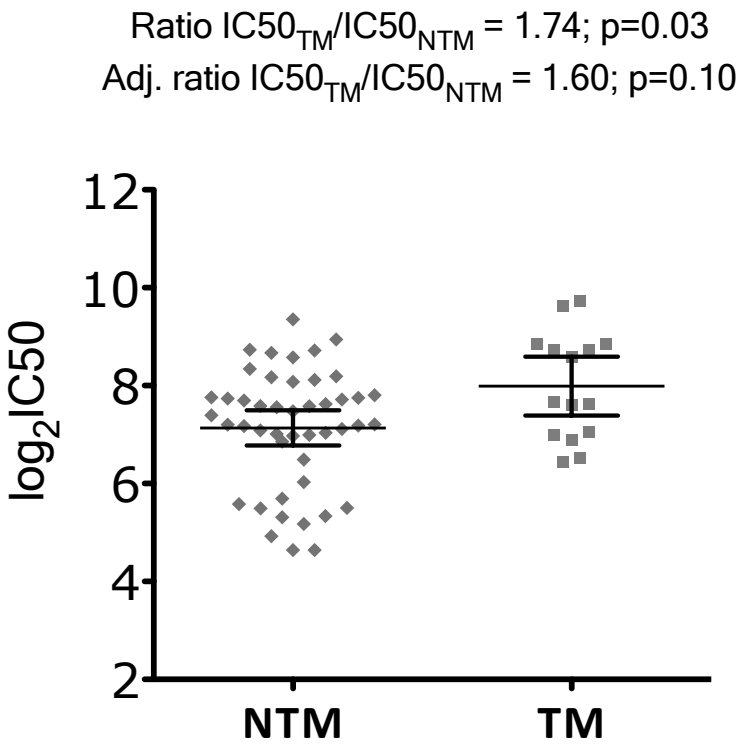


Fig. 2.2: Comparison of the average log_2IC50 values of the 46 non-transmitting (NTM) and 14 transmitting mothers (TM). The dot plots summarize the distribution of the average log_2IC50 for the 3 viruses, with mean and 95% confidence intervals for the log_2IC50 indicated by the horizontal bars for each. The unadjusted estimate for the fold-difference in IC50 and corresponding p-value are based on Welsh's t-test, and the adjusted fold-difference estimate is by linear regression controlling for log_{10} viral load, with the corresponding Wald p-value.

Discussion

Our findings demonstrate efficient transfer of HIV-specific antibodies from mother-to-infant. Infants had only 1.4- and 1.3-fold lower HIV-1 Envelope-specific binding and Nab levels, respectively, during the first week of life compared to the levels of these antibodies in their mothers near the time of delivery. This implies that protective Nabs, if present in an HIV-infected mother, could provide infant

prophylaxis, particularly late in gestation and during the early breastfeeding period, when the risk of infant infection is high(119).

The maternal Nab response against 3 heterologous HIV-1 variants was not associated with infant infection outcome after controlling for viral load, which is positively correlated with cross-reactive Nab responses and risk of transmission (reviewed in (40)). However, transmitting mothers showed a trend for having higher Nab levels than nontransmitting mothers in this analysis, a finding that is contrary to a protective role for maternal Nab in breastmilk HIV transmission. Similarly, infant Nab levels were not associated with infant infection risk, consistent with results of a prior study demonstrating a lack of association between infant infection and the breadth and potency of infant vertically acquired Nabs.(99) These data reinforce the notion that the Nab response elicited in a typical HIV-1 infection may not be adequate to provide significant protection from MTCT. However, this finding does not preclude the possibility that exquisitely broad and potent Nabs, such as those reported in a small subset of chronically infected individuals (reviewed in (40)) could provide some protection. In this context, the results presented here do suggest that in studies designed to test the potential of broad and potent antibodies to protect against MTCT, the therapeutic application of antibodies to the mothers could result in efficient transfer to the infant.

Chapter 3

Neutralization of distinct and representative HIV-1 variants by autologous antibodies is not associated with reduced risk of vertical HIV-1 transmission

Introduction

As mentioned in Chapter 1, there is evidence to suggest that Nabs can prevent HIV transmission in model systems under ideal circumstances, but little is known about the potential of such antibodies to prevent infection in real world settings where highly diverse virus populations circulate in humans. As outlined in chapter 2 of this dissertation, maternal heterologous Nabs are not associated with reduced risk of peripartum vertical HIV-1 transmission. However, in the context of MTCT, it has been previously shown that despite a highly diverse virus population present in the mother, only a limited subset of these viruses with low sensitivity to maternal Nabs are typically transmitted(86, 104, 149, 174), suggesting that Nabs can be protective against HIV-1 transmission, particularly against sensitive viral variants. These studies, where only transmitting mothers [TM] were analyzed, showed that although highly sensitive variants were present in the mother, they were not detected in the infants, rather, only the less sensitive variants were transmitted. But, in other recent studies, both the maternal and infants' variants had similar sensitivity to neutralization by maternal plasma(45, 75, 76, 140), contradicting the earlier studies. In support of the earlier studies that suggested protective effect of Nabs, several(36, 88, 149) but not all studies(61, 64, 71), have shown that infected mothers of uninfected infants have higher titers of autologous Nabs compared to transmitting mothers, suggesting that higher Nab titers are positively associated with reduced risk of vertical transmissions. Indeed, the study

by Wu and colleagues, maternal viruses that were neutralized with IC50 of ~100 or greater were never transmitted, suggesting that there may be a threshold level of neutralization in NTM that may protect against the transmission of the majority of their variants. This hypothesis can be addressed by examining whether there are differences in autologous Nabs responses against individual variants within the maternal viral population of transmitting versus non-transmitting mothers.

Many other early studies of autologous Nab responses in MTCT utilized HIV-1 primary isolates (cultured viruses) rather than individual variants cloned directly from patient samples (16, 71, 88, 149). It is important to note that the process of obtaining primary isolates involves in vitro co-culturing of patient's infected cells with uninfected donor PBMCs. While the major variants in the blood appear to be amplified in culture(162), the viral diversity can be altered(83, 142), archival HIV-1 variants reactivated(31), and minor circulating variants could disappear (162). It is possible that these in vitro viral dynamics may result in viruses that do not give neutralization profiles that fully capture the spectrum in the individual with regard to sensitivity to Nabs.

As noted above, studies of a collection of individual variants from the mother were only focused on transmitting mothers rather than both TM & NTM. To date, there has not been a detailed comparison of neutralization sensitivity of individual variants that are representative of viral population in TM versus NTM. Moreover, there is no consensus on how to define the relative number of variants that can sufficiently capture the quasispecies breadth in chronically infected individuals. It is possible that by

sampling only few variants as has been the case of previous studies, the responses to certain variants, particularly the less sensitive ones, whose neutralization could be predictive of the presence of protective Nabs, may be missed. Here, we have isolated 21-53 envelope variants from two transmitting mothers using limiting dilution PCR and used phylogenetic analysis of these variants to determine the minimum number of variants required to sufficiently represent the quasispecies diversity in these chronically infected women. We then isolated 4-8 Env variants from an additional 18 mothers (10 NTM & 8 additional TM), tested these variants against matched plasma isolated from near the time of delivery. We then compared aNabs responses between transmitting and non-transmitting mothers to dissect the efficacy of maternal aNab to block MTCT.

Material and Methods

Study subjects

As mentioned in the second chapter of this thesis, the women and their infants included in current study were among those who participated in a breastfeeding clinical trial between 1982 -1999 in Nairobi, Kenya . For the current study, mother-infant pairs were included based on the criteria that: plasma viral load of the mother >4.6 log10 copies/mL, which was the median for the original cohort(119) and the mother breastfed > 3 months and that a maternal sample was available during the period between the 32 weeks of pregnancy and delivery. For transmission pairs, another requirement was that the infants were HIV-1 DNA negative at birth and their samples were available at time-point when the infants were first detected HIV-1 positive, which ranged from 5 weeks (5Wks) to 14 weeks (14 Wks) after birth (Table 3). Twenty women who met these

requirements were included in the study.

Full-length envelope cloning

Full-length Env genes were cloned directly from DNA isolated from uncultured peripheral blood mononuclear cells (PBMCs). In addition, for two women (MF520 & MJ613), Envs were also generated from breast milk cells (BMC) as well as viral RNA isolated from plasma. DNA was extracted using the QIAamp Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Viral RNA was obtained from 140 μL of plasma by using a QIAmp viral RNA minikit (Qiagen, Germantown, MD) protocol. cDNA was generated by using Superscript III reverse transcriptase and an oligo(dT) primer, followed by RNase H treatment (Invitrogen, Carlsbad, CA). The copy number of HIV present in the DNA isolated from either PBMC or BMC or of cDNA generated from plasma RNA, was determined using a pol real time PCR assay(137). DNA and cDNA templates were diluted to near single copy based on the real time PCR estimates and envelope genes were then amplified using a nested PCR as outlined elsewhere(137). Briefly, env genes were amplified using the following primers: for first-round the primers vpr1 (5'-GAT AGA TGG AAC AAG CCC CAG-3') forward and nef24 (5'- TAC TTG TGA TTG CTC CAT GT 3') mixed in equal molar ratios with Nef34(5'- TAC TTG TGA CTG CTC CAT GT-3') reverse. The first round PCR was done in a final volume of 50uL containing 41.9ul of water, 5ul 10X Taq plus precision buffer, 0.4ul 100Mm dNTPs, 0.35ul of 1ug/ul primers, and 0.5 uL of Taq plus precision enzyme. The PCR cycling conditions were as follows: 94^0C for 4 min, followed by 35 cycles of 55^0C for 3 minutes and 68^0C for 4 minutes, with a final extension at 72^0C for 10

minutes. Two microliter of the 1st PCR product was used in the second round with equal molar ratios of two sets of primers: vpr21a1 (5'- TAA CCT AGA CGC GTG GAA TCA CCCGGG AAG TCA GCC TAC AAC ACC TTG TA-3') and Vpr21a2 (5'- TAA CCT AGA CGC GTG GAA TCA CCCGGG AAG CCG GCC TAC AAC ACC TTG TA-3') mix for forward and nef60a1(5'- CTT GTG GCG GCC GCA TGT TTA TCT AAA TCT CGA GAT ACT GCT CCT ACT CCT GGT GCT G-3' and nef60a2 (5'- CTT GTG GCG GCC GCA TGT TTA GCT AAA TCT CGA GAT ACT GCT CCT ACT CCT GGT GCT-3') for reverse primers. Gel electrophoresis was used to confirm an ~ 3kbp fragment, the expected size of HIV-1 Env amplified by this PCR reaction. Consistent with the expected copy number input, a frequency of about 30% of PCRs yielded product, further supporting that the product was the result of single copy amplification (144). The remaining PCR product from positive amplification reactions (~ 45uL) was gel isolated using Qiagen PCR gel extraction kit (Qiagen, Valencia, CA) and purified DNA was eluted into a volume of 35μL. Ten μL of the gel isolated product was digested with MluI & NotI, heat inactivated at 65°C for 20 minutes and analyzed on a 1% agarose gel to estimate the relative molar concentration to be incorporated in a ligation reaction. In most cases 1:3 vector to insert (Env) molar ratio was used in a ligation reaction with a MluI & NotI cut mammalian vector pCI-neo (Promega, Madison WI). Two microliters of the ligation product was used to transform Stbl-3 cells (Invitrogen) or XL-10 Gold cells (Promega). The transformed cells were plated on LB plates containing carbenicillin and incubated at 37°C overnight. Next day single colonies were picked, inoculated into 3ml cultures and grown overnight. Plasmid DNA was extracted using a Qiagen

miniprep kit and screened for the presence of Env by restriction digestion with Mlu1 and Not1, followed by gel electrophoresis.

Screening for functional Envs

Miniprep plasmids with Env of correct size were screened for functionality in the TZM-bl single cycle assay (discussed in chapter 2). We generated pseudoviruses from miniprep plasmids containing full length Env by co-transfecting these plasmids alongside Q23 ΔEnv (96) into 293-T cells using the Fugene-6 reagent (Hoffman- La Roche, Nutley, NJ), which aids in plasmid uptake by the cells. Forty-eight hours post transfection of 293T cells, 50uL of the transfection supernatant was directly used to infect $2x10^4$ TZM-bl cells in the presence of 20ug/mL DEAE in a final volume of 550uL complete media. Forty-eight hours post infection, TZM-lb cells were stained for ß-galactosidase activity and positive cells with blue foci were directly enumerated after staining with X-gal. Interestingly, only less than one-half of the Env clones were infectious, which is consistent with several previous reports (14, 18, 96). Long et al observed that such full-length but noninfectious Env clones were either defective or poorly expressed.

Phylogenetic analysis

The nucleotide sequence of all infectious Envs were determine using Sanger sequencing method performed in 10uL reaction using Big Dye (ABI, Foster City, CA) following the manufacturer's method. The Env sequence contigs both from forward and reverse reactions were assembled in sequencher (Gene Codes, Ann Arbor, MI) and

manually edited for base calling. Full-length, Env sequences were codon-aligned using MacClade version 4.01 and manually edited to exclude highly variable regions, which could not be unambiguously aligned. The aligned sequences were analyzed by a neighbor-joining tree using pairwise distance, based on the general time-reversible model, implemented in PAUP 4.0b10 (D. L. Swofford, Sinauer Associates, Inc., Sunderland, MA). One subtype K and other unrelated subtype sequences including subtype C, D & A obtained from the Los Alamos National Laboratory HIV database (http://www.hiv.lanl.gov.offcampus.lib.washington.edu) were also included as reference sequences. The subtype K reference sequence was set as an out-group. Neighbor joining trees were generated with reliability of branching orders checked using bootstrap with 1000 resampling events. Env sequences that differed by pairwise distance of 0.2 % were considered distinct as has been reported previously**(96)**

Generating Envelope Pseudotyped viruses

For the distinct infectious Envs, pseudovirus stocks were generated as outlined in chapter 2 of this dissertation. The titer of each viral stock was determined by a single-round infection of TZM-bl cell followed by directly counting ß-galactosidase positive 'blue' foci at 48 hours post-infection.

Neutralization assays

Neutralizing activity of maternal plasmas were measured as described in detail in chapter 2 of this thesis and elsewhere(131). Dose-response curves were used to derive the IC50, which was taken as the reciprocal plasma dilution at which 50% of the input

viruses were blocked from infecting the target cells.

Statistical analysis

We determined the difference in autologous Nab levels (log_2IC50) between transmitting and non-transmitting mothers to estimate transmission risk using logistic regression with general estimation equation (GEE) model that accounts for correlation between variants within a woman. Given our sample size, we used jackknife variance estimators as opposed to sandwich estimator, which is only valid when >40 subjects are included. In order to determine whether there is a threshold of Nab level that is associated with transmission risk, we used receiver operating characteristic [ROC] curve and likelihood ratios. In order to estimate the minimum number of variants sufficient to capture the quasispecies diversity among these women we used approximation of the width of 95% confidence intervals for log_2IC50 for a subset of the women from whom 21- 53 virus variants were obtained. These analyses were directed and performed by Katie Odem-Davis

Results

Characteristics of the study subjects

To determine if there is an effect of Nabs on vertical transmission, we selected women with high plasma viral loads (median = 5.2log cpies/mL), which is above the cohort median of 4.6log copies/mL(119). These women were therefore highly infectious because higher plasma viral load is positively associated with increased MTCT(37, 53). This subset of women breastfed for at least 3 months (mean duration 15 months, Table

3), which also put them at a high risk of transmission(119). For the 10 transmission pairs, the infants were all HIV-1 negative at birth and subsequently tested positive at average 6.9 weeks after birth, suggesting MTCT happened either during delivery or postpartum through early breastfeeding. The clinical characteristics including CD4+ T cell count and viral load did not differ between NTM versus TM

Table 3. Summary of the 20 women (10 NTM & 10 TM

Mother Idnum	infant's 1st HIV-1 DNA positive	infant's last HIV-1 DNA Negative	Duration of breastfeeding in months	CD4 count	Maternal log_{10} plasma VL	Virus Subtype[β]	No. of env clones obtained	cloning time point	Neutralization plasma time point	% Maximum pairwise distance[δ]
MO862	N/A	N/A	11	134	4.64	A	P33	7	P33	4.54
MB807	N/A	N/A	15	217	4.78	A	P32	8	P32	2.56
ML156	N/A	N/A	11	410	5.00	D	P32	6	P32	1.36
MF470	N/A	N/A	13	N/D	5.01	A	W3	7	W3	1.13
MP199	N/A	N/A	19	389	5.22	A	W0	7	W0	3.76
MM471	N/A	N/A	23	360	5.26	A	P32	8	P32	2.62
MM834	N/A	N/A	19	633	5.46	D	P32	6	W0	1.14
MF600	N/A	N/A	17	344	5.49	C	W2	5	W2	1.13
MA411	N/A	N/A	20	416	5.50	A	P33	6	P33	4.23
MG540	N/A	N/A	12	285	5.60	A	P34	6	P34	2.17
ML035	W5	WC	8	249	4.46	A	P32	5	P32	2.91
MI102	W6	WC	13	639	4.79	D	W0	8	W0	1.55
MJ412	W6	WC	23	293	4.86	C	P32	5	P32	7.30
MK184	W6	WC	3	568	4.89	D	P34	6	P34	2.26
MC046	W6	WC	3	255	5.05	A	P35	5	P35	1.65
MF403	W6	WC	30	213	5.07	A	W0	8	W0	0.94
MF535	W6	W1	20	690	5.53	A/C/D	W0	5	W0	3.08
MF520	W14	W1	28	511	5.59	A	P32	5	P32	1.27
MJ613	W6	W1	10	104	5.64	A	P32	6	P32	4.26
MB549	W6	WC	4	411	6.25	A	P30	7	P30	0.94

WC represents cord blood sample, W0, delivery; W#, weeks after delivery; P32: 32 weeks of pregnancy and N/A, not applicable. The last column (δ) represents the maximum pairwise distance among nucleotide sequences spanning the full-length envelope sequences within each mother. The virus subtypes (β) are derived from phylogenetic analysis and are inferred by clustering together with the reference sequences and confirmed by a previous study(120). The first 10 women (light gray) are NTM, while last 10 (dark gray) are TM.

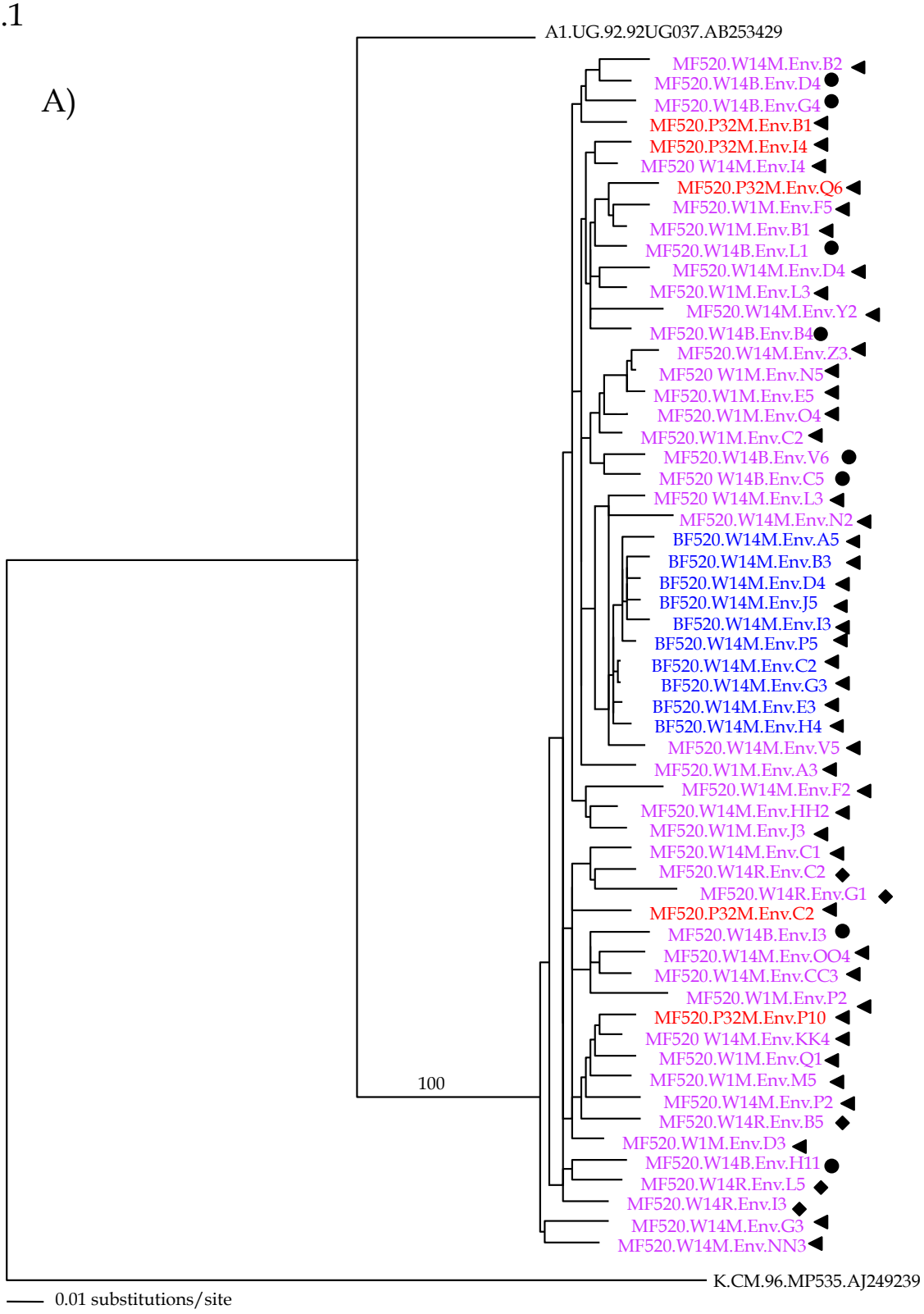
Defining the spectrum of neutralization sensitivity and HIV-1 envelope quasispecies diversity.

There is no gold standard in the HIV field for estimating the number of virus variants that would be sufficient to represent the viral quasispecies in a chronically infected patient. Here we determined, in two of the transmitting women (MF520 &

MJ613, Fig.3A & C), the minimum number of variants that would adequately represent the larger virus population of their variants. Typically between 5-10 clones are studied (174), and thus in this pilot analysis, we compared the sequences of the first 5 Env variants we isolated from PBMCs with an expanded collection of virus variants (N=53 for 1st woman & N= 21 from the 2nd woman), isolated from both blood and breast milk. We found in phylogenetic analyses that first 5 Env variants represented diverse subclades within the more complete spectrum of the virus population in the women analyzed (Fig.3.1A & C). These initial 5 variants generally clustered in different branches of the tree containing expanded number of clones from the same mother, suggesting that they were distinct and represented diverse component of the broader viral population in these two women. Moreover, the neutralization profiles of these variants, though not statistically significant, appeared to represent the neutralization profiles of the expanded clones as their average IC50s were comparable to the average IC50 of the expanded clones (Fig.3.1B & D)

In order to determine if the PBMC derived variants represent viruses in other compartments such plasma and breastmilk that are also relevant in MTCT(79, 80, 118, 137), we assessed the phylogenetic clustering patterns of the clones obtained from the three compartments; BMC, plasma and PBMCs. The RNA, PBMC and BMC Env sequences intermingled in a neighbor-joining tree, suggesting lack of compartmentalization (Fig.3.1A & C), which is consistent with the findings by other groups(59, 145).

Fig. 3.1


A)
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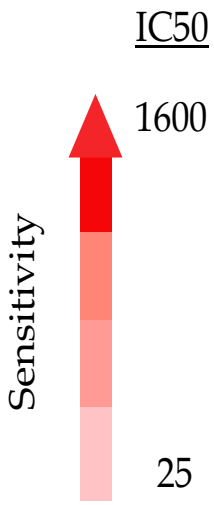
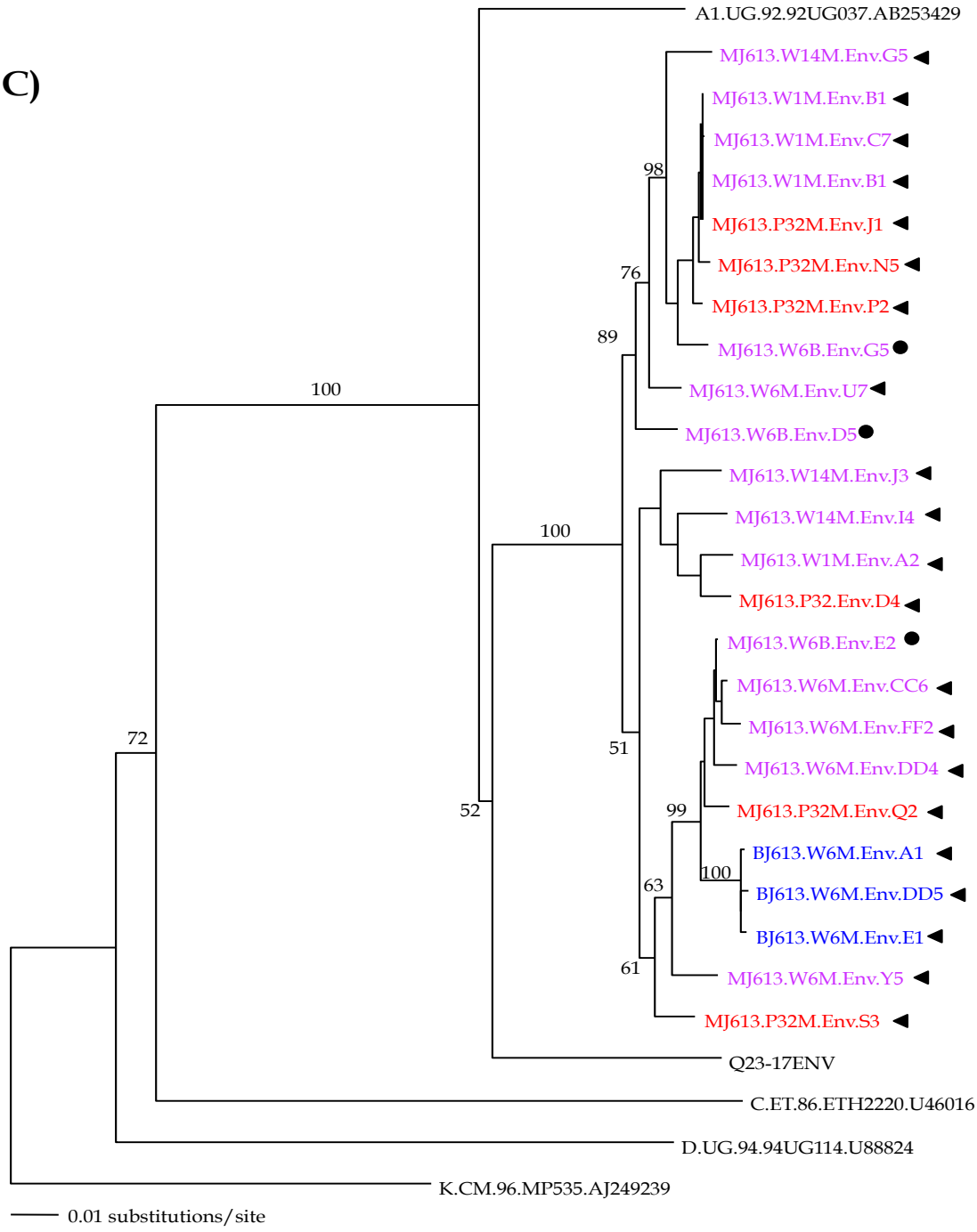
MF520.W14B.Env.D4
MF520.W14B.Env.G4
MF520.P32M.Env.B1
MF520.P32M.Env.I4
MF520 W14M.Env.I4
MF520.P32M.Env.Q6
MF520.W1M.Env.F5
MF520.W1M.Env.B1
MF520.W14B.Env.L1
MF520.W14M.Env.D4
MF520.W1M.Env.L3
MF520.W14M.Env.Y2
MF520.W14B.Env.B4
MF520.W14M.Env.Z3.
MF520 W1M.Env.N5
MF520.W1M.Env.E5
MF520.W1M.Env.O4
MF520.W1M.Env.C2
MF520.W14B.Env.V6
MF520 W14B.Env.C5
MF520 W14M.Env.L3
MF520.W14M.Env.N2
BF520.W14M.Env.A5
BF520.W14M.Env.B3
BF520.W14M.Env.D4
BF520.W14M.Env.J5
BF520.W14M.Env.I3
BF520.W14M.Env.P5
BF520.W14M.Env.C2
BF520.W14M.Env.G3
BF520.W14M.Env.E3
BF520.W14M.Env.H4
MF520.W14M.Env.V5
MF520.W1M.Env.A3
MF520.W14M.Env.F2
MF520.W14M.Env.HH2
MF520.W1M.Env.J3
MF520.W14M.Env.C1
MF520.W14R.Env.C2
MF520.W14R.Env.G1
MF520.P32M.Env.C2
MF520.W14B.Env.I3
MF520.W14M.Env.OO4
MF520.W14M.Env.CC3
MF520.W1M.Env.P2
MF520.P32M.Env.P10
MF520 W14M.Env.KK4
MF520.W1M.Env.Q1
100
MF520.W1M.Env.M5
MF520.W14M.Env.P2
MF520.W14R.Env.B5
MF520.W1M.Env.D3
MF520.W14B.Env.H11
MF520.W14R.Env.L5
MF520.W14R.Env.I3
MF520.W14M.Env.G3
MF520.W14M.Env.NN3
K.CM.96.MP535.AJ249239
0.01 substitutions/site

B)

MF520.W1M.Env.J3	1600
MF520.W1M.Env.N5	1600
MF520.P32M.Env.B1	1213
MF520.W14B.Env.L1	748
MF520.W14M.Env.Y2	594
MF520.W14M.Env.P2	563
MF520.W14M.Env.CC3	452
MF520.W14M.Env.G3	368
MF520.P32M.Env.P10	350
MF520.P32M.Env.Q6	305
MF520.W14B.Env.H11	304
MF520.W14B.Env.I3	281
MF520.P32M.Env.I4	187
MF520.P32M.Env.C2	180
MF520.W14M.Env.V5	174
MF520.W14B.Env.G4	162
MF520.W1M.Env.Q1	140
MF520.W14M.Env.B2	139
MF520.W1M.Env.P2	117
MF520.W1M.Env.L3	62
MF520.W14M.Env.NN3	25
MF520.W14B.Env.E9	25
MF520.W1M..Env.M5	25



C)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MJ613.W1M.Env.B1
MJ613.W1M.Env.C7
MJ613.W1M.Env.B1
MJ613.P32M.Env.J1
MJ613.P32M.Env.N5
MJ613.P32M.Env.P2
MJ613.W6B.Env.G5
MJ613.W6M.Env.U7
MJ613.W6B.Env.D5
MJ613.W14M.Env.J3
MJ613.W14M.Env.I4
MJ613.W1M.Env.A2
MJ613.P32.Env.D4
MJ613.W6B.Env.E2
MJ613.W6M.Env.CC6
MJ613.W6M.Env.FF2
MJ613.W6M.Env.DD4
MJ613.P32M.Env.Q2
BJ613.W6M.Env.A1
BJ613.W6M.Env.DD5
BJ613.W6M.Env.E1
MJ613.W6M.Env.Y5
MJ613.P32M.Env.S3
Q23-17ENV
C.ET.86.ETH2220.U46016
D.UG.94.94UG114.U88824
K.CM.96.MP535.AJ249239
0.01 substitutions/site
98
76
89
100
100
72
52
51
99
100
63
61

D)

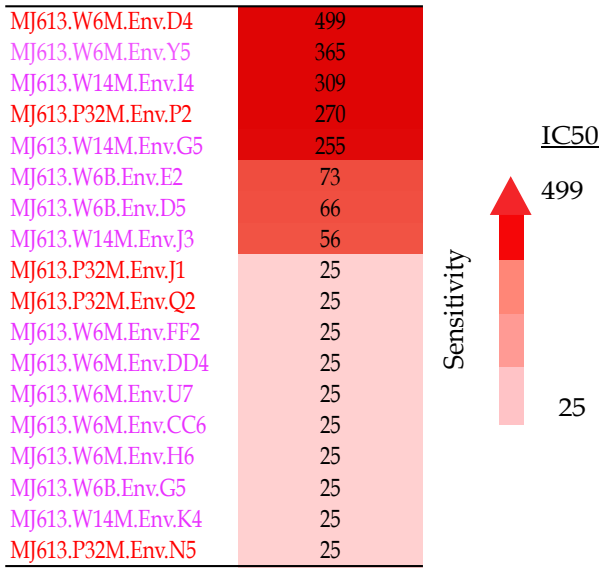

Fig.3.1. Analysis of quasispecies diversity in mother-infant pairs MF520 & MJ613. (A) & (C) represent neighbor- joining tree based on pairwise distance of full-length envelope sequences from two mother: MF520 and MJ613 respectively. The red sequences represent the first variants obtained from PBMC at P32; sequences in purple are additional sequences isolated from different time points including W1 & W14 for MF520 and W6 & W14 for MJ613; blue sequences are infant variants isolated at the first time (W14 for BF520 & W6 for BJ613) when the infant was detected HIV DNA positive. (B) & (D) represent heat-map of neutralization sensitivity (IC50) for the first variants (red) versus additional variants in purple. The variants tested for neutralization sensitivity were chosen based on the % maximum pairwise distance between the sequences. In the heatmap, which is based on quartile IC50 values, the darker the color the sensitive the variant is to neutralization by contemporaneous plasma and the lighter the color the less sensitive the variant. The shapes; ● represents clones from BMC, ◆ represents sequences from plasma and ◄ represents clones from PBMC

Diversity in envelope sequences

In order to estimate genetic diversity and check for potential intersample contamination, sequences from 4-8 full-length envelope clones from each of the 10 NTM and 10 TM were examined. The neighbor joining tree showed no evidence of cross-subject mixing of sequences (Fig.3.2A&B) and bootscan analysis with subtype reference

sequences from the Los Alamos HIV-1 database (http://www.hiv.lanl.gov/), revealed that 60% (N=12) of the subjects were infected with subtype A, which is the predominant subtype circulating in East Africa. A few (N=2) women were infected with subtype C and the remaining 30% (N=6) were subtype D infected (Table 3). For each woman, the HIV-1 Env variant sequence diversity was calculated as the maximum pairwise distance between Env sequences isolated at a single time-point. The maximum sequence diversity ranged from 0.93% to 7.3%. The transmitting mothers had a mean maximum nucleotide pairwise distance of 2.62 compared to 2.46 of NTM


A1.UG.92.92UG037.AB253429
MA411.P33M.Env.G10
MA411.P33M.Env.C2
MA411.P33M.Env.B2
MA411.P33M.Env.R1.
MA411.P33M.Env.S6
Q23-17ENV
MO862 P33M Env.D8
MO862 P33M Env.E6
MO862.P33M.Env.C7
MO862 P33M.Env.J9
MB807.P32M.Env.P2
MB807.P32M.Env.J3
MB807.P32M.Env. N5
MB807.P32m.Env.B2
MB807.P32M.Env.R4
MB807.P32M.Env.K4
MB807.P32M.Env.L5
MB807.P32M.Env.C5
MG540.P34M.Env.A6
MG540.P34M.Env.C3
MG540.P34M.Env.D1
MG540.P34M.Env.H2
MG540.P34M.Env.F1
MG540.P34M.Env.K4
MF074.W3M.Env.G10
MF074.W3M.Env.J8
MF074.W3M.Env.Q2
MF074.W3M.Env.S2
MF074.W3M.Env.T5
MF074.W3M.Env.J7
MF074.W3M.Env.H5
MM471.P32M.Env.C4
MM471.P32M.Env.L9
MM471.P32M.Env.M3
MM471.P32M.Env.G5
MM471.P32M.Env.B6
MM471.P32M.Env.E8
MP199.P38M.Env.F5
MP199.W0M.Env.B5
MP199.P38M.Env.L4
MP199.W0M.Env.I2
MP199.P38M.Env.M4
MP199.W0M.Env.F6
MP199.W0M.Env.G3
MP199.W0M.Env.H8
MP199.W0M.Env.D4
MP199.W0m.Env.E3
MF600.W2M.Env.B1
MF600.W2M.Env.II7
MF600.W2M.Env.C1
MF600.W2M.Env.GG10
MF600.W2M.Env.E3
C.ET.86.ETH2220.U46016
D.UG.94.94UG114.U88824
MM834.P32M.Env.F7
MM834.P32M.Env.H3
MM834.P32M.Env.M2
MM834.P32M.Env.B2
MM834.P32M.Env.I1
MM834.P32M.Env. P7
ML156.P32M.Env.J5
ML156.P32M.Env.I2
ML156.P32M.Env.F2
ML156.P32M.Env.H7
ML156.P32M.Env.K2
ML156.P32M.Env.G2
ML156.P32M.Env.D7
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88
64
100
100
100
61
78
100
84
100
100
100
98
100
100
0.01 substitutions/site

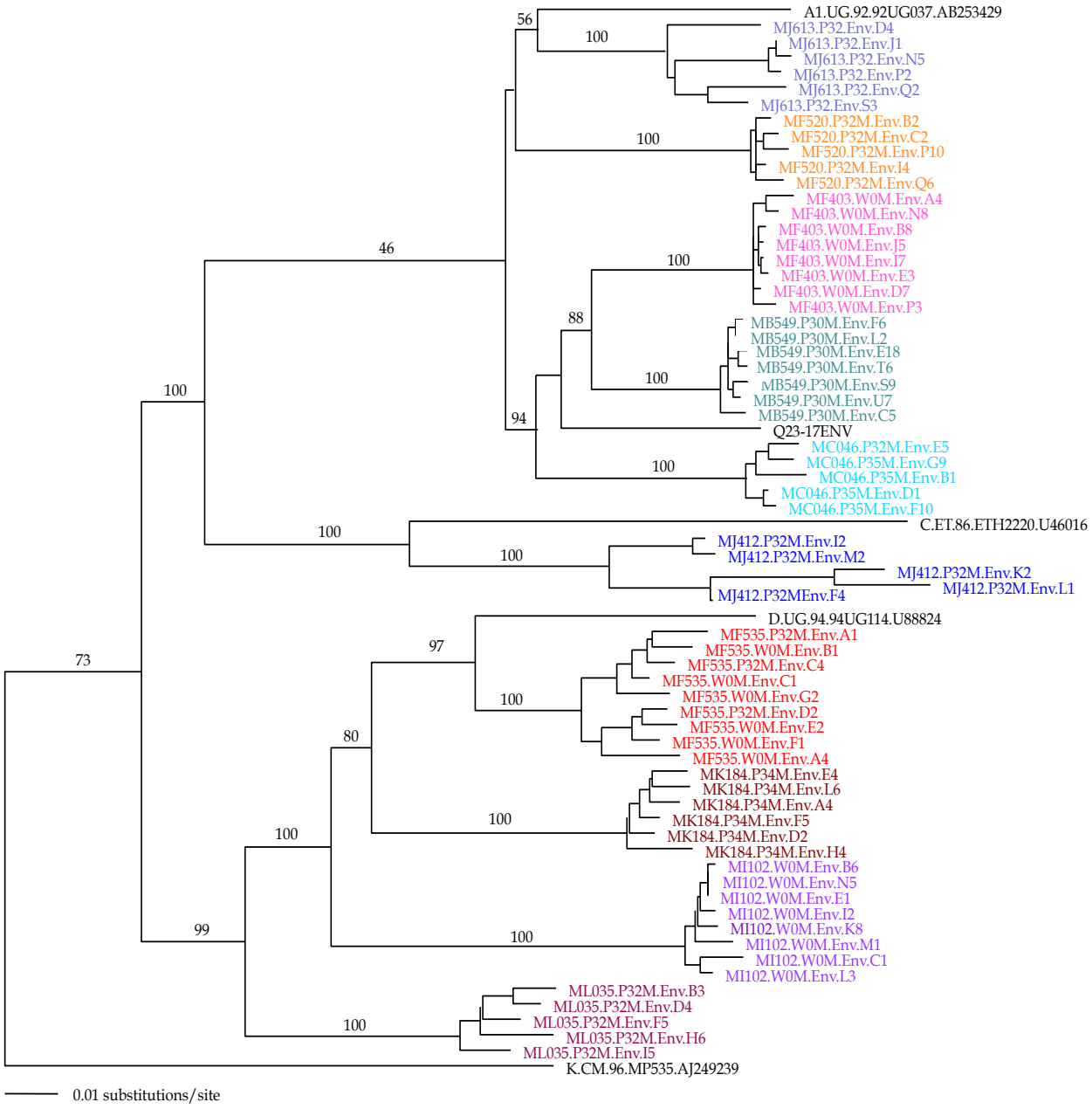


Fig.3.2. Neighbor-joining tree based on pairwise distance, showing 64 distinct envelope sequences for each **(A)** 10 NTM & **(B)** 10 TM. Different colors represent variants from different mothers. The sequences in black represent reference sequences. Bootstrap values are shown to the left of each node.

Neutralization sensitivity of maternal envelope variants.

To test if maternal autologous Nabs present near the time of transmission would impact MTCT, each of the cloned maternal envelope variants was tested for their ability to infect TZM-bl cells in the presence of matched autologous plasma as outlined previously(174). For the two cases where a large number of clones were obtained (MF520 and MJ613), only the first envelope sequences obtained from P32 were included in this analysis. P32 was the time-point most relevant for transmission and also when clones from most women were isolated (Table 3), hence synchronizing cloning time points between both TM and NTM. However, in our primary analysis, we included IC50s from all the clones tested for neutralization sensitivity (N=56 for NTM & N=57 for TM), while in a sensitive we considered only the first 5 clones per woman to avoid the risk of one woman with many clone overly influence the results. In both the analysis, TM and NTM mothers harbored virus variants with a broad spectrum of neutralization sensitivities (Fig.3.3A). For example MO862, who was NTM had a mix of neutralization sensitive and neutralization resistant viruses with IC50 ranging form 25 to 1467. This was also true for transmitting mother MI102 with IC50 values ranging from 25 -1600. On the other hand, some women (e.g. MA411 a NTM and MF520 a TM) had primarily sensitive variants, while others (e.g. MG540 a NTM and MJ412 a TM) had primarily resistant viruses. Overall, the mean IC50 = 242 (range 25 to 1842) for NTM was not significantly different from the mean IC50 = 322 (range 25 to 1644) for TM. There was also no apparent difference in the frequency of Nab-sensitive or resistant variants between NTM (25 resistant & 31 sensitive) versus TM (17 resistant & 40 sensitive) (Fig

3.3A & B).

Fig.3.3 A)

Maternal Idnum	Non-transmitting Mothers Env Variants	IC50
MO862	MO862.P33M.Env.J9	1467
	MO862.P33M.Env.K5	323
	MO862.P33M.Env.C7	96
	MO862.P33M.Env.L2	76
	MO862.P33M.Env.N5	25
MB807	MB807.P32M.Env.P2	221
	MB807.P32M.Env.L5	105
	MB807.P32M.Env.C5	25
	MB807.P32M.Env.B2	25
	MB807.P32M.Env.N5	25
	MB807.P32M.Env.R4	25
	MB807.P32M.Env.K4	25
	MB807.P32M.Env.J3	25
MB156	MB156.P32M.Env.G2	1842
	MB156.P32M.Env.F8	25
	MB156.P32M.Env.H7	25
	MB156.P32M.Env.J5	25
	MB156.P32M.Env.K2	25
NF074	MF074.W3M.Env.S2	1248
	MF074.W3M.Env.H5	250
	MF074.W3M.Env.J8	217
	MF074.W3M.Env.G10	185
	MF074.W3M.Env.Q2	25
MP199	MP199.W0M.Env.F6	132
	MP199.W0M.Env.D4	86
	MP199.W0M.Env.E3	67
	MP199.W0M.Env.H8	51
	MP199.W0M.Env.B5	25
	MP199.W0M.Env.G3	25
MM471	MM471.P32M.Env.C4	1600
	MM471.P32M.Env.B6	979
	MM471.P32M.Env.G5	315
	MM471.P32M.Env.M3	206
	MM471.P32M.Env.E4	114
MM834	MM834.P32M.Env.B2	116
	MM834.P32M.Env.H3	192
	MM834.P32M.Env.F7	107
	MM834.P32M.Env.I1	25
	MM834.P32M.Env.M2	25
	MM834.P32M.Env.P7	25
MF600	MF600.P32M.Env.C1	148
	MF600.P32M.Env.B1	115
	MF600.P32M.Env.E3	25
	MF600.P32M.Env.KK10	25
	MF600.P32M.Env.MM7	25
MA411	MA411.W0M.Env.C1	1252
	MA411.W0M.Env.T5	885
	MA411.W0M.Env.B2	151
	MA411.W0M.Env.S6	96
	MA411.W0M.Env.R1	25
MG570	MG540.P34M.Env.A6	212
	MG540.P34M.Env.C3	62
	MG540.P34M.Env.B5	25
	MG540.P34M.Env.D1	25
	MG540.P34M.Env.F1	25
	MG540.P34M.Env.K4	25

Maternal Idnum	Transmitting Mothers Env Variants	IC50
MI102	MI102.W0M.Env.K8	1600
	MI102.W0M.Env.I2	190
	MI102.W0M.Env.L3	134
	MI102.W0M.Env.N5	119
	MI102.W0M.Env.E2	64
	MI102.W0M.Env.B6	56
	MI102.W0M.Env.C1	25
	MI102.W0M.Env.M1	25
MF403	MF403.W0M.Env.E3	677
	MF403.W0M.Env.I6	359
	MF403.W0M.Env.J5	347
	MF403.W0M.Env.N8	113
	MF403.W0M.Env.P3	106
MF535	MF535.W0M.Env.B1	1600
	MF535.W0M.Env.F1	1600
	MF535.W0M.Env.C1	567
	MF535.W0M.Env.D1	554
MF520	MF520.P32M.Enn.B1	1213
	MF520.P32M.Env.P10	350
	MF520.P32M.Env.Q6	305
	MF520.P32M.Env.I4	187
	MF520.P32M.Env.C2	180
MJ613	MJ613.P32M.Env.D4	498
	MJ613.P32M.Env.P2	270
	MJ613.P32M.Env.J1	25
	MJ613.P32M.Env.N5	25
	MJ613.P32M.Env.Q2	25
MB549	MB549.P30M.Env. F11	122
	MB549.P30M.Env.C5	25
	MB549.P30M.Env.E18	25
	MB549.P30M.Env.L2	25
	MB549.P30M.Env.S9	25
	MB549.P30M.Env.T6	25
	MB549.P30M.Env.U7	25
MC046	MC046.P35M.Env.D1	1644
	MC046.P35M.Env.F10	442
	MC046.P35M.Env.E2	25
	MC046.P35M.Env.G10	25
MJ412	MJ412.P32M.Env.J5	328
	MJ412.P32M.Env.G6	152
	MJ412.P32M.Env.M2	119
	MJ412.P32M.Env.F8	25
	MJ412.P32M.Env.K2	25
	MJ412.P32M.Env.I2	25
	MJ412.P32M.Env.B1	25
ML035	ML035.P32M.Env.I5	1375
	ML035.P32M.Env.J5	580
	ML035.P32M.Env.C5	313
	ML035.P32M.Env.B5	264
	ML035.P32M.Env.F8	129
	ML035.P32M.Env.D4	84
	ML035.P32M.Env.H6	74
MK184	MK184.P34M.Env.A4	432
	MK184.P34M.Env.K2	288
	MK184.P34M.Env.R4	210
	MK184.P34M.Env.D2	201
	MK184.P34M.Env.H4	80

B) OR = 1.000, 95% CI: 0.999, 1.010; p = 0.201

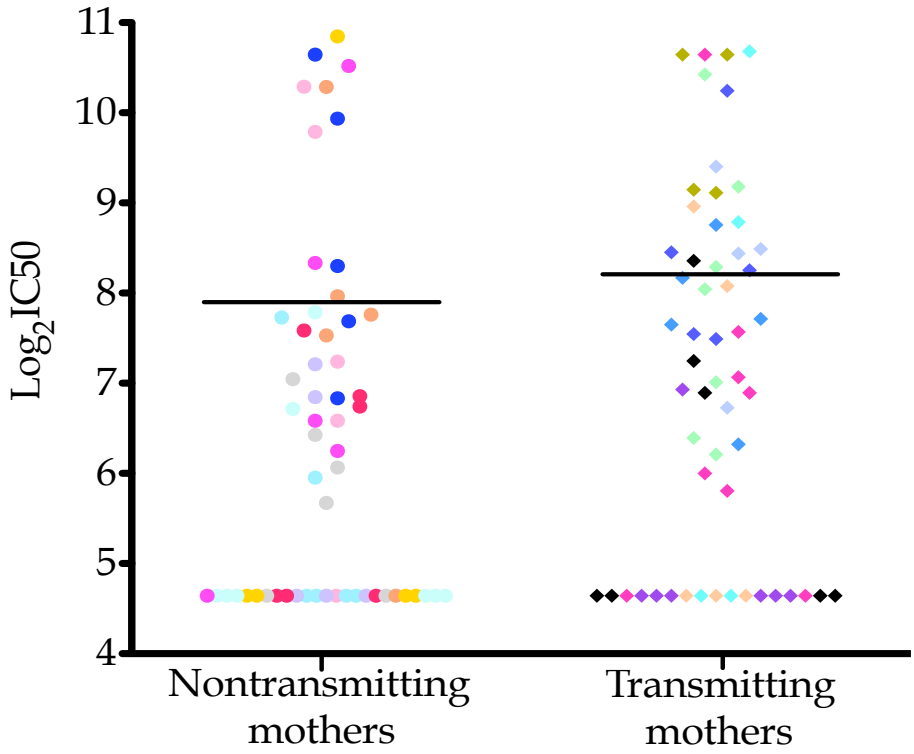


Fig.3.3. Summary of the virus variants with their neutralization profiles. **A)** A heat-map of neutralization sensitivity (IC50) for the individual variants from 10 NTM (N=56; left panel) and 10 TM (N=57; right panel). The 2nd & 3rd columns in both the left and right panels show the individual variants and IC50, respectively. The colors represent neutralization sensitivity, the colors are based on quartiles, the darker colors denote greater neutralization sensitivity to maternal contemporaneous autologous plasma. **(B)** Comparison of the distribution of IC50 values between NTM versus TM to assess for an association between IC50s values and the risk of MTCT - different colors represent variants from different mothers. The solid circles represent variants from NTM and the diamonds represent TM. The black horizontal line represents the means IC50. It is important to note that clones were chosen for neutralization based on the percent pairwise distance between individual sequences obtained in a woman.

To determine if potent neutralization of maternal diverse envelope variants was associated with reduced risk of MTCT, we compared log2-transformed IC50 (log transformation was done to normalize the IC50 values given the high variability in IC50 values). We used logistic regression with general estimating equation (GEE), which is suitable for a setting like this, where multiple samples are obtained from an individual, because it accounts for within-individual correlation of the data points. The risk of vertical transmission was not significantly associated with IC50 levels (OR = 1.14, 95% CI: 0.89, 1.47; p = 0.30). To further assess whether there is a threshold of IC50s that could predict transmission risk, we used receiver operating characteristic curve [ROC] analysis. Consistent with the finding above, IC50 values did not predict transmission risk (AUC < 0.5).

Discussion

Several vertical HIV-1 transmission studies that have focused on transmitting mothers suggest that maternal autologous neutralizing antibodies are able to block the transmission of a subset of variants circulating in the mother and that only variants that are less sensitive to Nabs found in the mother are transmitted to the infant. However, since these early studies only included transmitting mothers, the question arises of whether non-transmitting mothers have Nab that more potently neutralizes their viral quasispecies than transmitting mothers. In the current study, we isolated 128 unique virus variants that represent the quasispecies diversity in 10 NTM and 10 TM and determined if there were differences between the TM and NTM in the ability to neutralize their virus variants. We found no differences in the range of sensitivity of

virus variants isolated from NTM versus TM, rather both groups harbored variants with a broad spectrum of neutralization sensitivity and resistant variants were found in both TM and NTM. The presence of resistant variants in the NTM was surprising because if viral escape from neutralizing antibodies facilitates vertical transmission as suggested by several previous studies, then these variants could have been transmitted.

Our results suggest that Nab sensitivity alone does not determine MTCT because some NTM with high viral load harbored neutralization resistant variants. Given that vertical transmission is a multifactorial process that may be influenced by both host and viral features, other factors such as viral fitness that may override the impact of Nabs, may determine virus transmission during MTCT. Indeed several studies have shown that transmitted variants during MTCT have increased replicative fitness(78), CD4 receptor and co-receptor usage (122). However, differences in fitness and receptor usage were not observed in other studies that examine non-subtype B variants (46, 141). Future analysis may be required to determine whether the lack of transmission of the neutralization resistant variants present in NTMs is due to other features that impact transmission.

It is possible that the detection of neutralization resistant variants in NTM could reflect the fact that these variants represent archival rather that actively replicating viruses, since in all cases, the envelopes were isolated from integrated proviral DNA and not RNA, which ideally should represent currently circulating variants. However, for two women (MF520 & MJ613) from whom we cloned envelopes from both the plasma and PBMCs, the viruses from PBMC and RNA intermingled in a phylogenic

tree, suggesting limited compartmentalization. The similarity of virus variants circulating in different compartments including breastmilk, genital and plasma has been reported previously(144). Furthermore, latently infected resting T-cells are rare in circulation(28), suggesting that most infected cells among the PBMCs may harbor actively replication viruses. Moreover, studies using PBMC to derive viruses in culture suggest that most of the dominant HIV DNA sequences in PBMC represent viruses that can yield infectious virus(105, 155). Additionally, some previous studies have also shown that viruses cloned from RNA have similar phenotypic features as those from PBMCs(105, 155).

This is the first study to incorporate both TM & NTM with the primary intent of deriving conclusions on the capacity of autologous neutralizing antibodies to block HIV-1 transmission. We determined that autologous Nabs present near transmission do not correlate with reduced risk of peripartum MTCT. These findings are consistent with several previous studies that only utilized primary isolates and found that maternal autologous Nab induced in typical natural infection and that are present in the infant at the time of exposure, are not protective against MTCT(61, 64). However because during mother to child HIV-1 transmission, both cell associated and cell free viruses may be transmitted (149), I cannot rule out the possibility that our assay, which only measures the ability of Nab to block the transmission of cell-free viruses, may miss detecting the association with cell-to-cell spread. Indeed, a recent study by Abela and colleagues suggested that even a highly potent cross reactive neutralizing antibody like VRC01(175), that neutralizes up to 90% of circulating HIV-1

strains when tested against cell free viruses has up to 100-fold reduction in neutralizing cell-associated viruses(2). Therefore, assays that assess the ability to neutralize both cell-associated and cell-free virus may be required to definitively define the role of Nab in MTCT.

Given that Nabs specificity is critical during MTCT(146), we tested what number of sequences that were needed to detect the range of viral variants circulating in these women. This is important given the diversity inherent in chronic infection. Typically, within a few months of infection, almost all HIV-1 infected individuals generate Nabs that exert selective pressure on the virus, driving viral escape and generation of Env sequence diversity that can differ up to 10% within a chronically infected individual(3, 58, 134). Because of this diversity, the protective effect of autologous Nab could be missed if variants not representative of the broader quasispecies are analyzed. In the current study, the failure to detect an association between autologous Nab and reduced risk of MTCT is unlikely to be due to inadequate virus sampling because we first determined that a minimum 5 variants were adequate to capture the genetic diversity of the virus population within these women.

In conclusion, we have shown that autologous Nabs induced in natural infection and that target HIV-1 variants present near delivery, are not associated with a decreased risk of vertical HIV-1 transmission. However, this finding may not overrule the potential of exquisitely potent and broad Nabs, such as those that have recently been isolated from several chronically infected adults[(17, 166) and reviewed by Davide et al(32)], to protect against HIV-I transmission. Because the occurrence of such

antibodies is rare and have only been detected after several year of infection, it is possible that the current study that focused only on a limited number of individuals with unknown duration of infection, could have missed the occurrence of such antibodies. Moreover, apart from neutralization, maternal autologous Nab could also mediate other molecular functions such as antibody mediated cellular cytotoxicity (ADCC) that could also protect against MTCT. Future comprehensive studies may be necessary to determine whether such non-neutralizing activity of maternal antibodies correlate with reduced risk of MTC.

Chapter 4

The detection of HIV-1 virus escape during mother-to-child transmission depends on sample timing

Introduction

Multiple studies have reported the occurrence of a genetic bottleneck during most cases of MTCT(45, 75, 85, 104, 150, 173, 174, 179). These studies show that despite heterogeneous virus quasispecies present in the mother, only a limited number of viruses appear to initiate infection in the infant. Host genetics including certain maternal HLA types such as HLA B18* have been suggested to be associated with reduced risk of postpartum MTCT(42) and may restrict the transmission of a subset of maternal variants. Moreover, single nucleotide polymorphims in the HIV-1 co-receptors have also been reported to modulate the risk of infant infection (20, 103). Alternatively, bottleneck occurring during MTCT could be due to a stochastic event involving random transmission of a limited number of maternal variants. The observation that certain virus characteristics such better receptor (CD4) & co-receptor (CCR-5) usage as well as replicative fitness(179) may contribute to transmission during MTCT, argue against stochastic events facilitating the genetic bottleneck during vertical HIV-1 transmission and rather hints at the presence of a selective pressure that may block the transmission of certain variants. Host immune response particularly Nabs may be a source of selective pressure that restricts the transmission of certain variants but select for other antigenically distinct variants that escape such immune surveillance mechanism. The escape virus variants would typically have a survival advantage both in the mother as

well as in the infant, where passively acquired maternal antibodies exist. In chapter 3 of this dissertation we showed that autologous Nabs induced in natural infection are not sufficient to fully protect against MTCT. This observation argues the capacity of maternal Nabs to fully protect against vertical HIV-1 transmission. It is important to note that because of the rapid changes in immune response to the virus and the inherent virus escape, an association between Nab and protection could be missed if sample timing is not factored in a study. Consistent with this hypothesis, studies using samples near transmission have suggested that maternal viruses transmitted to the infant are those in the mother that are poorly neutralized by maternal plasma isolated within the window of MTCT(104, 174). In these studies, where individual variants were analyzed, sensitive variants were present in the mothers, but were not isolated from the infants, suggesting that maternal Nabs offered some protection against the transmission of such variants. However, in contrast to the findings of these early studies, some recent studies have suggested that maternal Nabs do not drive virus escape during MTCT. These studies show that the transmitted variants have similar neutralization profiles as the variants in the mothers(45, 75, 139, 157). The results from these recent studies are difficult to interpret because they were small and/or the time points (if it was reported) where antibodies and virus were analyzed were often after transmission had occurred (Table 4.1). For example in the study by Kishko and colleagues where 5 subtype B infected mother/baby pairs were analyzed, maternal viruses were isolated within one month of delivery (though the majority of clones were obtained from samples collected several weeks before parturition) and infants viruses were obtained within two months

of delivery (Table-4.1), but infection occurred intrapartum in most cases(75). These clones were tested with maternal plasma isolated 2 months after transmission(75). Similarly, in the study by Russel et al maternal virus variants isolated at delivery, which could be several weeks before or after the estimated time of MTCT (intarpartum/postpartum, Table 4.1), were tested against maternal plasma isolated about 2 months later (139). In addition to the differences in study design, other differences such as viral subtype analyzed may also explain the discrepant results in the detection of escape.

Table 4.1. Summary of the molecular studies focusing at detecting HIV-1 virus escape during MTCT

Study	Time of MTCT	Patient (N)	No. of functional Env clones analyzed	Virus subtypes examined	Time point when Env clones were obtained	Time of maternal plasma tested
Kisko *et al* (2011)	Intrapartum	Mother (5)	22	B	Within one month of birth	Not clear
		Infant (5)	8		1 months to or by 2 months after delivery	
Russel *et al* (2011)	In utero/Intrapartum	Mother (16)	50	C	Labor	Not mentioned
		Infant (16)	20		Within 6 weeks of delivery	
Thenin *et al* (2012)	In utero/Intrapartum	Mother (4)	11	AE	Delivery	4 -11 weeks after delivery
		Infant (4)	6		1st positive visit (range: 0-2.4months after birth)	
Wu *et al* (2006)	Intrapartum/breastmilk	Mother (12)	64	A, C, D, D/A & C/A	Mostly at W0 or the sample prior to the 1st positive visit	Contemporanous to materanal Env clones
		Infant (12)	32		1st positive visit (range: 0-15 months after birth)	

Table 4.1: (N) refers to total number of patients (mothers/infants) analyzed. It is important to note that the study by Wu et al is larger in terms of the number of clones & subtypes analyzed. The viruses and maternal plasma tested in Wu et al study were isolated close to the estimated time of MTCT.

In the current study, we hypothesize that discrepant findings regarding whether Nabs drive virus escape during vertical HIV-1 transmission in different studies are due to differences in sample timing in relation to transmission. In order to fill this important gap, we have tested maternal/infant envelope variants, obtained within the window of MTCT, against maternal plasma isolated from two time points including near (contemporaneous) and post transmission (later). We then performed an analysis

using the IC50 from each combination of virus/plasma pairs to determine if there are differences in neutralization sensitivity between mother and infant viruses.

Material and Methods & study design

Generation of pseudoviruses from mother-infant pairs: Envelope variants were from mother/baby pairs: J613, F403, F520, and I102 and are described in Chapter 3, Table 1. These four mother/baby pairs were chosen from the cases studied in chapter 3 based on availability of temporal maternal samples. Mother variants isolated from the sample closest to the last infant HIV-1 negative test (Table 4.2) and corresponding infant variants obtained at the first HIV-1 DNA positive visit were tested against both contemporaneous maternal plasma (near transmission sample) and plasma isolated on 10-20 weeks after transmission (post transmission sample, Fig.1). Neutralization assays were performed as previously described (Chapter 2). The differences in IC50 values between plasma/virus combination were estimated using GEE with independent working correlation (performed by Katie Odem-Davis)

Table 4.2. Summary of the 4 mother/baby pairs used in the Nab escape analysis

Patient Idnum	When infant was last tested HIV-1 DNA negative	When infant was 1st tested HIV-1 DNA positive	Duration of breastfeeding in months	Maternal CD4 count (prenatal)	Maternal prenatal plasma viral load (log_{10}copies/ml)	Env cloning time point	Virus Subtype	No. of env clones obtained	Maternal neutralization plasma time point	
									Early[γ]	Late[β]
MF403						W0	A	5	W0	M12
BF403	W0	W6	30	213	5.1	W6	A	4		
MF520						P32	A	5	P32	M6
BF520	W1	W14	28	511	5.6	W14	A	12		
MJ613						P32	A	6	W1	M3.5
BJ613	W1	W6	10	104	5.6	W6	A	4		
MI102						W0	D	9	W0	M6
BI102	W0	W6	13	639	4.8	W6	D	5		

Table 4.2: W0: delivery; W: week after delivery; P32: 32 weeks of pregnancy and M: months after delivery. [γ]Time-point for the maternal plasma used for neutralization, which is near estimated time of MTCT. [β]Time-point for the maternal plasma used for neutralization, which is post estimated time of MTCT.

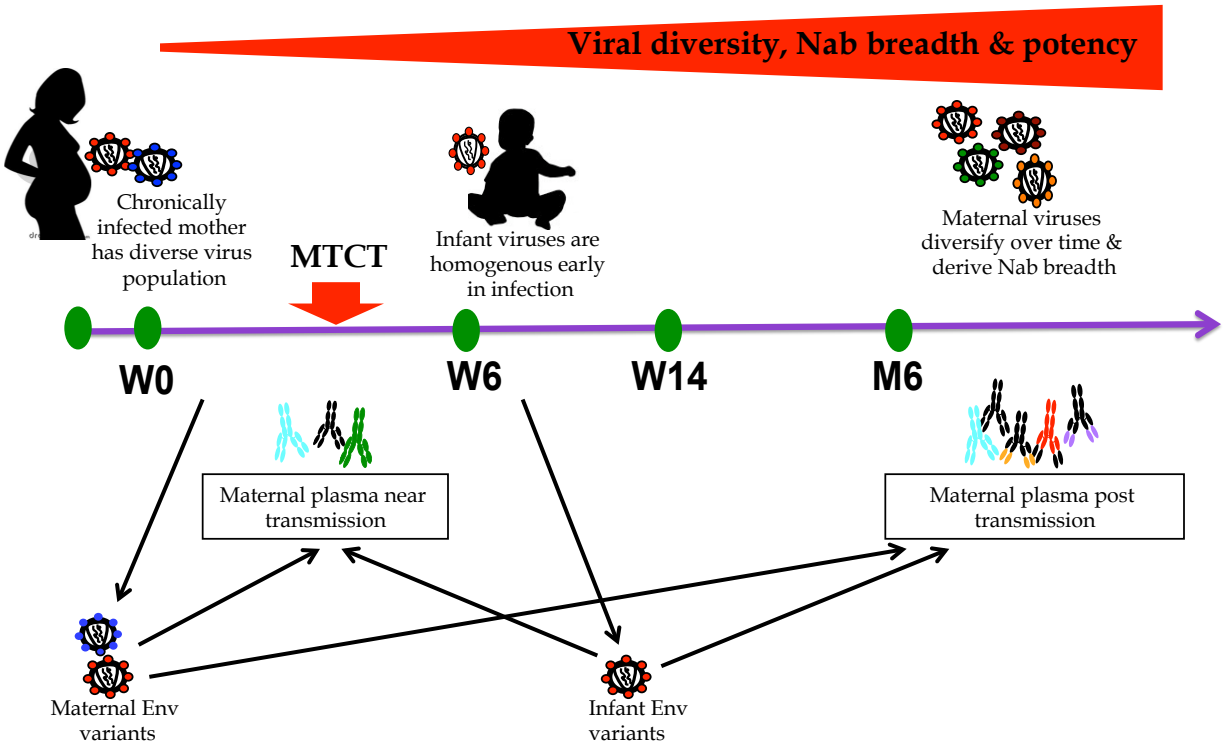


Fig.4.1: Study design highlighting the time when the maternal & infants clones (down pointing arrows) and maternal plasma (up-pointing arrows) were isolated and tested respectively. W0, W6, W14, M6 represent samples collected at 0, 6, 14, weeks and 6 month post delivery, respectively. The thick red arrow points to the median estimated time of MTCT, based on the mid point between the last HIV negative and first HIV positive test in the infant, which was between W0/W1 and W6 in all except one infant (BF520). This infant did not have a sample at 6 weeks, and first tested positive at 14 weeks.

Results

Infant variants are more resistant to neutralization by maternal plasma near transmission than maternal variants: We tested several distinct Env variants isolated from 4 mothers (N=23, average=6 clones/mother) and their corresponding infants (N=18, average=5 clones/infant, Table 4.2), against maternal plasma collected both near and post transmission to determine whether sample timing is critical in the detection of virus escape during MTCT. The neutralization results for the individual variants are shown in Table 4.3. Generally, infant variants were poorly neutralized by maternal plasma near transmission (average IC50 = 36, range 25-121) compared to later plasma (average IC50 = 109, range 25-335). While the majority of the infant variants [14/18] were not neutralized by maternal plasma isolated near time of transmission, only one of the 3/18 variants were resistant to neutralization by later maternal plasma from after transmission (Table 4.3). In contrast, most of the maternal variants [20/23] were sensitive to plasma near transmission and also to later plasma isolated after transmission time-point [17/23].

Table 4.3. Summary of Env variants with their corresponding IC50 and maternal plasma time-point.

Patient identification number	Individual envelope variants	IC50s near transmission		IC50s post transmission	
MJ613	MJ613.P32M.Env.D4	290		153	
	MJ613.P32M.Env.V1	103		696	
	MJ613.P32M.Env.P2	104	(W1)	101	(M3.5)
	MJ613.P32M.Env.J1	110		721	
	MJ613.P32M.Env.Q2	25		151	
BJ613	BJ613.W6M.Env.DD5	121		85	
	BJ613.W6M.Env.II1	25	(W1)	25	(M3.5)
	BJ613.W6M.Env.A1	25		120	
	BJ613.W6M.Env.E1	54		60	
MF403	MF403.W0M.Env.N8	112		259	
	MF403.W0M.Env.E5	677		530	
	MF403.W0M.Env.I6	359	(W0)	324	(M12)
	MF403.W0M.Env.J5	346		258	
	MF403.W0M.Env.P3	106		115	
BF403	BF403.W6M.Env.B6	25		183	
	BF403.W6M.Env.C2	25	(W0)	141	(M12)
	BF403.W6M.Env.E1	64		156	
	BF403.W6M.Env.F4	67		111	
MF520	MF520.P32M.Env.B1	1213		1600	
	MF520.P32M.Env.C2	180		400	
	MF520.P32M.Env.I4	187	(P32)	161	(M6)
	MF520.P32M.Env.Q6	305		242	
	MF520.P32M.Env.P10	350		346	
BF520	BF520.W14M.Env.A5	25		25	
	BF520.W14M.Env.B3	25		25	
	BF520.W14M.Env.H4	25	(P32)	25	(M6)
	BF520.W14M.Env.J5	25		335	
	BF520.W14M.Env.M1	25		67	
MI102	MI102.W0M.Env.B6	56		25	
	MI102.W0M.Env.C1	25		25	
	MI102.W0M.Env.E2	64		122	
	MI102.W0M.Env.I2	190	(W0)	25	(M6)
	MI102.W0M.Env.K8	1600		1600	
	MI102.W0M.Env.L3	134		25	
	MI102.W0M.Env.M1	25		25	
	MI102.W0M.Env.N5	119		25	
BI102	BI102.W6M.Env.A3	25		180	
	BI102.W6M.Env.C3	25		65	
	BI102.W6M.Env.D4	25	(W0)	74	(M6)
	BI102.W6M.Env.E5	25		112	
	BI102.W6M.Env.F6	25		174	

Table 4.3: The first column represents identification numbers for the mothers and their corresponding infants with maternal variants listed above the corresponding infant variants. The second column shows the variants, while column 3 contains their corresponding neutralization IC50 as well as the timepoints (near transmission) for the maternal plasma tested, which is shown in brackets. The last column represents neutralization IC50 derived from maternal plasma from after estimated time of transmission. The numbers shown in brackets represent months post delivery, which was further from estimated time of transmission. The horizontal panels represents different mothers (light blue) and babies (gray).

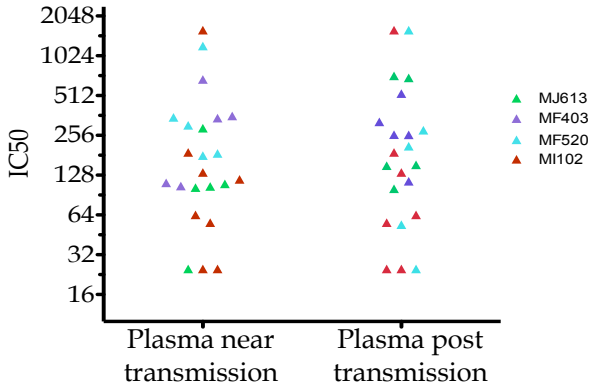
Both maternal plasma near and post transmission neutralized maternal variants at a similar level on average; IC50 ratio late/early was 1.12 (95% CI: 0.31, 3.09; p = 0.976, Fig.4.2A). In contrast, infant variants showed significant differences in sensitivity to plasma from the different time points, with plasma from near transmission yielding significantly reduced IC50 values compared to post transmission plasma (Fig.4.2B). When we compared the neutralization sensitivity of maternal and infant variants to plasma near the time of transmission, we found that compared to maternal variants, infants had reduced sensitivity to maternal plasma near transmission (Fig.4.2C), reconfirming the finding from previous studies(104, 131, 174). The maternal average IC50 value was 290 (range 25 -1600) compared to 36 for the infant (range 25-121). Using plasma near transmission, the ratio of maternal IC50 values to infant IC50s was approximately 12.67 (95% CI: 5.86, 27.38; p<0.0001, Fig.4.2C). When we tested maternal and infant variants against maternal plasma isolated post transmission, we found that maternal variants have similar sensitivity (average IC50 = 345, range = 25- 1600) as infant variants (average IC50 = 109, range = 25-335, Fig.4.2D). The ratio of maternal to infants IC50 values for later plasma was 1.29 (95% CI: 0.37, 4.47; p= 0.69, Fig 4.2D). For one infant in this study (MF520, Table 4.2), a sample at 6 weeks of life was not available for testing; thus the estimates date of infection in this infant is less precise than in the other cases. If we exclude this mother/baby pair from the analysis, we still saw similar results as outlined above (Fig. 4.2E & F).

Fig.4.2

Maternal viruses against maternal plasma from different time-points (near & post transmission)

A)

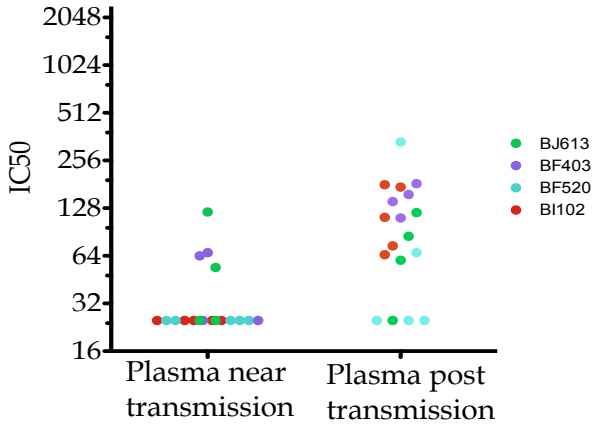
IC50 ratio = 0.98, (95% CI: 0.31, 3.09; p<0.976)



Infant viruses tested against maternal plasma from different time-points (near & post transmission)

B)

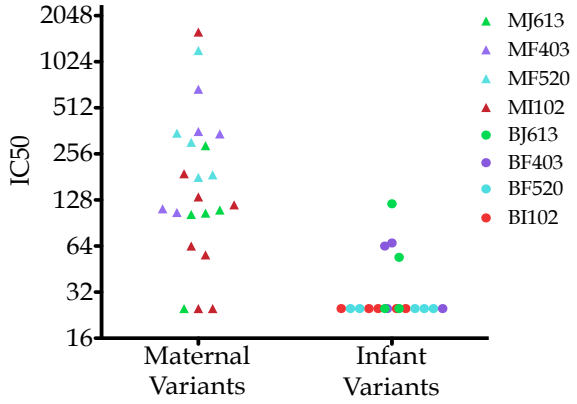
IC50 ratio = 5.5, (95% CI: 2.6, 11.6; p<0.0001)



C)

Maternal & infant viruses tested against maternal plasma isolated near transmission

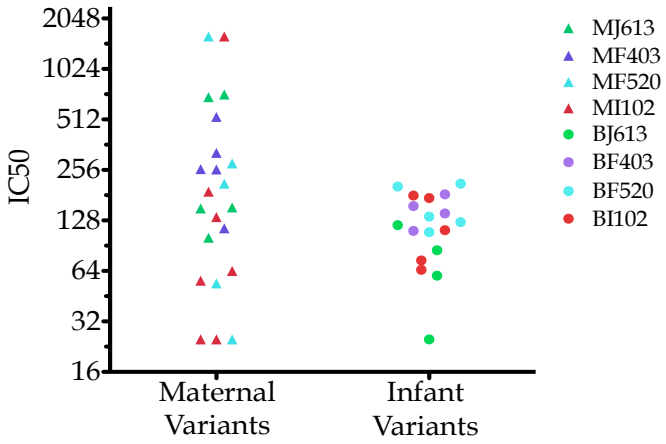
Mother/Infant IC50 ratio = 12.67 (95% CI: 5.86, 27.38; p<0.0001)



D)

Maternal & infant viruses tested against maternal plasma isolated post transmission

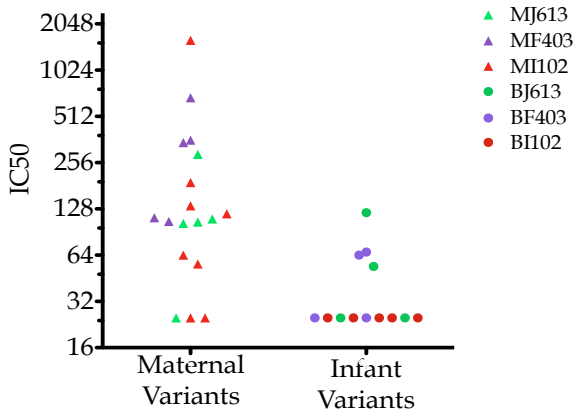
Mother/Infant IC50 ratio = 1.29, (95% CI: 0.37, 4.47; p=0.69)



E)

Maternal & infant viruses tested against maternal plasma isolated near transmission (excluding MF520/BF520 pair)

Mother/Infant IC50 ratio = 6.61 (95% CI: 3.64, 12.0; p<0.0001)



F)

Maternal & infant viruses tested against maternal plasma isolated post transmission (excluding MF520/BF520 pair)

Mother/Infant IC50 ratio = 1.39, (95% CI: 0.28, 6.90; p=0.69)

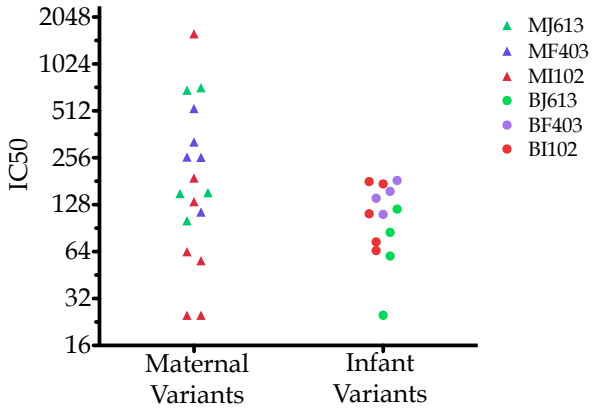


Fig.4.2. Comparison of neutralization sensitivity (IC50 values) of maternal and corresponding infant variants against maternal plasma isolated either near transmission (early plasma) or post transmission (late plasma). **A)** Maternal variants tested against early versus later plasma, **B)** Infants variants tested against early versus later plasma, **C)** Maternal versus infants variants tested against maternal early plasma, and **D)** Maternal versus infants variants tested against maternal later plasma; **E)** Maternal versus infants variants (excluding MF520/BF520) tested against maternal plasma near estimated time of transmission and **F)** Maternal versus infants variants (excluding MF520/BF520)

tested against maternal later plasma. The IC50 values represent the average IC50 values from experiments performed in duplicate on two different occasions.

Discussion

It has been proposed that the bottleneck that occurs during mother-to-child transmission may be in part attributable to the restrictive effect of maternal Nabs that limit transmission of sensitive variants (38, 104, 174). However several recent studies suggest that maternal Nabs may not play a major role in impacting HIV-1 transmission during MTCT(45, 75, 139, 157). It is not clear whether some of these discrepancies reflect inconsistencies in sample timing of viruses versus antibodies or just immunological differences that may vary by populations and different routes of MTCT. Here we show that the time of antibody sampling in relation to transmission is a determinant of the outcome of these studies and that sampling antibodies after the transmission event occurred is likely to reduce the chances to detect escape.

In the current study, we analyzed 4 mother/baby pairs with well characterized estimated time of MTCT as well as virus/plasma timing. We found that maternal variants transmitted to the infants are Nab escape variants that are less sensitive to maternal Nabs present near transmission, as observed previously, (38, 104, 149, 174) showing that maternal Nabs may select for virus escape during MTCT. However, when we analyzed the same maternal and infant variants using plasma from post transmission, we did not see evidence of escape. This finding is consistent with the results from several groups who did not find evidence that infant variants were escape viruses, suggesting that examining the presence of escape in plasma obtained after

transmission may be misleading(45, 75, 139, 157)

Typically virus escape during HIV-1 infection starts within a few months of acquiring the virus when autologous Nab response exerts selective pressure, forcing the virus to change the critical epitopes on envelope to allow for escape(116, 167). This happens through mutations including single amino acid substitutions, insertions and deletions as well as shifts in potential N-linked glycosylation sites (evolving glycan shield) to abrogate access of Nabs to their potential target sites on the Env(123). Given this dynamic between the host immune response and the continuous changing virus, inconsistency in the sample timing when the virus and Nab tested were obtained may yield different results. In our analysis of maternal Env virus variants and the transmitted variants isolated from the infants near transmission, we confirmed this hypothesis. Both the maternal and infant variants had similar sensitivity to maternal Nabs present several months post MTCT. The capacity of maternal plasma collected after transmission to neutralize infant variants, which were resistant to maternal Nab present near transmission may be due to several reasons including: 1. Broadening of maternal Nabs in response to novel epitopes revealed due to escape mutations arising from continued viral replication, and/or 2. Nab maturation against conserved epitopes present on the transmitted variant.

The capacity of maternal Nabs responses induced later after MTCT to neutralize infant variants contradicts the hypothesis of these responses being induced by new epitopes exposed on emerging variants. Rather, this result suggest that the recognition of infant escape variant by later maternal plasma is likely to be due to responses

directed against Env targets, which are conserved during the course of infection. Responses to such conserved epitopes are likely to get better over time most probably due to somatic hypermutation and antibody affinity maturation, allowing for better neutralization. Indeed it has been proposed in other previous studies that an immunological hierarchy of Nab responses may exist(116) hinting at bettering of Nab response over the course of infection. It is also possible that the recognition of the escape transmitted variant by later plasma reflects other changes in the envelope that affects the global conformation of envelope, resulting in exposure of the secondary Nab epitopes.

To the best of our knowledge, this study is the first study to demonstrate in a single study design, the significance of how sample timing may affect the interpretation of the role of Nab in virus escape during MTCT.

Chapter 5

Summary, Future direction and conclusion

It is generally believed that to prevent infection, an effective HIV vaccine will have to elicit antibodies that can neutralize diverse HIV-1 isolates circulating among humans. However, one major limitation of discovering an HIV-1 vaccine that would induce such antibodies is the lack of a clear definition of protective immune correlates of protection in natural exposure. The focus of this thesis is on studies that utilize natural HIV-1 infection models to probe whether Nabs induced in natural infection are correlates of protection with the idea that this be could be useful in defining which types of Nabs specificities may be required to be elicited by a candidate vaccine.

I have used the setting of mother-to-child HIV-1 transmission to explore in great detail, whether autologous Nabs, which are raised against distinct autologous representative HIV-1 variants circulating in an infected mother, correlate with reduced risk of vertical HIV-1 transmission. Since in MTCT, effective Nabs can block HIV-1 transmission by neutralizing maternal viruses within the mother or the infants (as vertically transferred antibodies), we first explored the relationship between maternal Nab levels and that of their infants (chapter-2). In chapter 3 of this thesis, we determined the relative number of HIV-1 envelope variants sufficient to capture the broader virus quasispecies diversity in the chronically infected women we examined. Using this information, we then examined whether there are differences in autologous Nab responses to these variants between transmitting versus non-transmitting women, to assess the presence of any association of such antibodies with reduced risk of vertical

transmission. Because Nabs drive viral escape, we examined in chapter 4 of this thesis how sample (virus & plasma) timing in relation to transmission affect the detection of escape during MTCT and how this impacts interpretation of results concerning the role of Nab in impacting the risk of vertical transmission. In the current chapter, I briefly review data on maternal plasma Nabs and their effect on vertical HIV-1 transmission. I also provide a summary of the neutralization profiles of maternal plasma compared to that of their corresponding infants. Further, a discussion on the possible implications of our findings in context of future studies will be explored.

Summary and implications:

In chapter 2 of this thesis our results showed a close linear relationship between placental transfer of both total HIV-1 specific IgG and Nabs. Our results also demonstrate that although there is efficient transfer of these antibodies, their transport is not uniform across all envelopes tested. We saw a hint that greater variability in transfer with less sensitive viruses having the least correlation, suggesting that the efficiency of transfer of HIV-1 specific antibodies may be affected by effectiveness of the antibody to recognize its epitope. This would be surprising because theoretically, the non-antigen binding Fc region of an antibody is typically involved in the transport of antibodies across the placenta. The Fc rather than the Fab (antigen binding fragment) segment of an antibody binds to the FcRn expressed by placental cells and facilitates its transport across the placenta. Hence, it is not clear why the ability of an antibody to recognize a pathogen would affect its selection for transfer to the infant. If the preliminary data bears out, then I would speculate that antibodies that bind well,

perhaps with higher affinity and avidity, are transferred to the infant because such antibody would be more effective at protecting the infants from potential infectious pathogens.

Nonetheless, our findings in chapter 2 have implication for immunotherapeutic interventions that may be employed to stop MTCT, because it suggests that protective Nabs, particularly those with broad cross neutralization capability, if present in an HIV-infected mother, could provide infant prophylaxis, especially late in gestation and during the breastfeeding period, when the risk of infant infection is highest**(119).** This is good news to the field because these findings form relevant basis for a larger study that evaluates whether recently isolated broadly neutralizing antibodies can be used either independently as immunotherapy or in combination with chemotherapy to block vertical HIV-1 transmission.

The study of neutralizing antibody responses to autologous virus requires the characterization of the variant virus population in the individual. Given that there is no gold standard in the HIV field that can be used to determine the minimum number of viruses required to capture all the relevant virus variants that represent the larger viral population in a chronically infected individual, we first chose to determine how many viruses would be sufficient to represent quasispecies diversity in the women we included in our analysis. We demonstrated in chapter 3 that at least 5 distinct envelope variants sampled through limiting dilution PCR, were sufficient to represent a broad range of genetic diversity of the virus population circulation in a chronically infected mother. We used this as a benchmark to isolate Env variants from an additional 17

women. It is important to note that isolating on average 6 variants from the twenty women analyzed in this thesis, was because of several reasons, including the fact that this number strikes a practical balance between sampling the major variants in a large number of women versus sampling very extensively in a more select subset of women and risking having the result from one woman overly influence the overall results.

In order to determine whether neutralization of the maternal variants that are representative of overall viral populations circulating in a mother, is associated with infant infection outcome, we compared neutralization IC50s between TM versus NTM. We showed that maternal Nabs present around the time of transmission are not associated with the risk of peripartum HIV-1 transmission, suggesting that Nab sensitivity is not the only factor that may determine whether MTCT occurs or not. Moreover, some NTM also harbored neutralization resistant variants, suggesting that escape from Nabs is not the sole determinant of MTCT. Given that vertical transmission is a multifactorial process that may be influenced by both host and viral features, other factors such as viral fitness that may override the impact of Nabs, may determine virus transmission during MTCT.

Typically there is genetic bottleneck during vertical HIV-1 transmission that allows only maternal viruses, which are less sensitive to the maternal plasma to be transmitted to the infant. However, in chapter 3 of this dissertation we showed that resistant viruses also exist in women who fail to transmit viruses to their infants. This suggests that escape from Nabs antibodies may not be the only factor that drives HIV-1 transmission, rather other mechanisms beyond maternal immune response may be

involved during MTCT.

Given our observation in chapter 3 that virus variants with broad a spectrum of neutralization sensitivities exist in TM, we determined in chapter 4 that only the less sensitive viruses are transmitted from a mother to her child. We then determined that the timing of virus and Nabs in relation to transmission time-point, is crucial in the detection of the occurrence of escape during MTCT. While this study included only 4 cases, its finding is the first to directly demonstrate the impact of insufficient consideration of temporal changes in autologous antibody responses on study design. We showed that the detection of viral escape during MTCT is likely to be missed if examined even after only fourteen weeks after transmission has occurred. Future studies using larger sample size with carefully selected temporal samples may be needed to determine the window within which to detect escape.

Given that high plasma viral load (VL) has been shown to be positively associated with increased risk of vertical HIV-1 transmission(119), neutralizing breadth and potency, the efficacy of Nabs to protect against MTCT would be better analyzed in women with these characteristics. Therefore, the women I analyzed in this thesis were among those with higher VL than the original cohort(119). It is important to note that NTM with low VL would likely have low Nab levels that could result in inability to make valid comparisons between the two groups (TM versus NTM). Moreover, in the case of NTM with low plasma VLs, the lack of transmission could have been due primarily to low infectiousness. Given that we have not demonstrated any clues of involvement of autologous Nabs in the lack of MTCT among these highly infectious

NTM women (high VLs) compared to TM, we can conclude that Nabs induced in natural infection may not be adequate to confer protection during MTCT. However this cannot be interpreted to mean that overall Nabs cannot protect against HIV-1 transmission in humans. In fact it has been recently shown that a combination of Nabs that target different epitopes on the HIV-1 Env may be protective against MTCT(45, 55, 141). These studies show that unlike the resistance to maternal autologous virus that is characteristic of transmitted variants(104, 174), these variants are potently neutralized by the recently isolated broadly neutralizing antibodies(40).

Future direction

An important next step for the passive transfer of Nabs is to determine whether Nabs specificity affect the transfer. We noted some suggestion that there was variability in the transfer Nabs depending on the virus tested, suggesting Nab specificity could be a factor in passive transfer of antibodies. One approach that can be used to test this would be to passively immunize pregnant nonhuman primates (rhesus macaques) with a cocktail of Nabs with different neutralization abilities. The antibodies in the cocktail should have equal concentration and target the same region on the Env such as CD4 binding site. To distinguish between these antibodies, they should be barcoded by introducing signature sequences in the proteins. After birth, antibodies would be isolated from both the mother and the infants and their levels analyzed. If neutralization potency of the an antibody affects the passive transfer then higher levels of the more potent Nab should be detected in the infants compare to less potent Nabs.

In chapter 2 we showed that maternal Nabs are efficiently transferred to the infant but such antibodies are not associated with reduced risk of peripartum MTCT. However, because the assay format we used could only detect protection against the transmission of cell-free virus, it still remains to be determined if maternal Nabs can protect against the transmission of cell associated viruses, which may be the major mode of breastmilk HIV-1 transmission(137). I propose a study similar in design to the one discussed in this thesis except for the assay format. Abela *et al*(2) recently demonstrated a robust assay that can be adapted to assess the effect of Nabs in curtailing cell-to-cell spreading of HIV-1. This takes advantage of the inability of most CCR-5 using HIV-1 viruses to infect susceptible cells in the absence cationic compounds such as Diethylaminoethyl Dextran (DEAE). During MTCT mostly viruses that utilize CCR-5 co-receptor are transmitted while CXCR4 viruses are rarely transmitted(161). In order to employ this assay to take advantage of Envs we have generated, we will need to assess the co-receptor usage of these Env to identify those that only use CCR-5 co-coreceptor for infectivity. We would then clone into HIV-1 genome deficient in Env to generate viruses capable of establishing spreading infection and use the same assay format outline in (2).

We observed in chapter 4 of this dissertation that maternal variants resistant to autologous Nabs existed in NTM just as in TM. If escape from neutralizing antibodies is a major prerequisite for transmission, these resistant viruses could have been transmitted. A study is needed that directly tests whether escape from autologous Nabs by such variants affect their infectivity. Kong and colleagues showed in a study

involving subtype C viruses that maternal viruses transmitted to the infants have higher replicative fitness compared to non-transmitted variants in the mother(78). My hypothesis is that escape from neutralizing antibodies may have fitness consequences to the virus. This hypothesis is supported by previous observation in a nonhuman primate model(163), which showed that viral variants newly emerging from the Nabs escape parent variant used to initiate infection, replicated more efficiently than their parent virus. This suggests that factors other than sensitivity to Nabs may be involved in determining viral fitness during transmission(74). In order to carry out an invitro experiment to determine if the lack of transmission of the resistant variants present in the NTM was due poor fitness, assay previously developed by our lab can be used(163). This will involve competition experiments to measure the relative fitness of escape variants isolated from NTM (non-transmitted variants) versus those obtained from TM (transmitted variants). Given the ex vivo versus in vivo differences such as rapid turnover of HIV-1 infected cells (life span of ~2day) resulting from cytopathic effect of the virus as well as cytotoxic CD8+ T cell responses, the in vitro assay conditions must be tailored towards mimicking the in vivo selective pressure imposed on virus. The fitness assay outlined by Veronin *et al* incorporates two cell culture systems (nonlimited life span of cells and rapid cell turnover cell culture systems), which relatively mimics the conditions that prevail both in vivo and in vitro. If from these experiments it is found that the escape variants are attenuated in replication fitness, this could explain why they are not transmitted in some cases. Further studies to discern the molecular markers of such attenuation may then be pursued. Such experiments will be guided by

the hypothesis that changes in the HIV-1 Env that coffer escape advantage may also abrogate replicative fitness. If such sites on the Env are identified, then they could be targeted with vaccines.

Conclusion

It is generally hoped that a vaccine that elicits cross-reactive neutralizing would be effective in stopping the spread of HIV-1 among humans. However, the data presented here suggest that Nabs induced in typical natural infection may not solely reduce the risk of HIV-1 transmission. However, our finding may not be generalized to other settings because we only analyzed chronically infected women with unknown duration of infection, and duration of infection has been demonstrated to be critical for generation cross reactive neutralizing antibodies(127, 147). Thus, many of these women may not have had very broad neutralizing antibodies. Nonetheless, our findings re-enforce the belief that for Nab based vaccines to be effective, they must be able to induce higher levels of Nab than that induced in natural infection.

Because several groups including our lab, have shown that the new generation of broadly Nabs are effective against the transmitted variants, our finding of efficient transfer passive Nabs, suggested that such antibodies may be given to the mother to provide prophylaxis in the infant. This may provide a way to test whether broad and potent neutralizing antibodies can reduce HIV transmission.

References

1. **Abdool Karim, Q., S. S. Abdool Karim, J. A. Frohlich, A. C. Grobler, C. Baxter, L. E. Mansoor, A. B. Kharsany, S. Sibeko, K. P. Mlisana, Z. Omar, T. N. Gengiah, S. Maarschalk, N. Arulappan, M. Mlotshwa, L. Morris, and D. Taylor. 2010. Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women. Science 329:1168-1174.**

2. **Abela, I. A., L. Berlinger, M. Schanz, L. Reynell, H. F. Gunthard, P. Rusert, and A. Trkola. 2012. Cell-cell transmission enables HIV-1 to evade inhibition by potent CD4bs directed antibodies. PLoS pathogens 8:e1002634.**

3. **Albert, J., B. Abrahamsson, K. Nagy, E. Aurelius, H. Gaines, G. Nystrom, and E. M. Fenyo. 1990. Rapid development of isolate-specific neutralizing antibodies after primary HIV-1 infection and consequent emergence of virus variants which resist neutralization by autologous sera. AIDS (London, England) 4:107-112.**

4. **Allan, J. S., J. E. Coligan, F. Barin, M. F. McLane, J. G. Sodroski, C. A. Rosen, W. A. Haseltine, T. H. Lee, and M. Essex. 1985. Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III. Science 228:1091-1094.**

5. **Amanna, I. J., I. Messaoudi, and M. K. Slifka. 2008. Protective immunity following vaccination: how is it defined? Human vaccines 4:316-319.**

6. **Arendrup, M., C. Nielsen, J. E. Hansen, C. Pedersen, L. Mathiesen, and J. O. Nielsen. 1992. Autologous HIV-1 neutralizing antibodies: emergence of neutralization-resistant escape virus and subsequent development of escape virus neutralizing antibodies. Journal of acquired immune deficiency syndromes (1999) 5:303-307.**

7. **Auvert, B., D. Taljaard, E. Lagarde, J. Sobngwi-Tambekou, R. Sitta, and A. Puren. 2005. Randomized, controlled intervention trial of male circumcision for reduction of HIV infection risk: the ANRS 1265 Trial. PLoS medicine 2:e298.**

8. **Baba, T. W., V. Liska, R. Hofmann-Lehmann, J. Vlasak, W. Xu, S. Ayehunie, L. A. Cavacini, M. R. Posner, H. Katinger, G. Stiegler, B. J. Bernacky, T. A. Rizvi, R. Schmidt, L. R. Hill, M. E. Keeling, Y. Lu, J. E. Wright, T. C. Chou, and R. M. Ruprecht. 2000. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. Nature medicine 6:200-206.**

9. **Bailey, R. C., S. Moses, C. B. Parker, K. Agot, I. Maclean, J. N. Krieger, C. F. Williams, R. T. Campbell, and J. O. Ndinya-Achola. 2007. Male circumcision for HIV prevention in young men in Kisumu, Kenya: a randomised controlled trial. Lancet 369:643-656.**

10. **Barin, F., G. Jourdain, S. Brunet, N. Ngo-Giang-Huong, S. Weerawatgoompa, W. Karnchanamayul, S. Ariyadej, R. Hansudewechakul, J. Achalapong, P. Yuthavisuthi, C. Ngampiyaskul, S. Bhakeecheep, C. Hemwutthiphan, and M. Lallemant. 2006. Revisiting the role of neutralizing antibodies in mother-to-child transmission of HIV-1. The Journal of infectious diseases 193:1504-1511.**

11. **Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier. 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science 220:868-871.**

12. **Bernstein, H. B., S. P. Tucker, E. Hunter, J. S. Schutzbach, and R. W. Compans. 1994. Human immunodeficiency virus type 1 envelope glycoprotein is modified by O-linked oligosaccharides. Journal of virology 68:463-468.**

13. **Binley, J. M., E. A. Lybarger, E. T. Crooks, M. S. Seaman, E. Gray, K. L. Davis, J. M. Decker, D. Wycuff, L. Harris, N. Hawkins, B. Wood, C. Nathe, D. Richman, G. D. Tomaras, F. Bibollet-Ruche, J. E. Robinson, L. Morris, G. M. Shaw, D. C. Montefiori, and J. R. Mascola. 2008. Profiling the specificity of neutralizing antibodies in a large panel of plasmas from patients chronically infected with human immunodeficiency virus type 1 subtypes B and C. Journal of virology 82:11651-11668.**

14. **Blish, C. A., Z. Jalalian-Lechak, S. Rainwater, M. A. Nguyen, O. C. Dogan, and J. Overbaugh. 2009. Cross-subtype neutralization sensitivity despite monoclonal antibody resistance among early subtype A, C, and D envelope variants of human immunodeficiency virus type 1. Journal of virology 83:7783-7788.**

15. **Blish, C. A., R. Nedellec, K. Mandaliya, D. E. Mosier, and J. Overbaugh. 2007. HIV-1 subtype A envelope variants from early in infection have variable sensitivity to neutralization and to inhibitors of viral entry. AIDS (London, England) 21:693-702.**

16. **Bongertz, V., C. I. Costa, V. G. Veloso, B. Grinsztejn, E. C. Joao Filho, G. Calvet, J. H. Pilotto, M. L. Guimaraes, and M. G. Morgado. 2001. Vertical HIV-1 transmission: importance of neutralizing antibody titer and specificity. Scandinavian journal of immunology 53:302-309.**

17. **Bonsignori, M., K. K. Hwang, X. Chen, C. Y. Tsao, L. Morris, E. Gray, D. J. Marshall, J. A. Crump, S. H. Kapiga, N. E. Sam, F. Sinangil, M. Pancera, Y. Yongping, B. Zhang, J. Zhu, P. D. Kwong, S. O'Dell, J. R. Mascola, L. Wu, G. J. Nabel, S. Phogat, M. S. Seaman, J. F. Whitesides, M. A. Moody, G. Kelsoe, X. Yang, J. Sodroski, G. M. Shaw, D. C. Montefiori, T. B. Kepler, G. D. Tomaras, S. M. Alam, H. X. Liao, and B. F. Haynes. 2011. Analysis of a clonal lineage of HIV-1 envelope V2/V3 conformational epitope-specific broadly neutralizing antibodies and their inferred unmutated common ancestors. Journal of virology 85:9998-10009.**

18. **Bosch, K. A., S. Rainwater, W. Jaoko, and J. Overbaugh. 2010. Temporal analysis of HIV envelope sequence evolution and antibody escape in a subtype A-infected individual with a broad neutralizing antibody response. Virology 398:115-124.**

19. **Broliden, K., E. Sievers, P. A. Tovo, V. Moschese, G. Scarlatti, P. A. Broliden, C. Fundaro, and P. Rossi. 1993. Antibody-dependent cellular cytotoxicity and neutralizing activity in sera of HIV-1-infected mothers and their children. Clinical and experimental immunology 93:56-64.**

20. **Brouwer, K. C., C. Yang, S. Parekh, L. B. Mirel, Y. P. Shi, J. Otieno, A. A. Lal, and R. B. Lal. 2005. Effect of CCR2 chemokine receptor polymorphism on HIV type 1 mother-to-child transmission and child survival in Western Kenya. AIDS research and human retroviruses 21:358-362.**

21. **Buchacher, A., R. Predl, K. Strutzenberger, W. Steinfellner, A. Trkola, M. Purtscher, G. Gruber, C. Tauer, F. Steindl, A. Jungbauer, and et al. 1994. Generation of human monoclonal antibodies against HIV-1 proteins; electrofusion and Epstein-Barr virus transformation for peripheral blood lymphocyte immortalization. AIDS research and human retroviruses 10:359-369.**

22. **Bukrinsky, M. I., N. Sharova, M. P. Dempsey, T. L. Stanwick, A. G. Bukrinskaya, S. Haggerty, and M. Stevenson. 1992. Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. Proceedings of the National Academy of Sciences of the United States of America 89:6580-6584.**

23. **Burton, D. R., C. F. Barbas, 3rd, M. A. Persson, S. Koenig, R. M. Chanock, and R. A. Lerner. 1991. A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. Proceedings of the National Academy of Sciences of the United States of America 88:10134-10137.**

24. **Centlivre, M., M. Sala, S. Wain-Hobson, and B. Berkhout. 2007. In HIV-1 pathogenesis the die is cast during primary infection. AIDS (London, England) 21:1-11.**

25. **Chackerian, B., L. M. Rudensey, and J. Overbaugh. 1997. Specific N-linked and O-linked glycosylation modifications in the envelope V1 domain of simian immunodeficiency virus variants that evolve in the host alter recognition by neutralizing antibodies. Journal of virology 71:7719-7727.**

26. **Chaillon, A., M. Braibant, S. Hue, S. Bencharif, D. Enard, A. Moreau, A. Samri, H. Agut, and F. Barin. 2012. Human immunodeficiency virus type-1 (HIV-1) continues to evolve in presence of broadly neutralizing antibodies more than ten years after infection. PloS one 7:e44163.**

27. **Chan, D. C., D. Fass, J. M. Berger, and P. S. Kim. 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell 89:263-273.**

28. **Chun, T. W., L. Carruth, D. Finzi, X. Shen, J. A. DiGiuseppe, H. Taylor, M. Hermankova, K. Chadwick, J. Margolick, T. C. Quinn, Y. H. Kuo, R. Brookmeyer, M. A. Zeiger, P. Barditch-Crovo, and R. F. Siliciano. 1997. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. Nature 387:183-188.**

29. **Cohen, M. S., Y. Q. Chen, M. McCauley, T. Gamble, M. C. Hosseinipour, N. Kumarasamy, J. G. Hakim, J. Kumwenda, B. Grinsztejn, J. H. Pilotto, S. V. Godbole, S. Mehendale, S. Chariyalertsak, B. R. Santos, K. H. Mayer, I. F. Hoffman, S. H. Eshleman, E. Piwowar-Manning, L. Wang, J. Makhema, L. A. Mills, G. de Bruyn, I. Sanne, J. Eron, J. Gallant, D. Havlir, S. Swindells, H. Ribaudo, V. Elharrar, D. Burns, T. E. Taha, K. Nielsen-Saines, D. Celentano, M. Essex, and T. R. Fleming. 2011. Prevention of HIV-1 infection with early antiretroviral therapy. The New England journal of medicine 365:493-505.**

30. **Cooper, D. A., A. A. Imrie, and R. Penny. 1987. Antibody response to human immunodeficiency virus after primary infection. The Journal of infectious diseases 155:1113-1118.**

31. **Cornelissen, M., E. J. Heeregrave, F. Zorgdrager, G. Pollakis, W. A. Paxton, and A. C. van der Kuyl. Generation of representative primary virus isolates from blood plasma after isolation of HIV-1 with CD44 MicroBeads. Arch Virol 155:2017-2022.**

32. **Corti, D., and A. Lanzavecchia. 2013. Broadly Neutralizing Antiviral Antibodies. Annual review of immunology.**

33. **Daecke, J., O. T. Fackler, M. T. Dittmar, and H. G. Krausslich. 2005. Involvement of clathrin-mediated endocytosis in human immunodeficiency virus type 1 entry. Journal of virology 79:1581-1594.**

34. **de Moraes-Pinto, M. I., F. Verhoeff, L. Chimsuku, P. J. Milligan, L. Wesumperuma, R. L. Broadhead, B. J. Brabin, P. M. Johnson, and C. A. Hart. 1998. Placental antibody transfer: influence of maternal HIV infection and placental malaria. Archives of disease in childhood. Fetal and neonatal edition 79:F202-205.**

35. **Deeks, S. G., B. Schweighardt, T. Wrin, J. Galovich, R. Hoh, E. Sinclair, P. Hunt, J. M. McCune, J. N. Martin, C. J. Petropoulos, and F. M. Hecht. 2006. Neutralizing antibody responses against autologous and heterologous viruses in acute versus chronic human immunodeficiency virus (HIV) infection: evidence for a constraint on the ability of HIV to completely evade neutralizing antibody responses. Journal of virology 80:6155-6164.**

36. **Dickover, R., E. Garratty, K. Yusim, C. Miller, B. Korber, and Y. Bryson. 2006. Role of maternal autologous neutralizing antibody in selective perinatal transmission of human immunodeficiency virus type 1 escape variants. Journal of virology 80:6525-6533.**

37. **Dickover, R. E., E. M. Garratty, S. A. Herman, M. S. Sim, S. Plaeger, P. J. Boyer, M. Keller, A. Deveikis, E. R. Stiehm, and Y. J. Bryson. 1996. Identification of levels of maternal HIV-1 RNA associated with risk of perinatal transmission. Effect of maternal zidovudine treatment on viral load. JAMA : the journal of the American Medical Association 275:599-605.**

38. **Dickover, R. E., E. M. Garratty, S. Plaeger, and Y. J. Bryson. 2001. Perinatal transmission of major, minor, and multiple maternal human immunodeficiency virus type 1 variants in utero and intrapartum. Journal of virology 75:2194-2203.**

39. **Englund, J. A. 2007. The influence of maternal immunization on infant immune responses. Journal of comparative pathology 137 Suppl 1:S16-19.**

40. **Euler, Z., and H. Schuitemaker. 2012. Cross-reactive broadly neutralizing antibodies: timing is everything. Frontiers in immunology 3:215.**

41. **Euler, Z., M. J. van Gils, E. M. Bunnik, P. Phung, B. Schweighardt, T. Wrin, and H. Schuitemaker. 2010. Cross-reactive neutralizing humoral immunity does not protect from HIV type 1 disease progression. The Journal of infectious diseases 201:1045-1053.**

42. **Farquhar, C., S. Rowland-Jones, D. Mbori-Ngacha, M. Redman, B. Lohman, J. Slyker, P. Otieno, E. Obimbo, T. Rostron, J. Ochieng, J. Oyugi, R. Bosire, and G. John-Stewart. 2004. Human leukocyte antigen (HLA) B*18 and protection against mother-to-child HIV type 1 transmission. AIDS research and human retroviruses 20:692-697.**

43. **Ferrantelli, F., R. A. Rasmussen, K. A. Buckley, P. L. Li, T. Wang, D. C. Montefiori, H. Katinger, G. Stiegler, D. C. Anderson, H. M. McClure, and R. M. Ruprecht. 2004. Complete protection of neonatal rhesus macaques against oral exposure to pathogenic simian-human immunodeficiency virus by human anti-HIV monoclonal antibodies. The Journal of infectious diseases 189:2167-2173.**

44. **Forthal, D. N., G. Landucci, and E. S. Daar. 2001. Antibody from patients with acute human immunodeficiency virus (HIV) infection inhibits primary strains of HIV type 1 in the presence of natural-killer effector cells. Journal of virology 75:6953-6961.**

45. **Fouda, G. G., T. Mahlokozera, J. F. Salazar-Gonzalez, M. G. Salazar, G. Learn, S. B. Kumar, S. M. Dennison, E. Russell, K. Rizzolo, F. Jaeger, F. Cai, N. A. Vandergrift, F. Gao, B. Hahn, G. M. Shaw, C. Ochsenbauer, R. Swanstrom, S. Meshnick, V. Mwapasa, L. Kalilani, S. Fiscus, D. Montefiori, B. Haynes, J. Kwiek, S. M. Alam, and S. R. Permar. 2013. Postnatally-transmitted HIV-1 Envelope variants have similar neutralization-sensitivity and function to that of nontransmitted breast milk variants. Retrovirology 10:3.**

46. **Fouda, G. G., N. L. Yates, J. Pollara, X. Shen, G. R. Overman, T. Mahlokozera, A. B. Wilks, H. H. Kang, J. F. Salazar-Gonzalez, M. G. Salazar, L. Kalilani, S. R. Meshnick, B. H. Hahn, G. M. Shaw, R. V. Lovingood, T. N. Denny, B. Haynes, N. L. Letvin, G. Ferrari, D. C. Montefiori, G. D. Tomaras, and S. R. Permar. 2011. HIV-specific functional antibody responses in breast milk mirror those in plasma and are primarily mediated by IgG antibodies. Journal of virology 85:9555-9567.**

47. **Freed, E. O. 2001. HIV-1 replication. Somatic cell and molecular genetics 26:13-33.**
48. **Freed, E. O., D. J. Myers, and R. Risser. 1991. Identification of the principal neutralizing determinant of human immunodeficiency virus type 1 as a fusion domain. Journal of virology 65:190-194.**

49. **Freed, E. O., D. J. Myers, and R. Risser. 1989. Mutational analysis of the cleavage sequence of the human immunodeficiency virus type 1 envelope glycoprotein precursor gp160. Journal of virology 63:4670-4675.**

50. **Frost, S. D., Y. Liu, S. L. Pond, C. Chappey, T. Wrin, C. J. Petropoulos, S. J. Little, and D. D. Richman. 2005. Characterization of human immunodeficiency virus type 1 (HIV-1) envelope variation and neutralizing antibody responses during transmission of HIV-1 subtype B. Journal of virology 79:6523-6527.**

51. **Gallo, R. C., S. Z. Salahuddin, M. Popovic, G. M. Shearer, M. Kaplan, B. F. Haynes, T. J. Palker, R. Redfield, J. Oleske, B. Safai, and et al. 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science 224:500-503.**

52. **Gallo, R. C., P. S. Sarin, E. P. Gelmann, M. Robert-Guroff, E. Richardson, V. S. Kalyanaraman, D. Mann, G. D. Sidhu, R. E. Stahl, S. Zolla-Pazner, J. Leibowitch, and M. Popovic. 1983. Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). Science 220:865-867.**

53. **Garcia, P. M., L. A. Kalish, J. Pitt, H. Minkoff, T. C. Quinn, S. K. Burchett, J. Kornegay, B. Jackson, J. Moye, C. Hanson, C. Zorrilla, and J. F. Lew. 1999. Maternal levels of plasma human immunodeficiency virus type 1 RNA and the risk of perinatal transmission. Women and Infants Transmission Study Group. The New England journal of medicine 341:394-402.**

54. **Goff, S. P. 2001. Intracellular trafficking of retroviral genomes during the early phase of infection: viral exploitation of cellular pathways. The journal of gene medicine 3:517-528.**

55. **Goo, L., Z. Jalalian-Lechak, B. A. Richardson, and J. Overbaugh. 2012. A combination of broadly neutralizing HIV-1 monoclonal antibodies targeting distinct epitopes effectively neutralizes variants found in early infection. Journal of virology 86:10857-10861.**

56. **Grant, R. M. 2010. Antiretroviral agents used by HIV-uninfected persons for prevention: pre- and postexposure prophylaxis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 50 Suppl 3:S96-101.**

57. **Gray, E. S., M. C. Madiga, T. Hermanus, P. L. Moore, C. K. Wibmer, N. L. Tumba, L. Werner, K. Mlisana, S. Sibeko, C. Williamson, S. S. Abdool Karim, and L. Morris. 2011. The neutralization breadth of HIV-1 develops incrementally over four years and is associated with CD4+ T cell decline and high viral load during acute infection. Journal of virology 85:4828-4840.**

58. **Gray, E. S., P. L. Moore, I. A. Choge, J. M. Decker, F. Bibollet-Ruche, H. Li, N. Leseka, F. Treurnicht, K. Mlisana, G. M. Shaw, S. S. Karim, C. Williamson, and L. Morris. 2007. Neutralizing antibody responses in acute human immunodeficiency virus type 1 subtype C infection. Journal of virology 81:6187-6196.**

59. **Gray, R. R., M. Salemi, A. Lowe, K. J. Nakamura, W. D. Decker, M. Sinkala, C. Kankasa, C. J. Mulligan, D. M. Thea, L. Kuhn, G. Aldrovandi, and M. M. Goodenow. 2011. Multiple independent lineages of HIV-1 persist in breast milk and plasma. AIDS (London, England) 25:143-152.**

60. **Greene, W. C. 2007. A history of AIDS: looking back to see ahead. European journal of immunology 37 Suppl 1:S94-102.**

61. **Guevara, H., J. Casseb, L. S. Zijenah, M. Mbizvo, L. F. Oceguera, 3rd, C. V. Hanson, D. A. Katzenstein, and R. M. Hendry. 2002. Maternal HIV-1 antibody and vertical transmission in subtype C virus infection. Journal of acquired immune deficiency syndromes (1999) 29:435-440.**

62. **Haigwood, N. L., D. C. Montefiori, W. F. Sutton, J. McClure, A. J. Watson, G. Voss, V. M. Hirsch, B. A. Richardson, N. L. Letvin, S. L. Hu, and P. R. Johnson. 2004. Passive immunotherapy in simian immunodeficiency virus-infected macaques accelerates the development of neutralizing antibodies. Journal of virology 78:5983-5995.**

63. **Hallenberger, S., V. Bosch, H. Angliker, E. Shaw, H. D. Klenk, and W. Garten. 1992. Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. Nature 360:358-361.**

64. **Hengel, R. L., M. S. Kennedy, R. W. Steketee, D. M. Thea, E. J. Abrams, G. Lambert, and J. S. McDougal. 1998. Neutralizing antibody and perinatal transmission of human immunodeficiency virus type 1. New York City Perinatal HIV Transmission Collaborative Study Group. AIDS research and human retroviruses 14:475-481.**

65. **Hessell, A. J., P. Poignard, M. Hunter, L. Hangartner, D. M. Tehrani, W. K. Bleeker, P. W. Parren, P. A. Marx, and D. R. Burton. 2009. Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques. Nature medicine 15:951-954.**

66. **Hessell, A. J., E. G. Rakasz, P. Poignard, L. Hangartner, G. Landucci, D. N. Forthal, W. C. Koff, D. I. Watkins, and D. R. Burton. 2009. Broadly neutralizing human anti-HIV antibody 2G12 is effective in protection against mucosal SHIV challenge even at low serum neutralizing titers. PLoS pathogens 5:e1000433.**

67. **Hirsch, V. M., R. A. Olmsted, M. Murphey-Corb, R. H. Purcell, and P. R. Johnson. 1989. An African primate lentivirus (SIVsm) closely related to HIV-2. Nature 339:389-392.**

68. **Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz. 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature 373:123-126.**

69. **Hofmann-Lehmann, R., J. Vlasak, R. A. Rasmussen, S. Jiang, P. L. Li, T. W. Baba, D. C. Montefiori, B. J. Bernacky, T. A. Rizvi, R. Schmidt, L. R. Hill, M. E. Keeling, H. Katinger, G. Stiegler, L. A. Cavacini, M. R. Posner, and R. M. Ruprecht. 2002. Postnatal pre- and postexposure passive immunization strategies: protection of neonatal macaques against oral simian-human immunodeficiency virus challenge. Journal of medical primatology 31:109-119.**

70. **Huet, T., R. Cheynier, A. Meyerhans, G. Roelants, and S. Wain-Hobson. 1990. Genetic organization of a chimpanzee lentivirus related to HIV-1. Nature 345:356-359.**

71. **Husson, R. N., Y. Lan, E. Kojima, D. Venzon, H. Mitsuya, and K. McIntosh. 1995. Vertical transmission of human immunodeficiency virus type 1: autologous neutralizing antibody, virus load, and virus phenotype. The Journal of pediatrics 126:865-871.**

72. **Keele, B. F., E. E. Giorgi, J. F. Salazar-Gonzalez, J. M. Decker, K. T. Pham, M. G. Salazar, C. Sun, T. Grayson, S. Wang, H. Li, X. Wei, C. Jiang, J. L. Kirchherr, F. Gao, J. A. Anderson, L. H. Ping, R. Swanstrom, G. D. Tomaras, W. A. Blattner, P. A. Goepfert, J. M. Kilby, M. S. Saag, E. L. Delwart, M. P. Busch, M. S. Cohen, D. C. Montefiori, B. F. Haynes, B. Gaschen, G. S. Athreya, H. Y. Lee, N. Wood, C. Seoighe, A. S. Perelson, T. Bhattacharya, B. T. Korber, B. H. Hahn, and G. M. Shaw. 2008. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. Proceedings of the National Academy of Sciences of the United States of America 105:7552-7557.**

73. **Kim, J. H., S. Rerks-Ngarm, J. L. Excler, and N. L. Michael. 2010. HIV vaccines: lessons learned and the way forward. Current opinion in HIV and AIDS 5:428-434.**

74. **Kimata, J. T., L. Kuller, D. B. Anderson, P. Dailey, and J. Overbaugh. 1999. Emerging cytopathic and antigenic simian immunodeficiency virus variants influence AIDS progression. Nature medicine 5:535-541.**

75. **Kishko, M., M. Somasundaran, F. Brewster, J. L. Sullivan, P. R. Clapham, and K. Luzuriaga. 2011. Genotypic and functional properties of early infant HIV-1 envelopes. Retrovirology 8:67.**

76. **Kittinunvorakoon, C., M. K. Morris, K. Neeyapun, B. Jetsawang, G. C. Buehring, and C. V. Hanson. 2009. Mother to child transmission of HIV-1 in a Thai population: role of virus characteristics and maternal humoral immune response. Journal of medical virology 81:768-778.**

77. **Kollmann, T. R., A. Rubinstein, W. D. Lyman, R. Soeiro, and H. Goldstein. 1991. Characterization of IgG and IgG subclass antibodies present in paired maternal and fetal serum which are directed against HIV-1 proteins. AIDS Res Hum Retroviruses 7:847-854.**

78. **Kong, X., J. T. West, H. Zhang, D. M. Shea, J. M'Soka T, and C. Wood. 2008. The human immunodeficiency virus type 1 envelope confers higher rates of replicative fitness to perinatally transmitted viruses than to nontransmitted viruses. Journal of virology 82:11609-11618.**

79. **Koulinska, I. N., E. Villamor, B. Chaplin, G. Msamanga, W. Fawzi, B. Renjifo, and M. Essex. 2006. Transmission of cell-free and cell-associated HIV-1 through breast-feeding. Journal of acquired immune deficiency syndromes (1999) 41:93-99.**

80. **Kourtis, A. P., A. M. Amedee, M. Bulterys, S. Danner, R. Van Dyke, M. J. O'Sullivan, R. Maupin, and D. J. Jamieson. 2011. Various viral compartments in HIV-1-infected mothers contribute to in utero transmission of HIV-1. AIDS research and human retroviruses 27:421-427.**

81. **Kourtis, A. P., F. K. Lee, E. J. Abrams, D. J. Jamieson, and M. Bulterys. 2006. Mother-to-child transmission of HIV-1: timing and implications for prevention. The Lancet infectious diseases 6:726-732.**

82. **Kowalski, M., J. Potz, L. Basiripour, T. Dorfman, W. C. Goh, E. Terwilliger, A. Dayton, C. Rosen, W. Haseltine, and J. Sodroski. 1987. Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. Science 237:1351-1355.**

83. **Kusumi, K., B. Conway, S. Cunningham, A. Berson, C. Evans, A. K. Iversen, D. Colvin, M. V. Gallo, S. Coutre, E. G. Shpaer, and et al. 1992. Human immunodeficiency virus type 1 envelope gene structure and diversity in vivo and after cocultivation in vitro. J Virol 66:875-885.**

84. **Kwiek, J. J., L. A. Arney, V. Harawa, B. Pedersen, V. Mwapasa, S. J. Rogerson, and S. R. Meshnick. 2008. Maternal-fetal DNA admixture is associated with intrapartum mother-to-child transmission of HIV-1 in Blantyre, Malawi. The Journal of infectious diseases 197:1378-1381.**

85. **Kwiek, J. J., E. S. Russell, K. K. Dang, C. L. Burch, V. Mwapasa, S. R. Meshnick, and R. Swanstrom. 2008. The molecular epidemiology of HIV-1 envelope diversity during HIV-1 subtype C vertical transmission in Malawian mother-infant pairs. AIDS (London, England) 22:863-871.**

86. **Lamers, S. L., J. W. Sleasman, J. X. She, K. A. Barrie, S. M. Pomeroy, D. J. Barrett, and M. M. Goodenow. 1993. Independent variation and positive selection in env V1 and V2 domains within maternal-infant strains of human immunodeficiency virus type 1 in vivo. Journal of virology 67:3951-3960.**

87. **Lasky, L. A., G. Nakamura, D. H. Smith, C. Fennie, C. Shimasaki, E. Patzer, P. Berman, T. Gregory, and D. J. Capon. 1987. Delineation of a region of the human immunodeficiency virus type 1 gp120 glycoprotein critical for interaction with the CD4 receptor. Cell 50:975-985.**

88. **Lathey, J. L., J. Tsou, K. Brinker, K. Hsia, W. A. Meyer, 3rd, and S. A. Spector. 1999. Lack of autologous neutralizing antibody to human immunodeficiency virus type 1 (HIV-1) and macrophage tropism are associated with mother-to-infant transmission. The Journal of infectious diseases 180:344-350.**

89. **Leonard, C. K., M. W. Spellman, L. Riddle, R. J. Harris, J. N. Thomas, and T. J. Gregory. 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. The Journal of biological chemistry 265:10373-10382.**

90. Lewis, P., R. Nduati, J. K. Kreiss, G. C. John, B. A. Richardson, D. Mbori-Ngacha, J. Ndinya-Achola, and J. Overbaugh. 1998. Cell-free human immunodeficiency virus type 1 in breast milk. The Journal of infectious diseases 177:34-39.

91. Li, B., J. M. Decker, R. W. Johnson, F. Bibollet-Ruche, X. Wei, J. Mulenga, S. Allen, E. Hunter, B. H. Hahn, G. M. Shaw, J. L. Blackwell, and C. A. Derdeyn. 2006. Evidence for potent autologous neutralizing antibody titers and compact envelopes in early infection with subtype C human immunodeficiency virus type 1. Journal of virology 80:5211-5218.

92. Li, H., P. C. Chien, Jr., M. Tuen, M. L. Visciano, S. Cohen, S. Blais, C. F. Xu, H. T. Zhang, and C. E. Hioe. 2008. Identification of an N-linked glycosylation in the C4 region of HIV-1 envelope gp120 that is critical for recognition of neighboring CD4 T cell epitopes. Journal of immunology (Baltimore, Md. : 1950) 180:4011-4021.

93. Li, Q., L. Duan, J. D. Estes, Z. M. Ma, T. Rourke, Y. Wang, C. Reilly, J. Carlis, C. J. Miller, and A. T. Haase. 2005. Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells. Nature 434:1148-1152.

94. Li, Y., L. Luo, N. Rasool, and C. Y. Kang. 1993. Glycosylation is necessary for the correct folding of human immunodeficiency virus gp120 in CD4 binding. Journal of virology 67:584-588.

95. Lifson, J. D., and N. L. Haigwood. 2012. Lessons in nonhuman primate models for AIDS vaccine research: from minefields to milestones. Cold Spring Harbor perspectives in medicine 2:a007310.

96. Long, E. M., S. M. Rainwater, L. Lavreys, K. Mandaliya, and J. Overbaugh. 2002. HIV type 1 variants transmitted to women in Kenya require the CCR5 coreceptor for entry, regardless of the genetic complexity of the infecting virus. AIDS research and human retroviruses 18:567-576.

97. Luzuriaga, K. 2007. Mother-to-child transmission of HIV: a global perspective. Current infectious disease reports 9:511-517.

98. Lynch, J. B., R. Nduati, C. A. Blish, B. A. Richardson, J. M. Mabuka, Z. Jalalian-Lechak, G. John-Stewart, and J. Overbaugh. 2011. The breadth and potency of passively acquired human immunodeficiency virus type 1-specific neutralizing antibodies do not correlate with the risk of infant infection. Journal of virology 85:5252-5261.

99. Lynch, J. B., R. Nduati, C. A. Blish, B. A. Richardson, J. M. Mabuka, Z. Jalalian-Lechak, G. John-Stewart, and J. Overbaugh. 2011. The breadth and potency of passively acquired human immunodeficiency virus type 1-specific neutralizing antibodies does not correlate with risk of infant infection. Journal of virology.

100. Lynch, R. M., R. Rong, S. Boliar, A. Sethi, B. Li, J. Mulenga, S. Allen, J. E. Robinson, S. Gnanakaran, and C. A. Derdeyn. 2011. The B cell response is redundant and highly focused on V1V2 during early subtype C infection in a Zambian seroconverter. Journal of virology 85:905-915.

101. Ma, B. J., S. M. Alam, E. P. Go, X. Lu, H. Desaire, G. D. Tomaras, C. Bowman, L. L. Sutherland, R. M. Scearce, S. Santra, N. L. Letvin, T. B. Kepler, H. X. Liao, and B. F. Haynes. 2011. Envelope deglycosylation enhances antigenicity of HIV-1 gp41 epitopes for both broad neutralizing antibodies and their unmutated ancestor antibodies. PLoS pathogens 7:e1002200.

102. Mabuka, J., R. Nduati, K. Odem-Davis, D. Peterson, and J. Overbaugh. 2012. HIV-specific antibodies capable of ADCC are common in breastmilk and are associated with reduced risk of transmission in women with high viral loads. PLoS pathogens 8:e1002739.

103. Mabuka, J. M., R. D. Mackelprang, B. Lohman-Payne, M. Majiwa, R. Bosire, G. John-Stewart, S. Rowland-Jones, J. Overbaugh, and C. Farquhar. 2009. CCR2-64I polymorphism is associated with lower maternal HIV-1 viral load and reduced vertical HIV-1 transmission. Journal of acquired immune deficiency syndromes (1999) 51:235-237.

104. MacDonald, M. G. 1994. Kliks, S.C. et al., Features of HIV-1 that could influence maternal-child transmission. JAMA 1994; 272:467-74. Pediatric AIDS and HIV infection 5:361-362.

105. Mahalanabis, M., P. Jayaraman, T. Miura, F. Pereyra, E. M. Chester, B. Richardson, B. Walker, and N. L. Haigwood. 2009. Continuous viral escape and selection by autologous neutralizing antibodies in drug-naive human immunodeficiency virus controllers. Journal of virology 83:662-672.

106. Mansky, L. M., and H. M. Temin. 1995. Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. Journal of virology 69:5087-5094.

107. Mascola, J. R., G. Stiegler, T. C. VanCott, H. Katinger, C. B. Carpenter, C. E. Hanson, H. Beary, D. Hayes, S. S. Frankel, D. L. Birx, and M. G. Lewis. 2000. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nature medicine 6:207-210.

108. Matsushita, S., M. Robert-Guroff, J. Rusche, A. Koito, T. Hattori, H. Hoshino, K. Javaherian, K. Takatsuki, and S. Putney. 1988. Characterization of a human immunodeficiency virus neutralizing monoclonal antibody and mapping of the neutralizing epitope. Journal of virology 62:2107-2114.

109. Mattapallil, J. J., D. C. Douek, B. Hill, Y. Nishimura, M. Martin, and M. Roederer. 2005. Massive infection and loss of memory CD4+ T cells in multiple tissues during acute SIV infection. Nature 434:1093-1097.

110. McClure, M. O., M. Marsh, and R. A. Weiss. 1988. Human immunodeficiency virus infection of CD4-bearing cells occurs by a pH-independent mechanism. The EMBO journal 7:513-518.

111. Merson, M. H., J. O'Malley, D. Serwadda, and C. Apisuk. 2008. The history and challenge of HIV prevention. Lancet 372:475-488.

112. Miyauchi, K., Y. Kim, O. Latinovic, V. Morozov, and G. B. Melikyan. 2009. HIV enters cells via endocytosis and dynamin-dependent fusion with endosomes. Cell 137:433-444.

113. Moog, C., H. J. Fleury, I. Pellegrin, A. Kirn, and A. M. Aubertin. 1997. Autologous and heterologous neutralizing antibody responses following initial seroconversion in human immunodeficiency virus type 1-infected individuals. Journal of virology 71:3734-3741.

114. Moore, J. P., Y. Cao, J. Leu, L. Qin, B. Korber, and D. D. Ho. 1996. Inter- and intraclade neutralization of human immunodeficiency virus type 1: genetic clades do not correspond to neutralization serotypes but partially correspond to gp120 antigenic serotypes. Journal of virology 70:427-444.

115. Moore, P. L., E. S. Gray, C. K. Wibmer, J. N. Bhiman, M. Nonyane, D. J. Sheward, T. Hermanus, S. Bajimaya, N. L. Tumba, M. R. Abrahams, B. E. Lambson, N. Ranchobe, L. Ping, N. Ngandu, Q. Abdool Karim, S. S. Abdool Karim, R. I. Swanstrom, M. S. Seaman, C. Williamson, and L. Morris. 2012. Evolution of an HIV glycan-dependent broadly neutralizing antibody epitope through immune escape. Nature medicine 18:1688-1692.

116. Moore, P. L., N. Ranchobe, B. E. Lambson, E. S. Gray, E. Cave, M. R. Abrahams, G. Bandawe, K. Mlisana, S. S. Abdool Karim, C. Williamson, and L. Morris. 2009. Limited neutralizing antibody specificities drive neutralization escape in early HIV-1 subtype C infection. PLoS pathogens 5:e1000598.

117. Muster, T., F. Steindl, M. Purtscher, A. Trkola, A. Klima, G. Himmler, F. Ruker, and H. Katinger. 1993. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. Journal of virology 67:6642-6647.

118. Ndirangu, J., J. Viljoen, R. M. Bland, S. Danaviah, C. Thorne, P. Van de Perre, and M. L. Newell. 2012. Cell-free (RNA) and cell-associated (DNA) HIV-1 and postnatal transmission through breastfeeding. PloS one 7:e51493.

119. Nduati, R., G. John, D. Mbori-Ngacha, B. Richardson, J. Overbaugh, A. Mwatha, J. Ndinya-Achola, J. Bwayo, F. E. Onyango, J. Hughes, and J. Kreiss. 2000. Effect of breastfeeding and formula feeding on transmission of HIV-1: a randomized clinical trial. JAMA : the journal of the American Medical Association 283:1167-1174.

120. Neilson, J. R., G. C. John, J. K. Carr, P. Lewis, J. K. Kreiss, S. Jackson, R. W. Nduati, D. Mbori-Ngacha, D. D. Panteleeff, S. Bodrug, C. Giachetti, M. A. Bott, B. A. Richardson, J. Bwayo, J. Ndinya-Achola, and J. Overbaugh. 1999. Subtypes of human immunodeficiency virus type 1 and disease stage among women in Nairobi, Kenya. Journal of virology 73:4393-4403.

121. O'Brien, W. A., Y. Koyanagi, A. Namazie, J. Q. Zhao, A. Diagne, K. Idler, J. A. Zack, and I. S. Chen. 1990. HIV-1 tropism for mononuclear phagocytes can be determined by regions of gp120 outside the CD4-binding domain. Nature 348:69-73.

122. Ochsenbauer, C., T. G. Edmonds, H. Ding, B. F. Keele, J. Decker, M. G. Salazar, J. F. Salazar-Gonzalez, R. Shattock, B. F. Haynes, G. M. Shaw, B. H. Hahn, and J. C. Kappes. 2012. Generation of transmitted/founder HIV-1 infectious molecular clones and characterization of their replication capacity in CD4 T lymphocytes and monocyte-derived macrophages. Journal of virology 86:2715-2728.

123. Overbaugh, J., and L. Morris. 2012. The Antibody Response against HIV-1. Cold Spring Harbor perspectives in medicine 2:a007039.

124. Parren, P. W., and D. R. Burton. 2001. The antiviral activity of antibodies in vitro and in vivo. Advances in immunology 77:195-262.

125. Parren, P. W., P. A. Marx, A. J. Hessell, A. Luckay, J. Harouse, C. Cheng-Mayer, J. P. Moore, and D. R. Burton. 2001. Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. Journal of virology 75:8340-8347.

126. Permar, S. R., A. B. Wilks, E. P. Ehlinger, H. H. Kang, T. Mahlokozera, R. T. Coffey, A. Carville, N. L. Letvin, and M. S. Seaman. 2010. Limited contribution of mucosal IgA to Simian immunodeficiency virus (SIV)-specific neutralizing antibody response and virus envelope evolution in breast milk of SIV-infected, lactating rhesus monkeys. Journal of virology 84:8209-8218.

127. Piantadosi, A., D. Panteleeff, C. A. Blish, J. M. Baeten, W. Jaoko, R. S. McClelland, and J. Overbaugh. 2009. Breadth of neutralizing antibody response to human immunodeficiency virus type 1 is affected by factors early in infection but does not influence disease progression. Journal of virology 83:10269-10274.

128. Pillay, K., A. Coutsoudis, D. York, L. Kuhn, and H. M. Coovadia. 2000. Cell-free virus in breast milk of HIV-1-seropositive women. Journal of acquired immune deficiency syndromes (1999) 24:330-336.

129. Pollakis, G., S. Kang, A. Kliphuis, M. I. Chalaby, J. Goudsmit, and W. A. Paxton. 2001. N-linked glycosylation of the HIV type-1 gp120 envelope glycoprotein as a major determinant of CCR5 and CXCR4 coreceptor utilization. The Journal of biological chemistry 276:13433-13441.

130. Popovic, M., M. G. Sarngadharan, E. Read, and R. C. Gallo. 1984. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science 224:497-500.

131. Rainwater, S. M., X. Wu, R. Nduati, R. Nedellec, D. Mosier, G. John-Stewart, D. Mbori-Ngacha, and J. Overbaugh. 2007. Cloning and characterization of functional subtype A HIV-1 envelope variants transmitted through breastfeeding. Current HIV research 5:189-197.

132. Raska, M., and J. Novak. 2010. Involvement of envelope-glycoprotein glycans in HIV-1 biology and infection. Archivum immunologiae et therapiae experimentalis 58:191-208.

133. Reitter, J. N., R. E. Means, and R. C. Desrosiers. 1998. A role for carbohydrates in immune evasion in AIDS. Nature medicine 4:679-684.

134. Richman, D. D., T. Wrin, S. J. Little, and C. J. Petropoulos. 2003. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proceedings of the National Academy of Sciences of the United States of America 100:4144-4149.

135. Rodriguez, B., A. K. Sethi, V. K. Cheruvu, W. Mackay, R. J. Bosch, M. Kitahata, S. L. Boswell, W. C. Mathews, D. R. Bangsberg, J. Martin, C. C. Whalen, S. Sieg, S. Yadavalli, S. G. Deeks, and M. M. Lederman. 2006. Predictive value of plasma HIV RNA level on rate of CD4 T-cell decline in untreated HIV infection. JAMA : the journal of the American Medical Association 296:1498-1506.

136. Rong, R., B. Li, R. M. Lynch, R. E. Haaland, M. K. Murphy, J. Mulenga, S. A. Allen, A. Pinter, G. M. Shaw, E. Hunter, J. E. Robinson, S. Gnanakaran, and C. A. Derdeyn. 2009. Escape from autologous neutralizing antibodies in acute/early subtype C HIV-1 infection requires multiple pathways. PLoS pathogens 5:e1000594.

137. Rousseau, C. M., R. W. Nduati, B. A. Richardson, G. C. John-Stewart, D. A. Mbori-Ngacha, J. K. Kreiss, and J. Overbaugh. 2004. Association of levels of HIV-1-infected breast milk cells and risk of mother-to-child transmission. The Journal of infectious diseases 190:1880-1888.

138. Ruprecht, R. M., R. Hofmann-Lehmann, B. A. Smith-Franklin, R. A. Rasmussen, V. Liska, J. Vlasak, W. Xu, T. W. Baba, A. L. Chenine, L. A. Cavacini, M. R. Posner, H. Katinger, G. Stiegler, B. J. Bernacky, T. A. Rizvi, R. Schmidt, L. R. Hill, M. E. Keeling, D. C. Montefiori, and H. M. McClure. 2001. Protection of neonatal macaques against experimental SHIV infection by human neutralizing monoclonal antibodies. Transfusion clinique et biologique : journal de la Societe francaise de transfusion sanguine 8:350-358.

139. Russell, E. S., J. J. Kwiek, J. Keys, K. Barton, V. Mwapasa, D. C. Montefiori, S. R. Meshnick, and R. Swanstrom. 2011. The genetic bottleneck in vertical transmission of subtype C HIV-1 is not driven by selection of especially neutralization-resistant virus from the maternal viral population. Journal of virology 85:8253-8262.

140. Russell, E. S., S. Ojeda, G. G. Fouda, S. R. Meshnick, D. Montefiori, S. R. Permar, and R. Swanstrom. 2013. HIV Type 1 Subtype C Variants Transmitted Through the Bottleneck of Breastfeeding Are Sensitive to New Generation Broadly Neutralizing Antibodies Directed Against Quaternary and CD4-Binding Site Epitopes. AIDS research and human retroviruses.

141. Russell, E. S., S. Ojeda, G. G. Fouda, S. R. Meshnick, D. Montefiori, S. R. Permar, and R. Swanstrom. 2013. Short Communication: HIV Type 1 Subtype C Variants Transmitted Through the Bottleneck of Breastfeeding Are Sensitive to New Generation Broadly Neutralizing Antibodies Directed Against Quaternary and CD4-Binding Site Epitopes. AIDS research and human retroviruses 29:511-515.

142. Sabino, E., L. Z. Pan, C. Cheng-Mayer, and A. Mayer. 1994. Comparison of in vivo plasma and peripheral blood mononuclear cell HIV-1 quasi-species to short-term tissue culture isolates: an analysis of tat and C2-V3 env regions. AIDS 8:901-909.

143. Sagar, M., X. Wu, S. Lee, and J. Overbaugh. 2006. Human immunodeficiency virus type 1 V1-V2 envelope loop sequences expand and add glycosylation sites over the course of infection, and these modifications affect antibody neutralization sensitivity. Journal of virology 80:9586-9598.

144. Salazar-Gonzalez, J. F., E. Bailes, K. T. Pham, M. G. Salazar, M. B. Guffey, B. F. Keele, C. A. Derdeyn, P. Farmer, E. Hunter, S. Allen, O. Manigart, J. Mulenga, J. A. Anderson, R. Swanstrom, B. F. Haynes, G. S. Athreya, B. T. Korber, P. M. Sharp, G. M. Shaw, and B. H. Hahn. 2008. Deciphering human immunodeficiency virus type 1 transmission and early envelope diversification by single-genome amplification and sequencing. Journal of virology 82:3952-3970.

145. Salazar-Gonzalez, J. F., M. G. Salazar, G. H. Learn, G. G. Fouda, H. H. Kang, T. Mahlokozera, A. B. Wilks, R. V. Lovingood, A. Stacey, L. Kalilani, S. R. Meshnick, P. Borrow, D. C. Montefiori, T. N. Denny, N. L. Letvin, G. M. Shaw, B. H. Hahn, and S. R. Permar. 2011. Origin and evolution of HIV-1 in breast milk determined by single-genome amplification and sequencing. Journal of virology 85:2751-2763.

146. Samleerat, T., S. Thenin, G. Jourdain, N. Ngo-Giang-Huong, A. Moreau, P. Leechanachai, J. Ithisuknanth, K. Pagdi, P. Wannarit, S. Sangsawang, M. Lallemant, F. Barin, and M. Braibant. 2009. Maternal neutralizing antibodies against a CRF01_AE primary isolate are associated with a low rate of intrapartum HIV-1 transmission. Virology 387:388-394.

147. Sather, D. N., J. Armann, L. K. Ching, A. Mavrantoni, G. Sellhorn, Z. Caldwell, X. Yu, B. Wood, S. Self, S. Kalams, and L. Stamatatos. 2009. Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection. Journal of virology 83:757-769.

148. Satomi, M., M. Shimizu, E. Shinya, E. Watari, A. Owaki, C. Hidaka, M. Ichikawa, T. Takeshita, and H. Takahashi. 2005. Transmission of macrophage-tropic HIV-1 by breast-milk macrophages via DC-SIGN. The Journal of infectious diseases 191:174-181.

149. Scarlatti, G., J. Albert, P. Rossi, V. Hodara, P. Biraghi, L. Muggiasca, and E. M. Fenyo. 1993. Mother-to-child transmission of human immunodeficiency virus type 1: correlation with neutralizing antibodies against primary isolates. The Journal of infectious diseases 168:207-210.

150. Scarlatti, G., T. Leitner, E. Halapi, J. Wahlberg, M. Jansson, H. Wigzell, E. M. Fenyo, J. Albert, M. Uhlen, and P. Rossi. 1993. Analysis of the HIV-1 envelope V3-loop sequences from ten mother-child pairs. Annals of the New York Academy of Sciences 693:277-280.

151. Sharp, P. M., and B. H. Hahn. 2011. Origins of HIV and the AIDS pandemic. Cold Spring Harbor perspectives in medicine 1:a006841.

152. Shioda, T., J. A. Levy, and C. Cheng-Mayer. 1991. Macrophage and T cell-line tropisms of HIV-1 are determined by specific regions of the envelope gp120 gene. Nature 349:167-169.

153. Simek, M. D., W. Rida, F. H. Priddy, P. Pung, E. Carrow, D. S. Laufer, J. K. Lehrman, M. Boaz, T. Tarragona-Fiol, G. Miiro, J. Birungi, A. Pozniak, D. A. McPhee, O. Manigart, E. Karita, A. Inwoley, W. Jaoko, J. Dehovitz, L. G. Bekker, P. Pitisuttithum, R. Paris, L. M. Walker, P. Poignard, T. Wrin, P. E. Fast, D. R. Burton, and W. C. Koff. 2009. Human immunodeficiency virus type 1 elite neutralizers: individuals with broad and potent neutralizing activity identified by using a high-throughput neutralization assay together with an analytical selection algorithm. Journal of virology 83:7337-7348.

154. Simister, N. 2003. Placental transport of immunoglobulin G. Vaccine 21:3365-3369.

155. Skrabal, K., S. Saragosti, J. L. Labernardiere, F. Barin, F. Clavel, and F. Mammano. 2005. Human immunodeficiency virus type 1 variants isolated from single plasma samples display a wide spectrum of neutralization sensitivity. Journal of virology 79:11848-11857.

156. Starcich, B. R., B. H. Hahn, G. M. Shaw, P. D. McNeely, S. Modrow, H. Wolf, E. S. Parks, W. P. Parks, S. F. Josephs, R. C. Gallo, and et al. 1986. Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. Cell 45:637-648.

157. Thenin, S., T. Samleerat, E. Tavernier, N. Ngo-Giang-Huong, G. Jourdain, M. Lallemant, F. Barin, and M. Braibant. 2012. Envelope glycoproteins of human immunodeficiency virus type 1 variants issued from mother-infant pairs display a wide spectrum of biological properties. Virology 426:12-21.

158. Tomaras, G. D., N. L. Yates, P. Liu, L. Qin, G. G. Fouda, L. L. Chavez, A. C. Decamp, R. J. Parks, V. C. Ashley, J. T. Lucas, M. Cohen, J. Eron, C. B. Hicks, H. X. Liao, S. G. Self, G. Landucci, D. N. Forthal, K. J. Weinhold, B. F. Keele, B. H. Hahn, M. L. Greenberg, L. Morris, S. S. Karim, W. A. Blattner, D. C. Montefiori, G. M. Shaw, A. S. Perelson, and B. F. Haynes. 2008. Initial B-cell responses to transmitted human immunodeficiency virus type 1: virion-binding immunoglobulin M (IgM) and IgG antibodies followed by plasma anti-gp41 antibodies with ineffective control of initial viremia. Journal of virology 82:12449-12463.

159. Trkola, A., M. Purtscher, T. Muster, C. Ballaun, A. Buchacher, N. Sullivan, K. Srinivasan, J. Sodroski, J. P. Moore, and H. Katinger. 1996. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. Journal of virology 70:1100-1108.

160. Van Dyke, R. B., B. T. Korber, E. Popek, C. Macken, S. M. Widmayer, A. Bardeguez, I. C. Hanson, A. Wiznia, K. Luzuriaga, R. R. Viscarello, and S. Wolinsky. 1999. The Ariel Project: A prospective cohort study of maternal-child transmission of human immunodeficiency virus type 1 in the era of maternal antiretroviral therapy. The Journal of infectious diseases 179:319-328.

161. van't Wout, A. B., N. A. Kootstra, G. A. Mulder-Kampinga, N. Albrecht-van Lent, H. J. Scherpbier, J. Veenstra, K. Boer, R. A. Coutinho, F. Miedema, and H. Schuitemaker. 1994. Macrophage-tropic variants initiate human immunodeficiency virus type 1 infection after sexual, parenteral, and vertical transmission. The Journal of clinical investigation 94:2060-2067.

162. Voronin, Y., B. Chohan, M. Emerman, and J. Overbaugh. 2007. Primary isolates of human immunodeficiency virus type 1 are usually dominated by the major variants found in blood. Journal of virology 81:10232-10241.

163. Voronin, Y., J. Overbaugh, and M. Emerman. 2005. Simian immunodeficiency virus variants that differ in pathogenicity differ in fitness under rapid cell turnover conditions. Journal of virology 79:15091-15098.

164. Walker, L. M., and D. R. Burton. 2010. Rational antibody-based HIV-1 vaccine design: current approaches and future directions. Current opinion in immunology 22:358-366.

165. Walker, L. M., M. Huber, K. J. Doores, E. Falkowska, R. Pejchal, J. P. Julien, S. K. Wang, A. Ramos, P. Y. Chan-Hui, M. Moyle, J. L. Mitcham, P. W. Hammond, O. A. Olsen, P. Phung, S. Fling, C. H. Wong, S. Phogat, T. Wrin, M. D. Simek, W. C. Koff, I. A. Wilson, D. R. Burton, and P. Poignard. 2011. Broad neutralization coverage of HIV by multiple highly potent antibodies. Nature 477:466-470.

166. Walker, L. M., S. K. Phogat, P. Y. Chan-Hui, D. Wagner, P. Phung, J. L. Goss, T. Wrin, M. D. Simek, S. Fling, J. L. Mitcham, J. K. Lehrman, F. H. Priddy, O. A. Olsen, S. M. Frey, P. W. Hammond, S. Kaminsky, T. Zamb, M. Moyle, W. C. Koff, P. Poignard, and D. R. Burton. 2009. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. Science 326:285-289.

167. Wei, X., J. M. Decker, H. Liu, Z. Zhang, R. B. Arani, J. M. Kilby, M. S. Saag, X. Wu, G. M. Shaw, and J. C. Kappes. 2002. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. Antimicrobial agents and chemotherapy 46:1896-1905.

168. Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw. 2003. Antibody neutralization and escape by HIV-1. Nature 422:307-312.

169. Weiss, R. A., P. R. Clapham, J. N. Weber, A. G. Dalgleish, L. A. Lasky, and P. W. Berman. 1986. Variable and conserved neutralization antigens of human immunodeficiency virus. Nature 324:572-575.

170. Weng, Y., and C. D. Weiss. 1998. Mutational analysis of residues in the coiled-coil domain of human immunodeficiency virus type 1 transmembrane protein gp41. Journal of virology 72:9676-9682.

171. Willey, R. L., R. A. Rutledge, S. Dias, T. Folks, T. Theodore, C. E. Buckler, and M. A. Martin. 1986. Identification of conserved and divergent domains within the envelope gene of the acquired immunodeficiency syndrome retrovirus. Proceedings of the National Academy of Sciences of the United States of America 83:5038-5042.

172. Williamson, S., S. M. Perry, C. D. Bustamante, M. E. Orive, M. N. Stearns, and J. K. Kelly. 2005. A statistical characterization of consistent patterns of human immunodeficiency virus evolution within infected patients. Molecular biology and evolution 22:456-468.

173. Wolinsky, S. M., C. M. Wike, B. T. Korber, C. Hutto, W. P. Parks, L. L. Rosenblum, K. J. Kunstman, M. R. Furtado, and J. L. Munoz. 1992. Selective transmission of human immunodeficiency virus type-1 variants from mothers to infants. Science 255:1134-1137.

174. Wu, X., A. B. Parast, B. A. Richardson, R. Nduati, G. John-Stewart, D. Mbori-Ngacha, S. M. Rainwater, and J. Overbaugh. 2006. Neutralization escape variants of human immunodeficiency virus type 1 are transmitted from mother to infant. Journal of virology 80:835-844.

175. Wu, X., Z. Y. Yang, Y. Li, C. M. Hogerkorp, W. R. Schief, M. S. Seaman, T. Zhou, S. D. Schmidt, L. Wu, L. Xu, N. S. Longo, K. McKee, S. O'Dell, M. K. Louder, D. L. Wycuff, Y. Feng, M. Nason, N. Doria-Rose, M. Connors, P. D. Kwong, M. Roederer, R. T. Wyatt, G. J. Nabel, and J. R. Mascola. 2010. Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1. Science 329:856-861.

176. Wyatt, R., N. Sullivan, M. Thali, H. Repke, D. Ho, J. Robinson, M. Posner, and J. Sodroski. 1993. Functional and immunologic characterization of human immunodeficiency virus type 1 envelope glycoproteins containing deletions of the major variable regions. Journal of virology 67:4557-4565.

177. Xiao, X., W. Chen, Y. Feng, and D. S. Dimitrov. 2009. Maturation Pathways of Cross-Reactive HIV-1 Neutralizing Antibodies. Viruses 1:802-817.

178. Yokoyama, M., S. Naganawa, K. Yoshimura, S. Matsushita, and H. Sato. 2012. Structural dynamics of HIV-1 envelope Gp120 outer domain with V3 loop. PloS one 7:e37530.

179. Zhang, H., F. Hoffmann, J. He, X. He, C. Kankasa, J. T. West, C. D. Mitchell, R. M. Ruprecht, G. Orti, and C. Wood. 2006. Characterization of HIV-1 subtype C envelope glycoproteins from perinatally infected children with different courses of disease. Retrovirology 3:73.

180. Zhang, M., B. Gaschen, W. Blay, B. Foley, N. Haigwood, C. Kuiken, and B. Korber. 2004. Tracking global patterns of N-linked glycosylation site variation in highly variable viral glycoproteins: HIV, SIV, and HCV envelopes and influenza hemagglutinin. Glycobiology 14:1229-1246.

181. Zwick, M. B., A. F. Labrijn, M. Wang, C. Spenlehauer, E. O. Saphire, J. M. Binley, J. P. Moore, G. Stiegler, H. Katinger, D. R. Burton, and P. W. Parren. 2001. Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. Journal of virology 75:10892-10905.