The Activity of Breast Milk and Monoclonal HIV-specific Antibodies in Mother-to-Child Transmission.

Jennifer K. Maroa

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Reading Committee:
Julie Overbaugh, Chair
Gael Kurath
Carey Farquhar

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Abstract

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Jennifer K. Maroa

Chair of the supervisory Committee:

Professor Julie Overbaugh

Pathobiology

Full Member: Division of Human Biology, Fred Hutchinson Cancer Research Center

Mother-to-child transmission of HIV is a unique setting that allows us to explore both the correlates of protective immunity and the characteristics of transmitted variants. This thesis first describes the levels and functional capacity of breast milk HIV-specific antibodies in 19 women with high plasma viral loads. Neutralizing antibodies (Nabs) were detected in breast milk supernatant (BMS) of 4 of 19 women examined, were of low potency and were not associated with infant infection. The low NAb activity in BMS was reflected in binding antibody levels with HIV envelope specific IgG titers being 2.2 $\log_{10}$ lower in BMS versus plasma. In contrast, non- neutralizing antibodies (nNAbs) capable of antibody dependent cell-mediated cytotoxicity (ADCC) were detected in the BMS from all 19 women. BMS ADCC activity was associated with envelope-specific IgG titers ($p = 0.014$) and was inversely associated with infant infection risk ($p = 0.039$). Our data indicate that BMS has limited HIV neutralizing activity, however, BMS ADCC activity is a correlate of transmission that may impact infant infection risk.

In the second part of this thesis the neutralization sensitivity of 111 variants of diverse subtypes obtained from mothers and infants was determined against 7 HIV-specific broadly neutralizing monoclonal antibodies (mAbs) (NIH45-46w, VRC01, PGT128, PGT121, PG9 PGT145 and b12). Maternal and infant variants did not differ in their neutralization sensitivity to these mAbs and neither did variants from transmitting versus those from non-transmitting women. However, subtype A viruses were

significantly more sensitive to neutralization by NIH45-46w and VRC01 ($p= 0.0001$ in both cases) and PGT145 ($p=0.03$) compared to non-subtype A viruses. Together, NIH45-46w and PGT128 neutralization profiles resulted in 100% coverage of the variants tested. These data suggest that the epitopes targeted by these mAbs are present and accessible in both circulating and transmitted variants and that a combination of antibodies would provide maximum coverage against diverse subtypes commonly found in HIV endemic regions.

Overall, this data suggest that an antibody based HIV vaccine capable of eliciting antibodies of multiple specificities that can mediate ADCC and/or neutralizing activity can provide protection and conquer the genetic diversity displayed by HIV.

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List of Abbreviations

ADCC: antibody-dependent cell-mediated cytotoxicity

ADCVI: antibody-dependent cellular and viral inhibition

AIDS: acquired immunodeficiency syndrome

ARV: anti-retroviral

cARV: combination ARV

AZT: zidovudine

BMS: breast milk supernatant

BM: breast milk

C1-C5: constant regions 1-5

CAV: cell associated virus

CFV: cell free virus

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

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

CD4bs: CD4 binding site

CTL: cytotoxic T lymphocyte

dsDNA: double stranded DNA

ELISA: enzyme linked immunosorbent assay

ER: rough endoplastic reticulum

GEE: generalized estimating equations

HIV: human immunodeficiency virus type-1

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

mAbs: broadly neutralizing monoclonal antibodies

MPER: membrane proximal external region

MTCT: mother-to-child transmission

NAbs: neutralizing antibodies

aNAbs: autologous neutralizing antibodies

hNAbs: heterologous neutralizing antibodies

nNAbs: non- neutralizing antibodies

NVP: nevirapine

PCR: polymerase chain reaction

PNLGs: potential N-linked glycosylation site

RCT: randomized clinical trial

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

WHO: world health organization

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Dedication

To my daughters Sandy and Sally

I love you!

Chapter 1

Introduction

HIV Epidemiology

Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS). It was discovered in 1981 predominantly in gay men and thus the disease was associated with homosexuality [1]. Thereafter, it was confirmed that HIV could be transmitted via heterosexual relationships, blood transfusions and vertically from mothers to their infants either during pregnancy, delivery or after birth via breast milk.

The proportion of women living with HIV continues to be substantial both globally (~50%) and in sub-Saharan Africa (> 59%). Of note, these infections are disproportionately distributed with the majority being found in women within the reproductive ages of 15-24 years old [2]. As a result, mother–to-child transmission (MTCT) of HIV also contributes significantly to new infections. For instance, there were ~370,000 new pediatric infections reported worldwide in 2009, 90% of which occurred in sub-Saharan Africa.

Currently, the total number of HIV infected persons is estimated to be 34 million worldwide. Sixty eight% of HIV infected people are residents of sub-Saharan Africa making it the region most affected by the pandemic in the world. To date, there have been an estimated >46 million deaths globally as a result of HIV, making it one of the most destructive diseases in the history of infectious diseases [3].

The HIV genome

HIV-1 belongs to the lentivirus group of retroviruses, named for the use of the viral reverse transcriptase (RT) enzyme, which converts viral RNA to double stranded DNA (dsDNA). The viral genome is ~9.7 kb and occurs as a positive sense single stranded RNA in virions [4,5]. There are two copies of HIV-1 RNA genomes per virion, allowing recombination during reverse transcription, which can contribute to viral escape

from host defenses, as discussed in detail later [6]. HIV-1 long terminal repeats (LTRs) flank the viral genes on the 5' and 3' ends and contain the viral promoter/enhancer and polyadenylation/transcription stop codon for cellular RNA polymerase. As in all retroviruses, HIV-1 encodes three major genes: *gag, pol*, and *env*. Besides these 3 core genes, HIV encodes several accessory genes *(vif,vpr,vpu,tat,rev and nef)* necessary for infection and pathogenesis.

HIV Envelope (env) protein

Env is the only viral protein found on the surface of the virus. It is processed from a precussor protein gp160 by host cell machinery in the rough endoplasmic reticulum (ER). During processing the gp160 undergoes modifications, which include glycosylation of the N-linked oligossacharides with host sugars at sites that meet the canonical sequence Asn-X- Ser/Thr (NxS/T; where x is any amino acid except proline) [7]

While still in the ER, gp160 monomers undergo folding followed by oligomerization into trimers that are trafficked into the golgi apparatus [8]. Here, cleavage of the gp160 protein by the host protein furin takes place to generate the surface unit (SU) gp120 and the trans-membrane (TM) gp41 domain. The 2 env domains (gp120 and gp41) remain non-covalently associated. The gp120 subunit is made up of constant regions C1-C5 interspersed with amino acid variable regions V1/V2-V5 that are constantly evolving due to neutralizing antibody (NAbs) pressure (Fig 1.1 and 1.2A). The gp41 consists of the ectodomain, the transmembrane anchor domain and the cytoplasmic tail [7](Fig 1.1). Three molecules each of gp120 and gp41 form a heterotrimeric protein (Fig 1.1), which is then transported through the golgi apparatus and incorporated into the plasma membrane of the host cell. This is followed by associations with other core viral proteins and finally budding of the new virus particle with the trimeric env spikes on the surface [7].

Role of Env in virus entry

Env is responsible for early interactions with the host target cell that initiate viral entry and therefore also define the host cell tropism. The gp120 domain contains the binding site for CD4, the primary receptor for HIV. Because of the CD4 requirement, HIV infects CD4 expressing cells such as CD4 +T cells, macrophages and/or microglia (Fig 1.3A). Infection and replication is much more efficient in activated CD4 T cells than in quiescent CD4 +T cells [9]. Interaction between gp120 and the CD4 molecule on target cells triggers a conformational change in the viral env protein that exposes the co-receptor binding site. The main co-receptors for HIV-1 are the CCR5 and CXCR4 members of the seven trans-membrane G protein coupled receptors, which function as chemokine receptors [10-12]. A vast majority of HIV variants use CCR5 but later in chronic infection, CXCR4 using variants are present [13]. Binding of gp120 to the chemokine receptor results in a second conformational change that exposes the fusion domain present in the gp41 ectodomain (Fig 1.3B). The fusion domain facilitates fusion between the viral and host cell membranes and the viral capsid is then deposited in the cytoplasm of the host cell a critical step towards productive infection[12](Fig 1.3C). Within the cytoplasm, the capsid is uncoated and viral RNA is converted to DNA in the process of reverse transcription. This DNA copy is then integrated within the host cell genome as a proviral DNA and represents cell-associated virus (CAV) measured by copies of HIV proviral DNA. Infection of cells can result in virus replication and subsequent production of new virions that bud out of the infected cell and these represent cell free virus (CFV) measured by copies of HIV RNA. CFV in plasma are typically referred to as viral load [4,5].

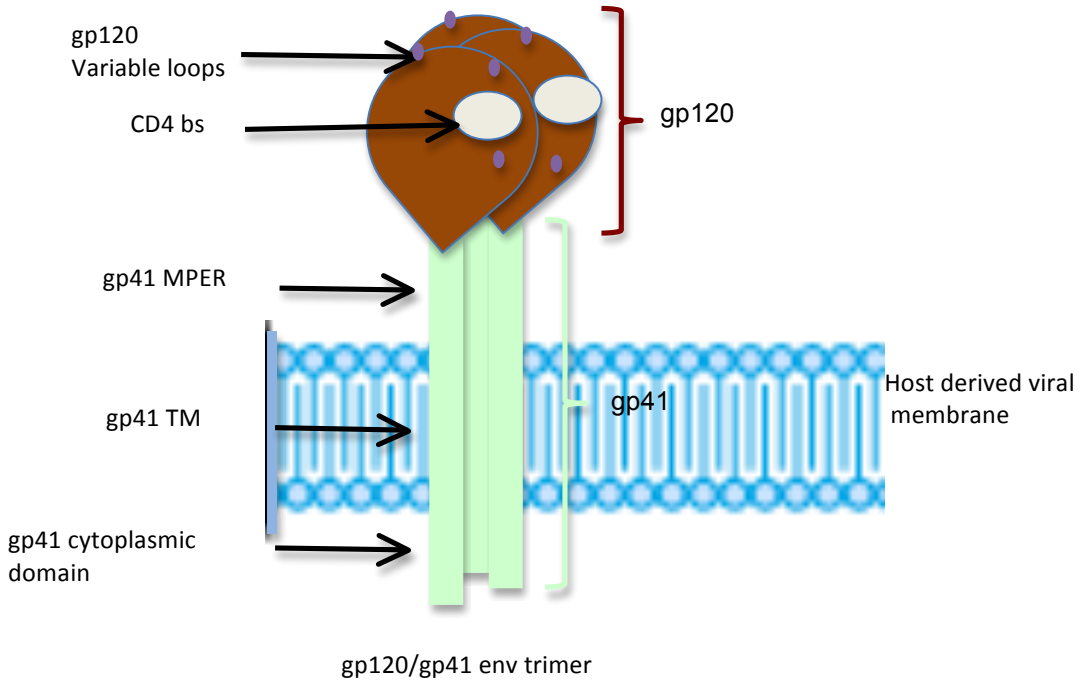

Figure 1.1. A schematic of trimeric envelope spike on the surface of the host-derived viral membrane. Three gp120 domains (brown) and 3 gp41 domains (light green) form the env trimer. Representative gp120 variable loops are shown in purple and the CD4 binding site in white. The gp41 TM domain anchors the env trimer into the host-derived viral membrane.

VL, variable loop; MPER, membrane proximal external region; TM, Transmembrane; CD4 bs; CD4 binding site

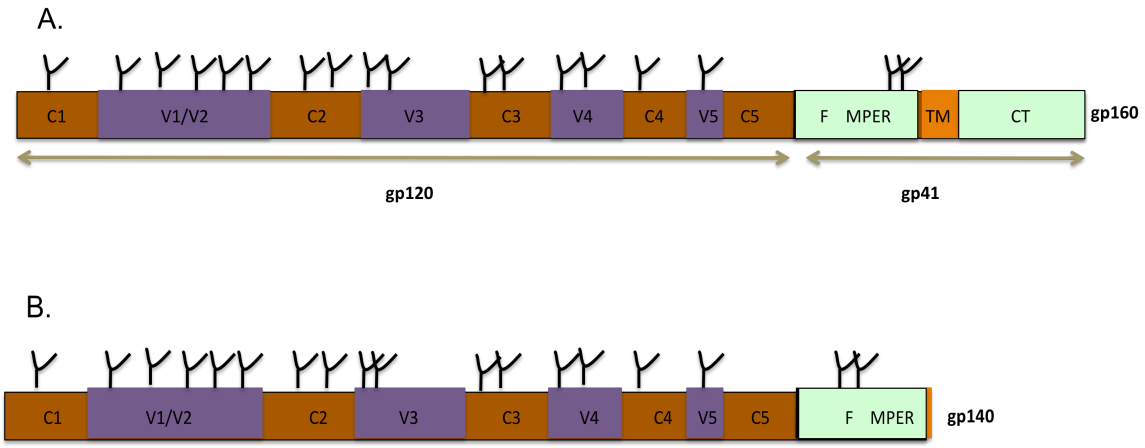

Figure 1.2. Illustration of envelope gp160 and gp140 protein domains. Panel A. shows the gp120 and gp41 env domains. The variable (purple) and constant (brown) regions of gp120 are shown. The gp41 domain is shown in light green and other regions F, MPER, TM and CT) are also indicated. The spikes in black represent host derived glycans, which can either be complex sugars or oligomannose hybrids. Panel B. shows a schematic of gp140 construct made by introduction of a stop codon at the end of the TM of gp41. Trimeric gp140 protein was used in assays described in chapter 2.
F, fusion domain; MPER membrane proximal external region; CT, cytoplamic tail; TM, transmembrane

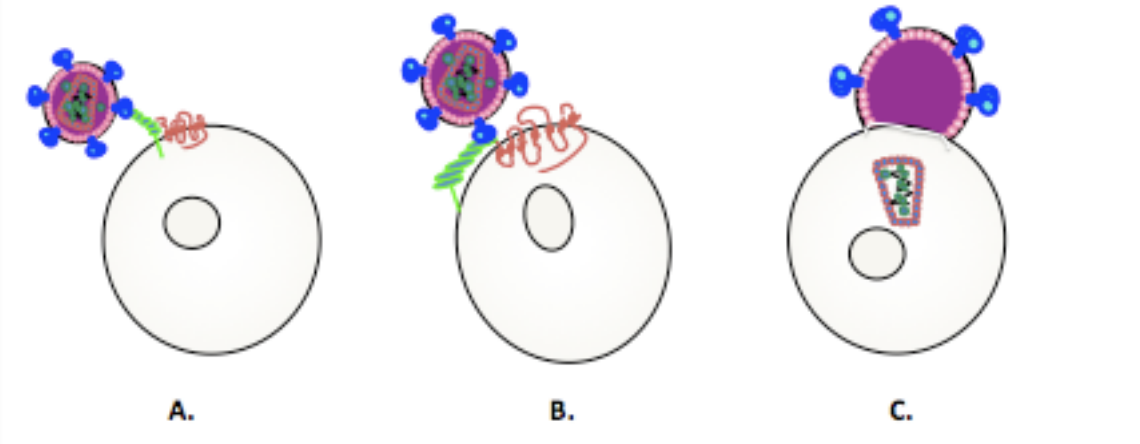

Figure 1.3. Role of envelope in target cell infection. A. gp120 (blue) contains the CD4 binding site that interacts with the CD4 molecule (green) on the target cell resulting in conformational changes that expose the co-receptor binding site in the V3 loop of gp120. B. gp120 then interacts with the co-receptor (orange) resulting in further conformational changes that expose the fusion domain in gp41. C. gp41 fuses with host cell membrane and the viral capsid is deposited in the cytoplasm.

Course of HIV infection

Mucosal membranes are the main sites of virus entry [14]. Soon after entry, the virus expands locally in founder CD4+ T cells followed by viral dissemination to mucosal lymphoid tissues, which are rich in activated CD4+ CCR5+ target cells [15]. These cells within mucosal lymphoid tissues are highly susceptible to infection, resulting in high numbers of infected cells soon after initial infection [16]. This initial massive infection of cells is followed by high rates of viral replication in which virus doubles every 6-10 hrs resulting in a distinct peak plasma viral load and rapid depletion of CD4+ T cells during acute infection (Fig 1.4) [15,17,18]. The mechanism by which massive destruction of CD4+ T cells occurs is a subject of continued debate and is thought to be either directly induced by virus –mediated killing of the infected cell or indirectly by apoptosis induced killing of bystander CD4+ T cells [15,18]. A subset of these infected cells become memory T cells and establish a latent reservoir of HIV [19].

After acute infection, there is a rapid decline of viral loads by several orders of magnitude to a steady state also referred to as viral set-point [20](Fig 1.4). High viral set-point is associated with disease progression [21-23]. Establishment of a viral set-point is also associated with a significant rebound of CD4+ T cells in the periphery (Fig 1.4). The immune mechanisms associated with viral control and CD4+ T cell rebound are discussed in detail below.

After establishment of a viral set-point replication is maintained at a steady state for several years (in adults), with no obvious signs of clinical infection (chronic infection). Typically, over a period of ~10 years after infection, there is progressive destruction of CD4+ T cells resulting in profound immune suppression in majority of adults marking the onset of AIDS (Fig 1.4). AIDS is characterized by among other things CXCR4 using viruses, low CD4 + T cell counts, increased opportunistic infections, high plasma viral load and ultimately death [24]. This is a typical disease cause in the absence of treatment.

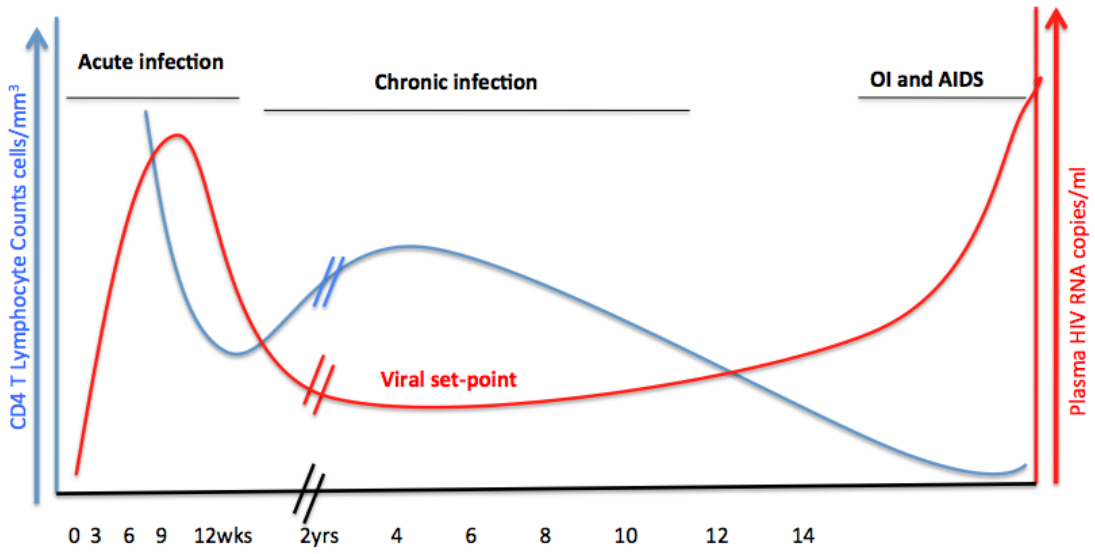

Figure 1.4. Schematic depiction of the course of HIV infection in the absence of treatment. The relationships between CD4+T cell counts (blue line) and plasma HIV RNA viral load (red line) over time since infection (X-axis) are shown. Initial infection results in high rates of virus replication leading to peak viremia and a decline in peripheral CD4+ T cells during acute stage. This is followed by rapid decrease in viral loads to a setpoint, which is maintained at a steady-state during chronic infection. Recovery of peripheral CD4+ T cells is observed with the decline in viral loads, however, over time progressive destruction of these cells results in immune suppression giving way to OIs and AIDS.

OIs, opportunistic infections; AIDS, acquired immunodeficiency syndrome.

Host immune responses to HIV infection

The host immune response to HIV infection is relatively early as evidenced by the rapid control of virus replication in acute infection resulting in a drastic decline of viral load to a set-point. Almost simultaneously, the levels of CD4+ T cells in the periphery display significant recovery (Fig 1.4). Available evidence suggests that the adaptive immune response is responsible for this rapid control of virus.

Cellular immunity:

CTLs: Cytotoxic T lymphocytes (CD8+ T cells) act by killing infected cells expressing antigen on their MHC class I on their surface. Consequently, CTLs though unable to stop establishment of infection can play a role in control of replication. There are several lines of evidence to suggest that CTLs play a role in control of viral replication during acute HIV infection. Soon after infection, an increase in the levels of CTLs in the peripheral blood precedes the decline in plasma viral load. This is also followed by an apparent rise of escape mutations in CTL targeted epitopes [25]. HIV also encodes an accessory protein *nef*, which functions to down-regulate HLA class 1 molecules from the surface of host cells [26,27]. The most convincing association however, is from studies conducted in animal models in which depletion of CD8+ T cells in macaques resulted in an increase in viral loads [28,29]. Taken together, these observations strongly suggest that CTLs might be playing an important role in controlling viral replication during acute HIV infection.

Humoral immunity:

B cells: B cells are the powerhouses of antibody production. The HIV antibody response has been a subject of intense research because compared to CTLs, antibodies have the capacity to block the virus before it establishes infection, hence providing protection. To achieve protection, antibodies can work indirectly through effector-mediated functions, which result in destruction of infected cells or opsonization of virus and/or directly by targeting and neutralizing CFV. Non-neutralizing antibody (nNAbs) can mediate effector cell functions via different mechanisms. Antibody-dependent cell-mediated cytotoxicity (ADCC) is a mechanism that targets infected cells that express

viral protein on their surface while antibody dependent cellular and viral inhibition (ADCVI), is mechanism that targets both cell free virus and infected cells. Neutralizing antibodies (NAbs) on the other hand act by binding to crucial viral epitopes and blocking the virus from accessing the host target cells.

For reasons that remain unclear, functionally distinct antibodies against HIV develop over time. HIV-specific binding antibodies can be detected as early as 8 days after infection. These binding antibodies form immune complexes with the virus but do not seem to control the virus (Fig 1.5 and 1.6) [30-32]. A few days later, binding antibodies against gp41 can be found and its not until weeks later that binding antibodies targeting gp120 and specifically the V3 can be detected [31]. These early binding antibodies do not appear to exert immune pressure on the env [33]. Non-neutralizing antibodies (binding antibodies with capacity to mediate effector function) on the other hand can be found ~13 days after initial infection with HIV hence can potentially have an impact during acute infection [34] (Fig1.5 and 1.7). Finally, NAbs that are directed at one's own virus also referred to as autologous NAbs (aNAbs) can be detected after ~3 months of infection (Fig 1.5 and 1.8 A) [30,31,35,36]. Over time and depending on the levels of viral antigen present, these aNAbs increase in titers, undergo rounds of affinity maturation and develop in breadth [37,38]. Consequently, NAbs that target diverse strains of virus referred to as heterologous neutralizing antibodies (hNAb), can be detected 2-3 years after infection and are characterized by the ability to neutralize a broad range of circulating viral strains. In some cases hNAbs can be highly potent (Fig 1.5)[39,40]. It is now known that ~25% of chronically infected individuals can develop cross-reactive and relatively potent antibodies [38,40,41]. A smaller subset (~1%) of HIV positive individuals termed as elite neutralizers develop antibodies that can neutralize over 90% of circulating strains of HIV (Fig 1.8 B) [42,43].

Although the ability of an antibody to bind is important, binding does not always lead to neutralization. The monoclonal antibody b6 for example competes with b12 for the CD4 binding site (CD4bs) but does not neutralize [44]. Nevertheless, other characteristics of binding antibodies have been useful in understanding the role of non-neutralizing antibodies. For example, IgG binding antibody titers and avidity have been correlated with ADCC and/or ADCVI, which in turn correlated with reduced acute

viremia in animal models [45-47]. More recently, immune correlate analysis from the first human HIV vaccine trial in which a 31.2 % protective efficacy was reported (RV144 trial), indicated that levels of plasma IgG binding antibodies to the V1/V2 region correlate with protection [48,49]. Given these results, binding antibodies to the V1/V2 region could be used as a primary endpoint to check for and develop hypothesis on protective antibodies in subsequent vaccine trials.

Non-neutralizing antibodies have the capacity to engage innate immune cells to perform antibody-effector cell functions such as ADCC and/or ADCVI. ADCC is a mechanism by which infected cells expressing env on their surface are coated with antibody that cross-links them via the Fcg receptor (CD16) on natural killer (NK) cells for destruction (Fig 1.7). High ADCC antibody titers in infected individuals have been associated with low viral set-point and subsequent slow disease progression [50-54]. In animal models, ADCC and ADCVI mediating antibodies have also been associated with reduced set point viral load [47,55,56]. Taken together, these studies indicate that if nNAbs of the right kind are present at the right amount they have a potential of blunting HIV infection by altering viral setpoint.

In contrast, there is limited evidence to suggest that NAbs impact the course of HIV disease following infection. Indeed, low titer NAbs that display minimum activity towards contemporaneous virus are detected during acute infection [36]. In general, NAbs in natural infection drive neutralization escape (discussed later), a process that is always a step ahead of the contemporaneous antibody response making contemporaneous viruses less sensitive to aNAbs although they remain sensitive to aNAbs from subsequent time-point [57-59].

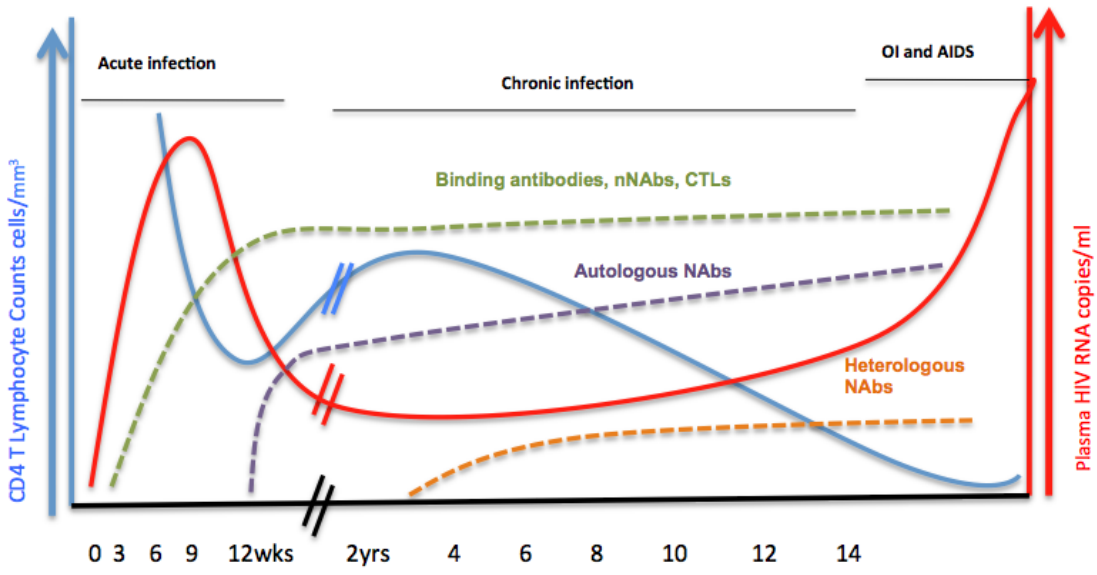

Figure 1.5. Adaptive immune response to HIV during the course of infection.
Binding antibodies, ADCC antibodies and CTLs are the first to appear soon after HIV infection (green) and are followed by a decline in viral loads and a rebound in peripheral CD4+ T cells. Month's later autologous antibodies capable of strain specific neutralization develop as shown in purple. After ~2 years, heterologous antibodies capable of neutralizing a broad range of circulating strains develop in a subset of individuals, this is shown in orange.
CTLs, Cytotoxic T lymphocytes; nNAbs, non-neutralizing antibodies; Nabs, neutralizing antibodies; OI, Opportunistic infections; AIDS, Acquired immunodeficiency syndrome

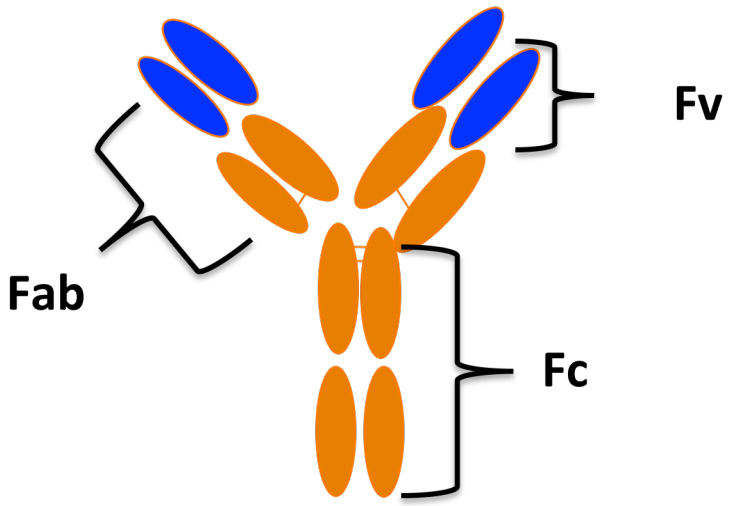

Figure 1.6. Schematic representation of an antibody. The Fab portion interacts with trimeric env and mediates NAb activity. The constant region (Fc) is recognized by Fc gamma receptors found on innate cells such as natural killer cells and mediates nNAb activity.

Fab, antibody antigen binding fragment; Fv, antibody variable fragment; Fc, antibody constant fragment; NAb, neutralizing antibody activity; nNAb non-neutralizing antibody activity.

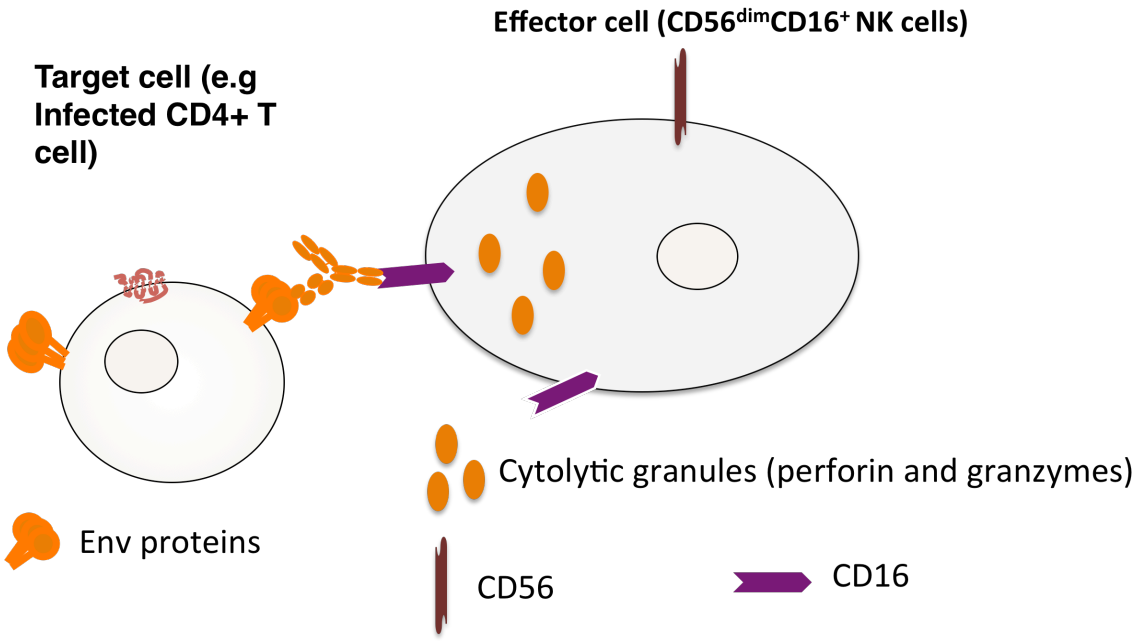

Figure 1.7. A schematic depiction of the mechanism of ADCC.

ADCC mediating antibodies bind to env on the surface of infected cells. Natural killer (NK) cells through their FcγR receptor interact with the Fc portion of the antibody. NK cells are pre-packaged with cytolytic granules containing perforins and granzymes. When an antibody capable of mediating ADCC binds to an infected cell, NK cells via their FcγR (CD16) can interact with the Fc portion of the antibody. Cross-linked NK cells release perforins, which form pores on the surface of the infected cells. Released granzymes can then enter the cells through the pores and activate enzymes that lead to apoptosis and/ or lysis of infected cells.

ADCC, antibody dependent cellular cytotoxicity; NK cell, natural killer cell; Fc, antibody constant fragment; FcγR, Fc gamma receptor (CD16)

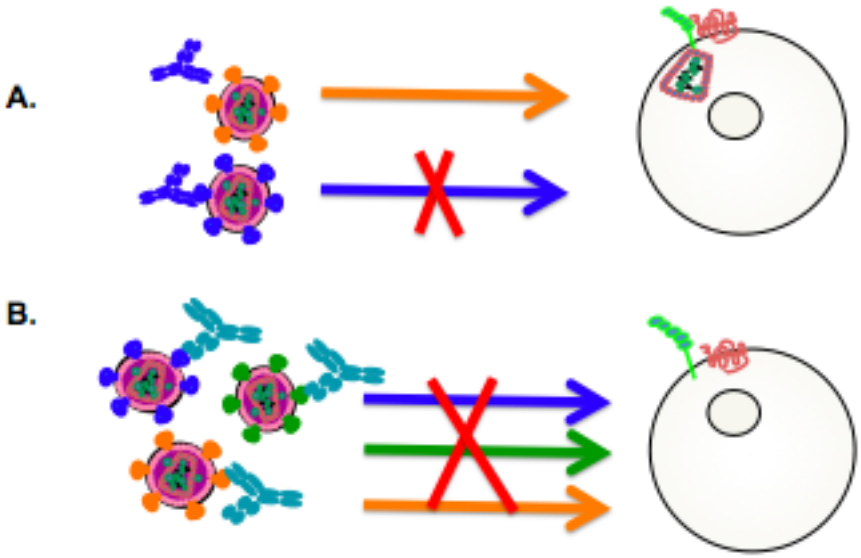

Figure 1.8. Schematic of aNAbs and hNAbs acivity. **A**. aNAbs are strain specific in their activity. In this schematic the aNAbs neutralize the strain specific virus (blue env) but the slightly diverse variant (orange env) is not neutralized and is thus capable of establishing infection. **B**. hNAbs have cross-reactive activity. In this schematic heterologous NAbs demonstrate breadth by their ability to completely block diverse viral variants (represented by color differences in env).
aNAb, autologous neutralizing antibody; hNAbs heterologous neutralizing antibodies.

Mechanisms of Env Escape from Antibody Response

Because env protein is exposed on the surface of the virus, it is the main target of both NAbs and nNAbs. As a result, the virus has evolved various mechanisms to evade this host mediated antibody response.

Amino acid sequence variation: Because of the critical role of the env protein in target cell infection, functionally relevant motifs such as the CD4bs have been well conserved across subtypes. These conserved regions are however, interspersed with hypervariable regions, which display an enormous amount of diversity between and within subtypes primarily to allow HIV to escape from the antibody response. Env diversity is mainly a result of point mutations due to RT, which besides being error prone, also has high recombination rates and lacks proof-reading ability. Diversity is also generated by insertions and/or deletions of nucleotides within the hypervariable regions also mediated by RT. Overall, based on amino acid sequence each env subtype differs from the other by about 30% while differences within a clade can be as high as 20% [60,61]. The sequence variability allows the virus to accumulate antibody escape mutants necessary to stay ahead of the host immune response [58,62]. Antigenic diversity within env has a huge impact on antibody reactivity and is largely responsible for the conundrum facing antibody based vaccine design efforts.

Epitope masking or hindrance: Env is one of the most heavily glycosylated proteins known with carbohydrates attached to ~25 N-linked glycosylation sites contributing ~50% of its mass [63]. The glycans are important to the virus because they act to mask conserved viral epitopes from antibodies. The virus also evolves by shifting the potential sites for glycosylation and by doing so it is able to develop resistance against autologous NAbs [57]. Furthermore, env variable loops are floppy making it difficult for antibodies to access conserved epitopes due to steric hindrance. For example, the V1/V2 of gp120 holds the virus in a conformation resistant to neutralization, a feature that is lost with deletion of this variable region [64,65].

Instability of the Env protein and spike density: The native env spike on the surface of the virus is highly unstable due to the non-covalent association between env gp120 and gp41 domains. As a result of this non-covalent interaction, gp120 readily dissociates from gp41 leading to expression of gp41 stumps on the surface of the virus

and soluable gp120 monomers. While the immune system is capable of generating binding antibodies directed at the dissociated gp120 and/or gp41 stumps and unprocessed gp160 oligomers (decoy antigens), these antibodies have limited relevance in the context of a functional env trimer on the surface of the virion [66]. On the other hand, the env glycoprotein displays a low spike density on the surface of the virus. This may result in reduced capacity of antibodies to cross-link env gp120s to increase avidity required for neutralization efficiency [67,68].

Mother-to-child transmission of HIV-1.

In the absence of intervention, vertical transmission occurs in ~30-40% of infants born to HIV infected women. This transmission can occur either in-utero, intra-partum or post-partum via breast milk. Of all the infants that become infected, in-utero and intrapartum transmission accounts for ~60 % of these cases while the rest are as a result of breast-feeding [69-72].

In-utero and intra-partum transmission of HIV-1

Maternal factors associated with in-utero and intra-partum transmission are relatively well defined and are summarized below.

Prenatal maternal virologic and immunologic status: High maternal plasma viral loads, high levels of genital viral loads and low CD4+ T cell counts are all associated with increased risk of vertical transmission [73-76,77,Garcia, 1999 #7622,78,79].

The immune composition of the placental milieu has been shown to impact transmission. There is evidence for increased HIV transmission with high levels of cytokines, which result in accumulation of activated cells in the placenta [80]. On the other hand, presence of high levels of beta-defensins, which are known to have various antiviral effects against enveloped viruses are associated with reduced transmission [81]. The physical integrity of the placenta has also been shown to be critical. Indeed, infections that perturb the integrity of the placental barrier such as chorioamnionitis result in an increased risk of transmission possibly due to an increase in the number of infected cells that traverse the placenta into the amniotic fluid [82,83].

Mothers with compromised health status due to co-infections such as in situations of pregnancy malaria and sexually transmitted diseases such as herpes simplex virus-2 also have an increase in the rate of HIV transmission [84,85].

Genetic factors: Mother-infant HLA concordance and certain HLA alleles such as A*2301 are associated with increased transmission risk [86,87]. Other genetic factors include presence of specific single necleotide polymorphisms in the HIV co-receptors (chemokine receptors), which can affect virus –chemokine interaction either directly or indirectly and thus alter transmission risk [88]. Recently, high levels of HLA-G, which is

expressed in the placenta and plays a role in immune tolerance in pregnancy have also been associated with increased transmission [89].

Obstetric factors: Prolonged rupture of membranes and invasive delivery procedures, which result in exposure of infant to infected fluids such as vaginal secretions along the birth canal contribute largely to intra-partum transmission [90].

Breast milk transmission of HIV:

Breast milk HIV transmission accounts for ~ 40% of all the infant infection cases [69-72]. There is some evidence to suggest that majority of breast milk transmissions occur in the 1st month of life [69,70,91]. Several maternal factors outlined below have been associated with breast milk transmission.

Maternal virologic and immunologic status: Breast milk from HIV infected women contains both CFV (RNA copies) and CAV (DNA copies). High levels of breast milk HIV RNA is associated with increased transmission [92-95]. In general, levels of breast milk HIV RNA are 2-3 logs lower than plasma and are highly correlated with plasma RNA viral load, which is also a correlate of breast milk transmission. A 10-fold increase in beast milk HIV RNA has been associated with a 2-fold increase in risk of transmission [91,92,95].

In addition, the levels of breast milk HIV DNA have also been associated with transmission [91,96,97]. Colostrum contains the highest concentration of cells (10^6/ml) compared to the cell numbers found in mature milk (10^4/ml) [98]. As a result, in HIV infected women the highest numbers of infected cells are also found in colostrum milk. A 10-fold increase in the levels of breast milk HIV DNA has been associated with a 3-fold increase in risk of transmission after controlling for breast milk HIV RNA, suggesting that infected breast milk cells may play a more important role in transmission via breast milk than CFV [97].

Maternal immune status defined by CD4+ counts also remains as predictor of breast milk transmission [99].

Genetics: Certain maternal HLA types such as HLA B18* have been associated with protection from breast milk transmission [100]. In addition, specific single nucleotide polymorphims in the HIV co-receptors can also modulate the risk of breast milk transmission as has been shown in some studies [101,102].

Other factors associated with breast milk transmission include presence of sub-clinical or clinical mastitis, which has been associated with increased breast milk viral loads and cell associated virus in some studies but not others [103-105].

Prevention of MTCT of HIV

One major area of success in the prevention of HIV transmission has been in the field of MTCT. This is in part because some correlates of MTCT are well defined, hence feasible prevention interventions have been forthcoming but not without challenges.

Antiretroviral (ARV) prophylaxis: As previously mentioned, high levels of maternal viral load are associated with increased risk of HIV transmission. ARV prophylaxis works by reducing viral loads in the mother and/or by providing prophylaxis to the unborn infant. Ziduvudine (AZT) was the first regimen used for prophylaxis to prevent MTCT in sub-Saharan Africa after it was shown to be effective when used for a period of 2 weeks (short course) preceding delivery in a breastfeeding cohort in Cote d'Iviore [106].

In efforts to simplify prophylactic regimens, a landmark study performed in Uganda HIVNET012 identified single dose nevirapine (NVP) administered on the onset of labor followed by a single dose to the infant within 72 hours of birth, to effectively reduce transmissions by 50% [107]. Consequently, short course AZT and/or single dose NVP became the standard of care in HIV prevention of MTCT programmes in developing countries.

Later, it became clear that these simple regimens were less effective at reducing breast milk transmission throughout the postpartum period. Moreover, randomized controlled trials (RCTs) that provided combination ART (cART) to HIV infected women showed an even greater reduction of HIV transmission (to <2%) even in the context of breastfeeding [108].

Although use of ARV prophylaxis as an intervention to prevent MTCT is very successful in reducing transmissions it comes with various limitations. Provision of ARVs are expensive therefore there is limited availability due to costs and /or poor coverage due to poor healthcare delivery systems with only a small fraction of women who qualify being able to access the services. In addition, short course interventions do

not significantly impact breast milk transmission. They also result in emergence of drug resistant strains in women and exposed infants who eventually become infected [109]. Development of drug resistance compromises the subsequent use of these regimens for treatment in affected individuals. To overcome issues related to drug resistance the most current WHO guidelines advocate for provision of cART to all women (if the mother isn't receiving treatment for her own health as yet) or infants throughout the breast-feeding period however whether most countries have the resources to implement these recommendations remains uncertain [110].

Caesarean-section: An elective C-section before the rupture of membranes reduces exposure of infant to infected fluids resulting in lower rates of vertical transmission when compared to vaginal delivery [111]. C-sections are however not readily acceptable nor affordable in regions of sub-Saharan Africa where they are most needed.

Alternative feeding methods: Breastfeeding is the norm for both HIV infected and uninfected women in resource-limited settings. To specifically tackle transmissions associated with breast-feeding, formula feeding was introduced as an option. This recommendation followed the evaluation of the magnitude of infections associated with breast-feeding reported in a meta-analysis published in 1992 and later confirmed in other studies [69,70,112]. In one such study, the Nairobi breast-feeding clinical trial, the frequency of breast milk transmission was 16.2%, which was 44 % of all transmissions in the breastfeeding women. Hence, provision of formula as an alternative to breast-feeding prevented 44% of infant infections [70].

In resource-limited settings however, formula feeding as an alternative to breast-feeding has met numerous challenges. In the Nairobi breast- feeding clinical trial in which women had access to clean water, infant mortality was similar in the 2 groups after 24 months. However, cumulative mortality at 3 and 6 weeks was higher in formula fed than breastfed infants [70,113]. Indeed more recent studies done in HIV infected breast feeding and non-breastfeeding cohorts in sub-Saharan Africa have shown that replacement feeding and early weaning are associated with significant increase in morbidity and mortality from gastroenteritis (GE) and GE associated complications [114]. Additionally, lack of breast-feeding has also been associated with stunted growth

observed in both HIV-infected and HIV-exposed uninfected infants with no benefit in overall survival [115-118]. Indeed the benefit of HIV-1 free survival in non-breastfed infants is diluted by the increased risk of morbidity and mortality due to infections during infancy [119,120]. Thus in sub-Saharan Africa, breast-feeding remains the best option for infants born to HIV-1 infected women.

In conclusion, despite tremendous progress in strategies to prevent MTCT, an estimated 370,000 new infections occurred in children in 2010. More than 90% of these children live in sub-Saharan Africa [3]. The United Nations in 2010 declared an ambitious goal to 'eliminate' MTCT of HIV-1 defined as <5% new infant infections, by the year 2015. Provision of ARVs to the mothers and their infants remains the best intervention available currently. However, it is clear that meeting this goal will be a challenge unless ARV intervention is scaled up to reach the majority of young women of reproductive age who are already HIV-1 infected. Besides, affordable new approaches such as vaccines are urgently needed to target prevention of HIV-1 new infections in this bracket of women in reproductive age, who are at the greatest risk of HIV acquisition [121,122].

The work presented here is an effort to fill the gaps in areas that could potentially lead to a breakthrough in prevention approaches, specifically vaccine development. In order to achieve this, we need a better understanding of the immune response to HIV and its impact on transmission in a natural setting. The setting of MTCT is unique because it allows us to examine among other things the potential correlates of immune protection and the characteristics of transmitted variants, both important in designing prevention strategies.

Antibodies and MTCT of HIV

HIV infected pregnant women have a robust HIV-specific antibody response. Furthermore, maternal IgG antibodies are passively transferred to the infants in-utero. Thus this setting of MTCT provides a scenario where viral exposure or transmission occurs in the presence of antibodies as would be in the case of a vaccine. This model is thus ideal in studying the role of antibodies in limiting HIV transmission.

Plasma binding and nNAbs and MTCT of HIV

As previously mentioned, there is some evidence to suggest that levels of binding antibodies can be a marker of a protective immune response. Studies looking at levels of maternal plasma binding antibody titers to specific env targets including gp41 and/or V3 loop peptides showed no association with HIV transmission in some studies [123-125]. In other studies however, levels of HIV-specific binding antibody titers were correlated with transmission. However, these results were not consistent as they varied in other studies depending on the choice of peptides used [126,127].

Other studies have looked at the levels of nNAb titers, specifically those that mediate ADCC in HIV transmitting women, their infants and non-transmitting women. In one study, there were no differences in the levels of ADCC titers in transmitting and non-transmitting women. However, infected infants born to women with high ADCC antibody titers had better clinical outcome [127]. Similarly, high ADCC antibody titers measured in infant plasma have directly been associated with better disease outcome and delayed disease progression [128,129]. Thus, although ADCC antibody titers could not distinguish between HIV transmitting and non-transmitting women, these few studies suggest that high ADCC antibody titers in the infected infant potentially impact HIV disease course.

The limitations in these studies include the fact that linear env peptides were used instead of trimeric env protein that preserves relevant and possibly protective conformational epitopes. Its possible that binding antibodies measured against linear peptides could be those directed at epitopes present on decoy antigens such as monomeric gp120 that are not relevant in the context of a trimer. Moreover, these peptides were obtained from lab-adapted strains of HIV and were not matched to relevant circulating

subtypes in populations under study given that HIV can vary by as much as 30% in sequence. Therefore additional studies employing use of trimeric env from circulating strains of relevance to populations under study would help clearly determine any relationship between HIV-specific binding and nNAbs in this context of MTCT.

Plasma NAbs and MTCT of HIV

Although HIV exists as a quasispecies, only a subset of the variants found in the mother are transmitted to the infant as characterized by low sequence diversity confirmed by pairwise diversity calculations and/or phylogenetic trees [130-135]. This severe bottleneck at transmission suggests a possible role of immune pressure in selecting for the transmitted variants.

To determine whether NAbs play a role in selection of the transmitted variants, several studies have looked at the sensitivity of maternal and infant variants against maternal plasma NAb. One such study looked at 12 mother- baby pairs infected with diverse subtypes (A, C, D and some subtype recombinants) representative of subtypes circulating in HIV endemic regions [132]. In this study, infants were HIV negative at birth but turned positive by 6 weeks post partum or later, hence transmission possibly occurred late in-utero or early via breast milk. Infant envelope variants were obtained at the first positive timepoint while maternal envelope variants were obtained around delivery, a timepoint close to the infant infection time. Although mothers harbored both neutralization resistant and sensitive variants, overall infant variants were less sensitive to maternal antibodies obtained at the timepoint closest to transmission. Similar results were also seen in other studies of subtype B in-utero infected infants and subtype C post-partum infected infants [136-138]. Taken together this data suggest that infants are susceptible to variants resistant to maternal antibodies or conversely that maternal NAbs with breadth provided infants with protection from highly sensitive variants.

One recent study that looked at 16 subtype C infected women whose infants got infected either in-utero or intra-partum reported no differences in neutralization sensitivity between maternal and infant variants [135]. Discrepancies in these results are possibly due to various identifiable differences such as HIV subtype examined, infant infection time and whether contemporaneous maternal plasma antibodies were used.

Indeed variants from different subtypes display differences in neutralization sensitivity and similarly differences have been observed between infant variants obtained from in-utero vs intra-partum transmissions [139,140]. To determine the capacity of NAbs to limit transmission, it is critical to pay attention to the study design; specifically maternal plasma antibodies present at/around the time of transmission should be tested against contemporaneous virus. The relationship between timing of maternal plasma tested in this study of subtype C infected women and infant timing of infection remains unclear.

One other way to examine the role of NAbs in MTCT is to look at differences in NAbs titers (defined by IC50s- plasma reciprocal dilution at which 50% of input virus is neutralized in an *in-vitro* neutralization assay) in transmitting and non-transmitting women. If NAbs are important one might hypothesize that non-transmitting women will have higher titers compared to transmitting women. Indeed, higher levels of plasma NAbs against autologous or heterologous virus (the latter is indicative of breadth) have been reported in transmitting as compared to non-transmitting women in some studies [137,139] but not others [135]. Again these studies cannot be directly compared given the differences in study design and the fact that they were conducted in populations infected with different subtypes (subtype B and circulating recombinant F01_AE vs C).

Infants passively acquire HIV specific IgG from their mother's in-utero. To test the hypothesis that uninfected infants acquire NAbs with higher breadth and potency compared to infected infants, a recent study from our laboratory evaluated the association between the breadth and potency of passively acquired antibodies in infants and transmission. A total of 100 infants, 32 of whom became infected after delivery were included in this study. Using a heterologous panel of viruses with diverse neutralization sensitivities, the breadth and potency displayed by these infant plasmas did not correlate with protection [141]. These results should however be interpreted with caution given that there is evidence for certain heterologous isolates being better at defining a protective antibody response than others [139,142].

Breast milk HIV-specific binding antibodies in MTCT of HIV.

HIV-specific binding antibodies can be detected in breast milk of HIV infected women [143,144]. There is some evidence to suggest that breast milk B cells locally produce these antibodies, although some antibodies could come in from the systemic circulation [145]. Studies conducted early in the epidemic looked to define the association between detection and/or levels of binding antibody and breast milk transmission. The first study conducted in Kigali Rwanda looked at breast milk samples obtained over time from 215 HIV+ women. Infant HIV infection status was determined by: AIDS defining illness, death as a result of HIV related cause or a positive serology test at 15 months of life. Because clinical and serological tests were used to define infant infection status, 32 (15%) of the infants were reported as indeterminate while 46 (21%) were defined as infected. Presence of HIV-specific antibody was determined by detection of anti p24 (gag) and/or gp120 or 160 antibodies on a preblotted nitrocellulose strip. In this study, detection of HIV-specific IgA and IgM antibodies over time was associated with protection from HIV transmission [96].

In another study, Duprat and colleagues determined the presence of HIV-specific IgA in breast milk of 63 HIV+ Kenyan women. Because infants acquire antibodies from their mothers' in-utero, infant serological testing was done at/after month 12 a time when antibodies acquired from the mother are thought to have waned. Infants were defined as HIV infected if they had a positive IgG serological test at or after their first birthday. Infants who had a negative test at 24 months were considered uninfected while infants who had no serum available for testing at 12 months and beyond were considered indeterminate. Fourteen (22%) of the women transmitted the virus to their infants, 30 (48%) were non-transmitters and 19 (30%) were termed as indeterminate. HIV-specific IgA against several HIV antigens was determined on preblotted nitrocellulose strips. In this study HIV-specific IgA was detectable in 59% of the breast milk samples tested but there was no association between detection of HIV-specific IgA and transmission possibly due to smaller sample size compared to the Rwanda study [146].

Several other factors could have influenced results from these early studies, but one main caveat is the mismatch in subtype between the antigen used for the antibody

ELISAs (subtype B) and that in circulation (non-subtype B) in the populations tested. Furthermore, these studies were conducted before nucleic acid tests for determining infant infection status were developed. As a result infant HIV-1 infection status in these early studies was determined by clinical manifestations and/or serology instead of DNA PCR, which could potentially lead to misclassification of infant infection status especially since infants passively acquire maternal IgG antibodies in-utero.

More recently, Kuhn and colleagues evaluated the presence of HIV-specific soluble IgA (sIgA) in breast milk of 26 transmitting and 64 non-transmitting Zambian women. In this study, infant HIV-1 infection status was determined by DNA PCR. Contrary to their original hypothesis, that HIV-specific sIgA is associated with protection, HIV specific sIgA was more commonly detected in transmitting (77%) compared to non-transmitting women (47%)[147]. Taken together, it remains uncertain whether detection and/or levels of breast milk HIV-specific binding antibodies can be used to predict vertical transmission.

Breast milk nNAbs and NAbs in MTCT of HIV

Until recently, little was known regarding the functional capacity of breast milk antibodies. Studies from animal models infected with SIV were the first to shed some light on the capacity of breast milk antibodies to neutralize virus. In a group of 4 rhesus macaques infected with SIVmac251, aNAbs were not detected in breast milk up to one year post infection despite a robust NAb response in plasma suggesting that overall, the magnitude of breast milk NAbs responses was lower compared to that in plasma [148].

Recent data from a group of subtype C infected, ARV exposed women confirmed the animal model results by reporting low levels of NAbs in breast milk of women from Malawi [149]. Interestingly, there was frequent detection of ADCC responses in breast milk of these same women [149]. This study however did not explore the role of BM ADCC antibodies in protection from infection. We are privileged to have samples from women enrolled in the Nairobi breast-feeding clinical trial (explained in details later) who did not receive ARV prophylaxis. We took advantage of the availability of transmission cases and asked the question whether breast milk nNAbs and NAbs are

associated with the risk of transmission and these findings are presented in chapter 2 of this thesis.

HIV broadly neutralizing monoclonal antibodies (mAbs)

Although HIV has devised various ways of evading the host immune system, the battle is not lost. As mentioned previously, ~1% of chronically infected people develop antibodies with relatively broad specificity with 'elite' neutralization activity. The reasons why broadly NAbs develop only in a subset of individuals is currently unclear. It also remains unclear whether the breadth displayed by these broadly NAbs is a result of a polyclonal set of antibodies that accrues over time and that targets multiple parts of the virus or whether it's due to highly affinity matured monoclonal antibodies that target a conserved viral epitope. Recent studies suggest it could be both with majority of the isolated broadly neutralizing monoclonal antibodies (mAbs) being responsible for the plasma breadth from which they were isolated [150,151].

Until 2009, only a few of such mAbs had been isolated including; the CD4 binding site (CD4bs) antibody IgGb12 (b12), membrane proximal external region (MPER) binding antibodies 2F5 and 4E10 and complex glycan binding antibody 2G12 [152-154]. These mAbs however, displayed limited breadth against non-subtype B HIV variants, which are of great interest because they are common in regions bearing the greatest burden of HIV disease [132,155-159]. Besides, 2F5 and 4E10 were found to be autoreactive to human protein and were thus not attractive for use in passive immunization studies [160].

However, in the recent past, several new mAbs that display great breadth and potency against non-subtype B isolates and which are non-autoreactive have been isolated. Monoclonal antibodies (mAbs) of superior breadth and potency are a powerful tool for designing effective vaccines. This is because by mapping the epitopes targeted by these elite antibodies, we can begin to appreciate vulnerable sites on the transmitted env for rational design of vaccines. In addition, they can potentially be used in passive immune prophylaxis and/or gene therapy to prevent HIV transmission.

The newly isolated mAbs include VRC01 and NIH45-46, which are clonal variants isolated from the same individual and that interact with the CD4bs in a manner

similar to the CD4 molecule [161,162]. NIH45-46 has been engineered by substituting a glycine for a tryptophan at position 54. The resulting engineered mAb NIH45-46w, displays increased interaction between the antibody and virus as a result of the tryptophan side chain inserting into a key hydrophobic pocket in the gp120. NIH45-46w portrays increased breadth and potency of >700 fold to some isolates that were originally resistant to wild type NIH45-46 [163]. Another pair of antibodies PG9 and PGT145 bind to a quaternary epitope in the V1/V2 region that is dependent on a glycan at position 160. This epitope is preferentially found on the envelope trimer and is absent in gp120 monomers [150,164,165]. Two other mAbs PGT121and PGT128 bind to an epitope in the V3 loop dependent on glycans at positions 301 and 332 [166,167]. All these mAbs neutralized over 70% of different virus panels tested representing the major circulating strains worldwide. The monoclonal antibody binding sites on env are shown in figure 1.9.

Isolation of these new mAbs with breadth and potency has rejuvenated the optimism for the development of NAb based HIV prevention strategies. The assumption is that these mAbs will be able to neutralize and hence block transmitted HIV variants with similar breadth and potency reported thus far. However, to set the stage on how to better harness the potential of these newly isolated antibodies for HIV prevention strategies, we can start by defining their neutralization profiles against transmitted variants of diverse subtypes *in-vitro* something that has not been specifically examined so far.

Table 1.1. Characteristics of mAbs relevant to this thesis.

Monoclonal Antibody	Target Site in gp120	Glycan Involvement	Refs.
IgGb12 (b12)	CD4 binding site	No	[152]
VRC01	CD4 binding site	No	[151]
NIH45-46w	CD4 binding site	No	[163]
PG9	V1/V2 loop	Yes	[150]
PGT145	V1/V2 loop	Yes	[166]
PGT121	V3 loop	Yes	[166]
PGT128	V3 loop	Yes	[166]

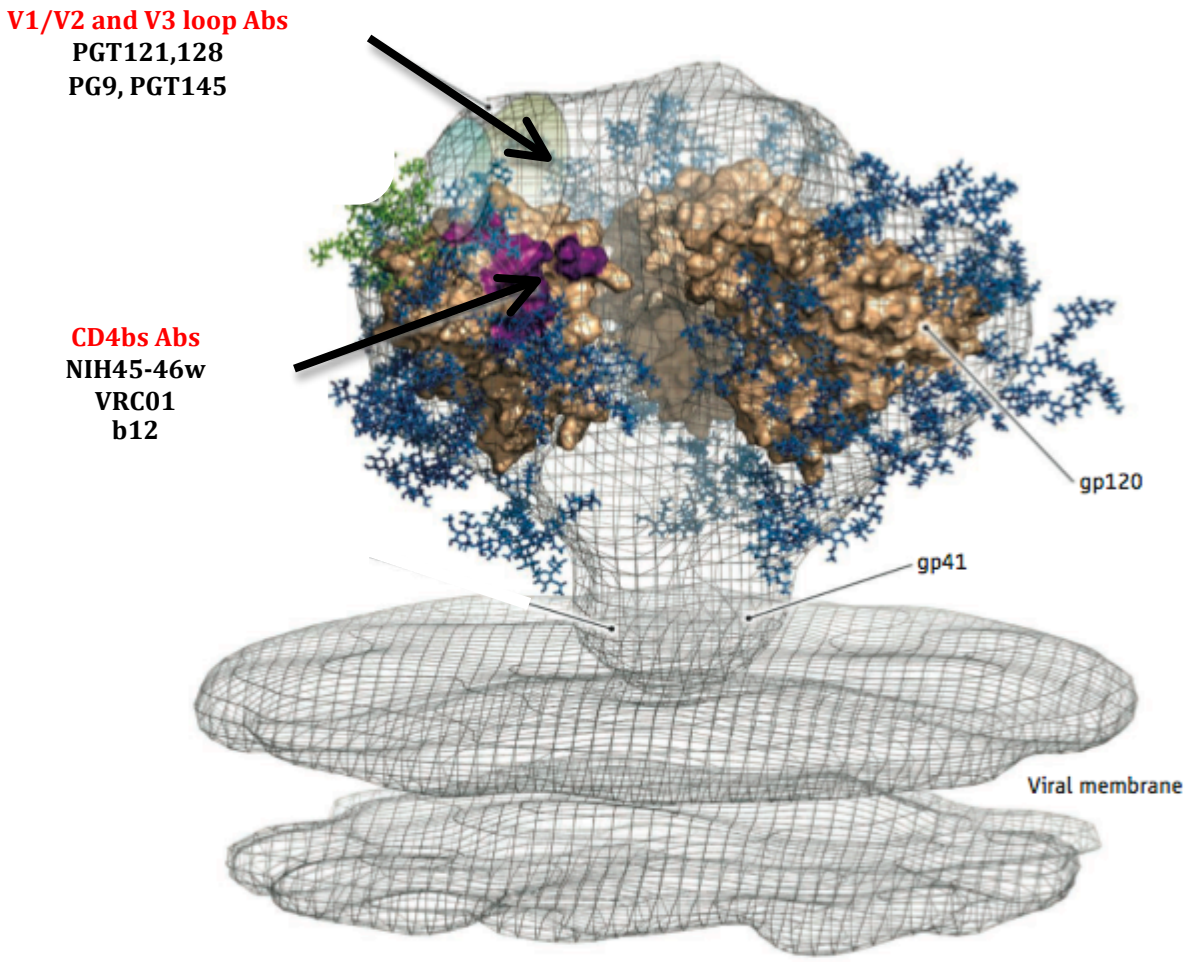

Figure 1.9: Selected HIV monoclonal antibody binding sites. The env CD4bs is shown in magenta. Schematic modified from [168]

Proof of concept for a protective role of mAbs in animal models

Passive immunization is a powerful tool for assessing correlates of protection *in-vivo*. Cumulative evidence from animal models suggests that if present at the right amounts at the time of exposure, mAbs have the capacity to prevent chimeric SIV/HIV challenge virus from establishing infection [169-177]. These early passive immunization studies however were performed in the face of some crucial limitations including; the use of high levels of NAbs which have not been observed in natural exposure –infection situations. Secondly, most studies were done with predetermined neutralization sensitive strains (matched pairs) despite the knowledge that infected individuals harbor both neutralization resistant as well as sensitive variants. To allow for infection of all control animals, animals were challenged with a high single dose of virus, which again is not representative of the multiple low dose exposures in nature.

Two of these limitations have been recently addressed simultaneously by using significantly lower amounts of antibodies 1mg/kg compared to 25mg/kg of body weight and low-dose repeated viral challenge of ~3% the traditional high dose challenge [178]. The results from this improved study were promising given that there was a significant difference between the number of exposures that were required for mAb treated animals to become infected as compared to controls suggesting that much lower titers of antibody than previously considered might be protective. This study however did not address how well this NAbs would work against strains from natural circulation.

The isolation of the new antibodies with greater breadth and potency when compared to those used in these studies, raises optimism for possible identification of vulnerable viral epitopes conserved across subtypes for use in vaccine design. By screening transmitted variants of diverse subtypes we can begin to tease out which of the specificities represented by these mAbs would be most likely to inhibit circulating HIV variants from establishing infection. This screen will also provide useful data that would be a basis of selecting mAbs that can be used in passive immunization studies in animal models to assess their ability to provide protection *in-vivo*. This is the topic presented in chapter 3.

Goals of this thesis:

The setting of MTCT provides a relevant scenario for us to investigate potential correlates of immune protection in HIV transmission including but not limited to antibodies. This is because we know the index person (infected mother) and the exposed person (infant) and with samples collected at close intervals, we can closely define the timing of transmission. By looking at the immune responses in the mother and infant around the time of transmission we can begin to decipher the potential immune correlates of protection. Two thirds of infants continuously exposed to infected breast milk do not become infected. Breast milk from HIV-1 infected women contains HIV-specific antibodies whose functions are only currently being explored. The presence of HIV-specific NAb and nNAbs in breast milk has only recently been shown [149]. Chapter 2 of this thesis will address the question whether breast milk NAbs and nNAbs play a protective role during MTCT via breast milk.

NAbs are a correlate of protection in many existing successful vaccines such as yellow fever, measles, small pox [179-181]. In the case of HIV the potential of NAbs is evidenced by complete protection from challenge virus in animals passively immunized with mAbs. Additionally, in a natural setting of MTCT, escape variants are more likely to be transmitted suggesting that maternal aNAbs can limit variants they recognize from possible transmission or conversely that mainly aNAb escape variants are capable of establishing infection. Given these observations, developing a NAbs based vaccine remains a high priority in the HIV field. Transmitted variants possess the viral characteristics that a NAb will have to conquer to offer protection. By screening transmitted variants against the new mAbs we can determine those antibodies with broad neutralization coverage. This information will shed a light on which epitopes should be considered in the rational design of immunogens or for other NAb based approaches to prevention such as passive immunization. In chapter 3 of this thesis the neutralization sensitivity of non-subtype B transmitted and circulating variants of diverse subtypes obtained from a well-characterized mother-baby cohort will be determined against the new generation of mAbs.

Chapter 2

HIV-Specific Antibodies Capable of ADCC are Common in Breastmilk and are Associated with Reduced Risk of Transmission in Women with High Viral Loads

Introduction:

Breast milk (BM) can be a vehicle for transmission of various pathogens, but the risk of infant infection is balanced by the potential clinical benefit of BM, which provides significant passive immunity and protection against many infectious agents [182-185]. In the case of HIV-1, exposure to virus through breastfeeding accounts for almost half of the 30-40% of vertical transmissions that occur in untreated, breastfed infants of HIV-1 positive women [69,70,72]. Replacement feeding, avoidance of breastfeeding and reduced BM exposure by early weaning can significantly reduce BM transmission, however, these interventions have been associated with significant increase in infant morbidity and mortality [114,117,186-189]. Additionally, HIV-1 infected as well as exposed uninfected infants who do not breast feed have been shown to exhibit stunted growth [116,118]. These observations highlight the challenges facing HIV-1 infected women in sub- Saharan Africa where prolonged breastfeeding could lead to HIV-1 transmission but no breast feeding could increase the risk of morbidity and mortality resulting in a diluted benefit of HIV-1 free survival [120,190,191]. Consequently, greater understanding of BM protective factors in HIV-1 infection may open promising new ways to make breastfeeding safe for infants born to HIV-1 infected women.

Approximately 15-20% of infants born to all HIV-1+ mothers in chronic infection acquire HIV-1 through BM [70,72,192,193]. This relatively low infection rate despite continued exposure suggests that either BM infectivity is low or that antiviral factors in BM may play a role in modulating transmission and/or acquisition of HIV-1 via the oral mucosa. Indeed, antiviral innate immune factors present in BM such as alpha defensins, bile salt-stimulated lipase, lactoferrin, and mucins have all been associated with modulating the risk of BM transmission [194-196]. BM is also composed of both innate and activated adaptive immune cells, presumably derived from other mucosal sites such as the gut associated lymphoid tissue. Indeed, HIV-1 specific CD8 T cells and B cells

have been reported in BM [145,197,198], but to date there have been no published studies that have explored the association between the functional immune responses in BM and risk of HIV-1 transmission through breastfeeding.

Vertical transmission, including BM transmission, is characterized by a transmission bottleneck [130-132,136-138,199-205]. In mother- to-child transmission (MTCT), it has been suggested that this bottleneck is in part a result of selection pressure from NAbs because the viruses that are transmitted tend to be relatively insensitive to neutralization by maternal autologous antibodies (Abs), even in mothers who harbor viruses with a range of neutralization sensitivities [132,137]. Consistent with the hypothesis that adaptive immunity plays a role in MTCT, several studies comparing levels of maternal plasma neutralizing antibody (NAb) titers reported that transmitting (T) mothers have lower levels of NAb in plasma compared to non-transmitting (NT) mothers [136,137,139,142,204,206] suggesting that maternal NAb may contribute to protection of the infant. However, the results of these studies are not consistent, particularly with respect to a role for NAb in protection by different routes of transmission [135,207,208]. Moreover, a recent study of passive Abs in 100 HIV-1 exposed infants did not find evidence for a protective effect of broadly NAb on infant infection [141].

Until recently, most studies of BM HIV-1 Abs focused primarily on determining the association between the levels or presence of binding Abs to env proteins and transmission. Several studies that have focused on BM IgG and IgA have showed no association between levels of these antibodies and transmission [146,209]. Notably, infant infection status in these early studies was determined by serology and/or clinical manifestation of AIDS, a situation that could result in misclassification of infant infection status. A more recent study that determined infant infection by DNA PCR showed increased levels of BM IgA in T compared to NT women suggesting that, rather than providing protection, BM HIV-1 env specific soluable IgA, is associated with increased risk of transmission [147]. However, all these studies used subtype B env proteins, in some cases from lab adapted viruses to detect HIV-1 binding Abs despite being conducted in sub-Saharan Africa where such variants are not typical of transmitted strains

of HIV-1[49,[210]. Taken together, the results from BM binding studies have not provided clear evidence of a role of BM Abs in vertical transmission.

BM Abs could provide benefit by directly neutralizing the virus within the milk or by non-neutralizing mechanisms such as antibody dependent cellular cytotoxicity (ADCC) that target infected cells. This could result in reduced levels of infectious cell-free virus and BM infected cells, which are both correlates of BM transmission [95,97,211,212]. The potential of Abs in BM to neutralize HIV-1 and/or mediate ADCC has only very recently been examined, and in this study of ARV-exposed, subtype C-infected women in Malawi, NAbs were detected in about half of the BM samples while ADCC activity was present in all BM samples obtained at 1 month after delivery [149]. There have been no studies to-date looking at BMS samples obtained from untreated T and NT women, particularly in colostrum and early milk, which is relevant given that virus levels are highest in colostrum [95] and the majority of BM transmissions occur early in life [70,193]. There has also been no study looking at how these BM Abs function in relation to MTCT.

We evaluated neutralizing, binding and ADCC activity in BMS or BMS-derived IgG and IgA and matched plasma from antiretroviral (ARV) naïve T and NT mothers with high plasma viral loads and systemic NAbs. Our data shows that BM NAbs are rare and their levels are significantly lower than in plasma. However, we report a high frequency of ADCC activity in BMS that was significantly higher in NT women compared to T women. These data suggest that BM ADCC mediating Abs but not NAbs may play a role in modulating HIV-1 transmission.

Materials and methods

Study subjects and sample collection

Between 1992-1998 pregnant women attending 4 city council clinics in Nairobi, Kenya were offered counseling and serological testing for HIV-1. Interested HIV+ women were further counseled on MTCT, risks and benefits of breast feeding and formula feeding and the structure of randomized clinical trials (RCTs). At enrollment women obtained standard physical examination including a pelvic examination to screen

for genital tract infections. 15 ml of blood was obtained to determine baseline CD4+ and CD8+ counts and plasma viral RNA levels.

The primary objective of the study was to define the magnitude of HIV-1 transmission via breast milk. Hence at 32 weeks women were randomized to either breast-feeding (n=213) or formula feeding arm (n=212). Free dried formula milk was provided to women in the formula feeding arm and they were taught how to prepare the milk and feed the infant. Fifteen ml of maternal blood, 25 ml of breast milk, 5 ml of infant blood and 5 drops of infant blood on filter paper were collected within the first week post-delivery, at 6 weeks, 14 weeks, 6 months and quarterly thereafter until 2 years. Infant HIV-1 status was determined using DNA PCR here in Seattle.

Breast milk was obtained by manual expression from both breasts into a 50ml falcon tube. Breast milk samples were centrifuged to remove the lipid layer and the supernatant was stored at -70°C before being shipped either on dry ice or in liquid nitrogen to Seattle, Washington for long term storage at -70°C until use. Plasma and BM viral loads were determined using the Gen-Probe HIV-1 RNA assay (Gen-Probe, La Jolla, Calf) [95,213]. The ethical review committees of the University of Nairobi, the University of Washington and the Fred Hutchinson Cancer Research Center approved this study and the Kenyan ministry of health gave permission for the original study to be conducted.

Generation of HIV-1 Env genes and corresponding pseudoviruses

HIV-1 envelopes present in the lab were used in this study. These clones were cloned directly from either, uncultured peripheral blood mononuclear cells (PBMCs) or cDNA obtained from plasma by amplifying HIV-1 env genes by nested PCR as described previously [132].

For this study breast milk clones were amplified from archived breast milk DNA samples extracted by Christine Rousseau [53]. Env sequences were obtained by nested PCR amplification of ~3000bp. Primers used to generate new clones include primer pairs vpr1 (5'-GAT AGA TGG AAC AAG CCC CAG-3') forward and nef50ab (5'-AGA GCT CCC TTG TAA GTC ATT GG-3' and 5'- AGA GCT GCT TTG TAA GTC ATT GG-3') reverse mix for round 1 and vpr21a1/a2 (5'- TAA CCT AGA CGC GTG GAA

TCA CCCGGG AAG TCA GCC TAC AAC ACC TTG TA-3' and 5'- TAA CCT AGA CGC GTG GAA TCA CCCGGG AAG CCG GCC TAC AAC ACC TTG TA-3') forward mix and nef60a1/a2 (5'- CTT GTG GCG GCC GCA TGT TTA TCT AAA TCT CGA GAT ACT GCT CCT ACT CCT GGT GCT G-3' and 5'- CTT GTG GCG GCC GCA TGT TTA GCT AAA TCT CGA GAT ACT GCT CCT ACT CCT GGT GCT-3') reverse mix for round 2. The first round PCR was performed in a total volume of 50ul using a standard gp160 PCR amplification protocol available in the lab. Each 50ul PCR amplification reaction contained 41.85ul water, 5ul 10X precision buffer, 0.4ul 100Mm dNTPs, 0.35ul of 1ug/ul primers, and 0.5 ul of taq precision enzyme. The thermocycling conditions were 94 x4min: 35x (60 x 3min, 68 x 4 min); 72 x 6 min.

Two ul of the first round PCR was used in the 2nd round PCR with similar conditions as above except for the differences in primers used. Five ul of the 2nd round PCR was then run on a gel to determine the presence of gp160 DNA and positive wells were PCR purified using qiagen columns to remove extra primers before restriction enzyme digestion using Mlu1 and Not1 in a final volume of 40ul (30ul PCR product, 1ul 10x NEB 3 buffer, 0.1ul 100x BSA, 0.5ul Mlu1 and 0.5ul Not1 and 7.9ul of water). The digested product was then gel isolated and the PCR amplicon was ligated at a ratio of 1:2 (vector:insert) into pCI-neo mammalian expression vector (Promega, Madison WI) at 16°C over night (O/N) (1ul vector, 2ul insert, 2ul 10x ligase buffer, 1ul T4 DNA ligase enzyme and 14ul water). The vector used has been engineered to contain the necessary Mlu1 and Not1 sites to facilitate ligation.

Two ul of the ligation product was used to transform STBL-3 cells at 37°C for an hour and then plated on LB agar plates supplemented with ampicillin over night (O/N). Individual clones were picked and expanded in 2ml LB supplemented with ampicillin O/N followed by plasmid DNA extraction using Qiagen mini-prep kit. Plasmid DNA was screened to confirm the presence of the correct insert by digesting using Mlu1 and Not1, followed by gel electrophoresis. An undergraduate student Dylan Peterson did some of this cloning.

Plasmid DNA encoding the env plasmid and a plasmid encoding an env-deficient HIV-1 subtype A proviral DNA, Q23Δenv [214], in the presence of a transfection reagent fugene were used to transfect 293T cells in a ratio of 1:2 to generate pseudotyped viral

particles, harvested 48hrs post-transfection. Pseudoviruses were also generated using envs from SIV and amphotropic murine leukemia virus (MuLV) for negative controls.

Pseudovirus infectivity;

The infectivity of the pseudoviruses generated was determined by single round infection of TZM-bl cells. Ten-fold dilutions of the neat supernatant containing pseudoviruses were incubated with 2x10e4 TZMbl cells in a 48 well plate for 48 hours. Cells were fixed using 500ul 1% formaldehyde for 5 min followed by 2 washes of 1x PBS. 200ul of 400ug/ml of bgal substrate in DMSO was then added to the cells and incubated at 37 for 55min. Cells were washed in 1X PBS and infectivity was determined by counting the number of b-gal positive cells (blue foci) under the microscope [215]

Neutralization assays

Neutralization was assessed using a single round infection of TZM-bl cells, a reporter cell line that is under the promoter of HIV-1 LTR [132]. In this assay, 500 infectious particles were incubated with 2-fold (or 5-fold see chapter 4) serial dilutions of heat inactivated plasma or BMS, purified BMS IgG or IgA fraction, FT fraction, media only or in a total volume of 50ul at 37°C for 1 hour. TZM-bl cells in 100ul of growth medium containing 30ug/ml of diethylaminoethyl-dextran were then added.

After 48 hours, neutralization was determined by measuring b-galactosidase activity present in the TZM-bl cell lysate. For each virus/antibody combination, at least two independent experiments were performed. Each experiment was performed in triplicate for plasma and BMS or duplicate for purified BMS antibody fractions and mAbs. Median inhibitory concentrations (IC50s) were defined as the reciprocal dilution of plasma, BMS, purified antibody or mAb that resulted in 50% inhibition, calculated by interpolation of the linear portion of the neutralization curve on the $\log_2$ scale as previously described [132,216]. For the purposes of analysis, in cases in which the IC50s were less than the lowest dilutions tested, the midpoint value between the lowest dilution and zero was assigned. IC50s from replicate experiments were averaged by the geometric mean. Here IC50s indicate the geometric mean IC50 estimates [217] .

Breast milk IgG Ab purification

BMS IgG was purified using NAb Protein G spin columns (Pierce, Biotech, Rockford, IL), with minimal changes to the manufactures instructions. Briefly, 250ul of heat-inactivated BMS was added to 250ul of binding buffer and the mixture was added to a protein G column followed by incubation at room temperature (RT) with end over end mixing for 30 min. Thereafter, the column was centrifuged to obtain the IgG flow through (IgG step FT) which was saved for subsequent IgA purification. The column with bound Ab was washed 3 times with 400ul of binding buffer. Bound Ab was eluted with 1ml of elution buffer (pH 2.8) and the eluate was neutralized by adding 100ul of 1M Tris.HCl (pH 8.5). Thus, the final purified IgG Ab was diluted 4-fold relative to the original BMS. The final eluted IgG and IgA was retained at a 1:4 dilution of the original BMS and this was used undiluted in further neutralization assays (Fig 2.1). Coomassie blue staining (Simply Blue, Invitrogen) (Fig 2.2) and ELISAs using Human IgG ELISA kit (E-80G) and human IgA ELISA kit (E-80A) (Immunology Consultants laboratory, Newberg, OR) were used to confirm the purity of Ab fractions.

Breast milk IgA Ab purification

BMS IgA was purified from the IgG step FT using the method outlined by Hirbod et.al with some modifications [218]. Spin columns (Thermo) were packed with 400ul of immobilized jacalin (Pierce biotech, Rockford, IL) and washed 3 times with 400ul of PBS to equilibrate. The column was then loaded with 500ul of the IgG step FT and incubated on an end over end roller for 2 hours at RT. After incubation, the column was centrifuged and a final flow through (FT- fraction lacking IgG and IgA) was collected and stored for analysis. The column was washed 3 times with PBS followed by a 3-hour incubation with 500ul of 1M Melibiose to elute bound IgA. The column was further washed with another 500ul of elution buffer to maximize recovery and bring the final dilution of purified IgA fraction to 1:4 relative to the original BMS, similar to the IgG fraction (Fig 2.1). As before, coomassie staining and ELISA were used to confirm the purity of Ab fractions (Fig 2.2).

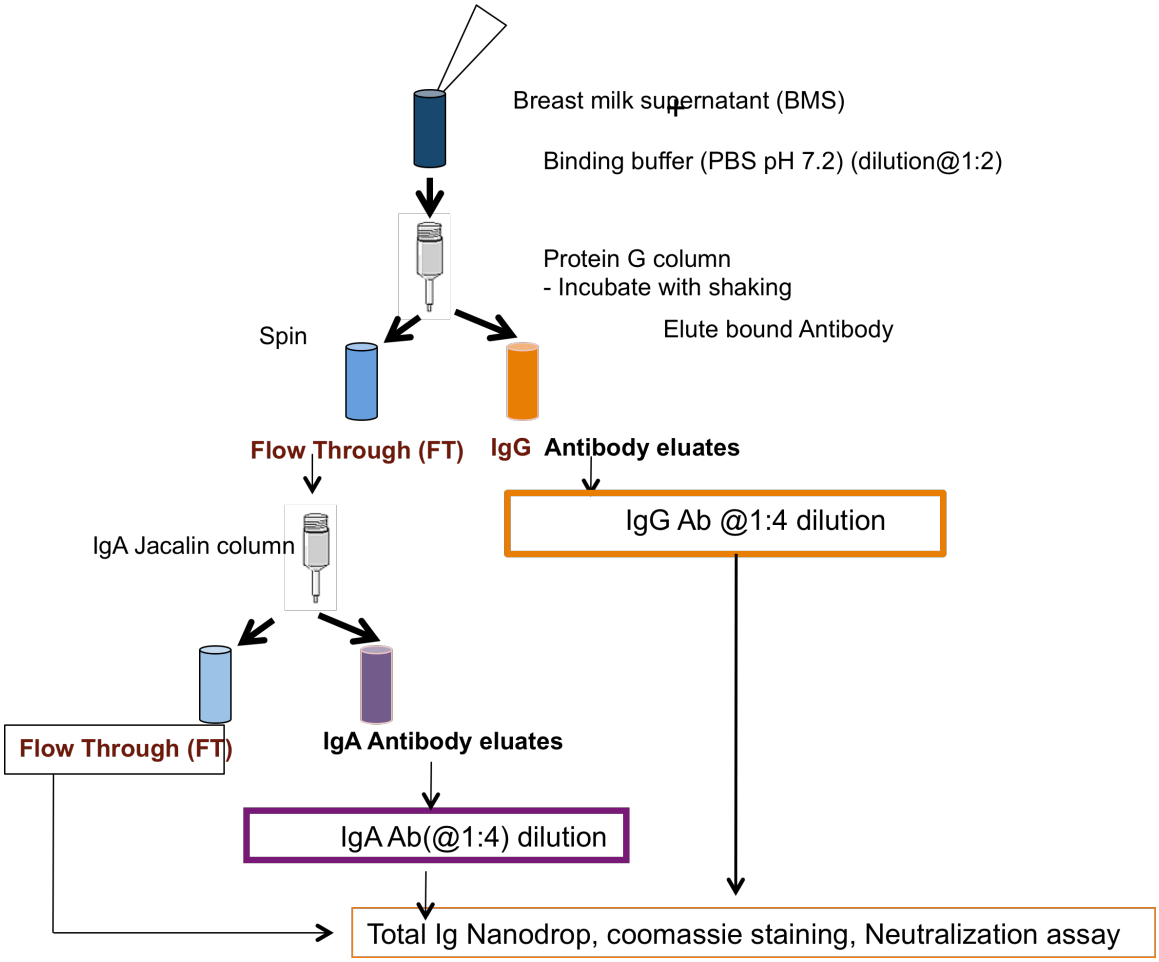

Figure 2.1. Schematic flow showing the steps in antibody purification. IgG and IgA were serially obtained from 250 ul of BMS and maintained at a 1:4 dilution relative to the original bm supernatant. Recovered fractions were used neat for the neutralization assays.

Ab, antibody

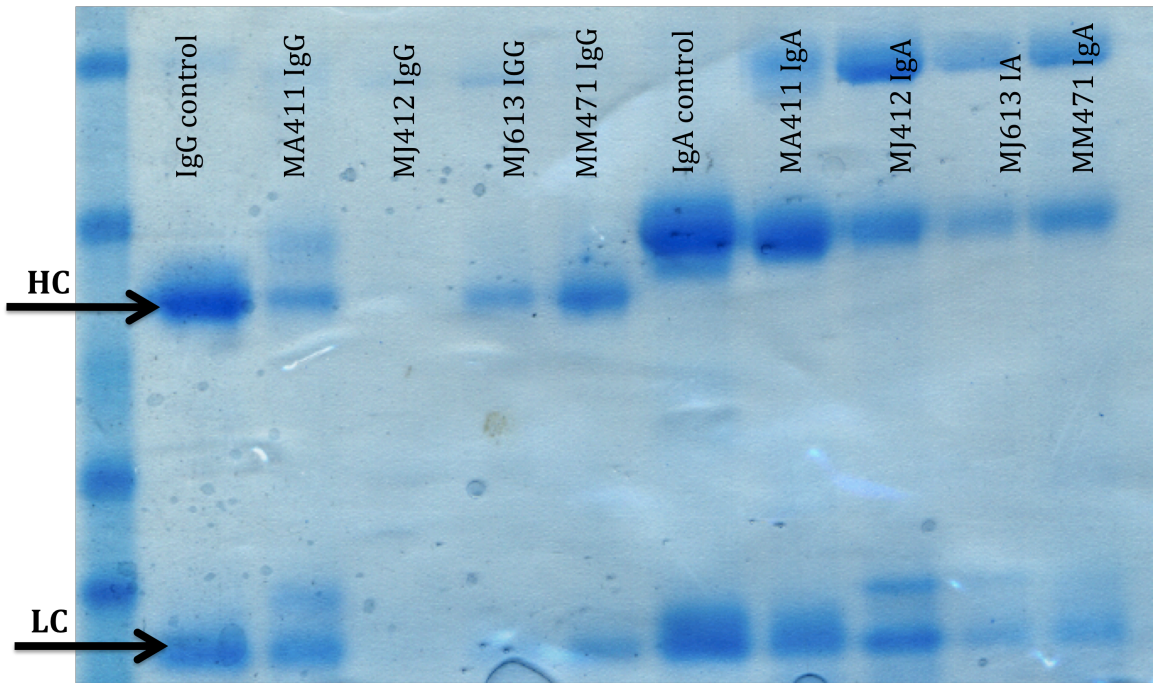

Figure 2.2. Purity of IgG and IgA fractions. Example of a coomassie stained gel, showing bands corresponding to the heavy and light chain of purified IgG and IgA. Commercially obtained human IgG and IgA (Bethyl laboratories) were used as controls. HC; Heavy chain, LC; Light chain

ELISAs for total IgG and IgA

Human IgG ELISA kit (E-80G) and human IgA ELISA kits (E-80A) (Immunology Consultants laboratory, Newberg, OR) were used to determine the levels of total IgG and IgA in un-purified BMS and plasma samples according to the manufacturers instructions.

IgG ELISA: Breast milk supernatants were diluted 1:10,00 and 1:125,000 or greater for BMS and plasma samples respectively. One hundred ul of diluted sample was incubated in duplicate in pre-assigned wells for 60 min at RT. Following incubation, the contents of the wells were discarded by gently flicking the plate into the sink. The plate was washed 4x with 400ul of wash solution.

After the last wash the plate was gently blotted on paper towels and 100ul of enzyme antibody conjugate was added to each well and incubated for 20 min at RT. Plate was then washed and blotted as described above.

100ul of TMB substrate solution was then added into each well and plate was incubated in the dark for 10min. Ten min later, the reaction was stopped by adding 100ul of stop solution (0.3M sulfuric acid) and absorbance (450 nm) was determined on an EL808 Ultra Microplate Reader (Bio-TEK Instruments.inc). The concentrations of IgG were determined by interpolation of the test value sample against a standard curve.

IgA ELISA: Breast milk supernatants were diluted 1:10,000 or 16,000 for BMS and plasma samples respectively. One hundred ul of diluted sample was incubated in duplicate in pre-assigned well for 30 min at RT. Following incubation, the contents of the wells were discarded by gently flicking the contents of the plate into the sink. The plate was washed 4x with 400ul of wash solution.

After the last wash plate was gently blotted on paper towels and 100ul of enzyme antibody conjugate was added to each well and incubated for 30 min at RT. The Plate was then washed and blotted as described above.

100ul of TMB substrate solution was then added into each well and plate was incubated in the dark for 10min. Ten min later, the reaction was stopped by adding 100ul of stop solution (0.3M sulfuric acid) and absorbance (450 nm) was determined using an EL808 Ultra Microplate Reader (Bio-TEK Instruments.inc). The concentrations of IgA were determined by interpolation of the test value sample against a standard curve.

Measurement of HIV specific IgG and IgA titers by in-house ELISA

HIV-1 env specific ELISAs were performed using the protocol outlined by Sather et.al with minimal modifications [38]. Briefly, Immulon 2HB ELISA plates were coated with 25ng/well of a HIV-1 subtype A Q461.d1 soluble trimeric gp140 (see schematic of region in Fig 1.2B) protein purified as described in [219] in 0.1 M $NaHCO_3$, pH 9.4 overnight at room temperature. Plates were blocked in phosphate buffered saline (PBS), supplemented with 10% dry milk and 0.3% Tween-20 for 1hr at 37°C. Unpurified BMS and plasma samples were diluted in 10% dry milk, 0.03% Tween in PBS. For detection

of HIV-1 env specific IgG and IgA, BMS samples were diluted at 1:100 and were titrated 2-fold up to a maximum dilution of 12,800. In cases where an end point titer could not be determined at this dilution, samples were diluted further up to a final dilution of 104,200. For HIV-1 env specific plasma IgG, samples were diluted at 1:100,000 followed by a 2-fold titration up to a maximum dilution of 12,800,000 while for IgA samples were initially diluted 1:200 followed by a 2-fold dilution up to 25,600. Samples were loaded in duplicate wells and incubated for 1hr at 37°C. Plates were washed in a plate washer and bound IgG Ab was detected at 37°C for 1hr with goat anti-human IgG- horseradish peroxidase (HRP) (Bio-Rad, Hercules, CA) diluted 1:3000 while IgA was detected by goat anti human IgA HRP (Invivogen, San Diego, CA) diluted 1:4000. Plates were developed with 50ul of 1-Step Ultra TMB-ELISA solution (Pierce Biotech, Rockford. IL) and stopped with 50 ul 1N H_2SO_4. Absorption at 450 nm was read on an EL808 Ultra Microplate Reader (Bio-TEK Instruments.inc). In this study, end point titer (EPT) was defined as the BMS or plasma reciprocal dilution at which the average OD value was greater than or equal to two times the average OD value of background.

Rapid fluorescence-antibody dependent cellular cytotoxicity assay (RF-ADCC)

The ability of BMS and their matched plasma to mediate ADCC activity was determined as described by Gomez-Roman et.al with a few modifications [220]. CEM.NKr cells, a natural killer resistant cell line obtained from AIDS Research and Reference Reagent Program, NIAID,NIH was maintained at ~2x10e6/ml in RP10 (RPMI +10% FBS, PSF and L-glutamine. For the assay, cells were double stained with a membrane dye, PKH-26 (Sigma,St. Louis,MO, USA) and a viability dye, carboxyfluorescein diacetate, succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR, USA) as recommended by the manufactures. After staining, $1x10^5$ cells were coated for 1hr at RT with 1.5ug HIV subtype A gp120 protein obtained from an infant in the Nairobi cohort at 6 weeks post-infection (BL035)[132]. Coated cells were then washed once and resuspended in 1ml of RPMI with 10%FBS.

Five thousand coated or uncoated CEM.NKr cells were added to the appropriate duplicate wells containing 100ul of 1:100 or 1:1000 heat inactivated BMS or plasma respectively. Similar experiments were performed using media only or HIVIgG (NIH

AIDS Research, Germantown, MD, USA) as negative and positive controls, respectively. The antibody-target cell mixture was incubated at RT for 10 min to allow the antibody to interact with the antigen on the surface of target cells. Following incubation, 50ul of effector cells (HIV negative donor PBMCs) were added to the mixture at an effector to target cell (*E/T)* ratio of 50:1 and incubated for 4 hours at 37°C. For all 19 BMS and plasma samples, PBMCs from the same donor were used in parallel assays. Cells were then washed and fixed in 150ul of 1% paraformaldehyde-PBS and stored at 4°C overnight. Fixed cells were analyzed within 24 hours of the ADCC assay using a BD LSRII instrument (Becton Dickinson, San Jose, CA, USA). Flow cytometry data was analyzed using Flojo version 9.4.6 (Tree Star Inc, Ashland, OR, USA). First, we used the uncoated cells media only wells (negative wells) to define CEM.NKr cells by forward and side scatter. From this subset of cells, we gated for PKH-26+ cells and determined the population of PKH+ cells that had lost CFSE by looking at PKH-26 (x-axis) and CFSE (y-axis). This gating from the negative well was then applied to all samples assayed on the same day. ADCC percent killing was defined as the percentage of membrane labeled cells ($PKH\text{-}26^{+}$) that had lost their viability dye ($CFSE^{-}$) after subtracting two times the level of killing in the media only wells (background), as described in (67).

Preparation of PBMCs :

Uninfected donor PBMCs were isolated from Pall filters obtained from the Puget Sound Blood Center using the ficoll gradient method and frozen in liquid nitrogen for long-term storage. PBMCs were thawed using the quick thaw method ~12hours before the set-up day and left to rest O/N at 37°C. The next day cells were washed, counted and resuspended at 5x10e6 cells/ml.

Statistical analysis

Odds ratios (OR) for assessment of associations between detection of HIV-1 specific and non-specific activity in BMS and transmission were estimated by Fisher's Exact Test. IC50s for HIV positive and HIV negative controls were compared by one-sided t-test on the $\log_2$ scale. All comparisons of Ab total concentrations and HIV-1 env specific titers were based on paired t-tests on the $\log_{10}$ scale, noting that differences on the log scale were approximately normally distributed, and corresponding multivariate adjustments were by linear regression. HIV-1 specific titers among those with detected virus neutralization by BMS IgG and IgA were each compared to titers among those with undetected neutralization using Welch's t-test on the $\log_{10}$ scale. All correlations were measured by Pearson's product moment correlation coefficient (PPMCC), denoted r, with p-values based on the Student's t approximation for the distribution of the corresponding standardized test statistic. The relationship between maternal clinical correlates and BMS Ab neutralization, HIV-env specific binding titers and ADCC activity were each individually assessed by Welch's t-test with corresponding adjusted estimates by linear regression. Statistical analysis was performed using R 2.13 ISBN 3-900051-07-0 and STATA version 11 edition, (College Station, TX) and was done by Katie Odem- Davis.

Results.

Characteristics of women in the study

The goal of this study was to determine the presence and functional capacity of BM HIV-specific antibodies and to determine if they impact MTCT. Therefore, we selected women who had high plasma viral loads (greater than the cohort median of 4.6 $\log_{10}$) and thus were at increased risk of transmission. Among these women, we identified those who exhibited potent plasma NAb responses (Majiwa and Overbaugh, unpublished data) to maximize the chances of detecting BM NAbs. From this subset of women, we selected those that breast-fed for greater than 3 months to capture cases of BM HIV exposure to the infant. Women whose infants were HIV-1 positive before 6 weeks of life were excluded to ensure that transmission was as a result of BM and not late in-utero, or intra-partum exposure. An additional criteria was that women had available BMS samples collected at less than 14 weeks after delivery because this early

period is the window within which the majority of BM transmissions occur [70] and protein concentrations are highest [221,222]. Nineteen women with a median CD4 count of 360 cells/uL met these criteria. The median plasma and BM viral loads were 5.22 and 2.44 $\log_{10}$ respectively, an ~ 2-log difference that was also observed in the larger cohort [95]. Nine of these women transmitted HIV-1 to their infants via BM at various time-points postpartum (Table 2.1).

Non-specific inhibition of viruses by BMS

The ability of heat inactivated BMS to neutralize virus bearing a highly sensitive env variant isolated from a Kenyan woman soon after her infection was determined. This heterologous HIV-1 subtype A env variant, Q461.d1, was chosen because >90% of plasma from individuals in the region showed detectable neutralization of this virus at a 1:100 plasma dilution [37]. The results with plasma from 4 representative women are shown in figure. 2.3A. All 4 plasma samples neutralized Q461.d1 with IC50 values of ~500 or greater. Importantly, 50% inhibitory activity was not achieved when testing plasma samples against SIV suggesting that the neutralization response was specific to HIV-1. Overall, virtually all19 plasmas displayed potent HIV-1 specific neutralization, with IC50s ranging from 185 to 3144 (Table 2.1).

We could not detect HIV-1 neutralization in any of the BMS at a similar starting dilution as plasma (1:100 data not shown). At a very low starting dilution (1:4) there was substantial non-specific inhibition of SIV and MuLV and preliminary assays suggested potential cytotoxic effect of more concentrated BMS, as reported previously [223]. BMS was therefore tested at a starting dilution of 1:20, hence 5x more concentrated compared to plasma. Results from BMS of 4 representative women against

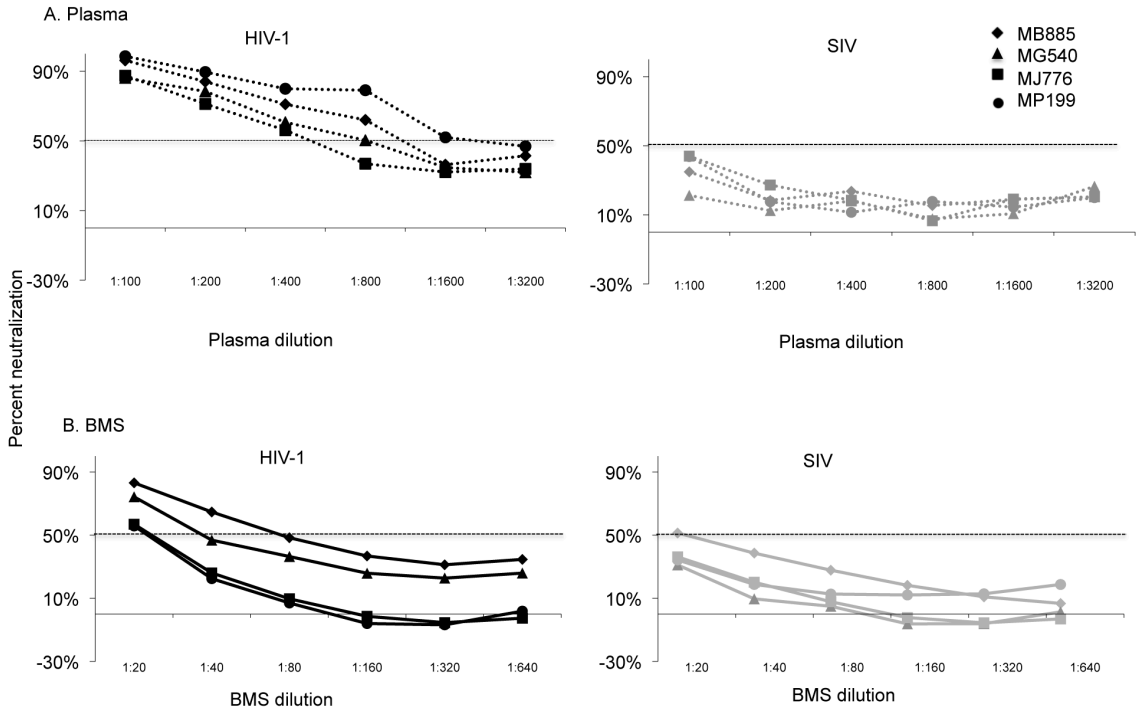

Figure 2.3: **Neutralization potency of plasma and BMS from four mothers against heterologous virus.**

The graphs show percent neutralization versus plasma (A) or BMS (B) dilution. Results using pseudovirus generated with heterologous Q461.d1 env (HIV-1 in black lines) are shown in the left graph and with SIVMneCl8 (SIV in grey lines) are shown in the right graph. The corresponding symbol for the data from each of the four mothers is shown in the upper right corner. The 50% neutralization level is shown with a dotted line. The results are from triplicate testing and are representative of at least two independent experiments. The average IC50s for the two experiments for all 19 women is reported in Table 2.1.

Table 2.1. The characteristics of transmitting and non-transmitting women in the study and the neutralization IC50s of their plasma and BMS.

								IC50s				
								Plasma[a]		BMS[b]		
	ID Number	Viral Subtype	CD4 Count	Log_{10} Plasma VL[c]	Log_{10} BMS VL[c]	Infant-Infection Wk[d]	Visit Wk[e]	HIV[f]	SIV	HIV	SIV	MLV
Transmitting Women	MB885	A	136	4.78	1.93	6	0	535	50	85	83	95
	MC046	A	255	5.05	3.17	6	0	1084	50	23	30	27
	MF520	A	511	5.59	2.73	15	1	327	50	23	25	26
	MF535	D	690	5.53	2.37	6	14	3144	50	21	10	10
	MI206	A	262	5.12	2.27	6	0	751	50	22	10	24
	MJ412	C	293	4.86	2.9	6	0	283	50	10	29	33
	MJ613	A	104	5.64	2.96	6	1	751	50	10	10	24
	MJ776	A	385	5.44	4.24	6	0	510	50	28	10	30
	MM596	nd[g]	392	5.75	2.26	6	6	469	50	10	10	10
Non-Transmitting Women	MA411	A	416	5.5	2.76	na[h]	0	1200	50	38	33	38
	MB727	C	416	4.70	2.54	na	8	314	50	10	10	10
	MB807	A	217	4.78	3.62	na	0	989	50	10	10	10
	MG540	A	285	5.6	3.95	na	0	762	50	37	27	22
	MH230	A	651	5.02	2.79	na	14	647	50	22	10	10
	MK371	D	352	4.62	bd[i]	na	2	354	50	10	10	10
	ML055	D	213	5.18	2.76	na	0	1107	50	10	10	45
	ML267	nd	551	5.68	3.77	na	0	185	50	10	10	10
	MM471	A	360	5.26	3.36	na	8	1963	50	10	10	10
	MP199	A	389	5.22	2.73	na	6	1253	50	10	10	28

[a]Plasma neutralization assays were performed at a starting dilution of 1:100; an IC50 of 50 was assigned in cases where 50% neutralization was not achieved.
[b]BMS neutralization assays were performed at a starting dilution of 1:20; an IC50 of 10 was assigned in cases where 50% neutralization was not achieved.
[c]Viral Load
[d]Indicates week since delivery when infant was first HIV-1 DNA positive.
[e]Indicates time-point after delivery at which BM sample was obtained.
[f]Q461.d1
[g]Not done
[h]Not applicable
[i]Below detection

Q461.d1 and SIV are shown in figure 2.3B. While a low level of inhibition of HIV-1 was observed with some BMS such as MJ776 and MP199, there was little difference in the magnitude of BMS neutralization of Q461.d1 and SIV in all 4 cases. Among all 19 women, 9 BMSs - 6 from T and 3 from NT women - showed HIV-1 inhibition with IC50 values ranging from 21-85; there was no detectable inhibition by BMS from 3 T and 7 NT women. BMS from the majority of women also inhibited SIV and MuLV pseudoviruses, with IC50 values ranging from 20-95 (Table 2.1). A paired comparison of BMS HIV-1 IC50s with the geometric mean of IC50s for corresponding negative control viruses (SIV and MuLV) showed that HIV-1 IC50s were not statistically greater than those of the negative controls ($p = 0.44$). This observation suggested that the majority of inhibition we observed with BMS was likely not due to HIV-1 specific Abs.

The presence of a non-specific inhibitor of HIV-1 in BMS could nonetheless be relevant to transmission risk. We thus examined the association between detection of non-specific activity and transmission and found that this relationship was not statistically significant (OR= 4.77; 95% CI: 0.51, 71.53; $p = 0.17$).

Limited ability of purified BMS IgG and IgA Abs to neutralize heterologous virus

To determine what portion of the non-specific inhibition observed with unfractionated BMS was due to Abs versus other factors, we separately purified IgG and IgA Abs from BMS for use in the neutralization assays (Figure 2.4). Bands of the expected sizes for IgG and IgA were observed in the respective purified fractions by coomassie staining as previously shown (Figure 2.2) and cross contamination between Ab isotype fractions by total Ig ELISA was below detection (data not shown). Purified Ab fractions were tested at a starting dilution of 1:8, which translated to a dilution 2.5 times higher than the most concentrated BMS we tested (1:20 dilution). Using the purified BMS IgG fractions, neutralization of greater than 50% was detected in only 2 (subjects MJ776 and MP199) of 19 purified BMS IgG tested, with IC50s of 9.4 and 9.9 respectively. (These two examples are shown in figure 2.4A and a summary of the 19 in Table 2.2). Of these women MJ776 transmitted HIV-1 to the infant while MP199 did not. In contrast, there was no detection of neutralization by purified BMS IgA fractions tested (Results from 4 representative women are shown in figure 2.4B and a summary of

the 19 in Table 2.2). Importantly, purified BMS IgG and IgA fractions did not inhibit viruses pseudotyped with SIV env including the two BMS IgG fractions from subjects MJ776 and MP199, which had detectable neutralization of virus pseudotyped with Q461.d1 env (Figure 2.3A, B, and Table 2.2). The FT fraction, which contained undetectable levels of BM IgG and IgA both by ELISA and coomassie staining, retained the non-specific activity displayed by BMS (Table 2.2).

Limited ability of purified BMS IgG and IgA Abs to neutralize autologous blood and breast milk-derived virus

To ensure that we were not missing NAb responses by using a heterologous virus, we examined the ability of BMS Ab fractions to neutralize autologous virus in a subset of the 19 women. BMS IgG and IgA Ab fractions and FT from a total of 8 women were each tested against 2 pseudoviruses bearing autologous env variants from blood [132]. Of the 8 women, 2 women both NTs, showed low potency neutralization of the blood-derived autologous virus to one of the two viruses tested. MM471 displayed low neutralization potency with an IC50 of 15 against one of her autologous viruses when using IgG but not the IgA fraction (representative experiment is shown in figure 2.5A). In contrast, MA411 displayed low neutralization potency with an IC50 of 9 against one of the autologous virus with IgA but not with IgG fractions (a representative experiment is shown in figure 2.5B). BMS IgG and IgA fractions from the remaining six women, all Ts did not neutralize their respective autologous viruses above 50%. Autologous viruses for MJ776 and MP199 were not available for testing

The ability of plasma and BMS purified Ab to neutralize variants obtained from BM was also determined for two subjects MF535 (T) and ML055 (NT). Autologous plasma from MF535 and ML055 diluted at 1:100 neutralized the respective BM viruses with IC50s of 152 and 718, respectively. In contrast, there was no detectable neutralization by BM Ab fractions against these autologous BM viruses (data not shown).

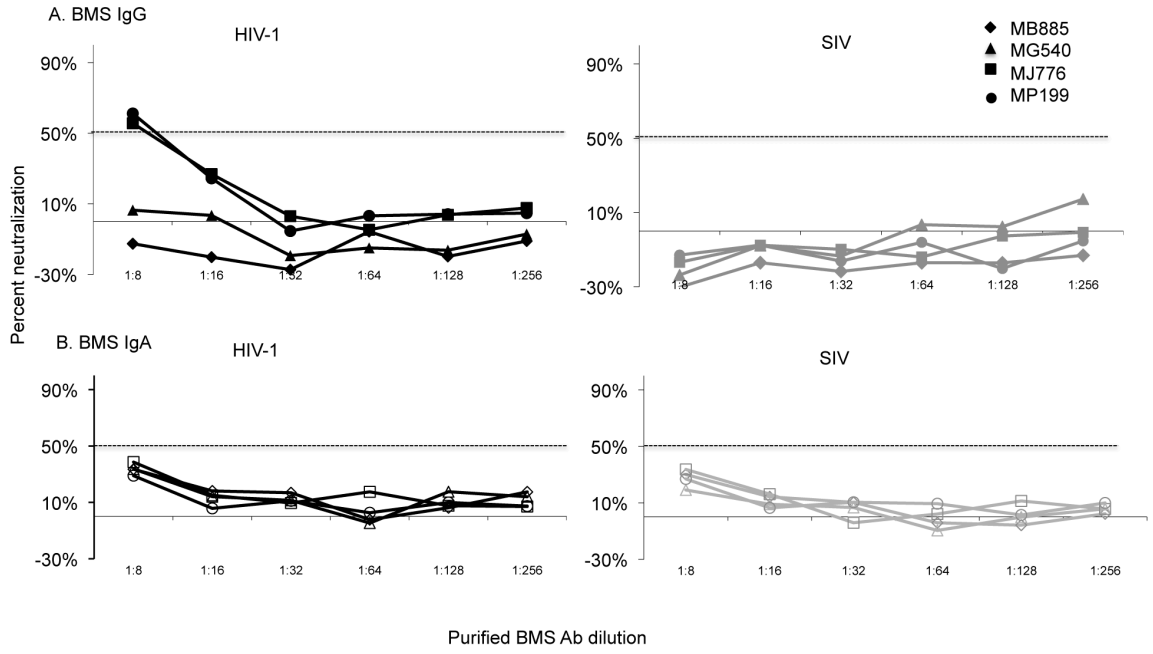

Figure 2.4: Neutralization potency of purified IgG and IgA from four mothers against heterologous virus.

The graphs show percent neutralization versus BMS purified IgG (A) or IgA (B) dilution. The corresponding symbol for the data from each of the four mothers is shown in the upper right corner. Neutralization by IgG and IgA is represented by filled and open symbols, respectively. Results using pseudovirus generated with Q461.d1 env (HIV-1 in black lines) are shown in the left graph and with SIVMneCl8 (SIV in grey lines) are shown in the right graph. The 50% neutralization level is shown with a dotted line. The results are from duplicate testing and are representative of at least two independent experiments. The average IC50s for the two experiments for all 19 women is reported in Table 2.2.

Table 2.2: Neutralization potency of purified BMS IgG, IgA and FT.

			IgG IC50[a]		IgA IC50[a]		FT[b] IC50[a]	
	ID Number	Visit Wk[c]	HIV[d]	SIV	HIV[d]	SIV	HIV[d]	SIV
Transmitting	MB885	0	<4	<4	<4	<4	**21**	**25**
Women	MC046	0	<4	<4	<4	<4	<4	**10**
	MF520	1	<4	<4	<4	<4	<4	<4
	MF535	14	<4	<4	<4	<4	<4	<4
	MI206	0	<4	<4	<4	<4	**11**	**8**
	MJ412	0	<4	<4	<4	<4	**21**	**10**
	MJ613	1	<4	<4	<4	<4	<4	<4
	MJ776	0	**9.4**	<4	<4	<4	<4	<4
	MM596	6	<4	<4	<4	<4	<4	<4
Non-Transmitting	MA411	0	<4	<4	<4	<4	**48**	**17**
Women	MB727	8	<4	<4	<4	<4	<4	<4
	MB807	0	<4	<4	<4	<4	<4	<4
	MG540	0	<4	<4	<4	<4	<4	<4
	MH820	14	<4	<4	<4	<4	**25**	**12**
	MK371	2	<4	<4	<4	<4	<4	<4
	ML055	0	<4	<4	<4	<4	<4	<4
	ML267	0	<4	<4	<4	<4	<4	<4
	MM471	8	<4	<4	<4	<4	<4	<4
	MP199	6	**9.9**	<4	<4	<4	<4	<4

[a]Purified antibody fractions neutralization assays were done at a starting dilution of 1:8; an IC50 of 4 was assigned in cases where 50% neutralization was not achieved.
[b]BMS flow through obtained after both IgG and IgA purification.
[c]Indicates time-point after delivery at which BM sample was obtained.
[d]Q461.

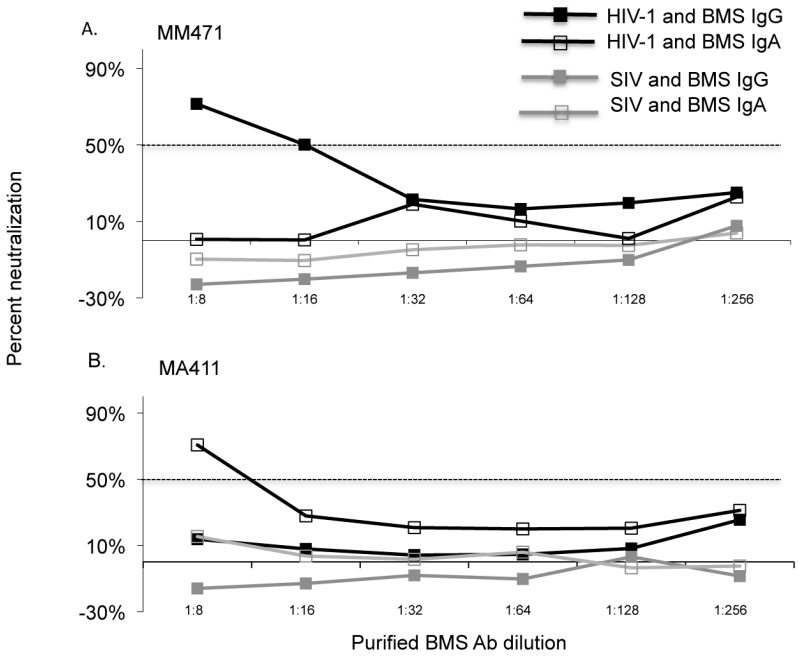

Figure 2.5: Neutralization potency of IgG and IgA from two mothers against autologous virus.

Representative graphs showing percent neutralization versus BMS purified IgG or IgA dilution. (a) Neutralization by IgG and IgA fractions from subject MM471 and (b) neutralization by IgG and IgA fractions from subject MA411. IgG (filled square) and IgA (open square) responses against pseudovirus generated with autologous HIV-1 env are shown in black lines and against SIVMneCl8 (SIV) are shown in grey lines. The 50% neutralization level is shown with a dotted line. The results are from duplicate testing and are representative of at least two independent experiments.

BMS IgG total and HIV-1 Env specific titers are lower than plasma IgG

To determine if low NAbs in BMS reflected lower total BM Ab levels, we measured the levels of total and HIV-1 env specific IgG and IgA Abs in BMS and compared them to plasma (Figure 2.6). The levels of total BMS IgG were 0.88 $\log_{10}$ lower than BMS IgA ($p < 0.0001$) (Figure 2.6A, black symbols). This is in contrast to plasma, where the IgG levels were found to be 1.02 $\log_{10}$ higher than IgA ($p < 0.0001$) (Figure. 2.6A, grey symbols). There was a pronounced difference between the magnitude of total IgG in BMS and plasma with BMS total IgG being 2.25 $\log_{10}$ lower than plasma IgG ($p <0.0001$). In contrast, the total IgA levels in plasma were only slightly higher than in BMS, with a modest 0.39 $\log_{10}$ difference between BMS and plasma ($p = 0.004$). We found statistically significant correlation between total BMS IgG and plasma IgG ($r = 0.67$; $p= 0.0034$) while the levels of BMS total IgA correlated with total plasma IgA ($r =0.78$; $p = 0.0003$). There was no significant correlation between BMS total IgG and BMS total IgA ($r = 0.39$; $p= 0.10$) (Table 2.3).

Next, we determined HIV-1 env specific IgG and IgA titers in unfractionated BMS and plasma against soluble gp140 protein derived from the subtype A variant, Q461.d1, that was used for the neutralization studies (Figure 2.6B). HIV-1 env specific IgG titers were obtained in 100% of BMS and plasma samples. In contrast, HIV-1 env specific IgA titers were obtained in 50% of BMS and 90% of plasma samples; the rest were below the cut off value for EPT as defined in this study. BMS HIV-1 env specific IgG titers were 1.96 $\log_{10}$ higher compared to env specific IgA ($p <0.0001$) (Figure. 2.6B, black symbols). Similarly, HIV-1 env specific IgG titers in plasma were higher by 3.63 $\log_{10}$ when compared to the env specific IgA titers ($p <0.0001$) (Figure 2.6B, grey symbols). Overall, similar to what we found for total IgG levels, BMS HIV-1 env specific responses were 2.22 $\log_{10}$ lower compared to that in plasma ($p <0.0001$) (Figure 2.6B). For HIV-1 env specific IgA, the $\log_{10}$ difference between BMS and plasma was 0.59 ($p = 0.0004$) (Figure 2.6B). BMS HIV-1 env-specific IgG titers were correlated with plasma HIV-1 env specific IgG titers ($r = 0.81$; $p < 0.0001$) and BMS total IgG ($r = 0.76$; $p = 0.0003$). There was no statistically significant correlation between BMS HIV-1 env specific IgG titers and BMS HIV-1 env specific IgA (Table 2.3). Similar to BMS

HIV-1 env specific IgG titers and BMS total IgG, BMS HIV-1 env specific IgA titers and BMS total IgA levels were also positively correlated ($r = 0.69$; $p = 0.015$) (Table 2.3)

We examined the relationship between the levels of HIV-1 env specific titers in BMS and detection of neutralizing activity. The three women with IgG neutralizing activity had a $\log_{10}$ IgG titer of 4.41 as compared to a mean of 3.83 among non-IgG-neutralizers ($p = 0.0001$). The one woman with IgA NAbs also had the highest IgA env specific titer, which was 1.10 $\log_{10}$ greater than the group median (Figure 2.7).

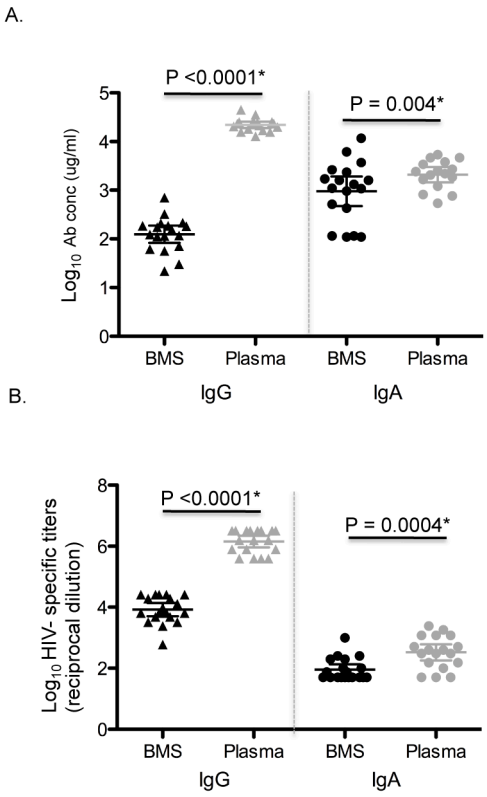

Figure 2.6: Levels of total and HIV-1 env specific IgG and IgA in unfractionated BMS and plasma. (A) Total IgG and IgA in unfractionated BMS and plasma. The Y-axis shows the $\log_{10}$ Ab conc (ug/ml) and the X-axis shows the sample type and Ab isotype. Black and grey symbols denote BMS and plasma, respectively. Triangles and circles represent IgG and IgA, respectively. (B) Unfractionated BMS and plasma HIV-1 env specific IgG and IgA titers. The Y-axis shows the $\log_{10}$ HIV-1 env specific titers (reciprocal dilution) and the X-axis shows the sample type and Ab isotype. Symbols are as described for A.

Table 2.3: Summary of the relationships between total and HIV-1 env specific IgG and IgA in BMS and Plasma.

	Difference in levels (log_{10}) [a]		Correlations[b]	
	Estimate (95% CI)	P value	r (95% CI)	P value
BMS[c] total IgG and IgA	-0.88 (-1.17, -0.60)	< 0.0001	0.39 (-0.09, 0.73)	0.1049
Plasma total IgG and IgA	1.02 (0.88, 1.17)	< 0.0001	0.42 (-0.09, 0.76)	0.1036
Plasma and BMS total IgG	2.25 (2.09, 2.40)	< 0.0001	0.67 (0.28, 0.87)	0.0034
Plasma and BMS total IgA	0.39 (0.15, 0.62)	0.0035	0.78 (0.47, 0.92)	0.0003
BMS env IgG and IgA	1.96 (1.74, 2.19)	< 0.0001	0.38 (-0.10, 0.71)	0.1133
Plasma env IgG and IgA	3.63 (3.38, 3.88)	< 0.0001	0.44 (-0.04, 0.75)	0.0688
Plasma and BMS env IgG	2.22 (2.09, 2.35)	< 0.0001	0.81 (0.55, 0.93)	< 0.0001
BMS env and total IgG	na[d]	na	0.76 (0.45, 0.90)	0.0003
Plasma and BMS env IgA	0.59 (0.31, 0.87)	0.00035	0.25 (-0.24, 0.64)	0.3117
BMS env and total IgA	na	na	0.69 (0.33, 0.88)	0.0015

[a]Difference in levels (log_{10}) indicates the average difference across the 19 women of the log_{10} levels for the first comparison measure minus the second.
[b]Correlations are described by the Pearson correlation coefficient, which indicates the strength of the linear association between the two variables, on the log_{10} scale.
[c]Breast milk supernatant
[d]Not Applicable

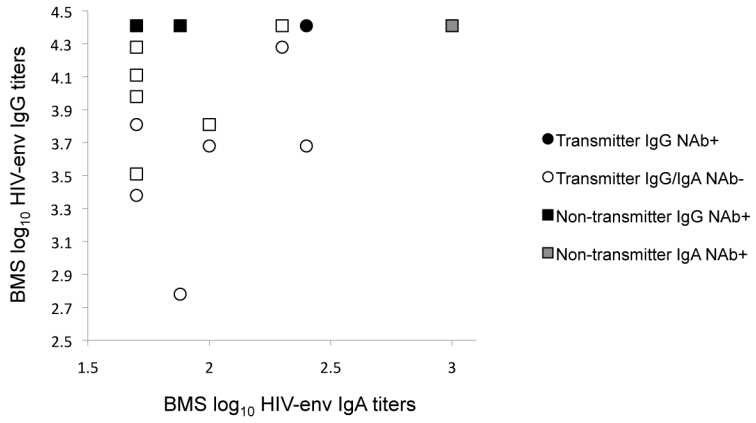

Figure 2.7: Levels of HIV-1 env specific IgG (Y-axis) and IgA (X-axis) titers and detection of NAbs in BM. Circles and squares represent transmitting and non-transmitting women, respectively. Symbols filled with black and grey correspond to detectable IgG and IgA neutralizing activity, while the open symbols denote no detection. One point might represent one or more values.

ADCC activity is common in BMS and it correlates with HIV-1 env specific IgG titers.

We determined the capacity of BMS binding antibodies and their matched plasma to mediate ADCC. The appropriate BMS and plasma dilution for the ADCC assay was determined by testing serial 10-fold dilutions of 4 representative BMS and plasma in the ADCC assay. The dilution that permitted detection of HIV-specific ADCC activity above background levels, but did not yield inhibition of ADCC activity that can occur with more concentrated samples [224] was chosen for testing (1:1000 for plasma and 1:100 for BMS) (Figures 2.8 and 2.9). Using a single dilution also allowed us to test all 19 BMS and plasma samples with effector cells obtained from a single PBMC donor, which is critical for avoiding bias due to differences in effector cell activity observed from donor to donor. Overall, ADCC activity was detected in all BMS and plasma samples tested (Figures 2.10 A and B). BMS ADCC mediated killing ranged from 1-27 % (median, 15%) while that of plasma ranged from 16-36% (median, 24%). BMS ADCC activity was correlated with gp140 env specific IgG titers ($r = 0.56$, $p = 0.014$) (Figure 2.11). A $\log_{10}$ increase in gp140 titers was associated with an absolute increase of 9.3 in % ADCC mediated killing by BMS (95% CI: 2.18, 16.41; $p = 0.013$).

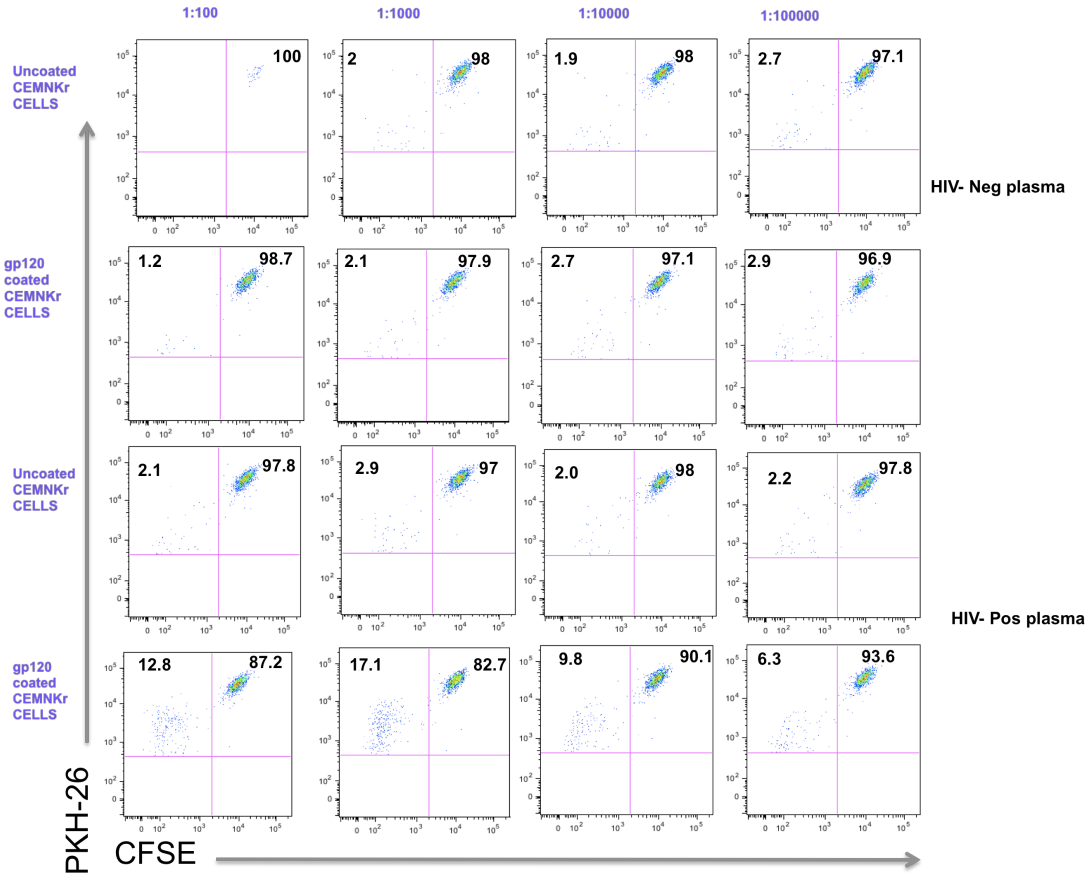

Figure 2.8: RFADCC data for HIV negative (1st 2 rows) and positive (last 2 rows) plasma controls. Samples were tested at various 10x dilutions to establish background and antibody inhibition effects. Uncoated CEM.NKr cells (negative control) and coated CEM.NKr cells were tested as indicated to the left. The X-axis represents PKH-26 membrane dye staining while the y-axis represents CFSE viability dye staining. A plasma dilution of 1:1000 was chosen for testing patient samples.

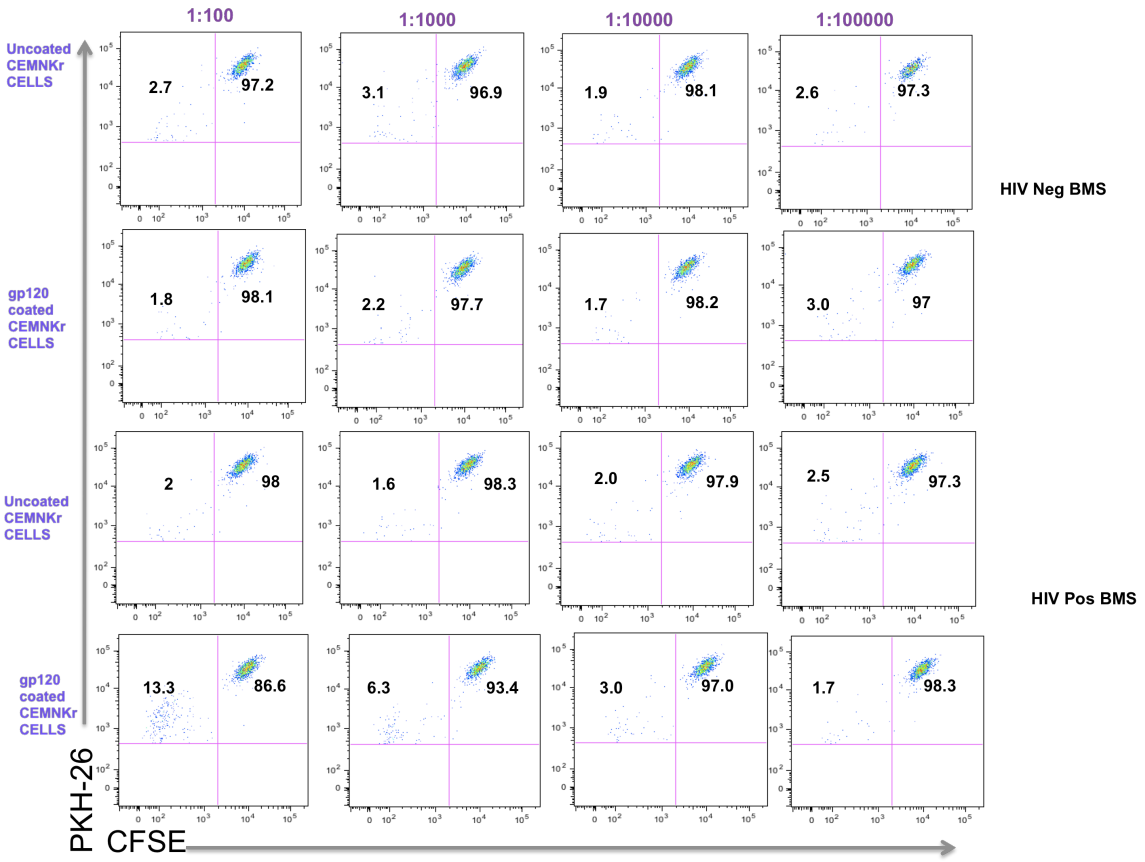

Figure 2.9: RFADCC data for HIV negative (1st 2 rows) and positive (last 2 rows) BMS controls. Samples were tested at various 10x dilutions to establish background and antibody inhibition effects. Uncoated CEM.NKr cells (negative control) and coated CEM.NKr cells were tested as indicated to the left. The X-axis represents PKH-26 membrane dye staining while the y-axis represents CFSE viability die staining.
A BMS dilution of 1:100 was chosen for testing patient samples.

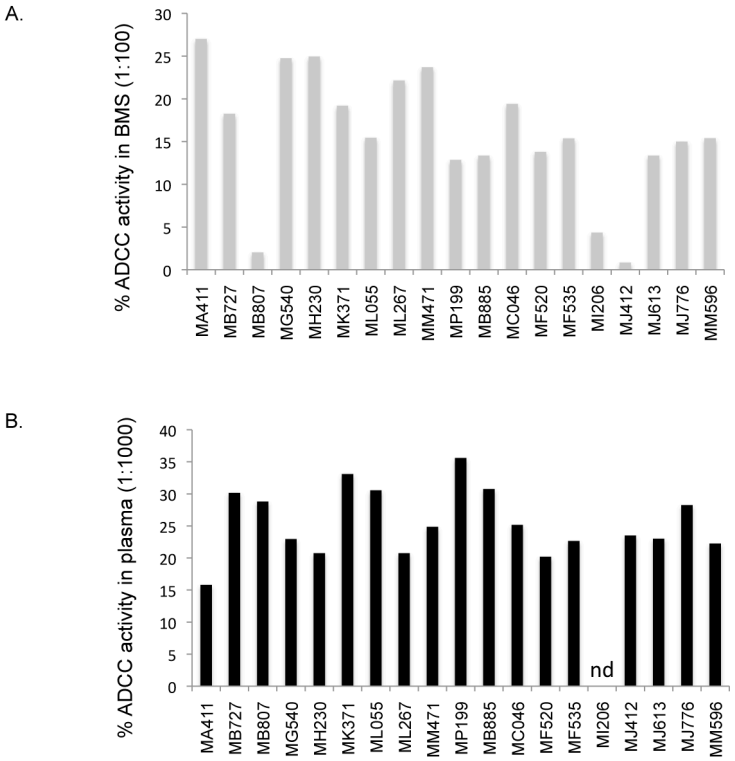

Figure 2.10: ADCC activity in BMS and plasma.

Percent ADCC activity in BMS (A) and plasma (B). BMS was tested at a 1:100 dilution and plasma at a 1:1000 dilution. The subject ID for the corresponding ADCC measure is shown below each bar. The results are from duplicate testing and are an average of at least two independent experiments each done using effector cells from a single donor. nd indicates not done

BMS ADCC activity is associated with risk of infant transmission.

The relationship between maternal clinical correlates and BMS Ab neutralization, HIV-env specific binding titers and ADCC activity were each individually assessed. There was no statistically significant association between antibody titers and any of the clinical parameters examined (Table 2.4).

There was no statistically significant association between detection of NAbs and infant infection (OR = 0.31; 95% CI: 0.0050, 4.94; p = 0.58). We observed a trend for statistical significance between infant infection and reduced BMS gp140 HIV-1 env specific IgG titers but not plasma titers (estimated mean $\log_{10}$ difference 0.35 95% CI: -0.07, 0.77; p= 0.098) in a univariate analysis (Figure 2.12A). This association was in similar direction after controlling for plasma viral load (p = 0.038). Importantly, NT women were more likely to have higher BM ADCC activity compared to T women (estimated mean % killing difference 6.89; 95% CI: 0.41, 13.37; p = 0.039) (Figure 2.12B). This relationship remained significant in a multivariate analysis controlling for plasma viral load (p = 0.011) and both plasma and BM viral load (P=0.012). There was no association between BM RNA viral load and BM ADCC activity (p=0.520) in these 19 women. There was also no significant difference between plasma ADCC in T and NT women (Figure 2.12B).

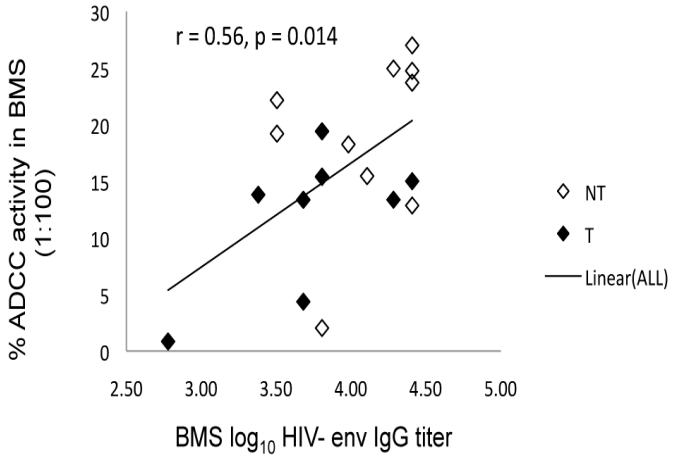

Figure 2.11: ADCC activity in relation to HIV-1 Env specific IgG titers in BMS.

The Y- axis shows % ADCC activity in BMS (1:100) and the X-axis shows the $\log_{10}$ BM HIV-1 env specific IgG titers. Filled and open symbols represent transmitting and non-transmitting women, respectively. The trend line 'Linear (ALL)' is the regression line including both transmitting and non-transmitting women.

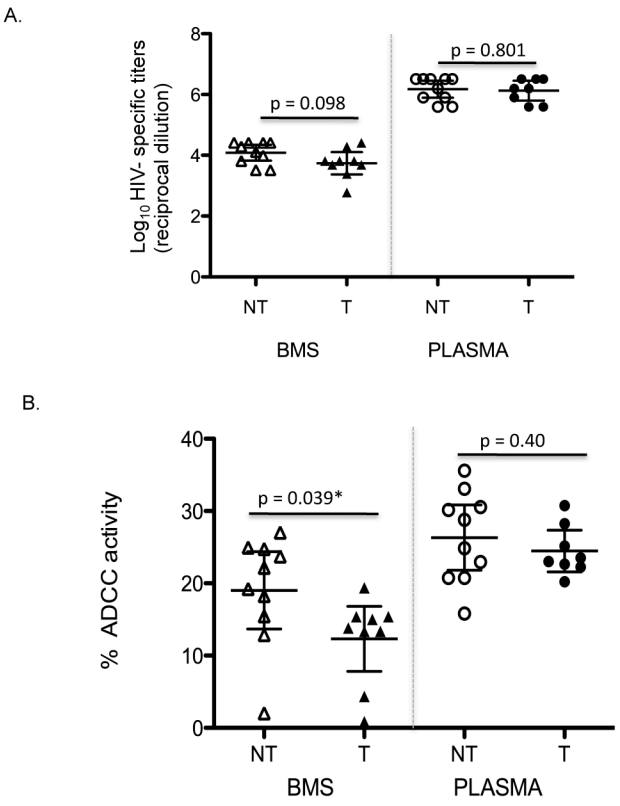

Figure 2.12: HIV-1 Env specific IgG titers and ADCC activity in BMS in relation to infant infection.

(A) Relation of HIV-1 gp140 specific IgG titers and maternal transmission. Results are from duplicate testing of unfractionated BMS and plasma and are an average of at least two independent experiments. The Y-axis shows the $\log_{10}$ HIV-1 env specific IgG titers (reciprocal dilution) and the X-axis shows the sample type (BMS versus plasma) and HIV transmission status (NT versus T). (B) BMS and plasma mediated ADCC activity in relation to transmission. The Y-axis shows the % ADCC activity and the X-axis shows the sample type and HIV transmission status. In both panels, triangles and circles represent BMS and plasma, respectively while open and filled symbols represented non-transmitting and transmitting women, respectively. Results are from duplicate testing of unfractionated BMS and plasma and are an average of at least two independent experiments.

Table 2.4: Summary of the associations for antibody levels and BM ADCC with clinical correlates of MTCT of HIV-1.

	Plasma Viral Load (log_{10})		Breastmilk Viral Load (log_{10})		CD4 Count (log_{10})	
	Estimate (95% CI)[a]	p value	Estimate (95% CI)	p value	Estimate (95% CI)	p value
Total BMS[b] IgG	0.19 (-0.30, 0.69)	0.421	0.08 (-0.20, 0.36)	0.566	0.27 (-0.59, 1.13)	0.518
Total Plasma IgG	0.08 (-0.10, 0.26)	0.355	-0.05 (-0.16, 0.05)	0.273	0.02 (-0.29, 0.34)	0.871
HIV BMS IgG	0.36 (-0.24, 0.96)	0.222	0.22 (-0.10, 0.55)	0.169	-0.05 (-1.13, 1.02)	0.916
HIV Plasma IgG	0.13 (-0.40, 0.66)	0.608	0.14 (-0.16, 0.43)	0.342	-0.43 (-1.32, 0.46)	0.319
Total BMS IgA	-0.04 (-0.91, 0.82)	0.914	0.12 (-0.36, 0.60)	0.615	-1.08 (-2.45, 0.30)	0.116
Total Plasma IgA	0.01 (-0.43, 0.44)	0.979	0.04 (-0.21, 0.28)	0.74	-0.58 (-1.29, 0.13)	0.103
HIV BMS IgA	0.24 (-0.26, 0.73)	0.326	0.13 (-0.15, 0.40)	0.335	-0.47 (-1.32, 0.37)	0.254
HIV Plasma IgA	0.31 (-0.42, 1.05)	0.377	0.21 (-0.20, 0.62)	0.295	-0.30 (-1.59, 1.00)	0.633
BMS ADCC	6.47 (-3.46, 16.39)	0.187	1.78 (-3.93, 7.49)	0.52	13.54 (-3.12, 30.19)	0.105

[a]Estimates for each clinical correlate (maternal plasma viral load, breastmilk viral load and CD4 count) correspond to the estimated 10-fold change in the correlate with a unit increase in the row-level variable. ADCC was modeled on an absolute percentage scale, while units for other variables are on the log_{10} scale.
[b]Breast milk supernatan

Discussion

The potential of HIV-1 specific Abs in BM to inhibit HIV-1 or impact transmission risk has not been well defined. Despite the fact that the levels of both IgG and IgA were low in BM compared to plasma, we observed a trend for inverse correlation between the levels of HIV-1 specific IgG and risk of infant infection in the 19 women examined here. The effect of these antibodies did not appear to be through neutralization, as only 4 of 19 women had any detectable neutralizing IgG or IgA Abs and there was no correlation between detection of NAb and risk of infant infection. Rather, the important functional activity of these antibodies was linked to ADCC activity, as there was a statistically significant inverse correlation between the levels of ADCC activity and risk of infant infection. These data suggest that antibodies capable of mediating ADCC may be one factor that impacts the risk of BM HIV-1 transmission.

We found that BM HIV-1 env-specific IgG titers were significantly higher than those of IgA but significantly lower when compared to IgG from matched plasma samples. A reduced IgA response at mucosal sites in HIV-1 infection is contrary to what is observed with mucosal responses to other pathogens but consistent with previous reports of a low HIV-1 specific binding IgA response in favor of IgG at various mucosal sites [225-228]. In general, low mucosal BM IgA might reflect an ability of HIV-1 to impair local immune responses as a means of evading the humoral immune system at the mucosal site. However, the observation that BM HIV-1 env specific IgG titers were correlated with total plasma IgG levels suggests that some of the BM IgG may originate from systemic circulation, a process that could help fight infection at the mucosal site.

Despite low HIV-specific antibody levels in BMS compared to plasma, antibodies capable of ADCC were detected in all BMS samples. We found that the capacity to mediate ADCC was associated with the levels of HIV-1 env specific IgG titers, which is in agreement with data from previous studies [51,54,149,229]. This is perhaps not surprising given that envelope binding is a required step for ADCC activity measured in the assay used here. Using purified BMS antibodies from a subset of these women, we further confirmed that ADCC activity in BM was exclusively mediated by IgG (data not shown). Thus, IgG mediated ADCC can be detected in unfractionated breastmilk, which includes IgA and other factors, as well as with purified antibody. ADCC titers have

previously been shown to be generally higher compared to NAbs titers in the same individual possibly due to the specificity required to overcome the constraints posed by env protein in a bid to escape neutralization and also the fact that virus neutralization requires that all of the functional trimers be occupied by at least one antibody [230,231]. Thus it may be possible to elicit high levels of antibodies capable of ADCC using an HIV-specific immunogen even in cases where neutralizing responses are limited.

BMS ADCC activity was significantly greater in NT compared to T women, suggesting a possible role in impacting infant infection. The mechanism by which BM ADCC might reduce transmission remains to be determined. ADCC would be expected to lead to effective clearance of infected cells. Given that the levels of HIV-infected cells in BM are correlated with transmission risk [97], it is plausible that HIV-specific ADCC responses within BM may act through reducing cell-associated viral transmission.

Other studies have implicated antibodies capable of ADCC in providing protection from infection and/or controlling an established infection. Several studies have shown that *de novo* ADCC responses to HIV and SIV infection are correlated with better viral control in chronic infection and/or clinical outcome. [50-54,232]. Vaccine-induced ADCC responses have also been correlated with reduced viral loads following SIV challenge [47,54-56,229], supporting a potential role of Fc-mediated antibody responses in blunting a new infection in SIV-infected macaques. A study by Forthal et al. also provided evidence that antibody-dependent cell-mediated virus inhibition, which is a measure of ADCC in combination with other antiviral activities, was correlated with infection rate in the Vax004 vaccine trial, although ADCC alone was not directly examined in this study[233]. In addition, studies of passive immunization using HIV monoclonal antibodies in macaques suggest that FcγR binding is required for optimal protective efficacy [169]. These findings support a potential role for antibodies that act through ADCC in providing protection from infection in the non-human primate model. The current study is the first that reports an association between HIV-specific ADCC activity and risk of HIV infection in humans.

This is the first study to examine BMS HIV-1 specific IgG and NAbs in relation to transmission risk using a relevant HIV-1 env representing recently transmitted virus from the dominant subtype in the population. This may explain our ability to detect a

trend in association between binding antibodies and transmission, which was not seen in prior studies using other env proteins less representative of viruses in the study population to measure binding [146,209].

We used the same highly neutralization sensitive (tier 1B) subtype A HIV-1 env representing the dominant subtype in the population under study to optimize our chances of detecting NAbs in BMS. Importantly, plasma from all subjects had a potent NAb response against this virus, indicating that all subjects had generated NAbs capable of specifically recognizing this test virus. Only 4 BMS had Abs that could neutralize >50% of either heterologous or autologous blood-derived viruses and the presence of HIV-1 specific NAbs was not associated with infant infection. The neutralizing activity was observed in women with higher levels of total IgG Abs in BMS. Therefore, it is possible that generally low IgG and IgA titers in BM might explain the limited neutralization capacity displayed by BM Abs.

The results of our study, showing low levels of HIV-1 env specific NAbs in BMS, are consistent with another recent study of BM HIV-1 NAbs [149]. In this study of a NVP-treated, clade C infected cohort, the levels of NAbs and HIV-1 env specific IgG were low in BM collected at 4 weeks post-delivery compared to plasma. We observed similarly low NAb levels in the breastmilk of ARV naïve women in a cohort that was enrolled prior to the availability of ARVs for prevention of MTCT [70]. Thus, collectively these studies indicate that the level of HIV-1 specific NAb are low in both early and mature milk, in both treated and untreated women and this is true no matter the infecting HIV-1 subtype.

We detected non-specific inhibition of HIV-1 and unrelated viruses (SIV, MLV) with several unfractionated BMS samples. This observation is perhaps not surprising because innate factors in BM such as defensins, lipids and lactofferin have documented activity against many viruses including enveloped retroviruses [185]. The ability of unfractionated BMS to inhibit HIV-1 in the in vitro TZM-bl assay used here did not correlate with risk of infant infection.

There are several limitations to our study, most notably the fact that we focused on a select group of women with high viral load and systemic NAbs in order to optimize our chances of detecting NAbs and to examine antibody levels in relation to transmission

risk. Thus it is unknown if these findings are applicable to women with low viral loads or low systemic NAbs levels. Interestingly, a correlation between ADCC activity and viral control in SIV- infected macaques was only observed when animals with low viral load were excluded [55]. These authors suggested that a threshold of antigen may be needed to elicit robust ADCC. Certainly, larger studies using relevant env antigens to examine HIV-1 specific BM antibody responses in other populations will be needed to verify these findings and determine if the findings apply to women with lower viral levels and/or systemic NAb responses. In addition, while we focused on breastmilk antibodies in relation to post-partum transmission, there could be some misclassification of time of infection in this study. Specifically, the cases of transmission examined here were all cases of relatively early post-partum transmission and we cannot exclude that some were the result of intrapartum transmission, where BM antibody levels would be less relevant. Finally, while we did not see an association between BM viral RNA levels in this small study, this does not rule out a relationship between ADCC and the cellular viral reservoir. Larger studies that include cell-associated virus levels and ADCC activity will be needed to clarify this issue.

In conclusion, we found that the capacity of BM to neutralize heterologous and autologous viruses obtained from blood and BM is limited. This observation can be explained in part by the low titers of Abs in BM compared to plasma in general, particularly IgG. It is unclear if such low NAb levels could play a role in protection, but no association was observed in this small study. However, the association between HIV-1 env specific IgG titers and ADCC activity with infant infection suggest that BM Ab could be playing some role in modulating infection through non-neutralizing mechanisms. To the best of our knowledge, this is the first study to report a positive association between BM transmission and ADCC capacity in BM. If these results are verified in a larger study of MTCT, then it would suggest that immunogens tailored at enhancing BM Abs capable of ADCC might be of potential benefit, particularly to HIV-1 infected women with high viral loads, who are at the greatest risk of transmission.

Chapter 3

Mother-Infant HIV-1 variants are similar in their sensitivity to broadly neutralizing antibodies but differ when grouped by subtype.

Introduction:

Neutralizing antibodies (NAbs) are a common feature of existing successful viral vaccines [179-181]. Consequently, since the discovery of human immunodeficiency virus-1 (HIV) ~3 decades ago, NAbs to this virus have been a subject of intense study. This far, NAb protection has been demonstrated in passive immunization studies of macaques challenged with a chimeric simian immunodeficiency virus (SIV)/HIV model. In these studies, pre-existing passive NAbs provided sterile immunity against parenteral, mucosal or oral viral exposure [169-177]. These studies and others were performed using viruses that were susceptible to the mAbs were passively infused, the prototype of which include IgGb12 (b12), 2G12, 4E10, 2F5 and F105 all of which were isolated early in the epidemic and demonstrate some breadth against circulating strains of HIV [132,155-159].

Recently, mAbs with improved breadth and potency have been isolated from HIV chronically infected individuals. Like b12, which has been tested in passive mAb studies, some of the new mAbs interact with the CD4 binding site (CD4bs) in a manner similar to the CD4 molecule [161,162,234]. Prototypes of CD4bs antibodies include VRC01 and NIH45-46, which are clonal variants isolated from the same individual. NIH45-46 has been further modified to increase its interaction with a hydrophobic pocket in the gp120 inner domain/bridging sheet by substituting the glycine at position 54 for a tryptophan [163]. The resulting mAb (NIH45-46w) portrays increased breadth and potency, in some cases by as much as 700-fold [163]. Another group of antibodies are those that target an epitope in the V1/V2 region. The prototypes of this group are PG9 and PGT145. These antibodies show specificity for a quaternary epitope in the V1/V2 region dependent on a highly conserved N-linked glycan at position 160. This epitope is formed on the envelope trimer but is absent in gp120 monomers [150,164,165]. Two other antibodies, PGT121 and PGT128 are examples of antibodies that target an epitope in the V3 loop.

The epitope for PGT121 and PGT128 is dependent on conserved N- linked glycans at positions 301 and 332 [166,167] (Chapter 1,Table1.1, Fig 1.9). In initial studies, all these mAbs neutralized over 70% of different HIV variants tested representing the major circulating strains worldwide [150,151,163,166,235].

Ideally, a protective NAb would block transmitted strains of HIV, which are a subset of relatively unique variants. This is because HIV transmission confers a severe bottleneck resulting in only a subset of the variants in the index person being transmitted to the exposed recipient [33,210]. This selection pressure not only drives transmission, but has also been demonstrated to have an effect on sensitivity of transmitted variants to NAbs [59,236]. In the case of vertical transmission, transmitted variants have been shown to be less sensitive to maternal autologous neutralizing antibodies (aNAbs) [132,134,137]. Other characteristics associated with vertically transmitted variants include fewer potential N-linked glycosylation sites (PNLGs) found on specific residues as shown in some studies [132,237]. In a recent study of mother- infant pairs from Rwanda infected with subtypes A and C, presence of PNLG-N339 and absence of PNLG-N295 were characteristic of infant variants [237]. Given these observations, one hypothesis could be that transmitted and circulating variants display distinct neutralization profiles defined by the frequency of PNLGs and types of glycans on their surface.

In addition to overcoming unique features in transmitted variants, a protective NAb response would have to overcome the complex genetic diversity typical of HIV. This requires a protective NAb based strategy to recognize common viral epitopes particularly those from areas worst hit by the HIV pandemic. Such regions of HIV endemicity are dominated by HIV subtypes A, C and D and more recently some emerging recombinants [3,238,239]. It is therefore critical for studies aimed at a NAb based protection to focus on transmitted and circulating diverse variants from these regions in order to determine the mAbs with appropriate protective specificity.

The discovery of HIV-specific mAbs with broad specificity provides a powerful tool to investigate multiple avenues towards NAb protection. The ideal approach would be to use the defined viral epitopes to develop immunogens that elicit antibodies of similar specificity, which can intercept genetically diverse virus soon after exposure and

hence provide sterilizing immunity. This approach however will have to overcome numerous barriers. So far, some of these impediments include the lack of knowledge on two key areas; how to stimulate the relevant necessary somatic hypermutations associated with these broad specificities and lack of a definition of the right structures that would confer maximum antigenicity and immunogenicity [168,240-242]. In the meantime, it may be possible to use these mAbs for prevention strategies by expression via gene delivery systems [243] or through passive immunization approaches [244,245]. For purposes of passive immunization, the challenge will be to identify mAbs with maximum coverage to match or exceed the current successful standard of care accorded by ARV prophylaxis used to prevent transmission in infants and adults [108]. Thus, a better understanding of the relative breadth and potency of these newly discovered mAbs against transmitted viruses and whether transmitted and chronic variants of diverse subtypes have distinct neutralization profiles will be important in considering how best to harness their potential.

We determined differences in neutralization sensitivity of env variants of diverse subtypes obtained from infants and from transmitting and non-transmitting women from a well-characterized Kenyan MTCT cohort. Sensitivity was tested against six new mAbs described above that target different epitopes on env gp120 and b12 a prototype from mAbs isolated earlier. We found that maternal and infant variants did not differ in their neutralization sensitivity to these mAbs. Similarly, variants from transmitting women did not differ in their neutralization sensitivity when compared to those from non-transmitting women. However, we observed that neutralization sensitivity of these variants was subtype-dependent with subtype A viruses being significantly more sensitive to neutralization by the CD4bs antibodies NIH45-46w and VRC01 and glycan dependent antibody PGT145 compared to non-subtype A viruses. Overall, combined neutralization profiles for NIH45-46w, PGT128 and PG9 provided the best coverage.

Materials and methods:

Study subjects and viruses.

The env variants described here were obtained from the Nairobi randomized breastfeeding clinical trial conducted between 1992-1998 [70]. Maternal env clones were

obtained from PBMC or BMC DNA, while infant envs were obtained from either PBMC DNA or plasma. Envs from 9 of the transmitting women have been previously described [132]. In addition to these 9 cases envelopes from 3 transmitters and 7 non-transmitters were provided by Majiwa M (Majiwa and Overbaugh unpublished). Five breast milk variants from two transmitters and one non-transmitter were cloned by Dylan and myself. HIV-1 env clones from the additional mother-infant transmission pairs and non-transmitting women were obtained as previously described except for differences in the primers used in some cases (See chapter 2 for primer sequences). A total of 111 variants consisting of 60 envs from 12 transmitting women, 26 envs from 7 non-transmitting women and 25 envs from 10 infants were included in this study. These included 78 variants from 10 mother- infant pairs. The ethical review committees of the University of Nairobi, the University of Washington and the Fred Hutchinson Cancer Research Center approved this study.

Neutralization assays:

Neutralization sensitivity was tested using a single cycle assay in TZM-bl target cells as described in chapter 2 (Mabuka J PLos 2012). Neutralization sensitivity was tested against mAbs: VRC01 (Wu X, Mascola, J; NIH), PG9, PGT121, PGT128 and PGT145 (IAVI Neutralizing Antibody Consortium), NIH 45-46w (Ron Diskin, Paola Marcovecchio and Pamela Bjorkman; California institute of technology, Pasadena California) and b12 (NIH AIDS Reagents). The highest concentration of antibody tested for the mAbs was 1ug/ml followed by a two-fold serial dilution to a lowest dilution of 0.033ug/ml except for b12, which was tested at 10ug/ml because it is less potent and is also available in high quantities. For viruses neutralized at <0.033ug/ml we retested them using a 5-fold dilution of 0.5ug/ml to a lowest dilution of 0.00008ug/ml. Median inhibitory concentrations (IC_{50}s) were defined as the reciprocal concentration of mAb that resulted in 50% inhibition, calculated by interpolation of the linear portion of the neutralization curve on the log base-2 scale as previously described [132,141]. At least two independent infections were performed in duplicate wells and the geometric mean of the two IC50s was used for the analysis. In cases were IC50s from independent runs showed a difference of greater than 2.5 fold, a third run was performed and the two close

values averaged. In cases where 50% neutralization wasn't achieved at the highest mAb concentration tested, an IC50 value corresponding to the highest concentration tested was assigned and this value was used in the analysis.

Statistical analysis;

Neutralization IC50 values were dichotomized, using the highest mAb concentration tested of 1ug/ml as the cut-off. Generalized estimating equation with a logit link and exchangeable correlation structure was used to analyze the data. To compare differences in sensitivities between maternal and infant variants, only data from the 10 mother-infant pairs with matched variants were used (n=78). Only maternal variants were used to compare differences in sensitivities of variants from transmitters versus non-transmitters. All maternal and infant viruses (n=111) were used to compare differences in sensitivities between subtypes. All analyses were done using R version 2.10.1 and were performed by Leslie Goo (University of Washington).

Results:

Neutralization sensitivity of mother-infant variants.

The goal of this study was to determine the neutralization profiles of maternal (circulating chronic) and infant (transmitted) variants against the newest HIV specific mAbs. We therefore screened 111 env clones from mothers and infants enrolled in the Nairobi randomized breastfeeding clinical trial (Nduati R 2000)**.** Infant envs were obtained from 10 breastfeeding infants who were HIV negative at birth, 9 of whom tested HIV positive at 6 weeks and 1 at 6 months postpartum. Maternal variants were obtained at either 32 weeks of pregnancy or at delivery [132] (Majiwa and Overbaugh Unpublished). The virus panel was comprised of envs from 3 major subtypes circulating in sub-Saharan Africa to-date (A, C and D) and some inter-subtype recombinants (C/D and D/A) Figure 3.1.

Overall, all variants were sensitive to at least two of the mAbs tested and several (n=13) were neutralized by all 6 of the new generation mAbs; however no variants were sensitive to all 7 mAbs including b12 (Figure 3.1). Each mAb except PGT145 and b12 neutralized greater than 60% of the variants tested (Figure 3.1 &3. 2). NIH45-46w, a

highly potent engineered clone of the CD4bs NIH45-46 [163] neutralized the highest fraction of viruses (88%) with a geometric mean IC50 of 0.135ug/ml**.** The glycan-dependent PGT128, neutralized 87% of the variants, and had the lowest geometric mean IC50 of 0.068ug/ml among all mAbs tested. PGT121, VRC01 and PG9 showed similar coverage neutralizing 69%, 67% and 66% of the variants with geometric IC50 means of 0.159, 0.384 and 0.264ug/ml, respectively. Surprisingly, PGT145, which shares a target site with PG9 and was previously reported to neutralize 40-80% of envs [166] , neutralized only 32% of the variants and was also the least potent of the new mAbs (geometric mean IC50 of 0.698ug/ml). Only 9% of the variants were neutralized by b12 (Figure 3.2).

There was variation in neutralization by mAbs that target the same epitope. For example, NIH45-46w neutralized 88% of the variants compared to 67% neutralized by VRC01 even though they both target CD4bs [151,163]. PGT128 and PGT121 share an epitope within the V3 loop[166] and neutralized 87% and 69% of the variants, respectively. Sixty six% of the variants were sensitive to PG9 compared to 32% sensitive to PGT145 even though they both target a similar epitope in the V1/V2 loop [166,246] (Figure 3.2).

Of note, 13 variants resistant to NIH45-46w were sensitive to PGT128 and similarly, 14 variants resistant to PGT128 were sensitive to NIH45-46w. Twelve of 14 (86%) variants resistant to PGT128 were sensitive to PG9 (Figure 3.3). In addition, 12 of 13 (92%) variants resistant to NIH45-46w were sensitive to PGT121 while 12 of 14 variants resistant to PGT128 were neutralized by VRC01 (Figure 3.1). On the other hand, all variants resistant to NIH45-46w were also resistant to VRC01. Of the 14 variants resistant to PGT128, 12 were also resistant to PGT121 while 33 of the 38 variants resistant to PG9 were resistant to PGT145 (Figure 3.4)

Figure 3.1. Summary of neutralization profiles of all mother –infant viruses against mAbs. Each row represents (from left to right) the virus name, virus subtype based on V1-V5 env sequence, the time of sample from which env was isolated and the IC50 values for all mAbs tested. IC50 values range from 0.001-1ug/ml except for b12 (2.39-10ug/ml) and are grouped by quartiles as shown in the key to the upper right. Darker shading indicates increasing mAb potency defined in the key to the upper right. White color indicates that 50% neutralization was not achieved at the highest concentration of mAb tested 1ug/ml or 10ug/ml for the case of b12. IC50s are an average of at least two independent experiments performed in duplicates. nd, Not done. (see attached pdf for figure 3.1)

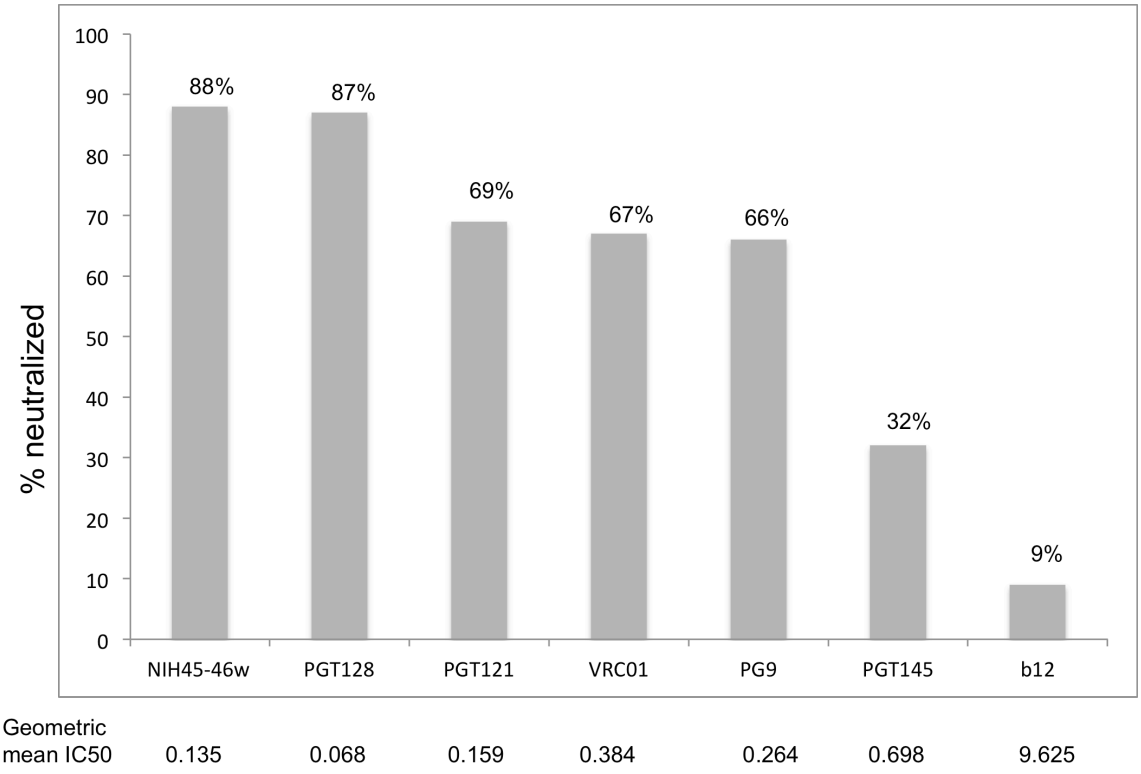

Figure 3.2. Neutralization sensitivity of all mother-infant variants to mAbs.

The Y-axis represents percentage of variants sensitive to the mAb indicated at the bottom of each bar on the X- axis. All mAbs were tested at a starting dilution of 1ug/ml except for b12 in which the starting dilution was 10ug/ml. The geometric mean IC50 for each mAb is shown at the bottom of each corresponding bar.

Note: All cases n=111 except with NIH45-46w where n=107 (4 Envs had consistently low titers that cold not be sufficient for use in the assay)

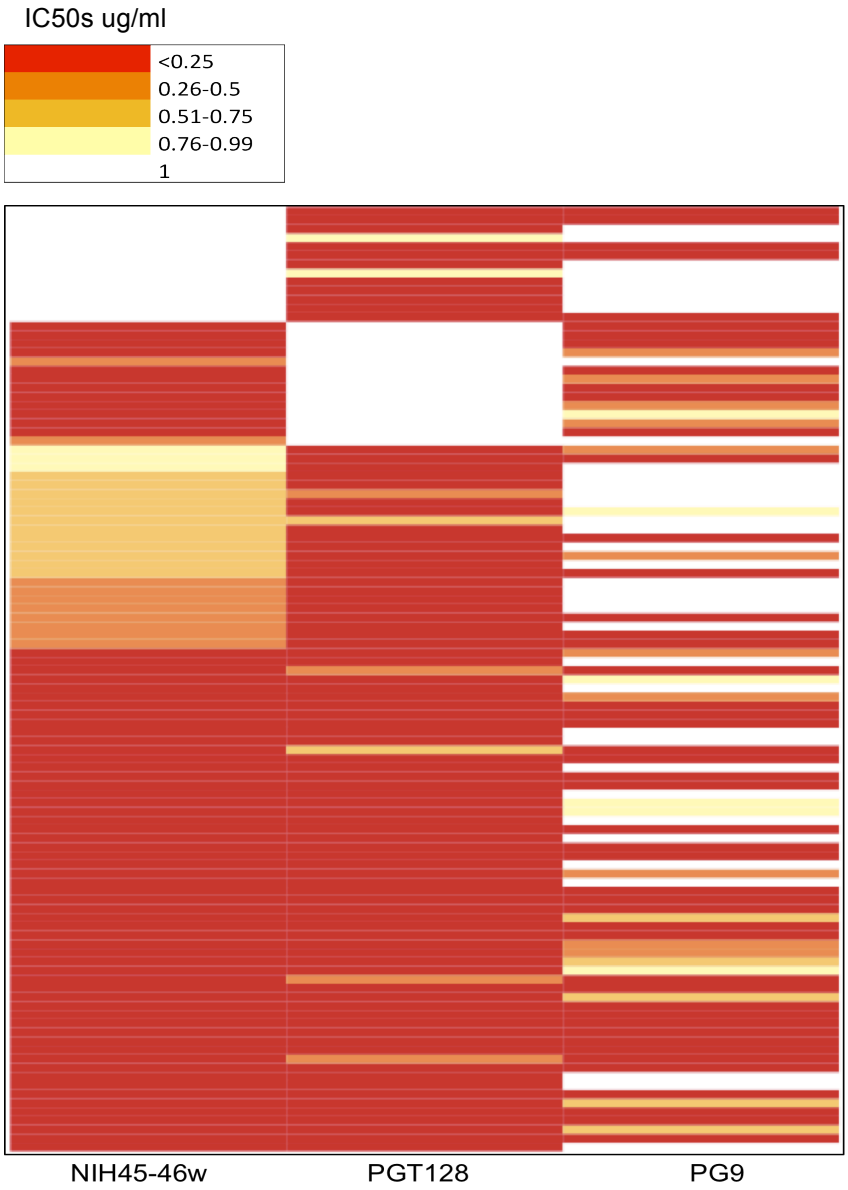

Figure 3.3. A heatmap showing sensitivity profiles of all variants to 3 mAbs with the best neutralization coverage in their class. Each row shows the IC50s of all maternal and infant variants from diverse subtypes for each of the 3 broad and most complementary mAbs arranged in columns. Darker shading indicates increasing mAb potency and white denotes variants resistant to neutralization by corresponding mAb at highest concentration tested (1ug/ml). Color key values are in the range from 0.001-1ug/ml and are grouped by quartiles as shown in the key to the upper left. Viruses resistant to NIH45-46w and PGT128 have been clustered together to emphasize the complete complementary activity of these mAbs. IC50s are an average of at least two independent experiments.

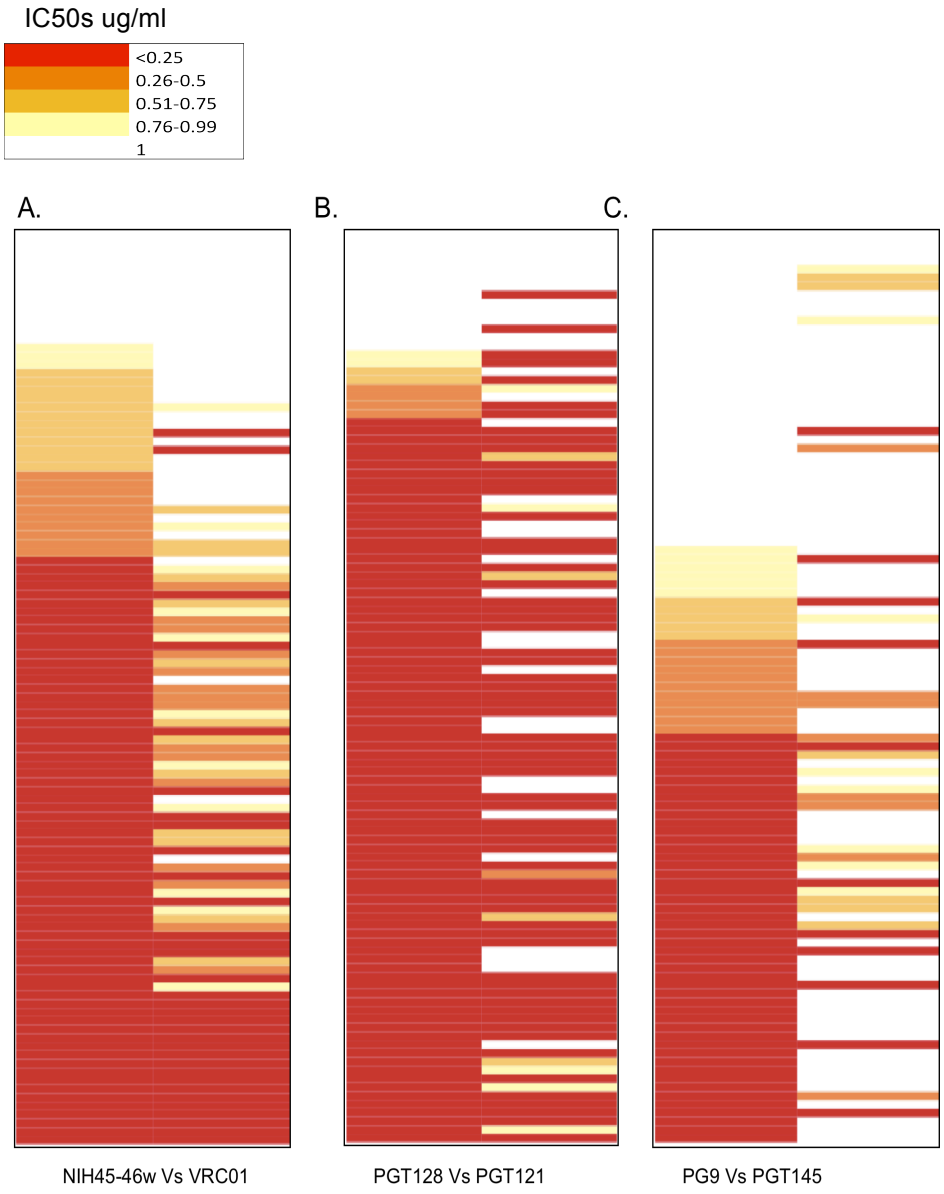

Figure 3.4 A heatmap showing sensitivity profiles of all variants to mAbs that target similar epitopes. Each row shows the IC50s from all maternal and infant variants for pairs of mAbs as indicated in panels A, B and C that target similar epitopes. Darker shading indicates increasing mAb potency and white denotes variants resistant to neutralization by corresponding mAb at highest concentration tested (1ug/ml). Color key values are in the range from 0.001-1ug/ml and are grouped by quartiles as shown in the key to the upper left. The white colored areas emphasize the non-complementary neutralization profile of these mAbs. IC50s are an average of at least two independent experiments.

Comparison between neutralization sensitivity of maternal and infant variants to mAbs.

Transmitted variants are selected for certain viral characteristics. Thus, we determined whether vertically transmitted non-subtype B envs from infants display neutralization profiles distinct from the env variants obtained from the transmitting and non-transmitting mothers. Eighty nine % of maternal and 83% of infant variants were sensitive to NIH45-46w (geometric mean IC50s; 0.16 Vs 0.069ug/ml respectively). Sensitivity of maternal and infant variants to PGT128 was very similar with 87% of the maternal and 88% of the infant variants being susceptible to neutralization (geometric mean IC50s 0.069 Vs 0.063ug/ml respectively). Sensitivity of both maternal and infant variants to all other mAbs was lower when compared to NIH45-46w and PGT128. Sixty-seven%, 64%, 62% and 31% of the maternal variants and 76%, 76%, 80% and 32% of infant variants being sensitive to PGT121, VRC01, PG9 and PGT145 respectively (geometric mean IC50s are shown in Figure 3.5). The least broad mAb was b12 neutralizing only 12% of the maternal variants and none of the infant variants (Figure 3.5). Overall, there was no consistent pattern of infants variants being more resistant to the mAbs than maternal variants.

To more formally examine differences in neutralization sensitivity between maternal and infant env variants, we compared IC50s from matched mother-infant pairs by GEE using a logit link and exchangable correlation structure. IC50 data from 78 variants comprising 53 from mothers and 25 from infants were used in this analysis. As in the analysis of all maternal verses infant variants, mAb PG9 showed the greatest difference in neutralization sensitivity between maternal (80%) and infants (57%) in a paired analysis, however, the difference did not reach statistical significance ($p = 0.13$). Overall, we did not find any statistically significant differences between neutralization sensitivity of maternal and infant variants against any of the mAbs used (Figure 3.6A).

We also determined whether there were differences in neutralization sensitivity between variants from transmitting and non-transmitting women. IC50 data from a total of 86 envs, 60 from transmitting women and 26 from non-transmitting women, were analyzed. Interestingly, in all cases except b12, the percentage of viruses neutralized was

higher in the non-transmitters compared to the transmitters, however, these differences were not statistically significant in any of the mAbs tested (Figure 3.6B).

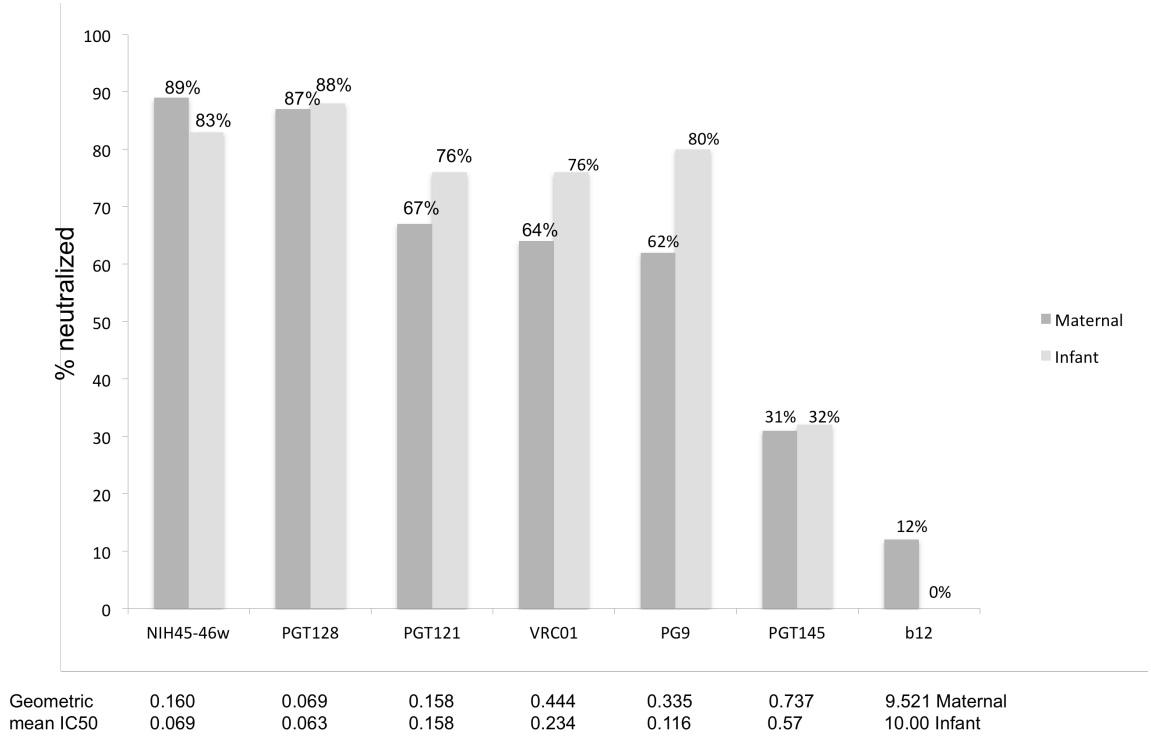

Figure 3.5. Neutralization sensitivity of all maternal and infant variants to mAbs. The Y-axis represents the percentage of maternal variants (dark grey bars) and infant variants (light grey bars) that are sensitive to each new mAb indicated at the bottom of each bar on the X-axis. All mAbs were tested at a starting dilution of 1ug/ml except for b12 in which the starting dilution was 10ug/ml. The geometric mean IC50 for each mAb is shown at the bottom of the corresponding bar.

A.

mAb	Mom % (n) n=53	Infant % (n) n=*	OR	95%CI	p-value
NIH45-46W	89% (47)	82% (18)	0.99	0.42-2.35	0.99
VRC01	66% (35)	76% (19)	1.34	0.52-3.48	0.54
PG9	57% (30)	80% (20)	2.58	0.75-8.86	0.13
PGT121	58% (31)	76% (19)	2.22	0.64-7.64	0.21
PGT128	87% (46)	88% (22)	1.42	0.27-7.41	0.68
PGT145	28% (15)	32% (8)	0.98	0.51-1.9	0.96
b12	17% (9)	8% (2)	0.46	0.03-6.79	0.57

B.

mAb	NTW % (n)	TW % (n)	OR	95%CI	p-value
NIH45-46W	96% (25)	86% (51)	0.2	0.02-1.8	0.15
VRC01	62% (16)	48% (29)	1.08	0.23-4.89	0.92
PG9	81% (21)	53% (32)	0.37	0.06-2.28	0.28
PGT121	85% (22)	43% (26)	0.39	0.08-1.95	0.25
PGT128	92% (24)	83% (50)	0.36	0.04-2.95	0.34
PGT145	42% (11)	27% (16)	0.56	0.15-2.13	0.4
b12	4% (1)	15% (9)	2.25	0.22-23.1	0.49

Figure 3.6. GEE analysis for the comparison between neutralization sensitivity of maternal and infant variants to mAbs. Comparison of neutralization IC50s for variants obtained from mother- infant pairs (A) and transmitting and non-transmitting women (B) performed by GEE using a logit link and exchangeable correlation structure. The *P* values for each mAb are shown. IC50s are an average of at least 2 independent experiments.

NTW, non-transmitting women, TW, transmitting women.

Influence of subtype on neutralization sensitivity of maternal and infant variants to mAbs.

Of the 111 envs tested here, 65 (59%) were subtype A, while 46 (41%) were non-subtype A (9 subtype C, 9 subtype D and 28 intersubtype recombinants) (Figure 3.1). These subtypes represent the major subtypes in circulation in many parts of sub-Saharan Africa [238,239]. We determined whether sensitivity of these variants to the various mAbs differed given their subtype. We found that subtype A variants were significantly more sensitive to the CD4bs antibodies NIH45-46w and VRC01 when compared to the non-subtype A variants ($p<0.0001$, in both cases). Indeed, NIH45-46w and VRC01 neutralized 100% and 94% of the subtype A variants compared to 73% and 24% of the non-subtype A variants, respectively. In addition, subtype A variants were more sensitive to PGT145 compared to non-subtype A variants ($p = 0.03$). There was no statistically significant difference in the neutralization sensitivity of the different subtypes to the glycan dependent antibodies PGT128, PGT121 and PG9. However, in the case of PGT128 and PGT121 there was a trend for a higher percentage of non-subtype A variants being neutralized than that of subtype As (Figure 3.7).

Relationship between presence of required asparagine (N) residues and neutralization activity of selected glycan dependent mAbs

Some residues that define PGT128, PGT121, PG9 and PGT145 epitopes have been previously defined [164,167]. N-linked glycans at positions 301 and 332 are required for PGT128 and PGT121 activity while positions 156 and 160 are important for PG9 and PGT145 binding [150,166,247]. Thus we examined variants for the presence of asparagine residues at positions 301 and 332 for one V3 binding antibody PGT128 one of the mAbs with greatest breadth and potency and positions 156 and 160 for PG9 a V1/V2 binding mAb with potential for complementing PGT128 activity. Of the 14 variants that were resistant to PGT128, the asparagine at position 332 was either absent (n=5) or shifted (n=5). Two of the variants with a shifted N also lacked the N at position 301. One variant had the N160 but lacked the N301, while the remaining variants (n=3) had both N332 and N301 present (Table 3.1).

mAb	Subtype A neut % (n)	non-Subtype A neut % (n)	OR	95% CI	p-value
NIH45-46W	100% (63)	73% (32)	Inf	4.96-inf	<0.0001
VRC01	94% (61)	24% (11)	0.03	0.007-0.11	<0.0001
PG9	78% (51)	48% (22)	0.3	0.07-1.3	0.11
PGT121	62% (40)	80% (37)	3.24	0.81-13	0.1
PGT128	85% (55)	89% (41)	1.59	0.42-6.01	0.49
PGT145	46% (30)	11% (5)	0.16	0.03-0.82	0.03
b12	6% (4)	17% (8)	3.63	0.58-22.7	0.17

Figure 3.7. GEE analysis for the comparison between neutralization sensitivity of different HIV subtypes to mAbs. Comparison of neutralization IC50s for all variants grouped as either subtype A or non-subtype A (C,D and inter-subtype recombinants C/D and D/A) performed by GEE using a logit link and exchangeable correlation structure. The *P* values for each mAb are shown. IC50s are an average of at least 2 independent experiments.

We determined the presence of N156 and N160 in 35 of the variants resistant to PG9 and found that N156 was absent in 7 of the variants. All variants had the N160 residue except in 2 variants (from a mother-baby pair) where although the residue was present, the canonical NxS/T sequence necessary for glycosylation was not fulfilled (sequence present was NV**A** in both cases) (Table 3.2).

Table 3.1. Shows amino acid residues at positions 301 and 332 in 14 variants resistant to PGT128

Virus	Subtype	N301	N332
BJ613.A1	A	N[a]	Shift[c]
BJ613.E1	A	N	Shift
MF535.A3	D/A	N	Shift
MF535.D11	D/A	N	**T**
MG505.E1	A	N	**T**
MG505.G2	A	N	**T**
MG505.H3	A	N	**T**
ML274.B1	A	**D**[b]	Shift
ML274.G3	A	**D**	Shift
MF600.B1	C	N	N
MF600.C1	C	**S**	N
MI102.C1	D	N	N
MC046.F10	A	N	**K**
BS208.B1	A	N	N

[a] PNLGs present.

[b] with bolded amino acid symbol, indicates PNLGs absent

[c] PNLGs is present but at position 334.

PNLGs, potential N-linked glycosylation site

Table 3.2. Shows amino acid residues at positions 156 and 160 in 35 of the variants resistant to PG9

Virus	Subtype	N156	N160
BK184.B1	C/D	N[a]	N
BK184.D2	C/D	N	N
BL035.B1	D/A	**K**	N
BL035.C1	D/A	**K**[b]	N
BF520.B3	A	N	*N*[c]
MF535.B1	D/A	N	N
MF535.C1	D/A	N	N
MF535.D11	D/A	N	N
MF535.E2	D/A	N	N
MF535.G2	D/A	N	N
MJ613.A2	A	N	N
MI206.D1	A	N	N
MI206.F3	A	N	N
MK184.E4	C/D	N	N
MK184.F2	C/D	N	N
ML035.A1	D/A	**K**	N
ML035.B1	D/A	**K**	N
ML035.E2	D/A	**K**	N
ML035.G2	D/A	N	N
ML035.J2	D/A	**K**	N
ML035.K4	D/A	**E**	N
MF535B.F22	D/A	N	N
MF055B.A1	D	N	N
ML055B.C6	D	N	N
MF520.C2	A	N	N
MF520.P10	A	N	*N*
MF520.Q6	A	N	N
MI102.B6	D	N	N
MI102.C1	D	N	N
MC046.E5	A	N	N
MC046.G9	A	N	N
MA411.B2	A	N	N
MA411.C2	A	N	N
MA411.G10	A	N	N

[a] PNLGs present.

[b] with bolded amino acid symbol, indicates PNLGs absent

[c] in italics and blue, PNLGs is absent (due to presence of a valine instead of the required serine or threonine to fulfill the NxS/T sequence)

PNLGs, potential N-linked glycosylation site

Discussion

Circulating HIV variants, particularly those found in HIV endemic regions such as subtype A, C and D viruses, are critical targets for prevention strategies including vaccines. As a result, the vulnerable sites displayed by these variants are important in defining potential protective epitopes and/or antibody specificities. In the recent past, new HIV mAbs have been isolated that show breadth against all HIV subtypes but their neutralization profile against vertically transmitted and circulating variants representing diverse subtypes from endemic regions has not been defined. We determined the neutralization sensitivities of 111 HIV variants from a vertical transmission cohort from Nairobi Kenya against mAbs NIH45-46w, PGT128, PGT121, VRC01, PG9, PGT145 and b12. These variants exclusively from the MTCT cohort displayed high sensitivity profiles similar to those observed in studies of other virus panels primarily composed of chronic strains of HIV variants. We did not observe any significant differences in neutralization sensitivity between the transmitted variants from the infants and variants obtained from the transmitting mothers. Similarly, there were no significant differences in sensitivity profiles of variants from transmitting mothers compared to those from non-transmitting mothers. However, we observed differences in neutralization sensitivity between subtypes, with subtype A being significantly more sensitive to several mAbs including the CD4bs antibodies NIH45-46w and VRC01 and glycan dependent PGT145 compared to the non-subtype As.

Neutralization sensitivity was greatest (>85%) when envs were tested against NIH45-46w and PGT128, two antibodies that recognize distinct epitopes. Interestingly, we observed that neutralization sensitivity of variants to these 2 mAbs was complementary. Viruses that were resistant to PGT128 were found to be sensitive to NIH45-46w and vice versa. Indeed, the capacity of NIH45-46w and PGT128 to complement each other's activity when used in combination has recently been demonstrated suggesting that these two antibodies can be used in combination to improve coverage across subtypes [248]. In this study, of the 14 variants that were neutralization resistant to PGT128, 12 were sensitive to PG9, suggesting that PG9 could also be combined with PGT128 and have broad coverage. Conversely, antibodies that shared epitopes neutralized overlapping variants and displayed poor capacity to complement one

another. These observations are in line with recent data by Doria-Rose and colleagues from 208 global envs that showed that additive viral neutralization was obtained when VRC01 and PG9, which target independent epitopes, were combined [235]. These observations emphasize the potential benefit of using a combination of mAbs with distinct specificities and/or the need to develop a multivalent immunogen capable of eliciting antibodies of distinct specificity to maximize coverage of the globally diverse HIV strains. Further, these data suggest that mAbs NIH45-46w, PGT128 and PG9 could be used in combination to offer maximum coverage.

The broadest antibody in this screen (NIH45-46w), is an example of a rationally designed antibody. By carefully studying the CD4 footprint during CD4-gp120 interaction, Diskin et.al were able to determine residues within NIH45-46 that if modified would improve its neutralization activity. This led to the creation of NIH45-46w, which is engineered to have a tryptophan at position 54 instead of the glycin found in the wildtype NIH45-46. The tryptophan accesses a hydrophobic pocket in gp120 resulting in overall improved interaction and neutralization breadth [163]. Thus the success of NIH45-46w gives hope that rationally designed antibodies and/or immunogens can be attained.

Our panel of 111 envs comprised of 3 subtypes (A, C and D) and some inter-subtype recombinants (C/D and D/A) that commonly circulate in sub-Saharan Africa. These non-subtype B envs have previously been shown to display limited sensitivity to antibodies directed to CD4bs and glycans such as b12 and 2G12, respectively [150,151,155,156]. Indeed, b12 could only neutralize 9% of variants tested in this screen. We found that subtype A variants were significantly more sensitive to neutralization by CD4bs antibodies NIH45-46w and VRC01 and glycan dependent antibody PGT145 than non-As. The interaction between mAbs NIH45-46w and VRC01 and env gp120 both mimic CD4-gp120 binding but with considerable differences. These differences are reflected in their neutralization capacities evidenced by lower neutralization capacity of soluble CD4 (sCD4) compared to VRC01 [161,162]. It remains unclear why subtype As are more sensitive considering these antibodies were obtained from a subtype B infected individual. We speculate that the antigenicity of the CD4bs differs in various subtypes. This could result in differences in interactions

between NIH45-46w and VRC01 and the different CD4bs with subtype A having superior interaction compared to non-subtype A variants.

We observed a significantly higher sensitivity of subtype As to PGT145 compared to all other subtypes grouped together. In a previous report however, the neutralization profile for PGT145 was somewhat similar between subtypes A and a combination of C and D (50% vs 46% respectively) [166]. PGT145 is a prototype V1/V2 binding antibody and thus its activity could potentially be altered by features that affect other prototype V1/V2 antibodies such as PG9 [164,247,249]. Although a lower percentage of non-subtype A compared to subtype A variants were sensitive to PG9, these difference did not reach statistical significance. This observation indicates that there are other neutralization determinants not shared by these 2 V1/V2 antibodies and thus a more elaborate study to map the molecular determinants for PGT145 activity is necessary.

The fact that NIH45-46w and VRC01 neutralize HIV variants in a subtype dependent manner could impact their utility in regions high in subtype C and D infections. However, sensitivity to PGT128, which complements NIH45-46w, was not dependent on subtype and thus it could be used in combination to overcome the constraints resulting from subtype differences.

We did not observe any significant differences in sensitivity profiles of transmitted variants from infants and variants from transmitting women. Likewise, there were no significant differences in neutralization sensitivity between variants from transmitting women and non-transmitting women. These data suggest that the epitopes targeted by these mAbs are present and accessible in both the circulating transmitted and non-transmitted variants of various subtypes.

Overall, the high sensitivity of our panel of viruses was very similar to previously reported data except in 2 instances or antibodies PGT128 and PGT145 [150,166,235]. In our hands PGT128 neutralized 87% of variants at 1ug/ml compared to 56% of variants previously reported by Walker, L et al 2011. In addition, in a recent study done in our lab, only 24% of heterosexually transmitted variants from acute infection were found to be sensitive to this mAb at 1ug/ml [248]. The variants used in these two studies were however different in their modes of transmission and time at which they were obtained in

relation to initial infection. We formally evaluated these differences as possible reasons for the discrepant results, by comparing IC50s from vertical transmission with those from heterosexually transmitted variants and found that vertically transmitted variants were significantly more sensitive to PGT128 ($p = 0.009$). Further, variants from chronic infection (maternal variants) were also significantly more sensitive than those from heterosexual acute infection ($p < 0.0001$). Thus the reasons for these discrepant results could be an indication of neutralization sensitivity differences in variants from different modes of transmission or acute verses chronic infection (or both) or other as yet unidentified factors.

In another instance, only 32% of the variants were sensitive to PGT145, in our hands compared to 48% neutralized in a previous study [166]. Only 2 of the 111 envs lacked the N160 glycan defined to be necessary for PGT145 activity. Thus these data suggest that the absence of N160 residue does not account for all the instances of resistance observed towards PGT145 as has been recently observed in another prototype V1/V2 binding antibody, PG9 [247]. Similarly, the absence of N301 and N332 did not account for all PGT128 resistance suggesting that in some rare instances other determinants, not as yet defined might be necessary.

We acknowledge that our study has some important limitations. Our panel of 111 envs mainly consisted of subtype A variants. Given that subtype C currently accounts for approximately half of HIV infections worldwide [3,238,239], similar studies with more subtype C variants are of high importance especially given its low sensitivity in our study. We were unable to determine the sensitivity of variants at concentrations >1ug/ml, due to limitations in the amounts of antibody at our disposal. Its therefore, possible that sensitivity reported here could be underestimated since some variants could have been sensitive at higher concentrations. The possibility that neutralization sensitivity of variants from transmitting is different from that of non-transmitting women cannot be ruled out given our small sample size of 86 variants. Studies with large sample size statistically powered to detect small differences should be considered. In addition, our study had only 25 transmitted variants hence studies with larger numbers of transmitted variants representing various modes of transmission will be necessary. Finally, there is evidence for increased resistance to mAbs over time and since the variants used in this

study were in circulation between 1992-1998, a screen involving variants from recent years will be informative [159].

In conclusion, we have shown that transmitted variants from infants and variants obtained from transmitting women and non-transmitting women are not different in their sensitivity to newly isolated mAbs. Sensitivity was greatest towards NIH45-46w and PGT128, two antibodies with the highest potency and with neutralization activities that complement each other. Our data suggests that a combination of NIH45-46w, PGT128 and PG9 would provide the best neutralization coverage. Importantly, an immunogen rationally designed to elicit multiple antibodies of specificities similar to those represented here is likely to provide coverage required to conquer the genetic diversity displayed by HIV.

Chapter 4

Conclusions and Future Directions

One of the deficiencies in HIV vaccine development is the lack of a clear definition of protective immune correlates in natural exposure. While cumulative data from animal models suggest that NAbs have the capacity to confer protection, only a limited number of HIV variants have been tested in that model. In reality, the ability of NAbs to offer protection depends largely on their capacity to target and block transmitted variants of diverse subtypes. Consequently, studies looking at the capacity of antibodies to block transmission during natural exposure and the neutralization sensitivity profiles of relevant circulating and transmitted variants by mAbs are needed to determine the types of antibody specificities required to be elicited by a candidate vaccine.

Using samples from an ARV naïve MTCT cohort from Nairobi, Kenya, this thesis has explored beast milk NAbs and nNAbs as potential protective immune correlates of breast milk HIV transmission (chapter 2). This thesis also determined neutralization profiles of transmitted variants to newly isolated mAbs (chapter 3). This chapter will briefly review data on breast milk NAbs and nNAbs and their role in limiting transmission. A summary of the neutralization profiles of transmitted variants to mAbs will also be provided. Further, a discussion on the possible implications of these data as well as areas of future study will be explored.

Summary and Implications

Our data described in chapter 2 indicates that while HIV infected women display a robust NAb response in plasma, NAbs are limited in breast milk. These low levels of NAbs in breast milk could be explained in part by a generally lower antibody level in breast milk compared to plasma (chapter 2). This limited NAb response in breast milk suggests that breast milk Abs are not likely to neutralize HIV-1 at a level that could contribute meaningfully to protection from HIV-1 transmission given that they are much lower than plasma NAb levels.

These results indicate that levels of NAbs in HIV infected people are not equally distributed between the systemic circulation and mucosal sites. The implications of these

data are relevant to both passive immunization studies and/or vaccine trial efforts in that they emphasize the importance of establishing the levels of NAbs associated with protection at different sites to maximize efficacy.

Contrary to limited HIV-specific NAbs levels in breast milk, nNAbs capable of ADCC were detected in all breast milk samples. We found that levels of ADCC activity were directly correlated with levels of breast milk IgG titers. Importantly, high levels of ADCC activity were associated with reduced breast milk transmission (chapter 2). Because levels of infected breast milk cells are a correlate of breast milk transmission, we hypothesize that ADCC activity acts by reducing the levels of infected breast milk cells. Indeed, in a later analysis looking at the levels of breast milk HIV DNA in samples from the 19 women, we found that log10 breast milk HIV-DNA is negatively associated with breast milk ADCC with data from the 19 women showing a trend towards statistical significance ($p = 0.09$). However, there is need to confirm this model in a larger subset of women.

It is interesting to note that higher levels of binding antibodies in breast milk trended towards reduced. Further analysis indicated that the levels of binding antibodies were correlated with the ADCC activity. This is very analogous to results from the RV144 immune correlate analysis study, which generated the hypothesis that HIV-specific levels of binding antibodies to V1/V2 could predict transmission. If these findings are replicated in other studies it implies that future studies can use a measure of HIV-specific binding antibodies as a primary indicator of a protective antibody response while pursuing more specific functional studies, which generally take a longer time.

An association between ADCC activity in breast milk and a reduced risk of transmission stresses the significance of infected breast milk cells in transmission. Consequently, interventions aimed at targeting breast milk transmission should be carefully evaluated to establish their effect on levels of infected breast milk cells. In addition, findings presented herein highlight the potential of ADCC mediating antibodies in limiting HIV transmission in a natural setting. Thus, while a lot of focus has been on understanding how to design a vaccine that can elicit effective NAbs, these data argue for a consideration of HIV immunogens that can elicit antibodies capable of ADCC.

In chapter 3 we demonstrate that similar to non-transmitted variants, transmitted variants are highly sensitive to newly isolated mAbs. Sensitivity was greatest towards NIH45-46w and PGT128, which target different epitopes on env gp120 (chapter 3). The data also suggested that in combination these two antibodies would provide 100% neutralization coverage while PGT128 and PG9 would provide 98% coverage of the variants tested. Sensitivity was found to be subtype dependent with subtype A variants being significantly more sensitive to CD4bs antibodies NIH45-46w and VRC01 than all other subtypes (C, D, C/D and A/D).

These data have some important implications for the HIV NAb field. The fact that we observed no differences in the neutralization profiles of transmitted variants from infants and circulating variants from the mothers suggests that the target epitopes for these mAbs are present and accessible in most variants from both chronic and acute infection. This is good news for the field because it allows us to use both circulating chronic and/or transmitted variants in evaluating the characteristics of mAbs of interest and of those antibodies generated in immunization studies.

Secondly, our data suggest that by combining NIH45-46w, PGT128 and PG9 we would provide wide coverage of circulating strains of HIV compared to if each was used singly. These observations emphasize the need for a multivalent vaccine capable of eliciting antibodies of multiple specificities to overcome the genetic diversity displayed by HIV.

Finally, although NIH45-46w displayed the best coverage overall, its activity was found to be subtype specific. This feature could impact its utility in regions dominated by other subtypes, especially subtype C, given that this subtype accounts for the majority of current HIV cases. This challenge can however be potentially overcome by using NIH45-46w and PGT128 in combination since together they display increased coverage.

Future Directions:

An important next step for the breast milk antibody study is to determine the epitopes associated with ADCC and subsequently protection. Immune correlate hypothesis generating analysis using samples from the Thai human vaccine trial (RV144) that showed ~31% efficacy [49], binding antibodies targeting the V1/V2 were associated with protection. This observation suggests a possible role of anti-V1/V2 antibodies. One approach to defining the specificity of ADCC mediating breast milk antibodies is to determine whether these antibodies display similar specificity to those detected in the RV144 vaccinees. A subtype A V1/V2 construct (possibly bearing Q461env.d1 V1/V2) can be generated using the same protocol used to generate gp70 V1/V2 that was used to detect binding antibodies in the RV144 hypothesis generating study (gp70 V1/V2-Murine leukemia virus gp70 protein and HIV V1/V2) [250]. This protein can then be used to define the presence of V1/V2 antibodies in two ways; 1.) Using ELISAs to determine V1/V2 binding antibody titers between transmitting and non-transmitting women, 2.) Performing competition assays using gp70 V1/V2 to determine the effect on the levels of ADCC activity before and after pulling out V1/V2 directed antibodies.

The mechanism of ADCC involves functional effector cell (NK cells) activity. Consequently, breast milk ADCC activity can only be effective in limiting HIV transmission in the presence of a robust effector NK cell activity in breast milk. So far, there are no data on breast milk NK cell frequencies from African women, although these cells have been reported in BM of HIV uninfected women from other populations [145,251]. I propose a study designed to test the hypothesis that breast milk from Kenyan women contains NK cells capable of mediating ADCC. The specific aims of such a study would be: to evaluate the frequency and phenotypes of breast milk NK cells in HIV infected and uninfected Kenyan women over time and to determine the ability of breast milk NK cells from these women to inhibit HIV through ADCC. Breast milk NK cells can be identified by cell surface staining by selecting for CD3-, CD56+, CD16+ cells. NK effector function can be measured using the RFADCC assay (described in chapter 2) against both autologous and heterologous breast milk antibodies. An understanding of the levels of NK cells in breast milk and their capacity to mediate

ADCC will shed more light on our current model which proposes that ADCC mediating antibodies in the breast milk recruit NK cells to destroy infected cells hence reducing risk of transmission.

One important question that remains unanswered given the mAb data is why subtype A variants are more sensitive to CD4bs antibodies compared to non-subtype A. The CD4bs is a conformational epitope and thus presents inherent challenges for further study. However, one pilot experiment to get at this question would be to determine the sensitivity of these variants to sCD4. This would shed a light on any possible differences in the exposure of the CD4bs in different subtypes.

Our data also suggests that more residues than previously defined might be required for the activities of the glycan dependent antibodies PGT128, PGT121, PG9 and PGT145. PGT128 is of particular importance given its breadth and ability to complement NIH45-46w. I propose that the variants resistant to PGT128 at 1ug/ml be tested at higher antibody concentration first to confirm if they are inherently resistant. If true, then determining other residues necessary for PGT128 activity will be necessary and this information can eventually be used for the radical design of an epitope optimal for this antibody. To further describe other potential determinants necessary for PGT128 recognition, overlapping regions of the V3 loop from sensitive to resistant variants and vice versa can be performed by site directed mutagenesis to determine loss and gain of function.

One exciting practical future direction is to design passive immunization studies to be conducted in animal models using a combination of NIH45-46w, PGT128 and PG9. These studies should focus on addressing other important questions regarding these antibodies such as their, formulation for *in-vivo* use, *in-vivo* half-life, appropriate dosing amounts that confer protection, most effective mode of administration and to evaluate their overall activity in-vivo (adverse effects). Availability of this information will then pave the way for a possible RCT in which infants can be randomized to receive either the mAb combination (NIH45-46w, PGT128 and PG9) plus ARV or ARVs alone during the breast-feeding period to determine their effectiveness in reducing transmission associated with breast-feeding.

The long-term efforts should be towards rationally designing a multivalent immunogen bearing epitopes targeted by these antibodies, which can then be tested in animal models to determine their immunogenicity.

Conclusions:

Effective antibody based vaccines have in the past been used to eradicate viral pathogens such as small pox from the human population. As a result, development of an antibody based HIV vaccine remains a high priority. The data presented here demonstrates that antibodies developed against HIV during natural infection can provide protection in a natural setting and can also inhibit transmitted variants *in-vitro*. This observation gives hope that a vaccine aimed at eliciting the appropriate kind of antibodies, either nNAbs or Nabs, could be effective at preventing HIV infection. Therefore, our data suggests that focusing on developing immunogens that elicit antibodies with specificities similar to those studied here would be a step in the right direction. In the meantime, the protective efficacy of a passively administered combination of NIH45-46w, PGT128 and PG9 should be evaluated in animal models.

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