Role of Antibodies in Mother-to-Child Transmission of HIV-1

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Abstract

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Designing an efficacious HIV vaccine remains an elusive goal. Most vaccines provide protection by eliciting protective antibodies. Vaccination studies have provided proof-of-concept that antibodies can protect against viral challenge. However, the efficacy of most promising HIV vaccine trial to date was modest. Therefore, identifying correlates of protection against natural HIV infections in humans may provide valuable information to help inform vaccine design.

Mother-to-child transmission (MTCT) is a unique natural setting analogous to vaccination. Infants receive passively-acquired antibodies from their mothers *in utero*, and therefore have pre-existing HIV-specific antibodies circulating prior to HIV exposure through breastfeeding. In this thesis, we utilized this unique setting of breastfeeding mother-to-child

transmission with the goal of identifying humoral correlates of reduced risk of MTCT, with a specific focus on characteristics of these pre-existing HIV-specific antibodies.

Anti-HIV antibody activity is determined by the specificity of the antibody for its target region on HIV (its epitope) and by the ability of the antibody to mediate effector functions by activating innate effector cells. Antibody-dependent cellular cytotoxicity (ADCC) is a well-studied effector function targeting infected cells for destruction. ADCC has been correlated with protection in both non-human primate and human vaccination studies. In the setting of MTCT, we previously reported that pre-existing passively-acquired ADCC in infants is associated with improved infant outcome. We hypothesized that pre-existing passively-acquired antibodies that mediate ADCC and/or bind to HIV target specific epitopes conferring protection against MTCT of HIV-1. Throughout this thesis, we investigated the complimentary roles that antibody epitopes and effector function play in risk of MTCT and infant outcome.

First, we investigated epitopes of antibodies capable of binding HIV, regardless of their potential to mediate effector function, from a cohort of breastfeeding mother-infant pairs with the goal of identifying epitopes targeted by antibodies associated with reduced risk of MTCT. While we did not observe any correlates of reduced risk of transmission, we did identify a correlate of increased risk. We observed that antibodies capable of binding to the ectodomain region of the HIV envelope transmembrane protein gp41 were correlated with increased MTCT. We hypothesize that the ectodomain of gp41 may be acting as an immune decoy to divert the immune response away from generating protective antibodies.

Next, we investigated whether epitopes of ADCC-mediating antibodies affected risk of MTCT and infant outcome. We studied three epitopes commonly targeting by ADCC-mediating antibodies in natural infection and vaccination. Interestingly, ADCC targeting these epitopes was

not correlated with reduced risk of MTCT or improved infant outcome. Surprisingly, our results indicate that ADCC targeting these epitopes is correlated with worse infant outcome. Our data suggest that ADCC targeting other epitopes or a polyclonal ADCC response warrant further investigation as potential correlates of protection.

Finally, we explored the role and mechanism of ADCC in MTCT in further detail using two approaches. In the first approach, we screened samples from our previous ADCC study in a newly-developed assay designed to detect the potential for antibodies to mediate effector functions in general, not limited to ADCC. Our goals were to understand the mechanism of the association of passively-acquired ADCC with improved infant outcome in more detail as well as to broaden our investigations to include effector functions other than ADCC. In the second approach, we measured ADCC in samples from a separate MTCT cohort with the goal of replicating the results of our initial ADCC study. In both approaches, we observed similar trends to our initial results that ADCC, and potential effector functions in general, are associated with improved infant outcome, although these trends did not reach statistical significance.

As a whole, the studies presented here provide information about the role that antibody epitopes and antibody effector functions play in the risk of MTCT and infant outcome. Future studies investigating the mechanisms of the associations described here would have the potential to inform vaccine and therapeutic design.

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ADCC: antibody-dependent cellular cytotoxicity

ADCD: antibody-dependent complement deposition

ADP: antibody-dependent phagocytosis

aOR: adjusted odds ratio

ART: antiretroviral therapy

BAMA: binding antibody multiplex assay

BSA: bovine serum albumin

CD4bs: CD4 binding site

CD4i: CD4-inducible

CI: confidence interval

ConA1: consensus A1 V3 peptide

ConB: consensus B V3 peptide

ConC: consensus C V3 peptide

ConD: consensus D V3 peptide

CT: cytoplasmic tail

CTL: Cytotoxic T Lymphocyte

Cum.: cummulative

EC: elite controller

Env: envelope

Fab: fragment antigen binding domain

Fc: fragment crystallizable

FcR: Fc receptor

FcγR: Fc gamma receptor

FP: fusion peptide

HEU: HIV-exposed uninfected

HIV+: HIV-infected

HR: hazard ratio

HR1: heptad repeat 1

HR2: heptad repeat 2

HSA: human serum albumin

IQR: interquartile range

LALA: L234A and L235A mutations

LTNP: long-term non-progressor

MC: multiple comparisons

MFI: median fluorescence intensity

MPER: membrane proximal external region

MTCT: mother-to-child transmission

NBT: Nairobi Breastfeeding Clinical Trial

NFDM: non-fat dry milk

NHP: non-human primate

NK: natural killer

NT: non-transmitting

OR: odds ratio

PBSE-EDTA: PBS with 1mM EDTA

PI: post-infection

Phip-seq: phage immunoprecipitation sequencing

PR: proximal region

RFADCC: rapid and fluorometric ADCC

RSC3: resurfaced Env core protein

RSC3Δ371I: resurfaced Env core protein CD4bs-defective mutant

RT: room temperature

sCD4: soluble CD4

SEM: standard error of the mean

SHIV: SIV encoding an HIV envelope

T: transmitting

TM: transmembrane domain

VC: viremic controller

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Chapter I

Introduction

HIV/AIDS Epidemic

Since the beginning of the HIV/AIDS epidemic, HIV/AIDS has claimed the lives of approximately 35.4 million people (1). The development and implementation of antiretroviral therapy (ART) was a cornerstone in the trajectory of the HIV/AIDS epidemic. Viral suppression due to ART-treatment has increased the lifespan of HIV-infected individuals dramatically worldwide, and to almost non-HIV levels with adherence to treatment in high income settings (2). In addition to the individual's benefit, an ART-treated individual with viral loads suppressed to undetectable levels is highly unlikely to transmit the virus horizontally to their partner (3). Moreover, when mothers are on ART, the risk of mother-to-child transmission is reduced from ~15-40% without treatment depending on the population and setting to approximately 1-2% in resource-rich settings and 10% in resource-poor settings (4-8). Finally, there has been a recent implementation of ART for use as pre-exposure prophylaxis for high-risk individuals, leading to direct prevention of new infections (9). The success of ART combined with other global efforts of increased education about safe sex practices and the use of other preventive strategies such as microbicides and condoms has dramatically reduced the rate of new infections by 47% and HIV/AIDS-related deaths by 51% since they reached their maximum in 1996 and 2004, respectively (1).

Despite this progress, HIV/AIDS still causes a massive burden of disease worldwide. As of 2017, almost 37 million people were living with HIV (1). In 2017 alone, there were

approximately 940,000 AIDS-related deaths and 1.8 million people newly-infected, of which 180,000 were children under 15 years of age (1). Development and worldwide implementation of an efficacious HIV vaccine is essential to prevent new infections and consequently reduce this global disease burden. Most licensed vaccines prevent infections by eliciting protective antibody responses (10, 11); therefore, to develop an efficacious vaccine, an understanding of the humoral response to HIV and humoral correlates of protection is necessary. The role of antibodies in HIV infection and prevention will be the focus of this thesis.

Antibody structure and function

Antibodies are antigen-specific proteins that are key effectors of the adaptive immune response. Antibodies are Y-shaped molecules that consist of two heavy and light chains covalently linked by disulfide bonds. Each heavy and light chain has a variable and constant region. Antibodies bind to their antigen of interest via two identical antigen binding domains (fragment antigen binding domain (Fab)). Antibodies also have a fragment crystallizable (Fc) domain, determined by the constant region of the heavy chain, which binds to Fc receptors on innate immune cells. Antibody structure and Fc-mediated effector functions are reviewed by Lu et al (12). Humans have 5 different antibody isotypes (IgD, IgM, IgG, IgA, IgE) which are produced through a DNA recombination process called class switch recombination (12, 13). Class switch recombination results in antibodies that can have the same antigen binding site and can thus bind to the same epitope but have different Fc domains.

The role of the Fc domain is to interact with the Fc receptor (FcR) on various effector cells and send signals that result in a variety of immune functions such as recruitment of other immune cells and killing or removing a pathogen or infected cell (12, 13). The different antibody

isotypes bind to different Fc receptors. For example, IgG antibodies bind to FcγRs, IgA antibodies bind to FcαRs, and IgE antibodies bind to FcεRs (13). FcRs are expressed in various patterns on innate immune cells leading to differential responses for each antibody isotype (12, 13).

Among the 5 isotypes, IgG is the most prevalent in the blood (14). IgG antibodies will be the focus of this thesis. There are 4 subclasses of IgG antibodies: IgG1, IgG2, IgG3, and IgG4, reviewed by Lu et al (12). There are a number of different FcγRs, some of which are activating, and one that is inhibitory. The various IgG subclasses bind with different affinities to the different FcγRs, and effector cells can express more than one type of FcγR (12, 13, 15). The combination of activating and inhibitory receptors that bind to antibody leads to either downstream effector cell function (if the activation signals outweigh the inhibitory signals), or lack of effector cell activity (if the inhibitory signal outweighs the activation signals) (12, 16). FcγR signaling is dependent on the affinity of the Fc region for the Fc receptor. Fc-FcγR affinity is dependent on Fc glycosylation patterns, single nucleotide polymorphisms of the FcγR, and IgG subclass (12, 13, 15). IgG1 and IgG3 tend to have higher affinities for FcγRs, and thus are stronger mediators of effector functions compared to IgG2 and IgG4 (13, 17). Importantly, for FcγRs to activate their signaling cascade, multiple antibodies must bind to neighboring FcγRs on the innate effector cell, which allows the FcγRs to cluster together and physically cross-link. Cross-linking of FcγRs, and FcRs in general, is necessary to trigger their signaling cascade and mediate their downstream effector function (12, 18, 19). Therefore, to mediate effector functions, both the antibody epitope is important to determine specificity of the target, and the angle of approach of the Fc region is important to determine the presence or absence of effector function.

Based on the Fc receptors an antibody binds, antibodies can mediate a variety of effector functions. These include antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent phagocytosis (ADP), and antibody-dependent complement deposition (ADCD) (12, 15, 20). One of the most well-studied non-neutralizing effector functions is ADCC, which has been of high interest to the HIV field and will be the Fc effector function focused on in this thesis. ADCC is a process by which antibodies bind to their antigen presented on infected cells and bind to FcγRs on innate effector cells, triggering the effector cell to kill the target cell. ADCC is primarily mediated by Natural Killer (NK) cells which primarily express FcγRIIIa and kill infected cells by releasing their cytolytic granules (12). ADCC can also be mediated by other innate effector cells such as monocytes and neutrophils, which express FcγRIIIa as well as other FcγRs (13, 21). Monocytes for example, express other Fc receptors such as FcγRIIa and FcγRI and can mediate phagocytosis as well as ADCC (12, 13, 21).

HIV-specific Antibodies

HIV antibodies targeting all of the HIV proteins have been identified (22). However, the vast majority of epitopes of HIV-specific antibodies identified to date are within the HIV envelope (Env) protein (22, 23), the viral glycoprotein present on the surface of the virion. HIV Env in its native form is a trimer of heterodimers made up of gp120 and gp41 subunits (Figure 1.1, top). HIV Env mediates viral entry into cells via CD4 and a chemokine co-receptor, typically CCR5. The Env surface unit, gp120 binds to cellular CD4, which triggers binding to the co-receptor and trimer opening (24, 25). This allows the gp41 subunit to mediate fusion between the viral and cellular membranes (24).

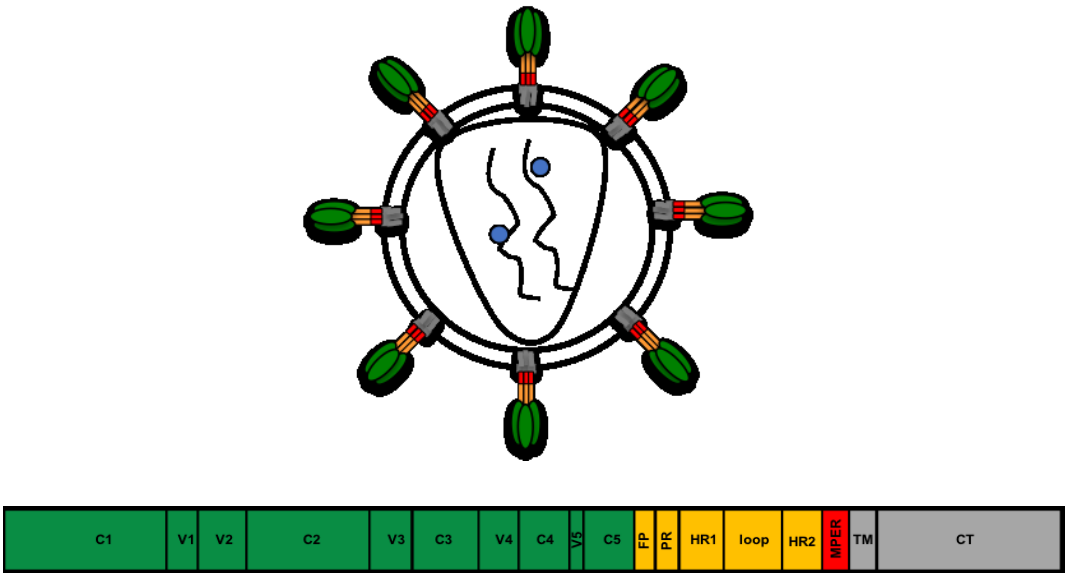


Figure 1.1. HIV structure.
Top: Schematic of an HIV-virion. HIV envelope is the viral protein on the surface of the virion. HIV envelope is a trimer of heterodimers of gp120 (green) subunits and gp41 (orange, red, gray) subunits.
Bottom: HIV gp120 has 5 variable regions and 5 constant regions (V1-V5 and C1-C5, respectively. Gp41 regions include the fusion peptide (FP), the fusion peptide proximal region (PR), N-terminal heptad repeat (HR1), the immunodominant C-C loop (loop), C-terminal heptad repeat (HR2), the membrane proximal external region (MPER), the transmembrane domain TM, and the cytoplasmic tail (CT).

Epitopes of HIV-specific antibodies

HIV-specific antibodies target many epitopes scattered across the Env glycoprotein (22, 23). Regions of Env are shown in Figure 1.1 and include 5 variable regions (V1-V5), 5 constant regions (C1-C5), a fusion peptide and its proximal region (FP, PR), two heptad repeats (HR1 and HR2), an immunodominant cysteine-cysteine loop, a membrane proximal external region (MPER), a transmembrane domain (TM), and a cytoplasmic tail (CT). The accessibility of epitopes for antibodies to bind varies widely across the protein and is dependent on the conformation of Env and glycosylation (25-27). Some epitopes are exposed on the native trimer or native-like trimeric antigens, such as the CD4 binding site and the V1V2 glycan-dependent epitopes (28-31). Interestingly, some epitopes which are immunodominant, and are targeted by very commonly elicited antibodies, are not exposed on native trimer but are exposed throughout the HIV entry process and life cycle. These include antibodies targeting V3, C1, and the gp41 C-C loop. The V3 region is occluded by V1V2, and C1 is buried in the middle of the native trimer. Both regions are exposed upon CD4 binding to Env which causes V1V2 to move toward the sides of the trimer leading to V3 exposure and the trimer to open up leading to C1 epitope exposure (25, 32). The immunodominant C-C loop of gp41 is a highly conserved epitope which becomes exposed after gp120 is shed from a virion or infected cell (30, 33-36). Antibodies targeting certain epitopes have been correlated with risk or protection in vaccination studies or with clinical outcome (discussed below).

Functions mediated by HIV-specific Antibodies

HIV-specific antibodies can mediate a variety of functions. The function that an antibody mediates is determined in part by its epitope and in part by its Fc region binding to Fc receptors. Antibody epitopes dictate where the antibody can bind on the virion or viral protein. The Fc region dictates the angle of approach, the specificity of FcγRs engaged, and thus the type of effector function (if any) initiated. HIV-specific antibodies can neutralize cell-free virus and prevent subsequent infection of a target cell. HIV-specific antibodies can also target infected cells by mediating non-neutralizing effector functions described above such as ADCC, ADP, and ADCD. For the purposes of this thesis, I will focus on ADCC, which has been extensively studied in the HIV field. In general, there is correlation between binding, neutralization, and ADCC, but the correlation is imperfect (28, 37-40). Some antibodies that can bind to HIV Env are unable to neutralize the virus or mediate ADCC (28, 41, 42). In general, neutralizing antibodies must bind to a site on the virus that is critical for viral entry and exposed on native trimer, with the CD4 binding site and V1V2 glycan apex being classic examples (28-31). Many neutralizing antibodies can mediate both neutralization and ADCC (37, 38, 40, 41), but others cannot (37, 41, 42), likely due to the orientation of their Fc regions not allowing for FcR engagement and/or cross-linking on effector cells (37, 43). Other antibodies are unable to neutralize but can mediate ADCC (37, 41, 42, 44). For these antibodies, many target epitopes on envelope that are buried in the native trimer and are exposed only after trimer opening and membrane fusion, such as the CD4-inducible gp120-specific antibodies (to be discussed in Chapter III) (45). These non-neutralizing antibodies are able to mediate their effector function primarily upon entry (where their epitopes can be exposed for as long as 2 hours in the case of the CD4-inducible antibodies) and exit (46, 47).

Antibody Responses During HIV Infection

During HIV infection, HIV-specific antibodies develop in a prescribed order based on epitope target and function (Figure 1.2) (48). Within the first two to three weeks of HIV infection, the first antibodies to develop are gp41-specific IgM antibodies. These are followed by gp41-specific IgG antibodies, then gp120-specific IgG antibodies.

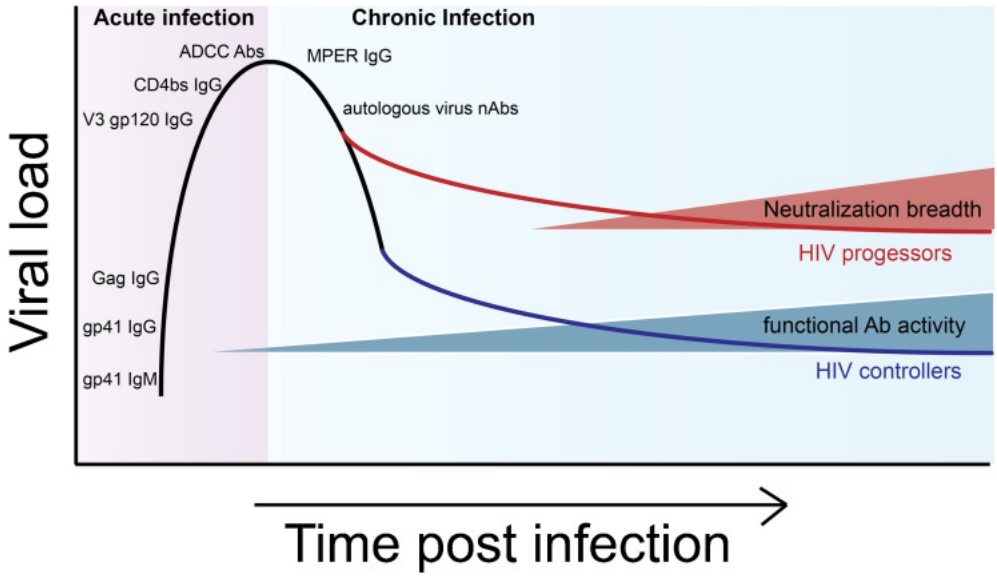


Figure 1.2. Antibody production over the course of acute and chronic infection. Figure reproduced from Butler, Fischinger, and Alter. The Antibodiome – Mapping the Humoral Immune Response to HIV. Current HIV/AIDS Reports (2019) 16:169–179. (48). http://creativecommons.org/licenses/by/4.0/

In terms of function, ADCC-mediating antibodies arise early in infection, and have been detected as early as 52 days post-infection (48-50). ADCC-mediating antibodies elicit some immune pressure on the virus as evidenced by viral escape from the autologous ADCC response (51). Despite viral escape, ADCC-mediating antibodies have been correlated with reduced set point viral load, reduced disease progression, delayed progression to AIDS, and improved clinical outcome in a variety of settings (52-67) (also reviewed in (48, 68, 69)).

After ADCC-mediating antibodies arise, the development of antibodies capable of neutralization follow. The first neutralizing antibodies to develop are generally specific for the infecting strain (autologous neutralizing antibodies) rather than other more genetically diverse circulating strains (neutralizing antibodies capable of neutralizing these diverse strains are called heterologous neutralizing antibodies) (48). As autologous neutralizing antibodies develop, the immune response is steered away from an ADCC response and towards a neutralizing response, and levels of ADCC-mediating antibodies wane (49). Over time, neutralizing antibodies undergo extensive mutations to co-evolve with the virus and become the predominantly antibody response throughout chronic progressive infection (48). Despite the production of increasingly specific neutralizing antibodies over time, neutralizing antibodies fail to contain viral replication as the virus rapidly escapes (48, 70). Since the virus can outrun the antibody response, there is a constant high level of antigenic stimulation which has been suggested to drive production of HIV-specific neutralizing antibodies (71). In contrast to ADCC-mediating antibodies, neutralizing antibodies do not correlate with improved clinical outcome (48). In fact, neutralizing antibody titer and breadth tends to directly correlate with viral load and disease progression, perhaps due to constant antigenic stimulation (48, 71). Despite not being effective in an established infection, it is generally thought in the HIV field that neutralizing antibodies can protect against HIV infection if they are present prior to HIV exposure in the setting of vaccination because the amount of virus they have to neutralize is much lower (vaccination studies will be discussed below).

A small percentage of HIV-infected individuals, termed long-term non-progressors (LTNPs) are able to control viral replication without antiretroviral treatment, maintain higher CD4 counts, and display reduced disease progression. LTNPs are comprised of elite controllers

(ECs) who maintain the virus at undetectable levels in the absence of therapy and viremic controllers (VCs) who maintain a low but detectable viral load and display slowed disease progression (72). Considerable emphasis has been placed on Identifying correlates that distinguish LTNP, EC, or VC status compared to viremic progressors (the majority of the population undergo normal rates of disease progression and high viral replication without treatment). Identifying these correlates would potentially provide insight into the mechanism by which viral replication is controlled and disease progression is reduced in these individuals. This mechanism may be mediated by the immune system, or be due to differences in infecting viral strains, genetic factors, or other factors (69, 72). Interestingly, multiple studies have shown that ADCC has been correlated to EC or LTNP status (58, 60, 61, 64), in contrast to neutralization which has been shown to be higher in viremic progressors compared to controllers, likely due to antigenic stimulation described above (60, 71). Additionally, even though a number of studies have not shown this association of ADCC individually with LTNP status (39, 73, 74), polyfunctional effector function responses, including a combination of ADCC, ADP, and ADCD, have recently been associated with elite control of HIV (73). Interestingly, a number of studies investigated epitopes of ADCC-mediating antibodies in LTNPs and have found that ADCC targeting HIV accessory proteins including vpu and tat, in addition to envelope (specifically V3 of envelope) are associated with LTNP status (58, 61, 64). Taken together, these data suggest that ADCC has the potential to control viral replication and reduce disease progression.

HIV Vaccine Trials

Vaccination in Non-Human Primates

Designing a preventive HIV vaccine remains a major, yet elusive, goal in the HIV field. Studies of macaques have repeatedly shown that antibodies can prevent infection with a SIV that encodes HIV envelope (SHIV) (reviewed in (75-77)). Specifically, studies have repeatedly shown that neutralizing antibodies can provide protection against viral challenge (77-79). However, this protection can be dependent, at least in part, on Fc-effector functions of the neutralizing antibodies. In a seminal study by Hessell et al, passive transfer of broadly neutralizing antibodies with a mutated FcR into non-human primates (NHP) provided incomplete protection compared to the wildtype antibody, indicating that protection mediated by neutralizing antibodies is partially dependent on Fc effector function (80). A follow-up study by the same group showed a trend toward reduced protection of NHP treated with wildtype compared to Fc-defective neutralizing antibody variants (81). In a more recent study where neutralization correlated with protection, disseminated foci of infections were detected in multiple tissues prior to viral clearance, suggesting that Fc effector functions were playing a role in clearing the foci of infected cells and in preventing the establishment of systemic infection (82).

Vaccine elicited ADCC-mediating antibodies have also been directly correlated with protection in multiple studies. For instance, vaccine-elicited ADCC responses have correlated with protection from viral challenge (83-85), even when neutralizing antibodies either were not correlated with protection or were not strongly elicited by vaccination (83, 84). These NHP studies have provided proof-of-principle that antibodies can prevent infection in the setting of vaccination and have shown that both neutralization and Fc effector functions can play roles in that protection.

One important consideration in extrapolating from the NHP models is that the challenge is often a single defined viral strain (75, 76), which does not recapitulate the complex diversity of HIV infection in humans. Indeed, the SHIV strains that are used are highly adapted to replicate in macaques (86, 87). In humans, the "challenge strain" is a viral quasispecies from the infected person, which can contain variants that have over 10% variation in envelope at the nucleotide level (88). To complicate matters further, the virus is incredibly diverse globally, and can vary by 20-36% in amino acid sequence between clades (88). Thus, while the NHP models are highly valuable for proof of concept studies, it remains unclear how studies from the NHP models will translate in the much more complex setting of human infection.

Vaccination in Humans

There have been 6 phase III vaccine trials in humans to date (89). An early HIV vaccine trial, Vax004 (AIDSVAX B/B; VaxGen protein vaccine) was not efficacious overall, but did have three humoral correlates of decreased risk: antibody-dependent cellular viral inhibition (which measures inhibition of viral release due to a combination of ADCC and antiviral activities mediated by B-chemokine secretion from effector cells), tier 1 neutralization, and CD4 blocking (the ability of plasma to block anti-CD4 antibodies from binding) (89-91). The only human trial to show any efficacy to date was the RV144 trial (92). This ALVAC-HIV (vCP1521) prime and AIDSVAX B/E boost vaccine elicited very weak neutralizing antibodies and thus these were not driving protection; rather, non-neutralizing antibodies appeared to play a protective role (92-94). IgG binding antibodies that targeted first and second variable regions (V1V2) of Env were correlated with protection, and surprisingly, HIV-specific IgA levels were correlated with increased risk (93, 95). Furthermore, V1V2 antibodies of the IgG3 subclass and antibodies

specific for linear V2 and V3 epitopes were also found to be correlated with protection in the RV144 trial (96, 97). In a secondary analysis, ADCC and tier 1 neutralization were correlated with protection in vaccinees that had low circulating IgA (93). ADCC-mediating antibodies from RV144 vaccinees were subject of further study (98-100). V1V2-directed ADCC-mediating antibodies from vaccinees were able to mediate ADCC against both tier 1 and tier 2 targets (98). A study by Tomaras and colleagues suggested that in vaccinees with high IgA, IgA inhibited IgG-mediated ADCC by competing for the C1 ADCC epitope (99). HIV-specific IgG3, which is often a strong effector of Fc-mediated functions as described above, was also a correlate of protection in the RV144 trial (96). Taken together, these data highlighted that antibody responses dependent on both the Fab domains and the Fc region may be important components of an effective vaccine response. However, the RV144 trial was only 31% efficacious (92), and the other 5 HIV vaccine trials did not show any efficacy (89). Thus, there is very limited data on correlates of overall protection from these trials. An improved knowledge of the correlates of protection or risk reduction from transmission and improved disease outcome in the setting of natural HIV infection has the potential to provide valuable information to improve rational vaccine and therapeutic design.

Mother-to-Child Transmission of HIV-1

Mother-to-child transmission (MTCT) provides a unique setting to examine correlates of risk reduction from HIV transmission as well as impact on disease progression, particularly because infants passively acquire maternal antibodies. Moreover, there is generally more clarity on the source and timing of transmission compared to sexual transmission. MTCT of HIV can occur *in utero*, peripartum, or via breastfeeding (101). As mentioned above, without

antiretroviral treatment, the risk of MTCT is ~15-40% depending on the population and setting (4-6). Among cases of MTCT, approximately 40-50% of transmissions are due to breastfeeding 20-25% are due to *in utero* transmissions, and 35-50% occur during delivery (102). Risk factors for MTCT of HIV include maternal viral load, maternal CD4, and gestational age, among others (101).

Antibodies in MTCT of HIV

Mothers passively transfer antibodies to their infants *in utero*, and these antibodies remain in infant circulation for months after birth (54, 103-113). IgG antibodies are the major isotype that crosses the placenta, and they cross at different rates based on subclass (IgG1>IgG4>IgG3>IgG2) (114). The majority of passive antibody transfer occurs in the third trimester and reaches levels close to or even exceeding antibody levels in the mother (114, 115).Therefore, in the setting of breastfeeding, infants who are uninfected at birth have pre-existing HIV-specific IgG antibodies elicited by natural infection (of the mother) circulating prior to HIV exposure through breastfeeding, analogous to what is expected as a result of vaccination. Thus, MTCT is a natural passive vaccination setting. Since the majority of HIV-exposed infants actually remain uninfected even without antiretroviral treatment, this may suggest that a maternal factor, such as antibodies, provides the infant with some protection against MTCT.

Many groups have investigated the role of antibodies in MTCT of HIV. Binding antibody epitopes, neutralization, and ADCC have been the focus of previous studies. Antibody binding to epitopes within V3, the CD4 binding site, gp41, gp120, and p24 (the capsid protein) have been associated with reduced risk of MTCT in some studies (55, 116-124), increased risk in other

studies (55, 122, 125-128), and not associated with risk in yet other studies (116, 119, 121, 126, 128-135). Studies focusing on neutralization have been equally inconsistent. Our group and others have shown that neutralizing antibodies are not correlated with risk of infant infection (54, 57, 103, 115, 128, 135-142). Other groups have shown that neutralization is correlated with reduced risk of infant infection (116, 119, 125, 143-150). Yet others have shown that neutralization is correlated with increased risk of MTCT (127, 151).

MTCT studies focusing on ADCC have been somewhat more consistent, with the caveat that there have been far fewer studies focusing on ADCC in MTCT of HIV compared to binding antibodies and neutralization. Our group showed that in a cohort of breastfeeding mothers with high viral load, breastmilk ADCC correlated with reduced risk of MTCT (53). While no other studies to our knowledge have reported plasma ADCC to be a correlate of reduced risk of MTCT (56, 57, 119, 137, 152, 153), three studies have reported ADCC activity to be associated with trends or non-significant associations with reduced risk of MTCT (54, 55, 138). Additionally, a number of studies have shown ADCC to be correlated with improved infant outcome such as reduced progression to AIDS and improved infant survival (54-57).

Even though passively-acquired antibodies are from the same repertoire as those circulating in the mother, it is possible that both antibodies circulating in the mother and passively-acquired maternal antibodies circulating in the infant are playing independent roles in MTCT of HIV, as they are acting in different landscapes. Maternal antibodies exist in the context of an established infection and may play a role in the transmissibility of viral variants. Passively-acquired maternal antibodies circulating in infants exist in a landscape without virus present and may play a role in determining the infectibility of infant cells and dissemination of the infection. Therefore, it is valuable to investigate the roles of both maternal and passively-acquired

antibodies in MTCT. However, the vast majority of the aforementioned studies investigated antibodies either circulating in the mother (55, 116-122, 126-135, 138, 140-150) or examined those circulating in the infant (56, 103, 153). Only a handful of studies examined antibodies from both maternal and infant samples at the same time (54, 57, 123-125, 136, 151, 152). Our group was one of the few to simultaneously characterize the associations of maternal and passively-acquired antibodies in infants with MTCT outcome (54). I took this approach for this thesis.

Additionally, the many of the aforementioned studies did not include breastfeeding transmissions and thus focused on earlier infections where passive antibodies in the infant may not have reached their peak levels (118, 119, 125, 135, 139, 140, 143, 146, 148). Some earlier studies may have included breastfeeding transmissions but were conducted using antibody assays where infant infection time or mode of transmission could not be accurately determined prior to 15-18 months of age and thus whether passively-acquired antibodies remained in infant circulation until transmission is unclear (116, 117, 122, 145). Our group was one of the few to include breastfeeding transmissions when timing of infection could be accurately determined using HIV nucleic acid testing, and the role of passively-acquired antibodies in breastfeeding transmissions could be more clearly discerned (54, 103, 128, 137, 142, 151). For this thesis, I focused on breastfeeding transmissions to investigate whether pre-existing HIV-specific antibodies could prevent MTCT and/or improve infant outcome because this MTCT setting is the most closely analogous to vaccination.

As mentioned above, antibodies that bind HIV do not necessarily mediate beneficial functions. In the following chapters, I will investigate the role that maternal and passively-acquired antibodies play in MTCT. I will investigate whether certain epitopes of binding antibodies are correlated with risk of infant infection and infected infant outcome (Chapters II

and III), and I will complement the investigation of antibody epitopes by exploring the role ADCC plays in MTCT risk and infant outcome (Chapters III-V).

MTCT Cohorts Utilized in this Thesis

To assess the effect maternal and passively-acquired antibodies have on MTCT and infant outcome, I utilized two cohorts of samples from the Nairobi Breastfeeding Clinical Trial (NBT) (5) and the Cytotoxic T Lymphocyte Study (CTL) (154-156). These two unique cohorts were ideal for this study. Both studies were conducted before long-term ART was the standard of care (NBT participants did not receive ART; mothers in the CTL study only received short course AZT near delivery, which has a short half-life and thus has limited impact on breastfeeding transmission (157-159)). This allowed antibody responses in the context of natural infection to be studied without the effects of long-term ART on viral replication and the humoral response. Infants in both cohorts did not have detectable HIV infection at birth, had samples available from near birth (prior to infection), and breastfed. This allowed us to study the effect of pre-existing HIV-specific passively-acquired antibodies on risk of MTCT of HIV and infant outcome in the face of continued HIV exposure through breastfeeding. Both maternal and infant samples were collected and available for use allowing us to compare the role of antibodies circulating in the mother to those passively-acquired and circulating in the infant in MTCT of HIV. Maternal plasma samples from the third trimester and infant plasma from the first week of life in breastfeeding mother-infant pairs were utilized in this thesis.

Goals for this thesis

The overall goal for this thesis is to investigate humoral correlates of reduced risk of transmission or improved clinical outcome in the setting of breastfeeding MTCT of HIV. This information can be informative for MTCT prevention, antibody-based therapeutic design and future vaccine design. As discussed above, both antigen specificity (determined by the Fab) and effector function (determined by the Fc region) are important to the immune response against HIV. In Chapter II, I will focus on antigen specificity of binding antibodies, and will screen maternal and infant plasma for binding to a panel of HIV antigens to determine whether certain epitopes are correlated with reduced risk of MTCT. In Chapter III, I will focus on the entire antibody, and determine whether epitopes of ADCC-mediating antibodies contribute to MTCT risk or infant clinical outcome. In Chapters IV and V, I will focus solely on the Fc region and effector function. In Chapter IV, I will investigate whether specific FcγRs have varying effects on the risk of MTCT and infant outcome to gain a more complete picture of the role antibody-mediated effector functions plays in the NBT cohort. In Chapter V, I will describe preliminary data on passively-acquired ADCC activity in the CTL cohort to examine whether our previously reported results with the NBT cohort (54) can be validated in a second cohort.

Chapter II

gp41 ectodomain-specific IgG is associated with increased mother-to-child transmission of HIV-1

Introduction

Mother-to -child transmission (MTCT) provides a setting to study the effect of pre-existing HIV-specific antibodies on the risk of HIV infection. Infants receive passively-transferred antibodies from their mothers *in utero*, which achieve maximal levels at birth (114) and these antibodies remain in infant circulation for months after birth (54, 103-113). Therefore, in the setting of breastfeeding, infants who are HIV-negative at birth have HIV-specific antibodies in their circulation prior to HIV exposure through breastmilk. This is generally similar to what is anticipated with vaccines, where the goal is to elicit antibodies that will be present during HIV exposure and provide protection. MTCT is thus a natural passive vaccination setting, where the antibodies were elicited by natural infection of the mother. MTCT is therefore a unique setting to identify characteristics of pre-existing antibodies correlated with reduced risk of transmission and improved disease outcome which may inform future vaccine and therapeutic design.

In order for a vaccine to provide protection from infection or disease, or an antibody-based therapy to reduce viral levels, antibodies associated with protection must target crucial epitopes on the virus which lead to a downstream effect upon binding, such as inhibition of a step in the viral life cycle, reduced viral fitness or which allows the antibody to bind in such a way that it can signal other effectors of the immune system to clear the virion or infected cell.

The epitopes recognized by HIV-specific antibodies are diverse (22, 23), and may vary depending on the functional activity of the antibody, such as for neutralizing versus non neutralizing antibodies, as described in Chapter I. There is not a consistent picture of which of these antibody specificities are correlated with protection or reduced disease progression. In the only partially protective human HIV vaccine trial to date, the RV144 trial, antibodies targeting the V1V2 region of envelope and linear epitopes within V2 and V3 of envelope were correlated with protection (92, 93, 95, 97). Multiple studies have investigated antibody specificities correlated with reduced risk of MTCT of HIV, but the results are inconsistent. There is no consensus as to which epitopes are targeted by binding antibodies that are associated with reduced risk of transmission. A number of studies have shown that antibodies which bind to the CD4 binding site (CD4bs), V3, gp120, p24, or gp41 were associated with reduced risk of MTCT (55, 116-124), but these associations were not observed in multiple other studies (55, 116, 119, 121, 122, 125-135). These inconsistencies could be due to many factors including differences in sample size, ART-treatment, methods of determining infant infection, or differences in protective mechanisms for different routes of transmission. Moreover, the majority of these studies included *in utero* and peripartum transmissions where it is impossible to sample infant antibody responses to investigate pre-existing passively-transferred antibodies present at the time of HIV-1 exposure prior to infection (55, 116-135), and most of these studies only measured maternal antibodies, and did not investigate epitopes of passively-acquired antibodies in infants (55, 116-122, 126-135). Measuring passively-transferred antibody responses from mother-infant pairs where transmissions are due to breastfeeding could allow pre-existing passively-transferred antibodies in infants to be characterized, identify protective characteristics of pre-existing antibodies, and clarify results from prior studies. In addition, examining both maternal and infant

passively-acquired antibodies could differentiate the role of maternal antibodies versus pre-existing passively-acquired antibodies in infants in MTCT of HIV.

In this study, we hypothesized that HIV-specific antibodies that target specific epitopes are associated with reduced risk of MTCT. We utilized a unique cohort of ART-naïve breastfeeding Kenyan mother-infant pairs from the Nairobi Breastfeeding Clinical Trial (5). We screened plasma from mothers and infants for binding against a panel of HIV antigens in the binding antibody multiplex assay (BAMA) to determine whether maternal or passively-acquired antibodies targeting specific epitopes were associated with reduced risk of MTCT.

Materials and Methods

Study Design and Plasma Samples

Plasma samples from the Nairobi Breastfeeding Clinical Trial (NBT) were utilized in this study. The NBT was conducted from 1992-1998, before anti-retroviral therapy was the standard of care; therefore, all of the participants were ART-naive (5). HIV-1 positive mothers were enrolled during the third trimester. Maternal blood samples were collected at the time of enrollment. Infant blood samples were collected at birth and at regular intervals until 2 years of age. HIV infection status of infant PBMCs was determined by single copy detection DNA PCR (160). Infants testing positive for HIV DNA, were retrospectively tested for HIV RNA from samples collected at prior timepoints using a prototype Gen-Probe/Hologic HIV viral load assay that detects diverse subtypes (161). For the purposes of this study, estimated time of infection was defined as the midpoint between the last HIV negative and first HIV positive test (54). The Kenyan Ministry of health gave permission for the Nairobi Breastfeeding Clinical Trial to be

conducted, and the Institutional Review Boards of the University of Nairobi, University of Washington, and the Fred Hutchinson Cancer Research Center approved the current study.

Plasma samples from a cohort of 72 mother-infant pairs from the Nairobi Breastfeeding Clinical Trial utilized in a previous study by our group were utilized in the present study according to identical selection criteria (54). Briefly, mother-infant pairs meeting the following selection criteria were included: the infant was HIV RNA and DNA negative at birth, breastfed for a minimum of 3 months, HIV-exposed uninfected (HEU) infants remained HIV-negative for a minimum of 6 months and at each follow up timepoint, and an infant plasma or cord blood sample was available from the first week of life (prior to estimated time of infection). Infant cord blood (N=60) or neonatal plasma from delivery (N=10) or week 1 (N=1) were tested in this study along with paired maternal plasma samples from the third trimester of pregnancy (N=68) or delivery (N=3). Of note, 1 infected infant and 1 non-transmitting mother from the 72 pairs studied by Milligan et al had no more plasma available (54); their corresponding transmitting maternal and HEU infant plasma samples were available and included in this study. The final cohort included 70 paired maternal and infant samples (50 non-transmitting pairs and 20 transmitting pairs), 1 unpaired transmitting maternal sample, and 1 unpaired HEU infant sample. All plasma and cord blood samples were heat inactivated at 56 degrees Celsius for 1 hour. This cohort will also be utilized in Chapters III and IV.

Binding Antibody Multiplex Assay

The binding antibody multiplex assay (BAMA) was performed as described previously (93, 162-164). Briefly, antigens were covalently conjugated to carboxylated fluorescent beads (Luminex) as described previously (163, 165). Antigen-conjugated beads were incubated with plasma samples for 30 minutes, washed, incubated with a secondary antibody to IgG

(biotinylated mouse anti-human IgG, Southern Biotec), washed, incubated with streptavidin-PE (BD Pharmingen) for 30 minutes, washed, and acquired with a Bio-Plex 200 instrument (Bio-Rad Laboratories). Washes were conducted with 0.1% BSA, 0.02% Tween20, and 0.05% sodium azide diluted in PBS. All other steps were conducted with 1% non-fat dry milk (NFDM), 5% goat serum (Sigma), and 0.05% Tween20 diluted in water. All steps were performed at room temperature (RT). A minimum of 50 beads per antigen per sample were assayed. The antigen panel included: monomeric gp120 proteins BG505.W6M.C2.T332N.L111A (clade A), Q461.d1 (clade A), BL035.W6M.ENV.C1 (clade A/D recombinant), SF162 (clade B), ZM109F.PB4 (clade C), C2-94UG114 (clade D), and SIV/mac239; clade A BG505 SOSIP Env trimer (courtesy of Marit van Gils, Rogier Sanders and John Moore) (28); resurfaced Env core protein (RSC3) and corresponding CD4-binding site defective mutant (RSC3 Δ371I) (construct from NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Drs. Zhi-Yong Yang, Peter Kwong, Gary Nabel) and produced as described previously (166); clade C 2J9C-ZM53_V1V2 and 1FD6-Fc-ZM109_V1V2 scaffolded peptides (167); 2J9C and 1FD6-Fc scaffolds (167); clade B caseA2 mulvgp70 scaffolded peptide (Immune Tech); mulvgp70 scaffold (Duke Protein Production Facility); V3 consensus peptides ConA1 (CTRPNNNTRKSIRIGPGQAFYATGDIIGDIRQAHC), ConB (CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC) ConC (CTRPNNNTRKSIRIGPGQTFYATGDIIGDIRQAHC), and ConD (CTRPYNNTRQRTPIGPGQALYTTRIKGDIRQAHC) (Genscript); clade A1 gp140 CF consensus (Duke Protein Production Facility); three gp41 antigens: clade B MN gp41 protein (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from ImmunoDX, LLC), clade C ectodomain ZA.1197/MB protein (Immune Technology Corp), and 6-helix gp41 stump mimetic

(courtesy of Peter Kim). BG505.W6M.C2.T332N.L111A gp120 was produced in 293F cells (Thermo Fisher) after transient transfection and purified by *Galanthus nivalis* lectin (Vector Laboratories) as described previously (168). The remining gp120 proteins were purchased from Immune Tech. Each conjugated bead preparation and assay were validated with monoclonal control antibodies (data not shown). Positive controls included VRC01, PG9, PGT121, 4E10, 2F5, anti-p24 antibody. All positive controls were obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, except anti-p24 antibody, which was produced as described previously (41, 164). Negative controls included both HIV-negative plasma (VA study) (169) and mock conjugated beads. Plasma samples or HIVIG were used at a dilution empirically defined by our group (163). Plasma samples or HIVIG were used at a 1:200 dilution for ConD V3, all V1V2 peptides and their scaffolds, BG505 trimer, all gp120 proteins except clade D C2-94UG114; a 1:800 dilution for C2-94UG114 gp120, conA1V3, conBV3, conCV3, RSC3, RSC3 Δ371I, and p24; and a 1:10,000 dilution for all three gp41 antigens. For each plasma sample, median fluorescence intensity (MFI) binding to each antigen was measured and averaged across duplicate wells. Plasma binding to an appropriate negative control antigen was measured and considered as background. Negative control antigens were as follows: 2J9C scaffold for the 2J9C-ZM53_V1V2, 1FD6-Fc scaffold for the 1FD6-Fc-ZM109_V1V2 peptide, mulvgp70 scaffold for the caseA2 mulvgp70 peptide, RSC3 Δ371I for RSC3, and SIV gp120 for all other antigens. For each antigen, background binding was subtracted from the plasma MFI. Since MFIs vary from experiment to experiment (170), in order to average data from biological replicates, we normalized the background subtracted MFI to that of the positive control HIVIG and converted it to a percent according to the following equation: (plasma $MFI_{HIV\ antigen}$ – plasma $MFI_{negative\ control\ angiten}$)/ (HIVIG $MFI_{HIV\ antigen}$ – HIVIG $MFI_{negative\ control\ antigen}$)*100%. Percentage

binding shown is averaged from two biological replicates, each performed in technical duplicate. Of note, 3 biological replicates were averaged for maternal plasma binding against 6-helix gp41 stump mimetic and the clade B MN gp41 antigen.

V1V2 ELISA

Immunolon 2-HB ELISA plates were coated with clade B V1V2caseA2-mulvgp70 protein at 1.25ug/ml overnight at 4 degress Celsius. The next day, plates were washed 4 times with PBS-0.1% Tween20 and then blocked with 10% NFDM in PBS-0.1% Tween20 for 1 hour at RT. Plasma samples (1:200 dilution, determined empirically to give a dynamic range between samples) were diluted in 10% NFDM in PBS-0.1% Tween20, added to the plate, and incubated for 1.5 hrs at RT. Plates were washed and incubated with goat anti-human IgG HRP (Sigma, 1:2500 dilution) diluted in 10% NFDM in PBS-0.1% Twen20 for 1 hour at RT. Plates were washed and incubated with Ultra-TMB (ThermoFisher) for 12 minutes. The reaction was stopped with 1N H_2SO_4 (Fisher Scientific). The absorbance was read at 450nm. The OD was normalized to that of the positive control HIVIG and converted to a percent to represent % binding. Percentage binding shown is averaged from two biological replicates, each performed in technical duplicate.

Soluble CD4 blocking assay

Immunolon 2-HB ELISA plates were coated with clade A BG505.W6M.C2.T332N.L111A (10ug/ml) or clade D C2-94UG114 gp120 (2.5ug/ml) overnight at 4 degrees Celsius. The next day, plates were washed 4 times with PBS-0.1% Tween20 and then blocked with 10% NFDM in PBS-0.1% Tween20 for 1 hour at RT. Plasma samples (1:200 dilution, determined empirically to give a wide dynamic range among plasma samples) were

diluted in 10% NFDM in PBS-0.1% Tween20, added to the plate, and incubated for 1.5 hours at RT. Plates were washed and incubated with soluble CD4 (sCD4) at 1ug/ml (determined empirically to be a saturating concentration) for 1.5hrs at RT. Plates were washed and incubated with biotinylated-OKT4 anti-CD4 antibody (ThermoFisher, 1:5000 dilution) diluted in 10% NFDM in PBS-0.1% Twen20 for 1 hour at RT. Plates were washed and incubated with streptavidin-HRP (ThermoFisher, 1:10000 dilution) for 1 hour at RT. Plates were washed and incubated with Ultra-TMB for 2 minutes for plates coated with clade D C2-94UG114 gp120 or 15 minutes for plates coated with clade A BG505 gp120. The reaction was stopped with 1N H_2SO_4. The absorbance was read at 450nm. Percent inhibition was calculated as follows: 100%-[(OD_{plasma}/$OD_{no\ plasma}$)*100%]. Inhibition was validated for each assay with the positive control antibody VRC01 and negative control HIV-negative plasma (VA study (169)) (data not shown). Percentage binding shown is averaged from two biological replicates, each performed in technical duplicate. Only one biological replicate with maternal plasma against clade D C2-94UG114 gp120-coated plates was performed.

Statistical Analysis

All statistical analyses were performed with Stata15SE (StataCorp, College Station, TX), GraphPad Prism7 (GraphPad Software, Inc., San Diego, CA), or R/RStudio (The R Foundation/R Studio Inc).To measure the association of plasma binding to each HIV antigen with risk of MTCT, we performed a logistic regression analysis on the normalized plasma binding or sCD4 inhibition value adjusted for maternal plasma RNA viral load, a known risk factor for MTCT and potential confounder in this study (101). Statistical significance was pre-specified as $p<0.05$. The Holm-Bonferoni method was used to correct for multiple comparisons.

Results

Plasma IgG binding to HIV antigen panel as measured by BAMA

To determine whether maternal or passively-acquired antibodies targeting specific regions of HIV were associated with risk of MTCT, we measured plasma IgG binding against a panel of HIV antigens in a binding antibody multiplex assay (BAMA) (Table 2.1).

Table 2.1. Plasma binding to BAMA antigen panel.
BAMA antigens are shown in the left-hand column. Normalized plasma binding (calculated as described in the methods) against each antigen is shown for HIV-exposed uninfected (HEU) infants, infants who acquired HIV (HIV+), non-transmitting mothers (NT), and transmitting mothers (T). Median and interquartile ranges (IQR) are shown.

Table 2.1	Infant Plasma % Binding (Median (IQR))		Maternal Plasma % Binding (Median (IQR))	
Antigen	HEU (N=51)	HIV+ (N=20)	NT (N=50)	T (N=21)
P24 (IIIB)	89 (58, 97)	54 (4, 94)	79 (47, 98)	52 (14, 94)
Clade C gp41 ectodomain (ZA1197)	17 (6, 31)	22 (13, 62)	22 (9, 40)	57 (26, 71)
Clade B gp41 (MN)	2 (1, 9)	3 (1, 13)	8 (2, 29)	24 (10, 37)
6-helix gp41 stump mimetic	6 (1, 20)	9 (2, 28)	15 (2, 28)	28 (17, 44)
Clade A1 gp140CF	61 (43, 81)	73 (61, 89)	60 (42, 86)	87 (58, 91)
Clade A trimer (BG505)	18 (5, 52)	26 (6, 76)	30 (18, 72)	69 (12, 89)
Clade A gp120 (BG505)	68 (18, 104)	73 (34, 109)	78 (40, 116)	101 (37, 119)
Clade A gp120 (Q461.d1)	77 (24, 111)	87 (30, 114)	85 (40, 123)	112 (46, 130)
Clade B gp120 (SF162)	7 (4, 20)	12 (6, 23)	21 (11, 40)	29 (13, 45)
Clade C gp120 (ZM109)	34 (4, 73)	46 (7, 88)	41 (22, 92)	78 (36, 107)
Clade D gp120 (C294UG)	7 (3, 39)	17 (5, 44)	13 (6, 88)	32 (14, 133)
Clade A/D gp120 (BL035)	90 (13, 135)	109 (30, 135)	99 (41, 150)	134 (55, 154)
Clade A1 V3	82 (8, 188)	79 (24, 176)	89 (12, 242)	140 (52, 242)
Clade B V3	26 (6, 57)	11 (2, 51)	25 (6, 57)	18 (3, 60)
Clade C V3	115 (13, 273)	103 (19, 220)	122 (10, 483)	172 (74, 522)
Clade D V3	2 (0, 317)	9 (0, 108)	8 (1, 261)	41 (3, 214)
Clade B V1V2caseA2	0.5 (0, 2)	0 (0, 6)	6 (1, 19)	4 (0, 20)
Clade C V1V2 (ZM109)	3 (0, 12)	7 (0, 15)	8 (0, 25)	17 (0, 41)
Clade C V1V2 (ZM53)	35 (1, 50)	24 (2, 70)	32 (1, 62)	24 (0, 90)
RSC3 CD4bs core	1 (0, 2)	1 (0, 4)	1 (0, 4)	2 (1, 8)

Maternal samples from the third trimester or delivery and infant plasmas from the first week of life, prior to estimated time of infection, were screened in this study. To measure the association of plasma IgG binding to the antigen panel with risk of MTCT, we performed a logistic regression analysis on the percent binding for each antigen in the BAMA adjusted for maternal plasma RNA viral load, an independent risk factor for MTCT and potential confounder in this study (Figure 2.1) (101).

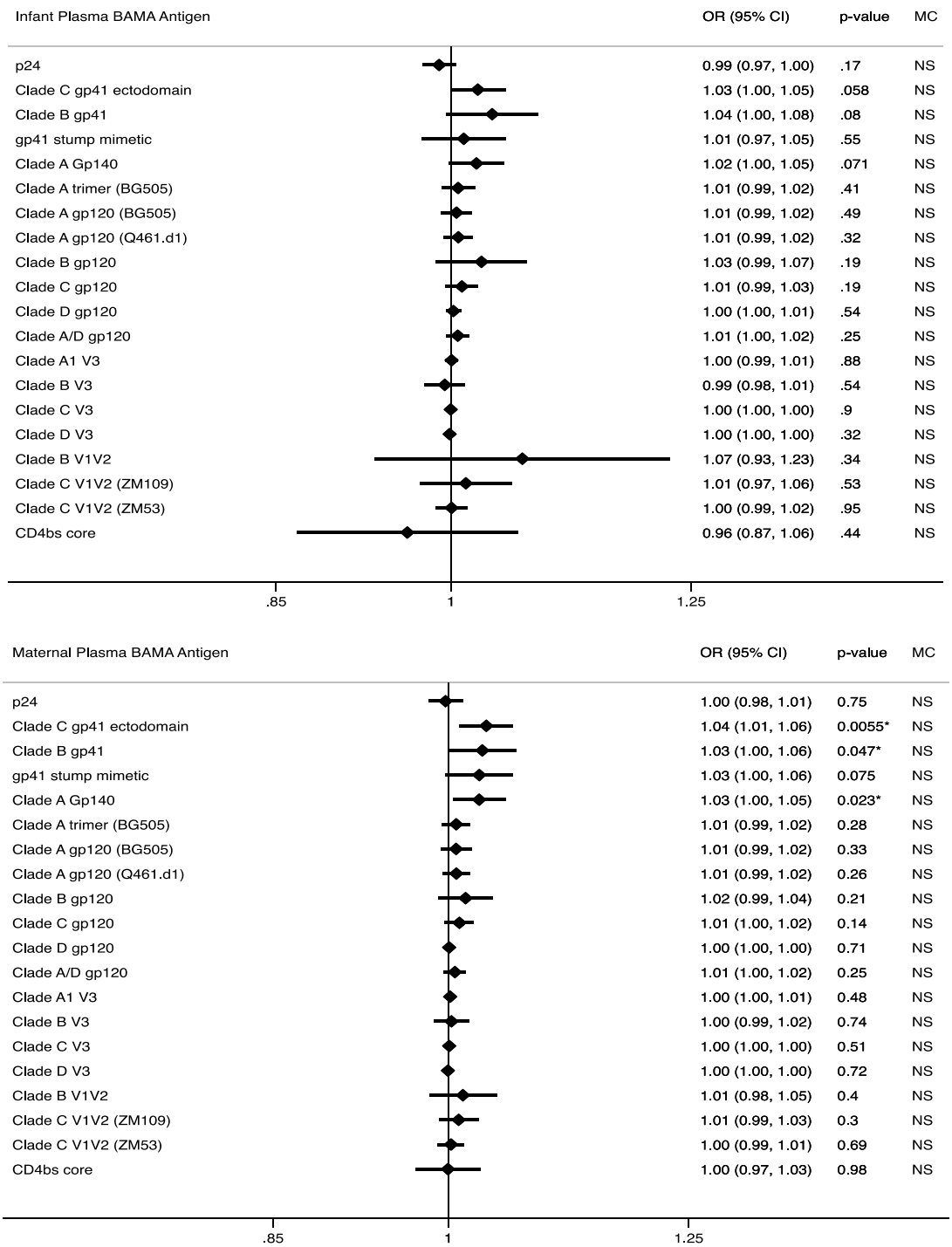


Figure 2.1. Association of plasma IgG binding with odds of MTCT.
The association of plasma IgG binding to each BAMA antigen with odds of MTCT was measured using a logistic regression analysis adjusted for maternal plasma viral load. Adjusted odds ratios (OR), 95% confidence intervals (CI), and p-values are shown for infant samples. Statistical significance was defined as $p<0.05$ (*). Results were corrected for multiple comparisons using the Holm-Bonferoni method (MC, right column).

Maternal plasma IgG binding to the clade C gp41 ectodomain antigen, clade A gp140CF, and clade B gp41 protein were significantly associated with increased odds of MTCT (ectodomain aOR=1.04, p=0.0055; gp140 aOR=1.03, p=0.023; gp41 aOR=1.03, p=0.047). Passively-acquired IgG binding to these antigens showed trends in the same direction (ectodomain aOR=1.03, p=0.058; gp140 aOR=1.02, p=0.071; gp41 aOR= 1.04; p=0.08). Finally, maternal plasma binding to the 6-helix gp41 stump mimetic was also associated with a trend towards increased odds of MTCT (aOR=1.03, p=0.075). Plasma binding to the remaining BAMA antigens showed no association with odds of MTCT. However, when we corrected for multiple comparisons according to the Holm-Bonferoni method, plasma IgG binding as measured by BAMA showed no significant or trending associations with odds of MTCT for any antigen.

Of note, the clade C gp41 ectodomain antigen contains a portion of the membrane proximal external region (MPER), as does gp140CF and MNgp41 (Figure 2.5). However, MPER positive control antibodies do not bind to the clade C ectodomain antigen, perhaps due to the C-terminal His-tag blocking the MPER epitopes, but they do bind to gp140CF and MNgp41 (Figure 2.2), as measured by BAMA.

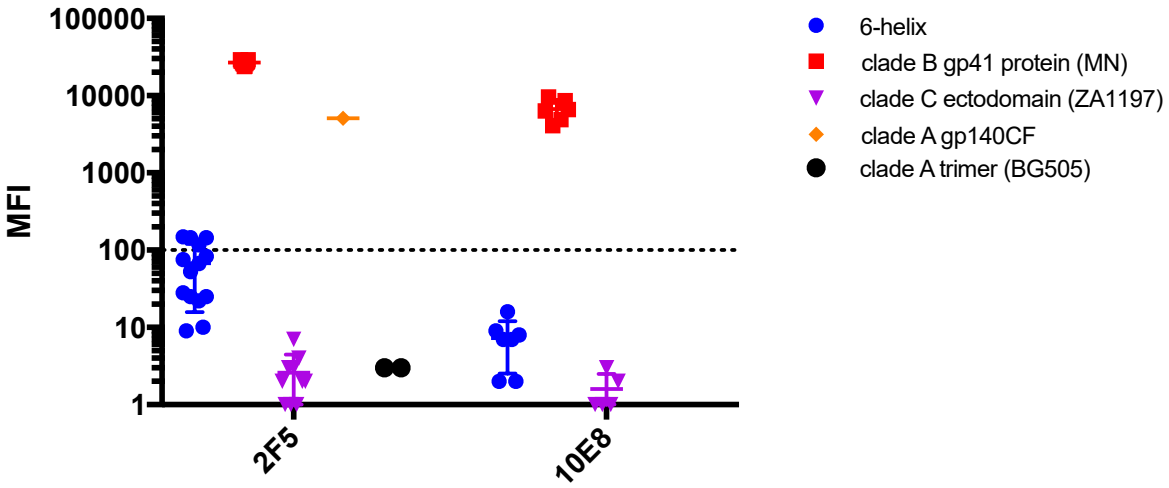


Figure 2.2. MPER antibodies do not bind the gp41 ectodomain antigen.
2F5 and 10E8 (25ug/ml) were used as controls in the BAMA. Binding of 2F5 and 10E8 to the 5 different gp41-containing BAMA antigens is shown. Y-axis is median fluorescence intensity (MFI). The line at 100MFI indicates the limit of the detection of the BAMA. Either Adam Dingens or I performed the experiments presented in this figure.

Plasma IgG binding to HIV antigens as measured by ELISA or soluble CD4 blocking assays

The majority of the BAMA antigens showed a wide dynamic range of plasma IgG binding above the limit of detection (100 MFI after subtracting out background) within each biological replicate (raw MFI data not shown). However, for antigens V1V2 ZM109, V1V2caseA2, conDV3, and RSC3, over 40% percent of the samples had binding below the limit of detection in multiple biological replicates (raw MFI data not shown), making it difficult to compare binding across transmission groups. For these antigens, we used alternative assays to measure plasma binding to those HIV regions. For the V1V2 antigens, we performed IgG ELISAs using V1V2caseA2 as the antigen to coat the plates (Figure 2.3 A and D). We chose to use V1V2case A2 as the antigen because IgG binding to this antigen was correlated with protection in the RV144 trial (93). Since RSC3 is a CD4 binding site core antigen, we used a

sCD4 blocking assay to measure plasma binding to the CD4 binding site on two different gp120 antigens (clade A BG505 gp120 and clade D C2-94UG114 gp120) as described previously (Figure 2.3 B, C, E, F) (119). Because there were three other V3 peptides in the BAMA antigen panel (ConA1, ConB, ConC), we did not use an alternative assay to measure plasma IgG binding to ConDV3. Plasma IgG binding to V1V2caseA2 and soluble CD4 blocking as measured by this assay showed a wide dynamic range across samples (Figure 2.3). The logistic regression analysis measuring the association of percent binding to V1V2 or percent sCD4 inhibition with odds of MTCT are shown in Figure 2.4. Neither plasma IgG binding to V1V2 nor plasma blocking of sCD4 were associated with odds of MTCT.

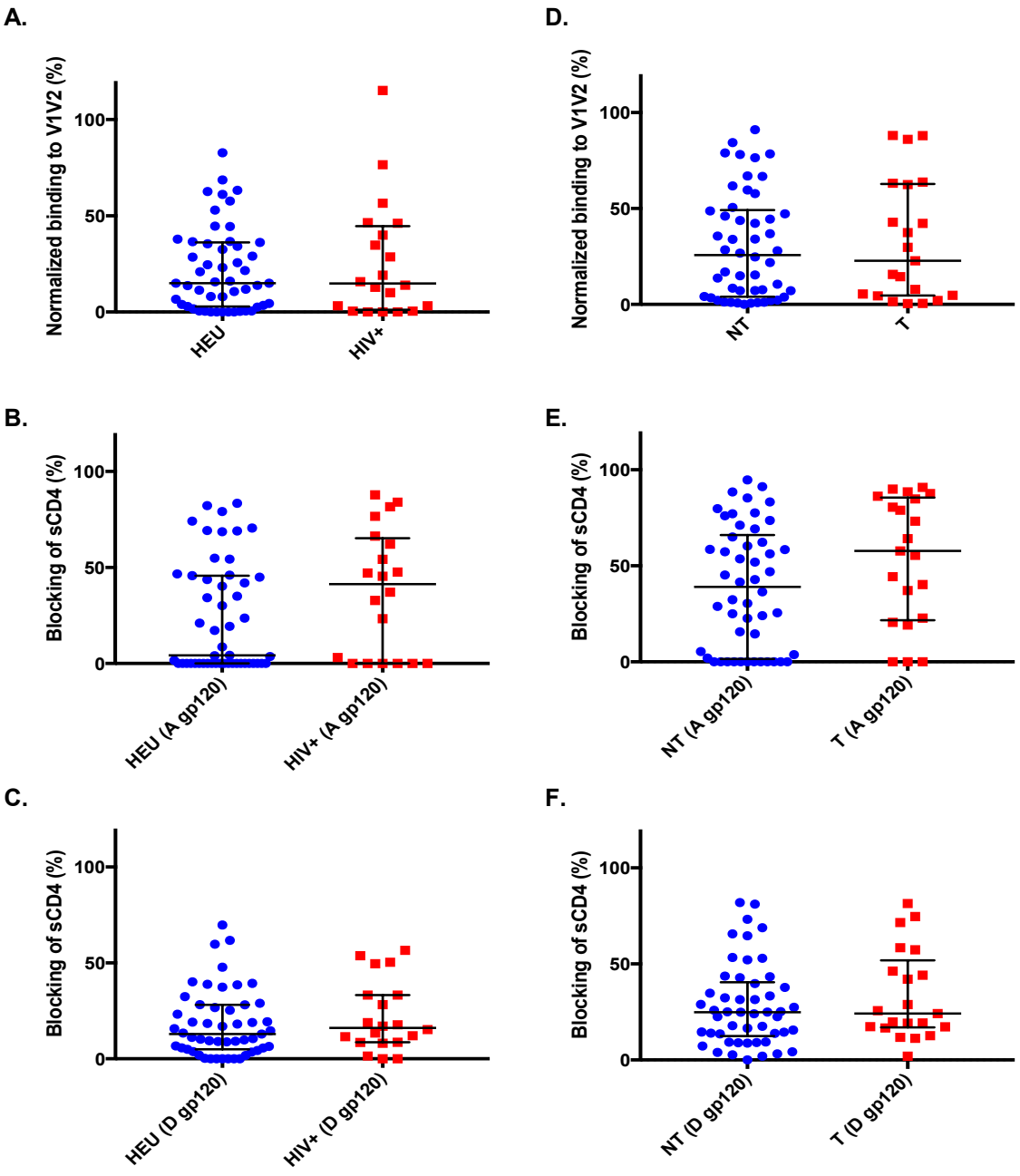


Figure 2.3. Plasma binding as measured by ELISA or sCD4 blocking assay. Normalized plasma binding to V1V2 as measured by V1V2 ELISAs for HEU versus HIV+ infants (A) and NT versus T mothers (D) are shown. The ability of plasma to block sCD4 from binding in a sCD4 blocking assay is shown for HEU versus HIV+ infants (B, C) or NT versus T mothers (E, F) against two gp120 antigens. Clade A BG505 (B, E) and clade D C294UG gp120 (C, F) were used as antigens in the soluble cD4 blocking assay.

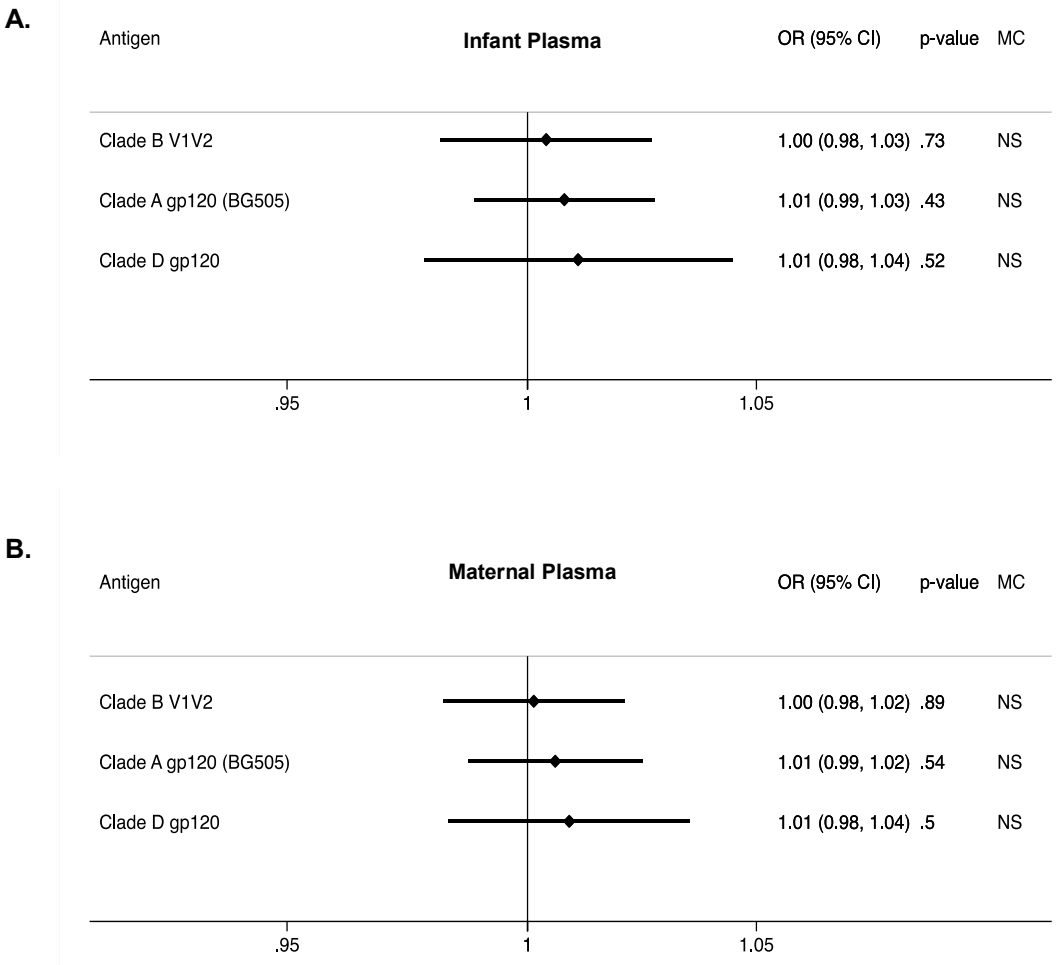


Figure 2.4. Association of V1V2-specific or CD4bs-specific antibodies with odds of MTCT.

The association of plasma binding to V1V2 or the ability of plasma to block sCD4 with odds of MTCT was measured using a logistic regression analysis adjusted for maternal plasma viral load. Adjusted odds ratios (OR), 95% confidence intervals (CI), and p-values are shown for infant samples (top) and maternal samples (bottom). Statistical significance was defined as $p<0.05$ (*).

Discussion

A detailed understanding of antibody epitopes correlated with protection in the context of natural infection would help inform rational vaccine design. This study utilized the unique setting of breastfeeding MTCT, in which the infant has pre-existing HIV-specific antibodies present at the time of HIV exposure through breastfeeding, to assess whether antibodies targeting certain epitopes or HIV regions are associated with reduced risk of transmission. Surprisingly, in this cohort, there were no correlates of reduced risk of transmission among the antigens tested here for plasma binding; rather, we found plasma binding to four antigens were associated with increased odds of MTCT. While not all of these associations were statistically significant, and none of these associations were significant after correcting for multiple comparisons, what is remarkable is that all of the associations, whether maternal or infant, were with proteins that included regions of gp41 (Figure 2.5), and all of the associations were with increased odds of MTCT. The consistency of these results, along with multiple cases of statistically significant individual associations supports a biologically relevant association rather than random chance.

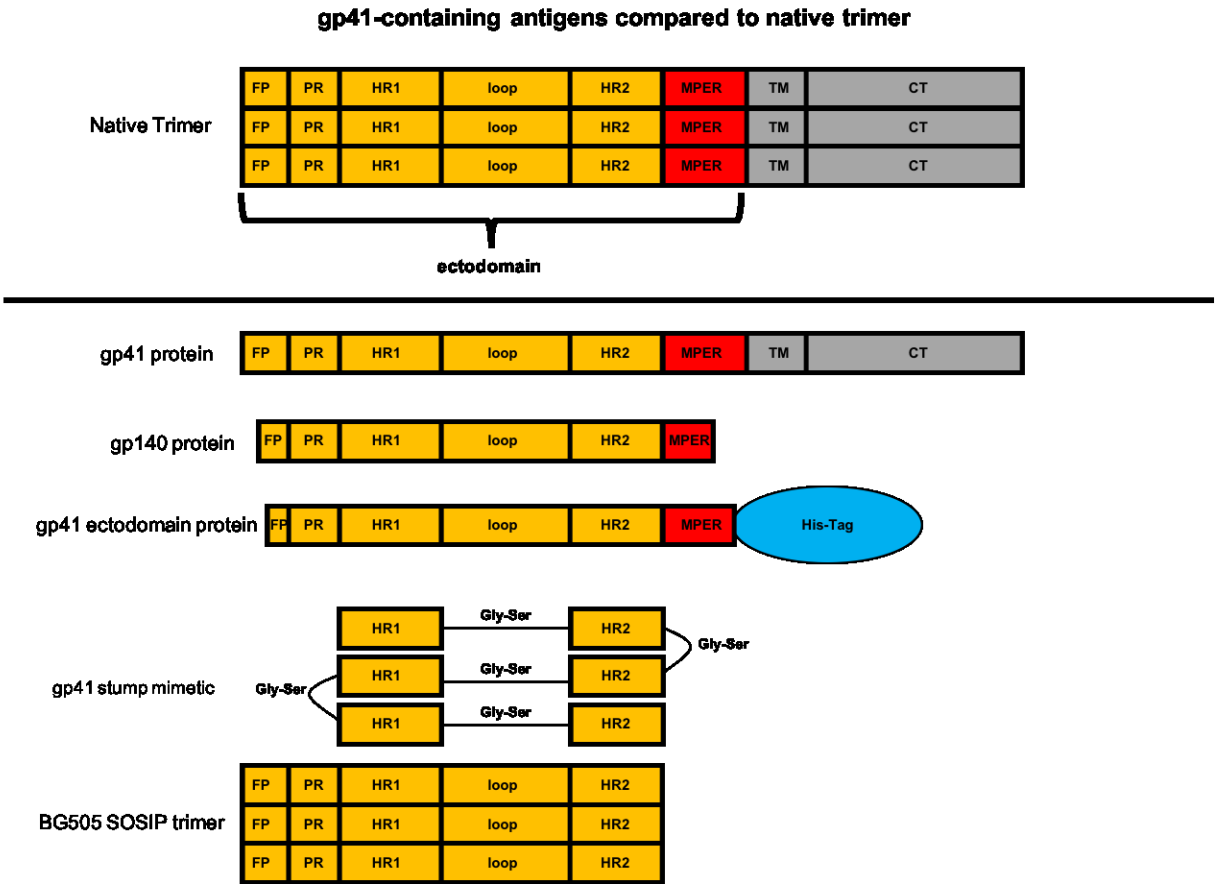


Figure 2.5. gp41-containing BAMA antigens.
Schematics of the gp41 structures present in native trimer (top, above line) or BAMA antigens (below line). The BG505 SOSIP trimer and gp41 stump mimetic are both trimeric. The gp41 stump mimetic is a continuous trimer of heterodimers of HR1 and HR2 regions connected with glycine-serine linkers. The gp41 ectodomain antigen contains a c-terminal His-tag. FP=fusion peptide. PR=proximal region. HR1=N heptad repeat. Loop=immunodominant C-C loop. HR2=C-terminal heptad repeat. MPER=membrane proximal external region. TM=transmembrane domain. CT=cytoplasmic tail.

The three antigens that showed similar MTCT associations for both infant and maternal plasma binding contained the fusion peptide (FP), fusion peptide proximal region (PR), N-terminal heptad repeat (HR1), the immunodominant C-C loop, C-terminal heptad repeat (HR2), and membrane proximal external region (MPER). The fourth antigen, which only showed a trend of association for maternal plasma binding, was 6-helix: a trimer of HR1 and HR2 heterodimers linked together by glycine-serine linkers (Figure 2.3). 6-helix is designed to mimic gp41 stumps, which are left on the surface of virions and possibly on infected cells after gp120 is shed (171). Plasma binding to the clade C gp41ectodomain antigen showed the strongest association with odds of MTCT suggesting that plasma binding to MPER is likely not driving this association because MPER antibodies do not bind to this protein, presumably due to the presence of a C-terminal His-tag. Interestingly, one other antigen in our panel, the BG505.SOSIP.664.D7342 trimer, includes part of the gp41 ectodomain (Figure 2.5), but plasma binding to this antigen was not associated with odds of MTCT (maternal aOR=1.01, p=0.28; infant aOR=1.01; p=0.41). While this antigen contains regions of the gp41 ectodomain, the gp41 ectodomain is actually occluded in the trimer as shown by the lack of binding of non-neutralizing ectodomain-specific antibodies (28). Additionally, none of the gp120-only antigens showed an association with odds of MTCT. Taken together, these data suggest that plasma binding to the gp41 ectodomain, but not to MPER, is driving the association with increased odds of MTCT.

Previous MTCT studies investigating the role of gp41 binding antibodies on MTCT risk are inconsistent. When looking in further detail at the studies that observed an association between binding antibodies and MTCT risk, antibodies targeting HR1 and the C-C loop were associated with increased risk of MTCT more often than not (55, 122, 126), but there were a few exceptions where antibodies targeting these epitopes were associated with decreased risk (55,

122). Antibodies targeting HR2 and the cytoplasmic tail tended to be associated with decreased risk of MTCT (116, 117, 122). Our study was not designed to look at epitopes in fine detail; however, we can preliminarily look at common regions shared by the four gp41 antigens discussed here in comparison to epitopes identified in other studies to see if any obvious patterns emerge. All four gp41-containing antigens showing an association with MTCT contained HR1 and HR2; the three antigens that showed an association for both maternal and infant plasma contained the C-C loop; but only 1 antigen contained the cytoplasmic tail. Therefore, it is possible that plasma binding to HR1 and/or the C-C loop of these antigens is driving the association with increased odds of MTCT, but as all of these antigens also contain HR2, further studies are required to identify the specific epitope(s) correlated with MTCT risk.

This study was designed to identify correlates of reduced risk of MTCT, but we surprisingly found that plasma binding to the gp41 ectodomain is a correlate of increased risk. However, as this was a correlation study, the association of plasma binding to the gp41 ectodomain with increased odds of MTCT could be a direct association or an indirect association and a marker of another variable that is driving the relationship. One example of a model that would explain a direct association is that perhaps ectodomain antibodies cause a conformational change upon binding that somehow makes the virion more infectious. Given that ectodomain epitopes are occluded in the trimer (28), this model is unlikely. A model that would explain a more indirect association is that the gp41 ectodomain is acting as an immune decoy. Virions can display envelope in native trimer or in non-native conformations such as monomers or gp41 stumps (30, 171). To mediate viral entry into cells, envelope must be displayed as native trimer (30). The antigens in our panel that showed an association with increased odds of MTCT upon plasma binding have conformations similar to non-native forms of envelope such as monomers,

uncleaved gp140, and stumps (171). In contrast, the trimeric antigen designed to have a native-like conformation had the ectodomain occluded and did not show an association with MTCT upon plasma binding (28). The gp41 ectodomain, especially the immunodominant C-C loop, is highly immunogenic (34-36). It is possible that the immunogenic ectodomain exposed on non-native envelope is easily recognized by naïve B cells and drives the immune system to primarily make antibodies against epitopes only exposed on non-native envelope at the expense of making antibodies against native trimer (as suggested by Moore and colleagues) (171). Thus, these antibodies would largely target forms of envelope which do not mediate viral entry. In this model, the association of ectodomain binding antibodies with increased MTCT would be a marker of lack of antibodies targeting protective epitopes. Further investigation is required to discern the mechanism of this association, and whether it is direct or indirect.

Our study is similar to a number of other studies which have investigated epitopes of binding antibodies in the context of MTCT to determine whether certain antibody epitopes are associated with reduced risk of transmission. Antibody binding to V3 (118, 119, 124), the CD4 binding site (119, 121), gp41 (55, 116, 117, 120, 122), gp120 (123, 124), and p24 (55, 123) have been correlates of reduced risk of MTCT in at least one study. However, in other studies, antibody binding to all of these epitopes or regions have also been correlates of increased risk (55, 122, 125-128), and not associated with risk in yet other studies (116, 119, 121, 126, 128-135). In our study, plasma binding to V3, the CD4 binding site, gp120, and p24 was not associated with odds of MTCT, but plasma binding to gp41, specifically to the ectodomain, was associated with increased risk. These inconsistencies may be due differences in timing and methods used to determine infant infection status, ART availability and treatment, infecting clades, maternal and/or infant sample availability, or mode of transmission (*in utero,* perinatal,

breastfeeding). All of the aforementioned studies included *in utero* and peripartum transmissions, whereas our study primarily focused on breastfeeding transmissions, as all infants were HIV RNA/DNA negative at birth. Perhaps different antibody responses are important for MTCT risk based on the mode of transmission, as suggested by Mutmucarama and colleagues (128).

Our study has a number of limitations, including low statistical power because of the small number of infants that acquired HIV. Additionally, of the 21 infants that acquired HIV, 7 had an estimated time of infection after 6 months of age, by which time passively-acquired antibody levels may have waned in the infant (54, 103). Therefore, the role of passively-acquired binding antibodies in these late transmissions is unclear. As this study was designed to be exploratory in nature, we tested many hypotheses, and did not find any significant correlates of increased or decreased risk after correcting for multiple comparisons. While our results are internally consistent and suggest that plasma binding to the gp41 ectodomain is associated with increased odds of MTCT, more focused, hypothesis-driven studies are necessary to determine the direct or indirect mechanism of this association, the specific gp41 ectodomain epitope(s) that are associated with increased MTCT risk, and to determine whether this association is reproducible.

The present study adds to the large body of literature investigating the role of antibody epitopes on risk of MTCT. As the conclusions are variable between studies and cohorts, further studies with larger cohorts are necessary to identify epitopes or patterns of multiple epitopes that correlate with increased or decreased risk across cohorts. Ours is one of only a few studies to investigate the role of passively-acquired binding antibodies on risk of breastfeeding MTCT. As this setting largely mimics vaccination, and is a natural passive vaccination setting, more studies specifically focused on identifying correlates of reduced risk for this mode of MTCT are warranted. Our results suggest that antibody binding to the gp41 ectodomain is a correlate of

increased risk of MTCT, rather than a correlate of decreased risk. Investigating the specific gp41 epitope(s) correlated with increased risk and the mechanism of this association has the potential to help guide future vaccine and therapeutic design.

Chapter III

Antibody-dependent cellular cytotoxicity targeting CD4-inducible epitopes predicts mortality in HIV-infected infants

The text in this chapter has been modified slightly from a manuscript entitled "Antibody-dependent cellular cytotoxicity targeting CD4-inducible epitopes predicts mortality in HIV-infected infants" which has been submitted to EBiomedicine and is currently under review.

Introduction

As discussed in Chapter I, the RV144 trial was the only human HIV vaccine trial to date to show any efficacy (89, 92). In the RV144 trial, non-neutralizing antibodies appeared to play a protective role, as the neutralizing antibody response was weak to this vaccine and was not correlated with protection (92-94). In a subsequent analysis, ADCC, a non-neutralizing Fc-mediated antibody function that targets infected cells for destruction by innate effector cells, was correlated with protection in vaccinees that had low circulating IgA (93). However, the RV144 trial was only modestly efficacious (92); thus, an improved knowledge of the correlates of protection from transmission and improved disease outcome in the setting of natural HIV infection may help inform rational vaccine and therapeutic design.

Our lab has previously reported that ADCC activity, but not neutralization, correlated with improved infant outcomes in the setting of breastfeeding. In a cohort of high-risk mother-infant pairs, breast milk ADCC activity was correlated with reduced risk of MTCT (53). In a larger cohort of breastfeeding mother-infant pairs, passively-acquired ADCC in infant plasma was significantly associated with improved survival of infected infants and associated with trends toward reduced risk of MTCT and reduced set point viral load (54). Studies from other

groups have also shown ADCC to be associated with improved infant outcomes, such as reduced disease progression to AIDS or death (55-57). These studies measured total ADCC; however, the epitopes of the ADCC-mediating antibodies that correlate with improved outcomes have not been investigated in the context of MTCT of HIV.

Major targets of ADCC-mediating antibodies are CD4-inducible (CD4i) epitopes, which are epitopes in the HIV envelope (Env) protein that are typically not well exposed in the native Env trimer (25, 172). CD4i epitopes become exposed as a result of a conformational change in Env after CD4 binding that is necessary to complete subsequent steps in entry, including co-receptor binding (24-26). CD4i epitopes have been further divided into clusters based on the specific epitope target in the CD4-induced state of Env, with cluster A, which targets the gp41-interactive region of gp120, and cluster C, which targets the co-receptor binding site, being the subject of intensive study (37, 41, 43, 44, 100, 173-186). ADCC activities targeting these epitopes are both common and potent (41, 44, 99, 100, 173, 177, 183, 185). In multiple studies that directly measured the amount of CD4i epitope-specific ADCC in plasma from small cohorts of infected individuals (N=9-14), over 90% of the participants had detectable cluster A-specific ADCC (41, 173, 185). The epitopes of two cluster A-defining antibodies, A32 and C11, are nearby each other but do not overlap (172). In the aforementioned studies, plasma ADCC specific for the A32 and C11 epitopes was dominant and ranged from 14-87% and 18-78% of total plasma ADCC from each participant, respectively (41, 173). The majority of the participants also had some detectable plasma ADCC activity to the CD4i cluster C co-receptor binding site epitope, as measured by ADCC specific for the epitope of a prototypical cluster C antibody, 17b (44, 173, 185). While less dominant than cluster A-specific ADCC responses,

ADCC targeting the 17b epitope accounted for 0-53% of total plasma ADCC in a cohort of infected individuals (173).

While CD4i epitope-specific ADCC is common and potent, especially cluster A-specific activity, the role of CD4i epitope-specific ADCC in protection or clinical outcome in humans has not been defined. A handful of non-human primate (NHP) studies have shown that antibodies targeting CD4i epitopes can provide protection from SHIV challenge, control viremia, and reduce transmitted/founder variants providing proof-of-concept that CD4i epitope-specific ADCC-mediating antibodies have the potential to play an important role in HIV vaccines and antibody-based therapies (83, 187, 188). We hypothesized that maternal and passively-acquired ADCC activity targeting cluster A (A32 and C11) and/or cluster C (17b) CD4i epitopes is correlated with reduced transmission and slower disease progression in the setting of MTCT. We utilized samples from a unique cohort of 72 antiretroviral (ART)-naïve breastfeeding mother-infant pairs from the Nairobi Breastfeeding Clinical Trial in which passively-acquired ADCC was significantly associated with improved survival of infected infants and a trend toward reduced risk of transmission (5, 54). In the present study, we investigated whether ADCC targeting the cluster A and C CD4i epitopes is associated with reduced risk of MTCT and/or improved infected infant outcome in this cohort.

Materials and Methods

Study Design and Plasma Samples

Plasma samples were from the Nairobi Breastfeeding Clinical Trial, conducted in Nairobi between 1992-1998, prior to the use of ART (5). HIV-1 positive mothers were enrolled during the third trimester, at which time maternal blood samples were collected. Infant blood samples

were collected at birth and at regular intervals thereafter until 2 years of age. Infant PBMC samples were tested for HIV infection by single copy detection DNA PCR (160). Infants testing positive for HIV DNA, were retrospectively tested for HIV RNA using a prototype Gen-Probe/Hologic HIV viral load assay that detects diverse subtypes from samples collected from previous timepoints (161). For this study, estimated time of infection was defined as the midpoint between the last HIV-negative and first HIV DNA and/or RNA-positive test (54). The Kenyan Ministry of Health gave permission for the Nairobi Breastfeeding Clinical Trial to be conducted, and the Institutional Review Boards of the University of Nairobi, University of Washington, and the Fred Hutchinson Cancer Research Center approved the current study.

Plasma or cord blood samples from 72 mother-infant pairs from the Nairobi Breastfeeding Clinical Trial meeting selection criteria described previously were utilized in the present study (54). Briefly, mother-infant pairs were included if the following criteria were met: the infant was HIV RNA and DNA negative at birth, breastfed for a minimum of three months, HIV-exposed uninfected (HEU) infants remained HIV-negative for a minimum of six months and at each follow up timepoint, and an infant plasma or cord blood sample was available from the first week of life (prior to estimated time of infection). Infant cord blood (N=60) or neonatal plasma from delivery (N=10) or week 1 (N=1) were tested in this study along with paired maternal plasma samples from the third trimester of pregnancy (N=68) or delivery (N=3). We verified that cord blood and plasma samples from infants in this cohort gave similar results by testing matched samples from infants in the NBT from whom both were collected. Of note, 1 infected infant and 1 non-transmitting mother from the 72 pairs studied by Milligan et al had no more plasma available (54); their corresponding transmitting maternal and HEU infant plasma samples were available and included in this study. The final cohort included 70 paired maternal

and infant samples (50 non-transmitting pairs and 20 transmitting pairs), 1 unpaired transmitting maternal sample, and 1 unpaired HEU infant sample. All plasma and cord blood samples were heat inactivated at 56 degrees Celsius for 1 hour. All experiments were performed in an unblinded fashion.

Antibodies

A32, C11, and 17b antibodies were produced by cloning A32 (light chain PDB: 3TNM_L; heavy chain PDB: 3TNM_A), C11 (light chain PDB: 4FZ8_L; heavy chain PDB: 4FZ8_H), and 17b (light chain PDB: 2NY1_C; heavy chain PDB: 2NY1_D) variable regions into IgG1 expression vectors (kindly provided by Michael Nussenzweig). Fc receptor binding defective variants called LALA variants were generated by mutagenesis, introducing two leucine to alanine changes, at L234A and L235A into the IgG1 expression vectors as described previously (41, 189, 190). All antibodies were expressed and purified as described previously (41, 164).

17b fab was produced by papain digestion of functional 17b using the Fab Preparation Kit (Pierce catalog#: 44985) according to the manufacturer's protocol as described previously (41).

Negative control influenza antibody Fi6V3 was produced by stably transfected 293F cells kindly provided by Jesse Bloom.

Competition ADCC Assay

The competition ADCC assay was performed by using a modified version of the rapid and fluorometric ADCC assay (RFADCC assay) with LALA variants as competitive inhibitors as described previously (41, 191). Briefly, CEM-NkR cells (NIH AIDS Reagent Program, catalog #: 458; RRID: CVCL_X622, contributed by Dr. Peter Cresswell) were double stained with PKH26 cell linker (Sigma Aldrich), a cell membrane dye, and CFSE (Vybrant CFDA SE Cell Tracer Kit, Life Technologies), a cytoplasmic dye, and coated with either clade A/D BL035.W6M.ENV.C1 gp120 protein (Immune Tech) or clade B SF162 gp120 (Immune Tech) for 1 hour at room temperature (RT). In the A32-LALA and C11-LALA competitive RFADCC assays, the target cells were coated with clade A/D BL035 gp120, the antigen used by Milligan et al, which was cloned from an infant in this cohort (54, 192). Functional 17b did not mediate measurable ADCC against BL035 gp120-coated target cells reproducibly. Because 17b consistently mediates ADCC against clade B SF162 gp120-coated target cells, target cells in the 17b-LALA competitive RFADCC assays were coated with clade B SF162 gp120. Cells were coated with 1.5ug gp120 per 100,000 cells. Coated target cells were washed and added at a concentration of 5000 cells/well to 96-well plates containing 50ul LALA antibody (at a concentration of 25ug/ml for A32-LALA and C11-LALA or 5ug/ml for 17b-LALA) or an equivalent volume of media and incubated for 15 minutes in the dark to allow the LALA antibody to bind gp120. Following this pre-incubation, 50ul of plasma at a 1:5000 dilution or 100ng/ml of the monoclonal control antibodies were added to the plate and incubated for another 15 minutes in the dark at RT to allow the plasma to bind to available sites on gp120. HIV-negative donor PBMCs (Bloodworks Northwest) were added at a 50:1 effector:target ratio to each well. The plates were incubated for 4 hours at 37 degrees Celsius and fixed with 150ul of

1% paraformaldehyde in PBS (Affymetrix). 100ul of fixed cells were analyzed by flow cytometry using an LSR II (BD) (for assays with maternal samples against BL035 gp120-coated target cells) or a Symphony (for all other assays) (BD). PKH was detected in the PE channel and CFSE was detected in the FITC channel. ADCC was measured as the percent PE+, FITC- cells of total PE+ cells after subtracting out background (ADCC against uncoated target cells), which was set to 3-5% as analyzed by FlowJo (Treestar). ADCC was normalized to killing mediated by pooled anti-HIV immune globulin (NIH AIDS reagent program, catalog #3957, contributed by NABI and National Heart Lung and Blood Institute (Dr. Luiz Barbosa)), which was set to 100%. Results are averaged from two biological replicates. Each biological replicate contained two technical replicates. Within each biological replicate, samples with total ADCC below our limit of detection (pre-specified as 2*total ADCC mediated by negative control influenza Fi6v3 antibody) were excluded from this analysis. Seven maternal samples (six non-transmitters, one transmitter) and six infant samples (five HEU, one HIV-infected) met this exclusion criteria for at least one biological replicate.

Relative ADCC was defined as:

$$\frac{\text{ADCC in the presence of the LALA variant}}{\text{ADCC in an equivalent volume of media}} \times 100\%$$

CD4i epitope-specific ADCC, referred to as CD4i antibody-like ADCC activity, is defined as: 100%- relative ADCC.

Enhancement of ADCC is defined as: relative ADCC-100%.

Total cluster A-specific ADCC is defined as: A32-like ADCC + C11-like ADCC.

Negative values were treated as zeros.

All cells were cultured in RPMI complete (RPMI (Gibco) supplemented with 10% FBS (Gibco), 1% PSF antibiotic-antimycotic (Life Technologies), and 1% 4.0mM Glutamax (Gibco)). All antibodies and plasma samples were diluted in RPMI complete.

Statistical Analysis

Statistical analyses were performed with Stata15SE (StataCorp, College Station, TX) and GraphPad Prism7 (GraphPad Software, Inc., San Diego, CA). All graphs were generated by GraphPad Prism7. To determine whether CD4i-specific ADCC affected risk of MTCT, relative ADCC or CD4i antibody-like ADCC activity of HEU vs HIV-infected infant plasma or non-transmitting vs transmitting maternal plasma were compared using a Mann-Whitney U test and logistic regression analysis adjusted for maternal plasma HIV RNA viral load. The effect of ADCC targeting CD4i epitopes on survival of HIV-infected infants was assessed by a Cox-proportional hazards model and a log-rank test comparing Kaplan-Meier survival curves of HIV-infected infants with CD4i-specific ADCC at/above vs. below the HIV-infected infant cohort median as noted in the figure legends. Statistical significance was defined as $p<0{\cdot}05$.

Results

Quantification of CD4i epitope-specific ADCC activity

To measure ADCC activity that targets known CD4i-specific epitopes, we used variants of A32, C11, and 17b, containing L234A and L235A (LALA) mutations that abrogate binding to the Fc gamma receptors (Fc□R) as competitive inhibitors in the RFADCC assay (189). Each LALA variant inhibited at least 90% of the ADCC activity mediated by its fully functional

counterpart (Figure 3.1) but did not inhibit ADCC activity of the unmatched antibodies, demonstrating their specificity.

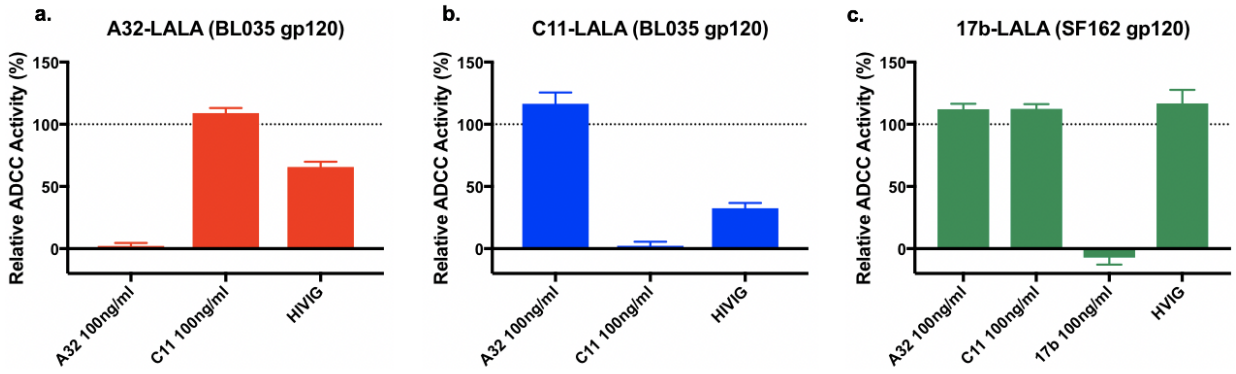


Figure 3.1. Validation of LALA antibodies in the competition RFADCC assay. 25ug/ml A32-LALA (a), 25ug/ml C11-LALA (b), or 5ug/ml 17b-LALA (c) was pre-incubated with target cells in the competition RFADCC assay. Target cells in the A32-LALA and C11-LALA competition ADCC assays were coated with clade A/D BL035 gp120. Target cells in the 17b-LALA competitive ADCC assay were coated with clade B SF162 gp120. Relative ADCC mediated by fully functional A32, C11, and 17b antibodies (100ng/ml) or the positive control HIVIG (1:5000 dilution) in the presence of each LALA antibody is shown. Relative ADCC is defined as ADCC in the presence of the LALA variant normalized to ADCC in an equivalent volume of media (represented by the dashed line at 100%). Error bars represent mean+SEM. Results are averaged from six replicates (a and b) or eight replicates (c).

We utilized samples from a cohort of 72 breastfeeding mother-infant pairs of which all infants tested HIV-negative at birth (54). We measured ADCC of infant cord blood or plasma from the first week of life, which captures passively acquired maternal antibodies, as well as paired maternal plasma from the third trimester in the presence or absence of A32-LALA, C11-LALA, or 17b-LALA. For all maternal and infant samples in the cohort, we normalized the ADCC activity in the presence of the LALA variant to ADCC activity in the absence of the LALA variant to calculate the percent relative ADCC for each sample in the presence of the LALA competitor variant (Figure 3.2 A-C). Both A32-LALA and C11-LALA inhibited plasma ADCC for the majority of plasma samples (Figure 3.2 A, B, orange points below dotted line).

Conversely, 17b-LALA weakly inhibited plasma ADCC in only a small number of samples (Figure 3.2 C, orange points below dotted line).

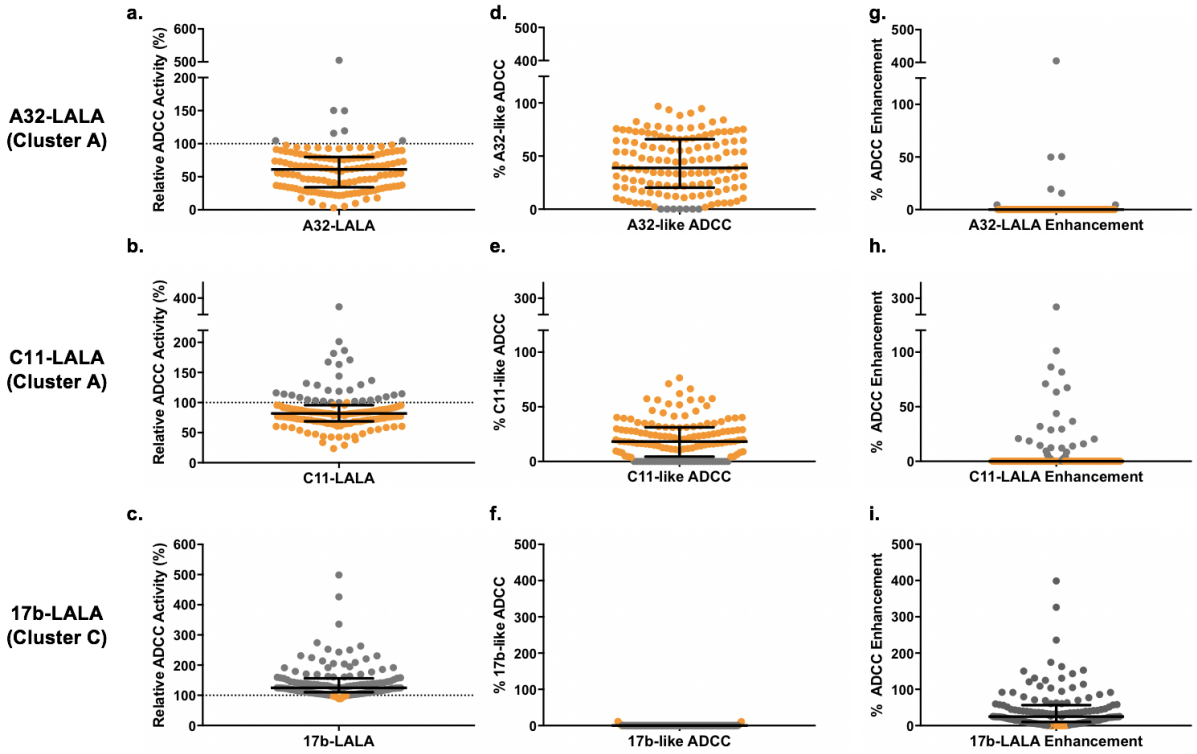


Figure 3.2. Relative ADCC and CD4i epitope-specific ADCC in MTCT cohort. Results of the competition RFADCC assay performed on 142 plasma samples from breastfeeding Kenyan mother-infant pairs. a-c: Relative ADCC in the presence of A32-LALA (a), C11-LALA (b), or 17b-LALA (c) normalized to ADCC in the presence of media alone (dotted line at 100%) is shown for each plasma sample. d-f: Percent CD4i epitope-specific (referred to as CD4i antibody-like) ADCC activity (100% - relative ADCC) was calculated for each plasma sample in the presence of A32-LALA (d), C11-LALA (e), and 17b-LALA (f) Negative values were treated as zeros (grey points). g-i: Percent LALA-mediated ADCC enhancement (relative ADCC – 100%) was calculated for each plasma sample in the presence of A32-LALA (g), C11-LALA (h), and 17b-LALA (i) Negative values were treated as zeros (orange points). Results are averaged from two biological replicates. Error bars represent median + interquartile range.

Relative ADCC is a measure of the ADCC activity targeting epitopes other than that of the LALA competitor variant, as it is the activity that is not blocked by the LALA variant. Therefore, we calculated the percent reduction in ADCC activity in the presence of the LALA variant, as described in the materials and methods (referred to as CD4i antibody-like ADCC: i.e. A32-like ADCC, C11-like ADCC, or 17b-like ADCC) to define the fraction of activity due to that antibody specificity in each sample. The cohort average for A32-like ADCC, C11-like ADCC and 17b-like ADCC was 42.0%, (maximum 96.8%), 20.8%, (maximum 76.5%), and 0.35%, (maximum 11.3%) respectively (Figure 3.2 D-F). Surprisingly, in the case of 17b-LALA, the majority of plasma ADCC was enhanced in the presence of 17b-LALA (Figure 3.2 C, grey points). The cohort average ADCC enhancement mediated by 17b-LALA was 44.0% (maximum 398.7%) (Figure 3.2 I).

Effect of ADCC activity targeting CD4i epitopes on risk of MTCT

To measure the association of CD4i epitope-specific ADCC with risk of MTCT, we compared relative ADCC between 51 HIV-exposed uninfected infants (HEU) and 20 infants who acquired HIV over the course of the follow up period, or between their paired 50 non-transmitting and 21 transmitting mothers (Figure 3.3). There was no statistically significant difference in relative ADCC activity in the presence of any of the three competitor LALA variants between the HEU and HIV-infected infants or between the corresponding non-transmitting and transmitting mothers (Figure 3.3). Because maternal viral load is a known risk factor for MTCT, we also performed a logistic regression adjusting for maternal plasma HIV RNA viral load (Table 3.1). We found no statistically significant association between passively-acquired or maternal A32-like or C11-like ADCC and MTCT. Because A32-like and C11-like

antibodies are common and have close but non-overlapping epitopes (41, 44, 99, 100, 172, 173, 177, 183, 185), we also examined their combined effect on outcome. There was no statistically significant association between total cluster A-like ADCC activity (sum of A32-like ADCC + C11-like ADCC) with MTCT risk (Table 3.1).

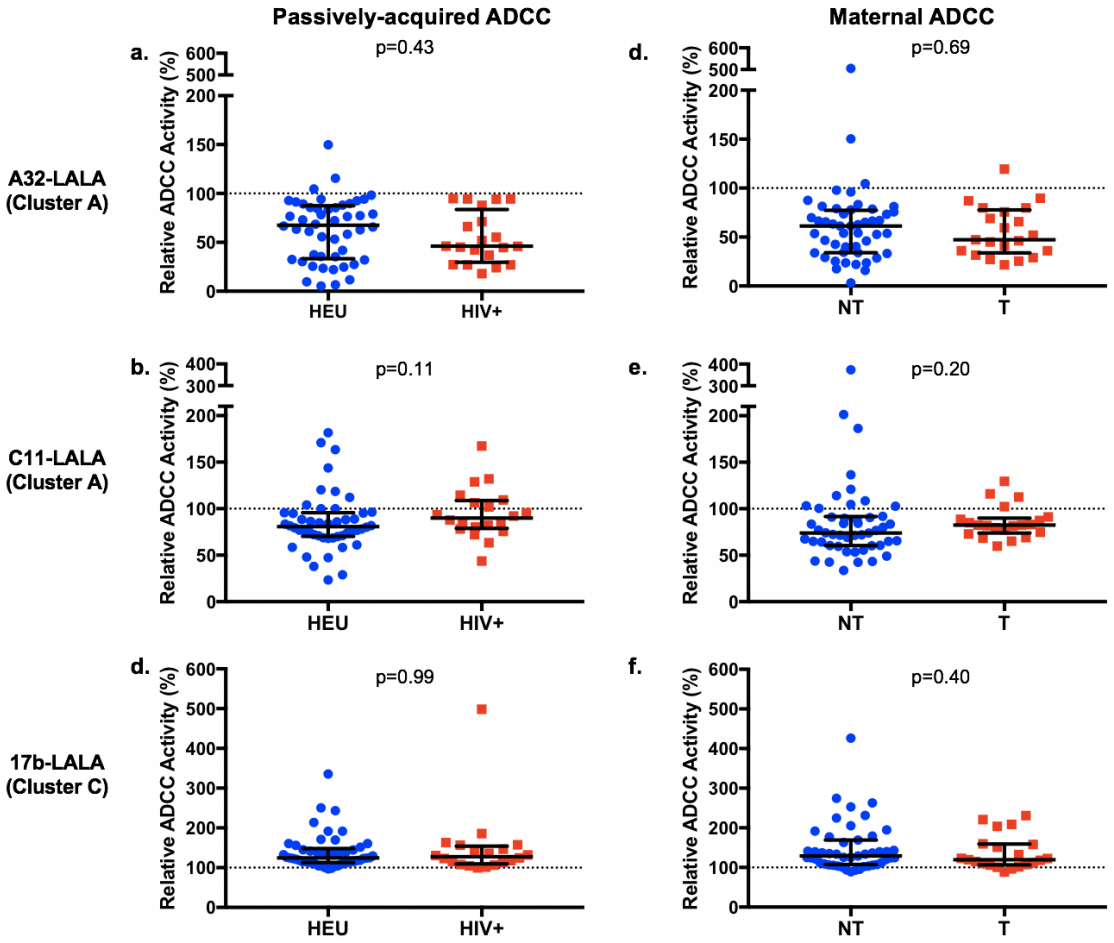


Figure 3.3. Relative ADCC among transmission groups.
Results of the competition RFADCC assay for passively acquired ADCC in 71 infant plasma samples (a-c) and maternal ADCC in 71 corresponding maternal plasma samples (d-f). Relative ADCC in the presence of A32-LALA (a, d), C11-LALA (b, e), or 17b-LALA (d, f) is shown for HIV-exposed uninfected infants (HEU) compared to HIV-infected (HIV+) infants or non-transmitting (NT) compared to transmitting (T) mothers. Relative ADCC was compared between the infection or transmission groups by a Mann-Whitney U test. Statistical significance was defined as $p<0.05$ (*). Error bars represent median + interquartile range. Results are averaged from two biological replicates. Negative values were treated as zeros.

Table 3.1. Association of CD4i antibody-like ADCC or ADCC enhancement with risk of MTCT.
The association of CD4i epitope-specific ADCC (A32-like ADCC, C11-like ADCC, or total cluster A-specific ADCC (sum of A32-like ADCC + C11-like ADCC)) and 17b-LALA-mediated enhancement of plasma ADCC with odds of MTCT was measured using a logistic regression analysis adjusted for maternal plasma viral load. Adjusted odds ratios (aOR), 95% confidence intervals (CI), and p-values are shown for infant samples (passively-acquired ADCC, left) and maternal samples (maternal ADCC, right). Statistical significance was defined as $p<0.05$ (*).

	Passively-Acquired ADCC			**Maternal ADCC**		
	aOR	**95% CI**	**p-value**	**aOR**	**95% CI**	**p-value**
A32-like ADCC	1.005	0.985-1.026	0.61	1.013	0.989-1.038	0.29
C11-like ADCC	0.967	0.929-1.006	0.096	0.977	0.943-1.013	0.21
Total cluster A-specific ADCC	0.995	0.977-1.013	0.60	1.001	0.982-1.021	0.90
17b-LALA-mediated enhancement	0.997	0.988-1.007	0.58	0.990	0.978-1.002	0.12

Because 17b-like ADCC as measured by LALA competition was only detected rarely, we could not assess whether 17b-like ADCC was associated with risk of MTCT. Since 17b-LALA enhanced ADCC activity in a majority of the cohort samples (91.2% of maternal samples and 95.7% of infant samples showed enhancement of plasma ADCC), we assessed the association between 17b-LALA-mediated enhancement of plasma ADCC and MTCT adjusting for maternal HIV RNA viral load. We did not find a statistically significant association between 17b-LALA-mediated ADCC enhancement and risk of MTCT (Table 3.1).

Passively-acquired ADCC is often undetectable in infants by six months of age (54). When the analysis was restricted to infants who were infected prior to six months (N=14/21), associations between ADCC and MTCT remained non-significant (data not shown).

Effect of overall ADCC on infant survival in this cohort

In this cohort, which includes 21 infants who went on to acquire HIV after birth, there was an association of passively-acquired ADCC activity and improved survival of infants who subsequently acquired HIV (54). The present study was conducted with data from 20 of these 21 HIV-infected infants who had remaining samples available, of whom seven died during the two-year follow-up. In this independent evaluation of ADCC activity and infant survival, the results from our prior study showing an association between passively-acquired ADCC activity against infant-derived, clade A/D BL035 gp120 antigen with improved HIV-infected infant survival were confirmed (hazard ratio (HR)=0.948, p=0.031; log-rank X^2=5.05, p=0.025; Figure 3.4 A). We also tested a clade B gp120 antigen in this study, SF162 gp120, which demonstrated an association between passively-acquired ADCC activity and improved HIV-infected infant survival with a different antigenic variant (HR=0.965, p=0.044; X^2=1.70, p=0.19; Figure 3.4 B).

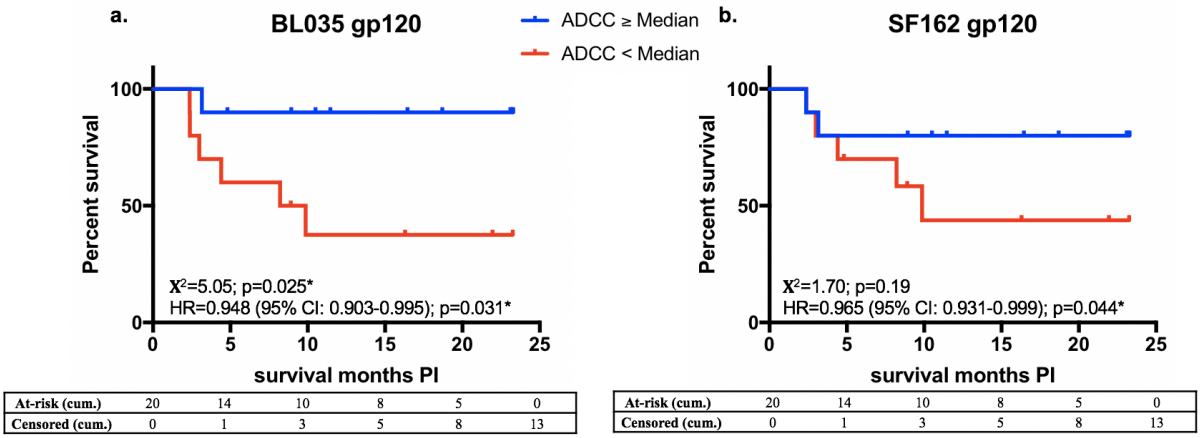


Figure 3.4. Association of passively-acquired ADCC with HIV+ infant survival using two different gp120 antigens.

Passively acquired ADCC in HIV+ infant plasma (N=20) was measured in the control (no LALA competitor) condition of the competition RFADCC assay (1:5000 dilution of plasma added to target cells pre-incubated with media only). Target cells were coated with clade A/D BL035 gp120 (a) or clade B SF162 gp120 (b). Kaplan-Meier survival curves between infants that had passively-acquired ADCC at/above the HIV-infected infant cohort median (blue lines) or below the HIV-infected infant cohort median (red lines) were compared by a log-rank test (X^2 values and p-values are shown). The x-axis shows months survival post infection (PI). The association of passively-acquired ADCC with risk of HIV+ infant mortality was measured by a Cox-proportional hazards model. Hazard ratios (HR), 95% confidence intervals (CI), and p-values are shown on the graphs. Statistical significance was defined as $p<0.05$ (*). Cumulative (cum.) number of infants at-risk or censored by the end of each month on the x-axis are shown in the tables.

Effect of CD4i Cluster A-specific ADCC on survival of HIV-infected infants

We next measured the association between cluster A-specific (A32-like or C11-like) ADCC and survival of infected infants (Figure 3.5). There was no statistically significant association of A32-like ADCC (maternal or infant) with risk of infected infant mortality in Cox-proportional hazards models. Consistent with this result, there was no difference in length of infected infant survival between HIV-infected infants who had high or low passively-acquired A32-like ADCC, nor was there a difference in infected infant survival between infants born to mothers who had high versus low A32-like ADCC, as measured by log-rank tests (Figure 3.5 A, D). There was no statistically significant association between passively-acquired C11-like ADCC in infants and infected infant mortality (Figure 3.5 B). However, higher maternal C11-like activity was associated with a trend toward higher risk of mortality in HIV-infected infants (HR: 1.069, p=0.067) (Figure 3.5 E). Interestingly, higher maternal total cluster A-specific ADCC was statistically significantly associated with shorter infected infant survival (X^2=5.65, p=0.017) and associated with a trend for increased risk of infected infant mortality (HR: 1.036, p=0.089) (Figure 3.5 F). Higher passively-acquired total cluster A-specific ADCC showed a similar relationship with shorter HIV-infected infant survival but this association was not statistically significant (X^2=2.20; p=0.14) (Figure 3.5 C). When these analyses were restricted to infants who acquired HIV prior to six months of life, results were similar with maternal total cluster A-specific ADCC being statistically significantly associated with poorer infant outcome (HR=1.108 (95% CI: 1.019-1.205), p=0.016; X^2=5.84, p=0.016) and maternal C11-like ADCC showing trends towards poorer infant outcome (HR=1.083 (95% CI: 0.999-1.174), p=0.052; X^2=3.54, p=0.060).

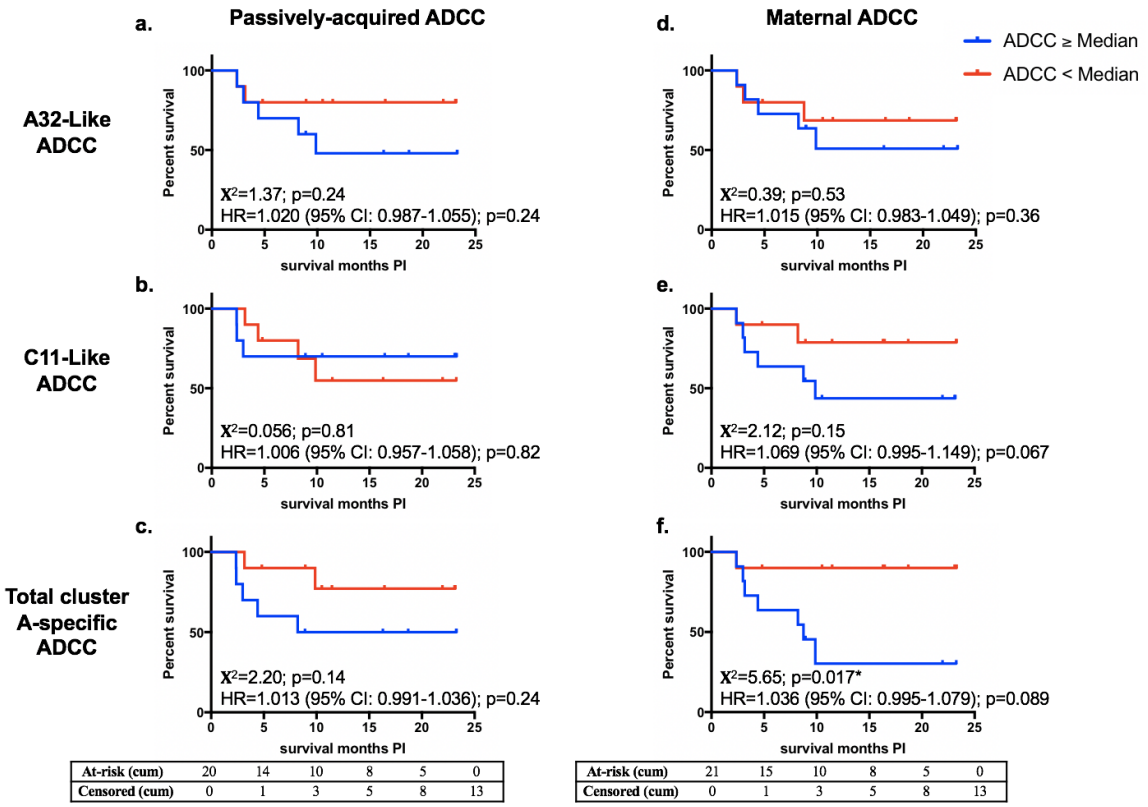


Figure 3.5. Effect of Cluster A-specific ADCC on HIV-infected infant survival. a-c: Kaplan-Meier survival curves between infants (N=20) that had passively-acquired cluster A CD4i epitope-specific ADCC at/above the HIV-infected infant cohort median (blue lines) or below the HIV-infected infant cohort median (red lines) were compared by a log-rank test for A32-like ADCC (a), C11-like ADCC (b), or total cluster A-specific ADCC (sum of A32-like ADCC + C11-like ADCC, c). d-f: Kaplan-Meier survival curves between infants whose mothers (N=21) had maternal cluster A CD4i epitope-specific ADCC at/above the transmitting mothers cohort median (blue lines) or below the transmitting mothers cohort median (red lines) were compared by a log-rank test for A32-like ADCC (d), C11-like ADCC (e), or total cluster A-specific ADCC (A32-like ADCC + C11-like ADCC, f). X^2 values and p-values are shown. The x-axis shows months survival post infection (PI). The association of passively-acquired or maternal cluster A CD4i epitope-specific ADCC with risk of HIV+ infant mortality was measured by a Cox-proportional hazards model. Hazard ratios (HR), 95% confidence intervals (CI), and p-values are shown on the graphs. Statistical significance was defined as $p<0.05$ (*). Cumulative (cum.) number of infants at-risk or censored by the end of each month on the x-axis are shown in the tables.

Effect of ADCC enhancement on survival of HIV-infected infants

We also assessed the association of 17b-LALA-mediated ADCC enhancement with survival in the HIV-infected infants (Figure 3.6). 17b-LALA-mediated enhancement of maternal ADCC was statistically significantly associated with reduced survival of HIV-infected infants and associated with a trend for increased risk of mortality (X^2=6.41, p=0.011; HR=1.013, p=0.091; Figure 3.6 B). Similarly, we found non-significant associations between 17b-LALA-mediated enhancement of infant passively-acquired ADCC and increased mortality of HIV-infected infants (HR=1.008, p=0.12; X^2=1.70, p=0.19, Figure 3.6 A).

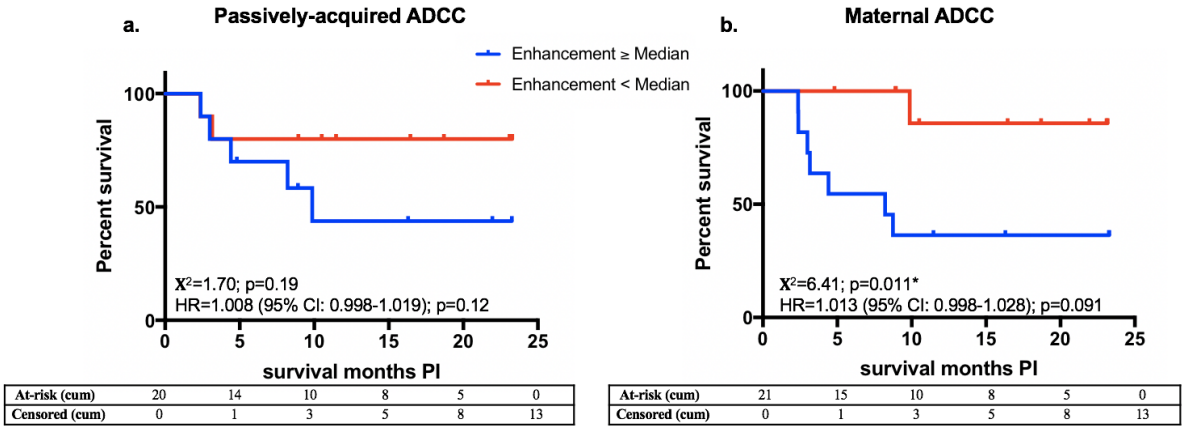
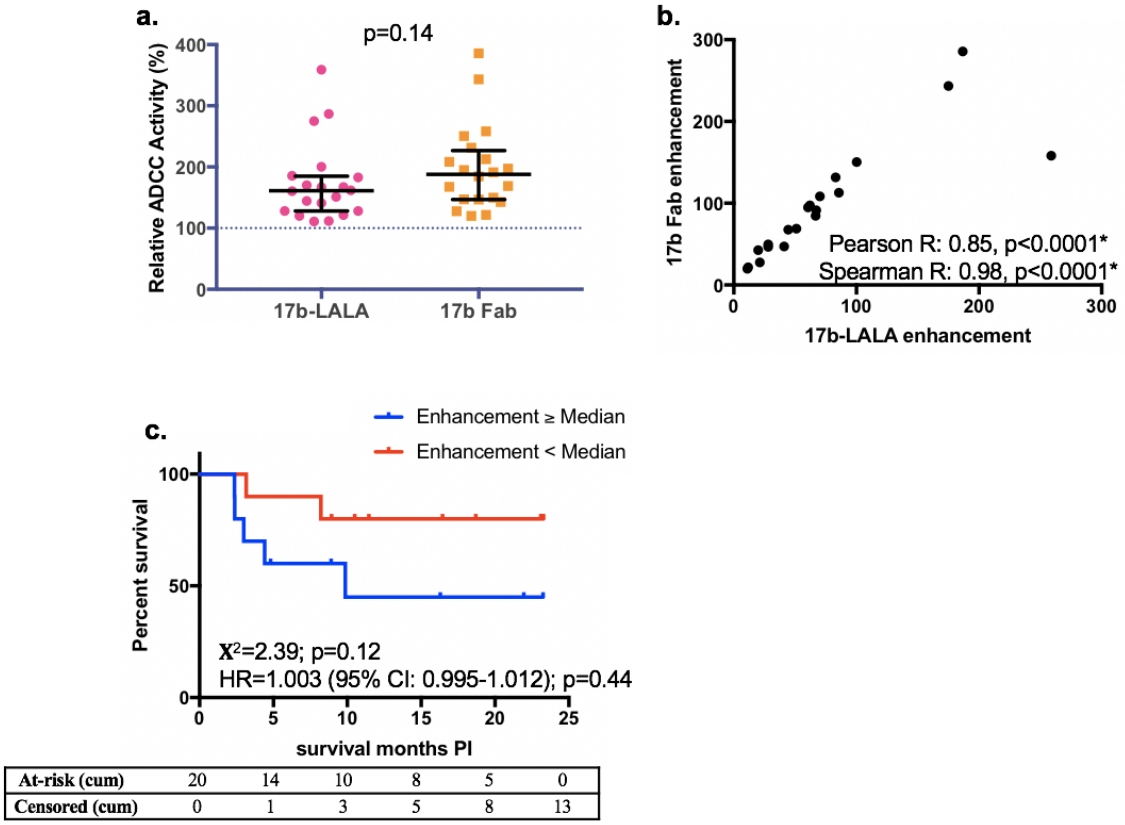


Figure 3.6. Effect of 17b-LALA-mediated ADCC enhancement on HIV-infected infant survival.

a: Kaplan-Meier survival curves between infants (N=20) that had 17b-LALA-mediated enhancement of passively-acquired ADCC at/above the HIV-infected infant cohort median (blue line) or below the HIV-infected infant cohort median (red line) were compared by a log-rank test. b: Kaplan-Meier survival curves between infants whose mothers (N=21) had 17b-LALA-mediated enhancement of maternal ADCC at/above the transmitting mothers cohort median (blue line) or below the transmitting mothers cohort median (red line) were compared by a log-rank test. X^2 values and p-values are shown. The x-axis shows months survival post infection (PI). The association of 17b-LALA-mediated enhancement of passively-acquired or maternal ADCC with risk of HIV-infected infant mortality was measured by a Cox-proportional hazards model. Hazard ratios (HR), 95% confidence intervals (CI), and p-values are shown on the graphs. Statistical significance was defined as $p<0.05$ (*). Cumulative (cum.) number of infants at-risk or censored by the end of each month on the x-axis are shown in the tables.

To further explore the mechanism of the association between 17b-LALA-mediated ADCC enhancement and infant outcome, and to rule out possible indirect steric effects or an artifactual increase in avidity due to pre-incubation with 17b-LALA, we compared plasma ADCC activity of the HIV-infected infants in the presence of 17b-LALA compared to 17b Fab (Figure 3.7). Plasma ADCC was enhanced to a similar degree by both 17b-LALA and 17b Fab (mean LALA enhancement: 73.7%; mean Fab enhancement: 97.5%) and the enhancement observed in the presence of the Fab competitor correlated with the enhancement mediated by the 17b LALA variant (Pearson $R=0.85$, $p<0.0001$; Spearman $R=0.98$, $p<0.0001$). Similar to the results with 17b-LALA, enhancement mediated by 17b Fab was associated with shorter survival of HIV-infected infants, although this association was not statistically significant ($X^2=2.39$, $p=0.12$).



At-risk (cum)	20	14	10	8	5	0
Censored (cum)	0	1	3	5	8	13

Figure 3.7. Enhancement of plasma ADCC by 17b Fab compared to 17b-LALA. a: Either 5ug/ml 17b-LALA or 10ug/ml 17b Fab was used as the competitive inhibitor in the competition RFADCC assay with HIV-infected infant plasma (N=20). Relative ADCC in the presence of 17b-LALA or 17b-Fab is shown and was compared by a Mann-Whitney U test. Error bars represent median + interquartile range. Relative ADCC in the presence of 17b-LALA is averaged from four biological replicates, relative ADCC in the presence of 17b-Fab is averaged from two biological replicates. b: Correlation between enhancement mediated by 17b-LALA and enhancement mediated by 17b-Fab is shown. Pearson and Spearman correlation coefficients and corresponding p-values are shown. d: Kaplan-Meier survival curves between HIV-infected infants (N=20) that had 17b Fab-mediated passively-acquired ADCC enhancement at/above the HIV-infected infant cohort median (blue lines) or below the HIV-infected infant cohort median (red lines) were compared by a log-rank test. X^2 values and p-values are shown. The x-axis shows months survival post infection (PI). The association of 17b Fab-mediated enhancement of passively-acquired ADCC with risk of HIV+ infant mortality was measured by a Cox-proportional hazards model. The hazard ratio (HR), 95% confidence intervals (CI), and p-values are shown on the graph. Cumulative (cum.) number of infants at-risk or censored by the end of each month on the x-axis are shown in the tables. Statistical significance was defined as $p<0.05$ (*).

Correlations between various ADCC activities

To gain insight into interactions among the ADCC activities to specific epitopes and the overall ADCC activities in the absence of competitor, we compared correlations of A32-like ADCC, C11-like ADCC, total cluster A-specific ADCC, 17b-LALA-mediated ADCC enhancement, and 17b-Fab-mediated ADCC enhancement with overall ADCC (without the competitor) and with each other (Figure 3.8). Interestingly, passively-acquired A32-like ADCC, total cluster A-specific ADCC, 17b-LALA-mediated ADCC enhancement, and 17b-Fab-mediated ADCC enhancement are statistically significantly inversely correlated with total plasma ADCC and directly correlated with each other, with a similar but weaker pattern for maternal plasma. C11-like ADCC showed a different pattern and was not statistically significantly correlated with overall ADCC or with ADCC enhancement. The inverse relationship between 17b-LALA mediated ADCC enhancement and overall ADCC was quite striking and strongly statistically significant for both infant and maternal plasma (infant spearman $R=-0.959$, $p<0.0001$; maternal spearman $R=-0.728$, $p<0.0001$).

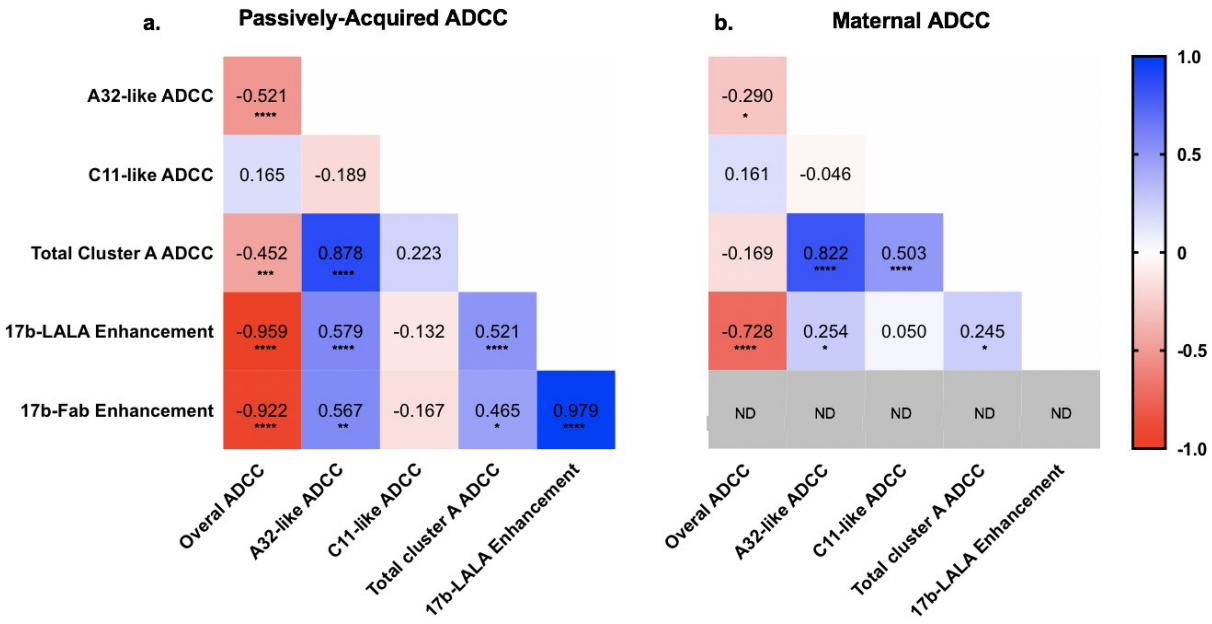


Figure 3.8. Correlations between various ADCC activities.
Correlation matrix for ADCC activities of passively-acquired infant plasma (a) or maternal plasma (b). Spearman rank correlations are shown on the heatmap. Red indicates a negative correlation, white indicates no correlation, and blue indicates a positive correlation. ND= not done. Statistical significance levels are shown as:
$p<0.05$ =*
$p<0.01$ =**
$p<0.001$=***
$p<0.0001$=****

Discussion

ADCC has been shown to correlate with reduced risk of infection, reduced disease progression, and improved clinical outcome in MTCT, chronic infection, and vaccination (52-64, 66, 67, 93, 193, 194). However, there are limited data on which epitopes of ADCC mediating antibodies are correlated with protection or improved clinical outcome in humans (58, 61, 64). This study aimed to address this question in the context of breastfeeding MTCT in a cohort where ADCC activity has been reported as a correlate of improved infant outcome (53, 54). To our knowledge, this is the first study designed to measure the association of ADCC targeting CD4i epitopes with infection risk and disease outcome in humans. We did not find any associations between these responses and HIV transmission risk. Unexpectedly, rather than explaining the overall beneficial effect of ADCC-mediating antibodies, we found that commonly elicited anti-cluster A-activities were associated with increased mortality among those infants who contracted HIV infection.

Both A32-like and C11-like ADCC responses targeting the gp41-interactive region of gp120 were common and dominant in mothers and their infants, consistent with non-MTCT human studies (41, 44, 99, 100, 173, 177, 183, 185). While these specificities were common, there was no detectable association of A32-like ADCC, C11-like ADCC, or total cluster A-specific ADCC with risk of infant infection. This lack of association with transmission is perhaps not surprising given our previous finding of a weak and non-significant association between high total plasma ADCC and reduced risk of infant infection in this cohort (54).

In this study, we extended prior findings showing that passively-acquired ADCC in infants, but not maternal ADCC, was associated with improved survival of HIV-infected infants using two different antigens (54). Neither passively-acquired nor maternal A32-like or C11-like

ADCC were significantly associated with HIV-infected infant survival; rather, there was evidence from multiple analyses that high levels of total cluster A-specific ADCC was associated with reduced HIV-infected infant survival. These data indicate that ADCC-mediating antibodies targeting the cluster A CD4i epitopes are not driving the association of passively-acquired ADCC with improved survival of HIV-infected infants, and that ADCC targeting cluster A CD4i epitopes may be associated with increased mortality among those infants who acquire HIV-infection.

There was little detectable 17b-like ADCC activity in this cohort, perhaps reflecting the fact that cluster C-specific antibodies are less potent than cluster A (44, 173, 175). Our lack of detection of 17b-like ADCC is in contrast to cohorts of chronically infected individuals in which cluster C-specific plasma ADCC was readily detectable and common (173, 185). However, this difference in detection of cluster C-specific ADCC may be due to differences in methods between our study and those conducted by Ferrari et al and Alsahafi et al, which used the chromium release ADCC assay and a FACS-based assay against infected target cells, respectively (173, 185). Instead of detecting cluster C-specific plasma ADCC, we saw a surprising effect of preincubation of the target cells with 17b-LALA in which plasma ADCC was enhanced compared to preincubation with no competitor antibody. This was also observed when we used 17b Fab fragment as the competitor. Due to this enhancement phenomenon, we cannot rule out that the enhancement of non-17b-like ADCC was masking the 17b-like ADCC activity.

Interestingly, 17b-LALA-mediated enhancement of plasma ADCC measured in this assay was associated with decreased survival among the HIV-infected infants. Notably, 17b-LALA-mediated enhancement of ADCC was strongly inversely correlated with total plasma ADCC, raising the possibility that 17b-LALA-mediated ADCC enhancement may be a proxy for low

overall ADCC, which was correlated with poorer infant outcome in this cohort, as shown here and in the prior study of this cohort conducted by Milligan et al (54).

We hypothesized the following mechanism to explain the unexpected enhancement of ADCC mediated by 17b-LALA: during the preincubation step with 17b-LALA, binding of a 17b Fab arm to gp120 increases exposure of other epitopes of ADCC-mediating antibodies. Of note, enhancement of plasma ADCC by 17b-Fab and 17b-LALA were significantly directly correlated with A32-like ADCC and total cluster A-specific ADCC, but not with C11-like ADCC, suggesting that 17b binding to its co-receptor binding site epitope leads to further exposure of the A32 epitope. This model is consistent with structure studies conducted by Tolbert et al in the context of trimer, showing that after 17b antibody or 17b fab binding, A32 binds more efficiently to envelope than C11 does, and the authors suggest that the A32 epitope is exposed earlier in the viral entry process compared to the C11 epitope (182). These findings were replicated by Alsahafi et al (185). Taken together these data support a model that antibody binding to the co-receptor binding site leads to differential exposure of the non-overlapping cluster A A32 and C11 epitopes, with the A32 epitope becoming better exposed upon 17b binding. Thus, the 17b enhancement data, which may be indicative of increased cluster A-specific activity, is consistent with the finding that cluster A-specific activity is associated with worse infant outcome. However, we cannot rule out that the 17b fab was forming bivalent complexes after long-term storage (195), in which case bivalent 17b fab would resemble 17b f(ab)'2, and be expected to act similarly to 17b-LALA.

These findings add to limited studies of ADDC epitopes in relation to clinical outcomes (58, 61, 64, 98-100). A handful of studies have identified ADCC epitopes associated with elite controller or long-term non-progressor (LTNP) status compared to viremic progressors (58, 61,

64). Additionally, CD4i-specific antibodies have been isolated from natural viral suppressors and RV144 vaccinees (44, 100), but these studies did not compare epitopes between controllers vs progressors or HIV-infected vs uninfected vaccinees. In contrast to these promising data, our data in the setting of MTCT suggests that ADCC targeting the cluster A CD4i epitopes was not associated with protection from infection and was associated with accelerated HIV progression in infants who acquired HIV infection.

Cluster A-specific antibodies, while common, also have some unusual properties and have been shown to poorly mediate ADCC against cells infected with primary isolates due to the reduction of CD4 and Env on the cell membrane upon productive infection (37, 40, 174-177, 180, 181, 186, 196-205). Because they can recognize the open conformation of Env and thus the gp120 monomer, cluster A-specific antibodies can also target uninfected cells that have bound gp120 that has been shed from virus or infected cells (176, 177, 206). It is interesting that A32-like ADCC measured in the assay used here, which uses target cells with bound gp120, was inversely correlated with total ADCC. This could suggest that A32-dominant responses typically arose when ADCC responses overall were weak due to the high immunogenicity of the A32 epitope, and that the positive association between A32-like activity and poor infant survival actually reflects a relatively weak overall ADCC response. In this scenario, the presence of A32 responses per se are not accelerating HIV progression but represent a misdirection of the immune response away from more protective antibody epitopes. Interestingly, ADCC responses *in vivo* are often polyclonal (41, 173, 177, 183), and combinations of monoclonal antibodies have been shown to be important for ADCC (174, 184-186). Since most plasma samples had some A32-like activity, an alternative, plausible explanation for the negative correlation between A32-like activity and overall ADCC is that the strongest ADCC responses associated with improved infant

outcome are polyclonal, where A32-like activity would be detectable, but would make up a lower percentage of the overall ADCC response compared to a monoclonal response, which could have a high percentage of A32-specific activity, but weak total activity.

Our study has a number of limitations. Our study is limited by relatively low statistical power because of the small number of infants that acquired HIV. Additionally, of the 21 infants that acquired HIV, 7 had an estimated time of infection after six months of age, by which time passively-acquired antibody levels may have waned in the infant. Therefore, the relevance of passively-acquired ADCC in these late transmissions is unclear. Notably, when our data analyses were restricted to the 14 infants who acquired HIV prior to six months of age, results were similar to those of the entire cohort. Another caveat to this study is that we used the RFADCC assay with coated target cells. The RFADCC assay has been shown to predominantly measure a "monocyte-mediated ADCC" or trogocytosis process (207-209). It has also been argued that coated cells are not as biologically relevant as infected cells. While the exact activity captured in this assay remains controversial, results of this assay have repeatedly shown a correlation between activity and outcome in humans (53, 54, 59, 62, 194). Specifically, we chose to take this approach to replicate the methods used by Milligan et al that showed a correlate of RFADCC activity and outcome (54). The aim of the current study was to identify the epitopes of plasma ADCC initially measured in that prior study that are associated with the observed clinical outcome as these associations can help inform mechanisms of protection.

Although CD4i epitopes are highly conserved and immunodominant, our findings indicate that ADCC targeting these epitopes by themselves may not be beneficial, as CD4i epitope-specific ADCC was not associated with either reduced risk of infant infection or improved HIV-infected infant survival. These findings highlight a need for further research to

clarify the role of CD4i epitope-specific ADCC-mediating antibodies in protection and clinical outcomes and whether they are beneficial or detrimental. Additionally, ADCC targeting other epitopes, including the V1V2 epitope which was a correlate of protection in the RV144 trial (93, 95, 98), non-gp120 epitopes, novel epitopes yet to be defined, and polyclonal ADCC responses should also be explored as potential correlates of protection to inform vaccine and therapeutic design.

Chapter IV

Measuring potential antibody Fc-mediated effector activity using a non-cell-based assay

Introduction

As discussed in Chapters I and III, antibody effector functions including ADCC have been shown to correlate with protection, slowed disease progression, and improved outcome in vaccination (93), chronic infection (52, 58-67), and MTCT (53-57). ADCC is one of a number of antibody effector functions, such as antibody-dependent phagocytosis (ADP) and antibody-dependent complement deposition (ADCD), in which the antibody binds to the antigen with its fab, and triggers innate effector cell activity by binding to Fc receptors on the effector cell membrane (12, 20). The type of effector function triggered is dependent on which cell type and Fc receptor the antibody binds with its Fc region (12, 13, 208). NK cells, which express high levels of FcγRIIIa, are the classical effector cells for ADCC (12, 13). Other effector cells express a variety of FcγRs and can mediate multiple effector functions (12, 13, 208). For example, monocytes, which express high levels of FcγRIIa and lower levels of both FcγRIa and FcγRIIIa, can mediate both phagocytosis and ADCC (12, 13, 21). Multiple alleles of Fc receptors are present in the population. FcγRIIIa has a high and low affinity variant (V158 and F158, respectively) and FcγRIIa is also present as two variants with high and low affinity, especially for IgG2 (H131 and R131, respectively) (15, 208, 210).

Unlike neutralization assays which have been standardized in the field, assays measuring ADCC vary widely both in their methods and in the biological function they are measuring (208, 210-212). Some ADCC assays measure ADCC according to target cell death, gain/loss of a

soluble marker in target cells, or loss of viral expression in target cells. Others measure the activation of effector cells (NK cell activation assay). Some assays use coated target cells, and others use infected target cells. Effector cells can very between PBMCs or purified NK cells among different labs and assays. See reviews by Kramski et al, Veillette et al, Lewis et al, and Wines et al for description and comparisons of these assays (208, 210-212). This variability among assays can lead to results that are inconsistent and difficult to interpret across the field. It is important to use a number of different assays to measure ADCC among the same set of samples to gain a more complete understanding of the mechanisms of ADCC, and of effector functions in general, that are biologically relevant to HIV infection.

As described in Chapter III, our lab previously reported that passively-acquired, but not maternal, ADCC was associated with improved survival among infants from the NBT cohort who acquired HIV through MTCT (54). This study used the cell-based rapid and fluorometric ADCC assay (RFADCC assay) to measure ADCC (191). Although originally thought to measure classical NK-cell mediated ADCC, it has been found that this assay largely measures monocyte uptake of target cell membrane in a "monocyte-mediated ADCC" or trogocytosis process, with only a minority of the measured activity being due to NK cell-mediated ADCC (207-209). With the above caveats in mind, in this chapter we aimed to further investigate the biological mechanism measured by the RFADCC assay that is correlated with improved outcome. To do so, we utilized a newly-developed non-cell-based ELISA designed to measure the potential for antibodies to mediate ADCC and other effector functions (213). This assay uses a soluble dimeric Fc receptor to bind two antibodies at once, mimicking the Fc receptor cross-linking that must occur to trigger Fc receptor signaling and subsequent effector functions (12, 18, 19, 213), and it has been shown to correlate well with cell-based ADCC assays (213, 214). We tested the

maternal and infant NBT cohort samples in this soluble dimeric Fc receptor ELISA using high and low affinity variants of soluble dimeric Fc gamma receptors IIa and IIIa. We measured the association of dimeric FcγR binding with risk of MTCT and infected infant mortality to gain a more complete understanding of the biological mechanism of the association of ADCC with improved outcome, as measured previously by the RFADCC assay.

Materials and Methods

Study Design and Plasma Samples

Plasma samples were from the NBT, conducted in Nairobi between 1992-1998, prior to the use of anti-retroviral therapy (5). HIV-1 positive mothers were enrolled during the third trimester at which time maternal blood samples were collected. Infant blood samples were collected at birth and at regular intervals thereafter until 2 years of age. Infant PBMC samples were tested for HIV infection by single copy detection DNA PCR (160). Infants testing positive for HIV DNA, were retrospectively tested for HIV RNA using a prototype Gen-Probe/Hologic HIV viral load assay that detects diverse subtypes from samples collected from previous timepoints (161). As described in Chapters II and III, estimated time of infection was defined as the midpoint between the last HIV negative and first HIV positive test for this study (54). The Kenyan Ministry of health gave permission for the Nairobi Breastfeeding Clinical Trial to be conducted, and the Institutional Review Boards of the University of Nairobi, University of Washington, and the Fred Hutchinson Cancer Research Center approved the current study.

Plasma samples from 72 mother-infant pairs from the NBT meeting selection criteria described by Milligan et al and in Chapters II and III were utilized in the present study (54). Briefly, mother-infant pairs were included if the following criteria were met: the infant was HIV

RNA and DNA negative at birth, breastfed for a minimum of 3 months, HIV-exposed uninfected (HEU) infants remained HIV-negative for a minimum of 6 months and at each follow up timepoint, and an infant plasma or cord blood sample was available from the first week of life (prior to estimated time of infection). Infant cord blood (N=58) or neonatal plasma from delivery (N=10) were tested in this study along with paired maternal plasma samples from the third trimester of pregnancy (N=68) or delivery (N=3). A subset of paired neonatal plasma and cord blood from infants who had both sample types available were compared in the soluble dimeric FcγR ELISA, and no major differences between sample types were observed (data not shown). Of note, 2 HIV-infected infants, 2 HEU infants, and 1 non-transmitting mother from the 72 pairs studied by Milligan et al had no more plasma available (54); their corresponding transmitting maternal, non-transmitting maternal, and HEU infant plasma samples were available and included in this study. The final cohort included 67 paired maternal and infant samples (48 non-transmitting pairs and 19 transmitting pairs), 2 unpaired transmitting maternal samples, 2 unpaired non-transmitting maternal samples, and 1 unpaired HEU infant sample. All plasma and cord blood samples were heat inactivated at 56 degrees for 1 hour.

Soluble dimeric FcγR ELISA

Maxisorp 384-well ELISA plates (ThermoFisher) were coated with clade A/D BL035.W6M.ENV.C1 gp120 (ImmuneTech) at 1ug/ml overnight at 4 degrees Celsius in PBS. (This was the same antigen used by Milligan in the RFADCC assay with the NBT cohort samples and here in Chapter III (54)). Two positive control wells were coated with HIVIG at 5ug/ml (NIH AIDS reagent program, catalog #3957, contributed by NABI and National Heart Lung and Blood Institute (Dr. Luiz Barbosa)) to measure maximum dimeric FcγR activity used to normalize OD values (as described below). The next day, plates were washed 4 times with

PBS-0.02% Tween20 and then blocked with 1% human serum albumin (HSA, Sigma) in PBS with 1mM EDTA for 1 hour at 37 degrees Celsius. Plates were washed 4 times, then plasma samples or controls diluted in 1% bovine serum albumin (BSA, Sigma) in PBS with 1mM EDTA (PBSE-BSA) were added to the plate and incubated for 1 hour at 37 degrees Celsius. Plates were washed and incubated with 0.1ug/ml biotinylated soluble dimeric receptor FcγRIIIa (V158 or F158) or 0.2ug/ml biotinylated soluble dimeric receptor FcγRIIa (H131 or R131) diluted in PBSE-BSA for 1 hour at 37 degrees Celsius. Plates were washed and incubated with high sensitivity streptavidin-HRP (ThermoFisher) at a 1:10000 dilution in PBSE-BSA for 1 hour at 37 degrees Celsius. Plates were washed and incubated with Ultra-TMB (ThermoFisher) for 7 minutes (for soluble dimeric FcγRgIIIa V158) or 15 minutes (for all other receptors) at RT. The reaction was stopped with 1N H_2SO_4 (Thermo Scientific). The absorbance was read at 450nm. Background was defined as the OD of BL035 gp120-coated wells without plasma. Dimeric FcγR activity was calculated as follows: $[(OD_{plasma} - OD_{background})/(OD_{HIVIG\text{-}coated\ wells})]*100\%$. Averaged results from two biological replicates performed in technical duplicate are shown.

The plasma samples were run at a 1:100 dilution. This dilution was determined empirically to be the highest dilution (lowest concentration of antibody) to give a wide dynamic range of ODs across all 4 soluble dimeric FcγRs in 3 pilot experiments with a subset of samples. HIV-negative serum (VA study,(169)) at a 1:100 dilution in BL035 gp120-coated wells was used as a negative control. Positive controls were VA positive plasma pool (VA study,(169)) and HIVIG (NIH AIDS reagent program, catalog #3957, contributed by NABI and National Heart Lung and Blood Institute (Dr. Luiz Barbosa)) run in a two-fold dilution curve (1:100-1:800) in BL035 gp120-coated wells.

Statistical Analysis

Statistical analyses were performed with (StataCorp, College Station, TX) and GraphPad Prism7 (GraphPad Software, Inc., San Diego, CA). All graphs were generated by Prism7 (GraphPad Software, Inc., San Diego, CA). To determine whether dimeric FcγR activity was associated with risk of MTCT, dimeric FcγR activity of HIV-exposed uninfected (HEU) vs HIV-infected (HIV+) infant plasma or non-transmitting (NT) vs transmitting (T) maternal plasma was compared by a Mann-Whitney U test and logistic regression analysis adjusted for maternal plasma RNA viral load. The association of dimeric FcγR activity with HIV+ infant survival was assessed by a Cox-proportional hazards model and a log-rank test comparing Kaplan-Meier curves of HIV+ infants with high or low dimeric FcγR activity in infant plasma (or corresponding maternal plasma) dichotomized at the HIV+ infant cohort median (or T maternal cohort median) as noted in the figure legends. Statistical significance was defined as $p<0.05$. All reported correlations are Spearman rank correlations.

Existing data from the NBT cohort for correlation analysis

RFADCC activity from the NBT cohort was measured by Milligan et al (54) and by me (Chapter III). Total HIV-specific IgG was measured by ELISA by Milligan et al (54), and by me via binding antibody multiplex assay (BAMA) (Chapter II). Levels of IgG1 and IgG3 were measured by cell-surface binding assays and standard binding ELISAs, respectively, by Milligan et al (54).

Results

Association of dimeric FcγR activity with risk of MTCT

To measure the association of dimeric FcγRIIa and dimeric FcγRIIIa activity with risk of MTCT, we measured the ability of plasma from the NBT cohort to bind four biotinylated soluble dimeric FcγRs (IIIaV158, IIIaF158, IIaH131, IIaR131) in a newly-developed soluble dimeric FcγR ELISA (213). As expected, dimeric FcγR activity was higher for the high affinity dimeric FcγR variants, IIIaV158 and IIaH131, compared to the low affinity variants, IIIaF158 and IIaR131, for both infant and maternal plasma (Figure 4.1, compare A, C, E, G to B, D, F, H). We compared dimeric FcγR activity between 49 HIV exposed uninfected infants (HEU) and 19 HIV+ infants, or between 50 non-transmitting (NT) and 21 transmitting (T) mothers, by a Mann-Whitney U test (Figure 4.1) (see methods for description of unpaired samples). There was no difference in dimeric FcγR activity between the infection or transmission groups for any of the 4 dimeric FcγRs. Because maternal viral load is a known risk factor for MTCT (101), we also performed a logistic regression analysis comparing dimeric FcγR activity between HEU and HIV+ infants, or between NT and T mothers, adjusting for maternal plasma viral load. There was no association of dimeric FcγRIIa or FcγRIIIa activity of infant plasma containing passively-acquired antibodies or paired maternal plasma with MTCT risk (Table 4.1).

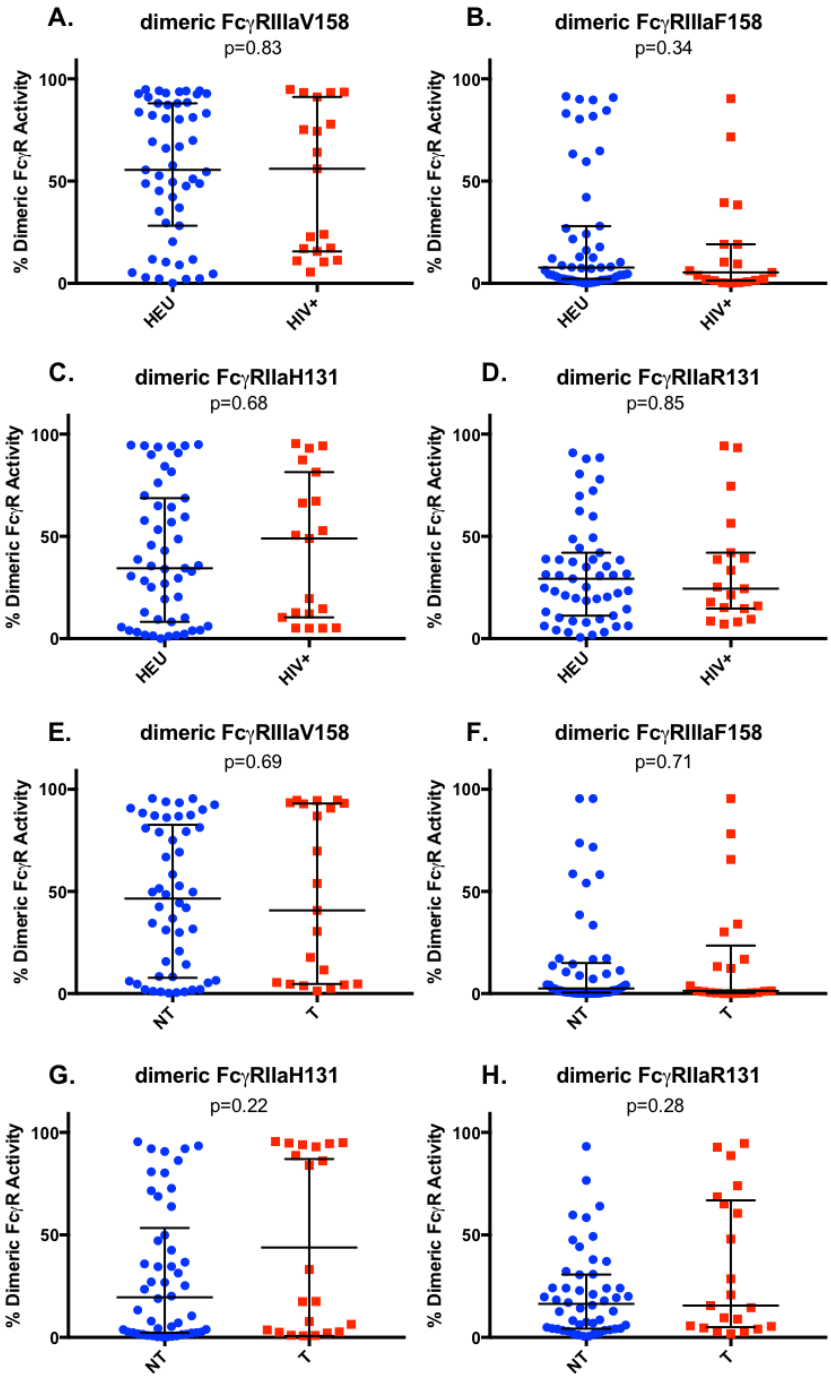


Figure 4.1. Soluble dimeric FcγR activity in NBT cohort.
The soluble dimeric FcγR ELISA was performed on 68 infant plasma samples (A-D) and 71 corresponding maternal plasma samples (E-H). % dimeric FcγR activity is shown for HIV-exposed uninfected infants (HEU) compared to infected (HIV+) infants or non-transmitting (NT) compared to transmitting (T) mothers. Dimeric FcγR activity was compared between the infection or transmission groups by a Mann-Whitney U test. Statistical significance was defined as $p<0.05$ (*). Error bars represent median + interquartile range. Results are averaged from two biological replicates. Negative values were treated as zeros.

Table 4.1. Association of dimeric FcγR activity with odds of MTCT.
The association of dimeric FcγR activity with odds of MTCT was measured using a logistic regression analysis adjusted for maternal plasma viral load. Adjusted odds ratios (aOR), 95% confidence intervals (CI), and p-values are shown for infant samples (passively-acquired ADCC, left) and maternal samples (maternal ADCC, right). Statistical significance was defined as $p<0.05$ (*).

	Infant Plasma			Maternal Plasma		
	aOR	**95% CI**	**p-value**	**aOR**	**95% CI**	**p-value**
Dimeric FcγRIIIaV158	.998	.981 - 1.014	0.77	1.004	.989 - 1.020	0.57
Dimeric FcγRIIIaF158	.992	.972 - 1.013	0.46	1.006	.985 - 1.027	0.58
Dimeric FcγRIIaH131	.999	.983 - 1.016	0.90	1.010	.995 - 1.025	0.18
Dimeric FcγRIIaR131	.996	.975 - 1.017	0.70	1.015	.994 - 1.036	0.16

Association of dimeric FcγR activity with infected infant survival

To measure the association of dimeric FcγR activity on infant survival in this cohort, we performed the same two types of survival analyses described previously for the NBT cohort (Figure 4.2) ((54), Chapter III). Briefly, we measured the association of dimeric FcγR activity with mortality risk for the infected infants (infant plasma samples: N=19, 7 deaths; maternal plasma samples N=21, 8 deaths) with a Cox-proportional Hazards model, in which dimeric FcγR activity is a continuous variable to determine whether or not there is change in mortality risk for each percentage increase of dimeric FcγR activity. We also determined if there was an association of high or low levels of dimeric FcγR activity with infected infant survival times by comparing Kaplan-Meier curves of infected infants with dimeric FcγR activity at/above the infected cohort median or below the cohort median by a log-rank test. There was no significant association of dimeric FcγR activity for any of the 4 receptors with infected infant survival in either analysis method for infant or maternal plasma (Figure 4.2). However, dimeric FcγR activity of infant plasma containing passively-acquired antibodies showed weak non-significant associations with improved infected infant survival for three of the four dimeric FcγRs in the log-rank test (H131 X^2=2.43, p=0.12; R131 X^2=2.43, p=0.12; F158 X^2=2.84, p=0.092).

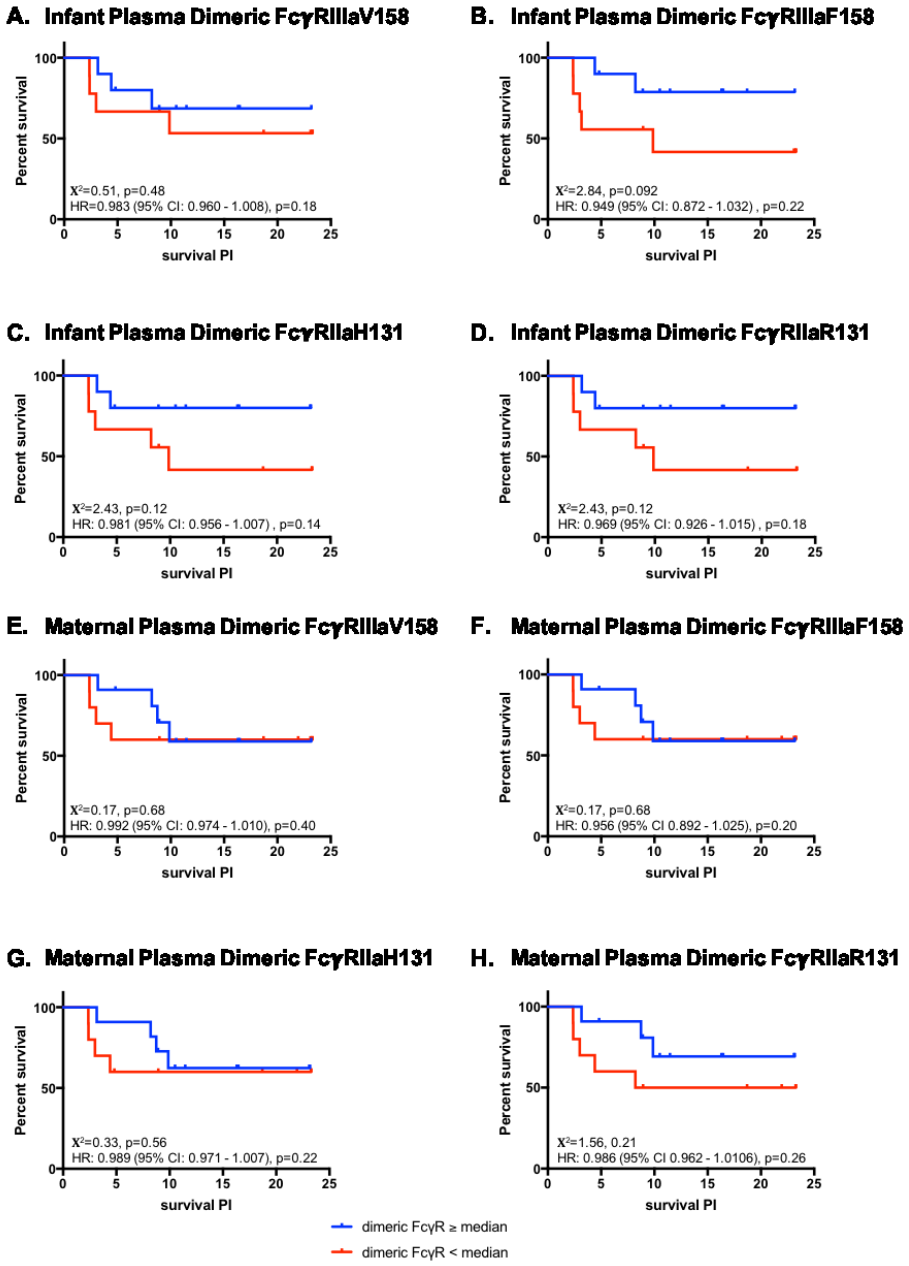


Figure 4.2. Soluble dimeric FcγR activity and HIV-infected infant survival.
A-D: Kaplan-Meier survival curves between infants (N=19) that had passively-acquired dimeric FcγR activity of at/above the HIV+ infant cohort median (blue line) or below the HIV+ infant cohort median (red line) were compared by a log-rank test. E-H: Kaplan-Meier survival curves between infants whose mothers (N=21) had dimeric FcγR activity at/above the T mothers cohort median (blue line) or below the T mothers cohort median (red line) were compared by a log-rank test. X^2 values and p-values are shown. The x-axis shows months survival post infection (PI). The association of passively-acquired or maternal dimeric FcγR activity with risk of HIV+ infant mortality was measured in a Cox-proportional hazards model. Hazard ratios (HR), 95% confidence intervals (CI), and p-values are shown on the graphs. Statistical significance was defined as $p<0.05$ (*).

To determine if a combination of dimeric FcγR IIa and IIIa activity was associated with improved HIV+ infant survival, we grouped plasma samples that had high dimeric FcγR IIa and high dimeric FcγR IIIa activity, low dimeric FcγR IIa and low dimeric FcγRIIIa activity, or high dimeric FcγR activity for one receptor type only (Figure 4.3). We compared infected infant survival between infants who had both high passively-acquired dimeric FcγR IIa and IIIa activity or low activity for both receptor types by log-rank tests (Figure 4.3 A and B). We did the same analysis for infants born to mothers who had high maternal dimeric FcγR IIa and IIIa activity compared to low activity for both receptor types (Figure 4.3 C and D). We could not perform log-rank tests with the survival curves of infants who had passively-acquired or maternal high dimeric FcγR activity for only one receptor type, because these curves only had Ns of 1 or 2 infants as shown in the figure (Figure 4.3 red and green lines). We did this analysis for both high affinity alleles (Figure 4.3 A and C) and both low affinity alleles (Figure 4.3 B and D); we did not look at mixed combinations of high and low affinity alleles. There was no statistically significant association of a combination of dimeric FcγR IIa and IIIa activity with infected infant survival for maternal or infant plasma. However, there was a trend for high passively-acquired dimeric FcγR IIa and IIIa activity being associated with improved infected infant survival for both low affinity alleles (Figure 4.3 B), and a weaker non-significant association for high passively-acquired dimeric FcγR IIa and IIIa high affinity allele activity being associated with improved infected infant survival (Figure 4.3 A).

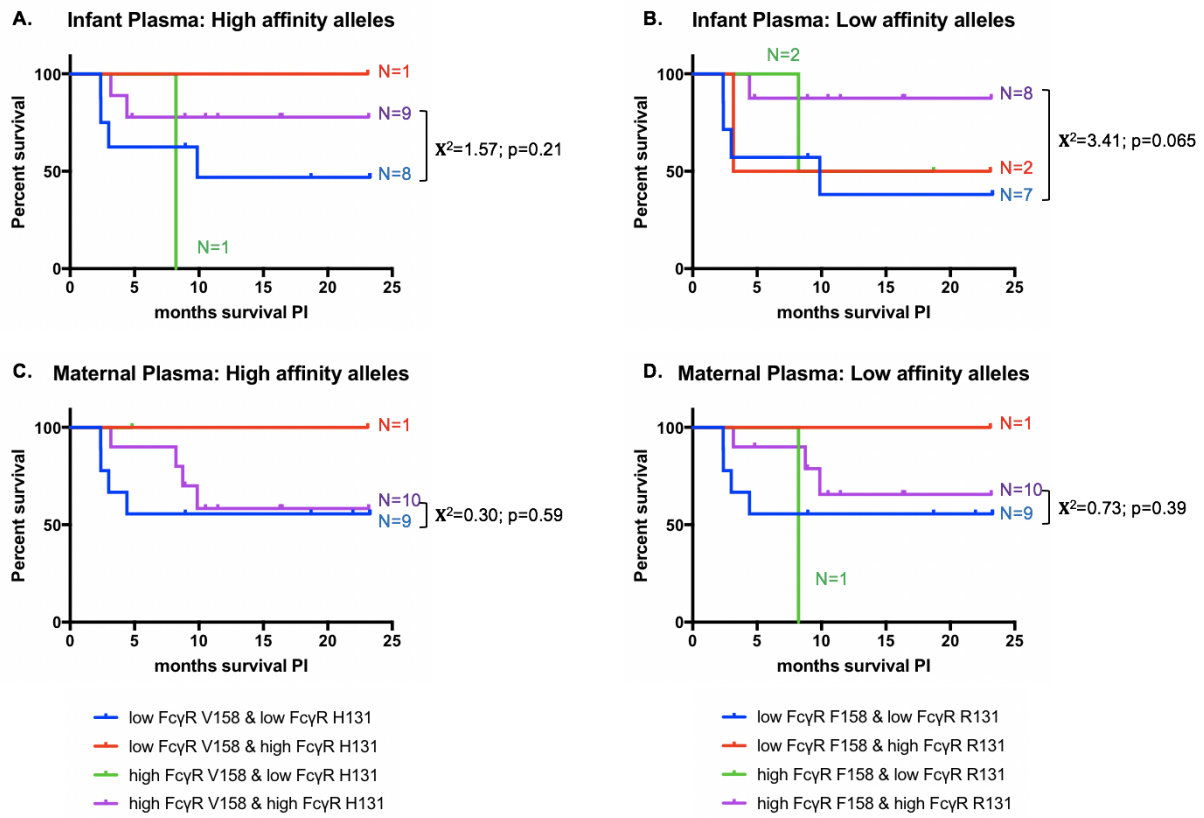


Figure 4.3. Combination of soluble dimeric FcγR IIa and IIIa activity and HIV-infected infant survival.

A-B: Kaplan-Meier survival curves between infants (N=19, 7 deaths) that had passively-acquired dimeric FcγR activity of at/above the HIV+ infant cohort median for both dimeric FcγR IIa and IIIa (purple line), below the HIV+ infant cohort median for both dimeric FcγR IIa and IIIa (blue line) were compared by a log-rank test. Survival curves between infants that had high passively-acquired dimeric FcγR activity for only receptor IIa (red line) or only receptor IIIa (green line) are also shown. C-D: Kaplan-Meier survival curves between infants whose mothers (N=21, 8 infant deaths) that had maternal dimeric FcγR activity at/above the transmitting mothers cohort median for both dimeric FcγR IIa and IIIa (purple line), below the transmitting mothers cohort median for both dimeric FcγR IIa and IIIa (blue line) were compared by a log-rank test. Survival curves between infants whose mothers had high maternal dimeric FcγR activity for only receptor IIa (red line) or only receptor IIIa (green line) are also shown. X^2 values and p-values are shown. The x-axis shows months survival post infection (PI). Statistical significance was defined as $p<0.05$ (*).

Correlations of NBT cohort dimeric FcγR activity with RFADCC activity and binding data

We used Spearman rank correlations to determine the correlations among dimeric FcγR activity with existing infant data from the NBT cohort of passively-acquired RFADCC activity, total passively-acquired HIV-specific IgG levels, and levels of passively-acquired IgG1 and IgG3 (Figure 4.4). Infant dimeric FcγR activity for all 4 variants were highly significantly correlated with each other (all p's<0.0001) and significantly correlated with both sets of existing infant RFADCC data with this cohort (all p's <0.05) (one dataset was generated by Milligan et al (54) and the other by me (Chapter III)). The strength of the correlation with RFADCC activity was: IIIaV158 > IIaH131 > IIaR131 ~ IIIaF158, consistent with the stronger correlations among the high affinity dimeric FcγR variants compared to the low affinity variants. Additionally, the infant dimeric FcγR activity was significantly correlated with total passively-acquired HIV-specific IgG as measured by ELISA (54) and BAMA (Chapter II) (all p's<0.01). Milligan et al previously measured IgG1 and IgG3 activity in this cohort and showed that passively-acquired ADCC activity is largely mediated by IgG1 (54). Here, dimeric FcγR activity was significantly correlated with IgG1 for dimeric FcγRs IIIaV158, IIaH131, and IIaR131. Levels of IgG3 were not correlated with any measure of passively-acquired antibody activity.

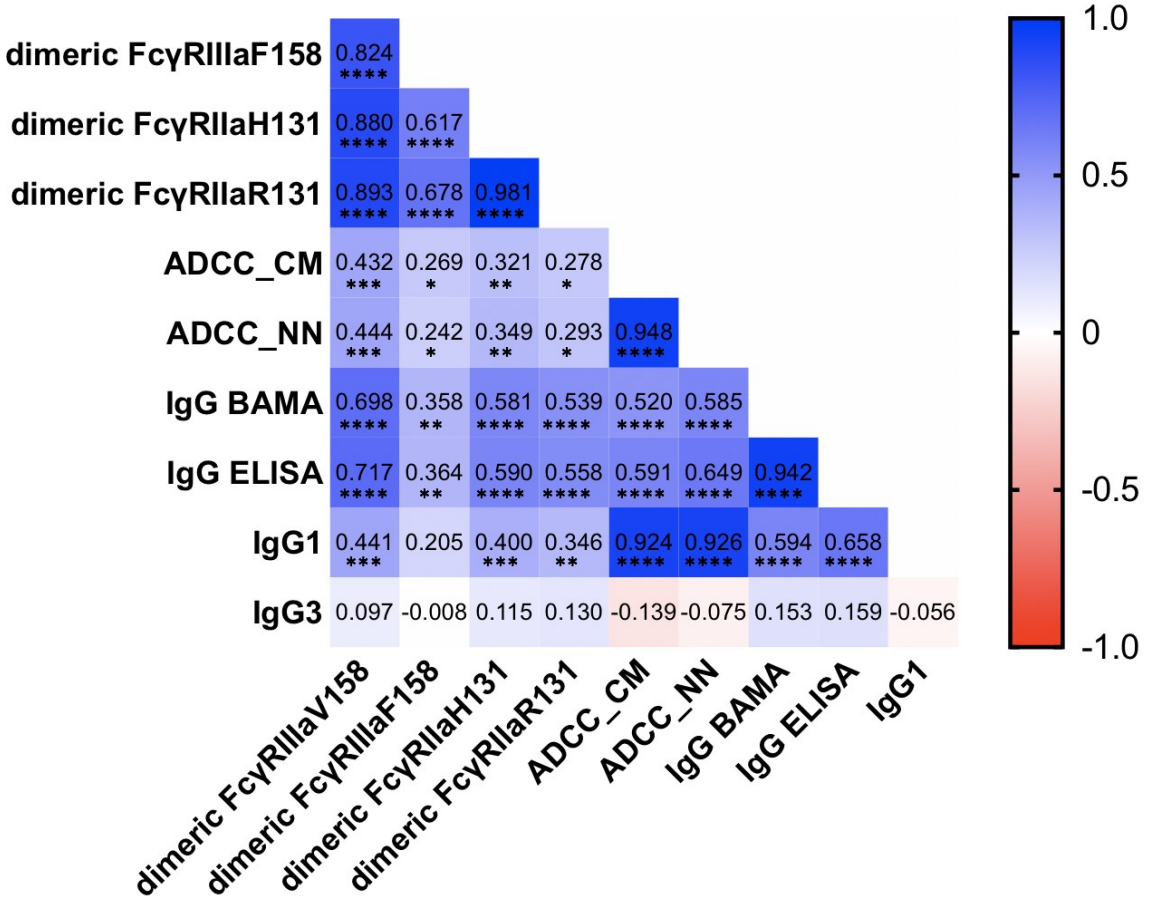


Figure 4.4. Correlation matrix for infant dimeric FcγR activities with each other and with previously collected NBT infant ADCC and binding data.
Data from HIV+ infants from the NBT cohort were correlated with each other. Data included in this correlation analysis are: dimeric FcγR activities with each of the four soluble dimeric FcγR variants, RFADCC data from Milligan et al (54), RFADCC data collected by me (Chapter III, Figure 3.4), total HIV-specific IgG as measured by BAMA (Chapter II, Figure 2.1), total HIV-specific IgG measured by Milligan et al via ELISA (54), HIV-specific IgG1 measured by Milligan et al via cell-surface binding assay (54), and IgG3 end point titers measured by Milligan et al via ELISA (54). BL035 gp120 was the antigen used for all of the assays shown here. Spearman rank correlations are shown on the heatmap. Red indicates a negative correlation, white indicates no correlation, and blue indicates a positive correlation. Statistical significance levels are shown as:
$p<0.05$ =*
$p<0.01$ =**
$p<0.001$=***
$p<0.0001$=****

Discussion

Our group previously showed that passively-acquired ADCC activity as measured by the RFADCC assay is associated with improved infected infant survival ((54); validated in Chapter III). NK cell-mediated ADCC is responsible for a minority of the effector function measured in the RFADCC assay while the majority of activity is actually monocytic uptake of target cell membrane via a trogocytic or monocyte-mediated ADCC process different from phagocytosis (207-209). The biological mechanism of this activity and its role in the setting of HIV is not fully understood. To investigate the mechanism of the correlate of improved outcome measured in the RFADCC assay (54), we tested the same cohort of NBT samples in a soluble dimeric Fc receptor ELISA designed to measure potential for effector function by using soluble dimeric Fcγ receptors that mimic cross-linked Fcγ receptors on the surface of effector cells (213). We used high and low affinity variants of FcγRIIIa and FcγRIIa. FcγRIIIa is considered the classical mediator of ADCC, as it is highly expressed on NK cells (12, 13, 208). FcγRIIIa is also weakly expressed on monocytes and other cell types and can mediate other functions such as ADP and cytokine release in addition to ADCC (12, 13, 15, 21, 208). Conversely, FcγRIIa is highly expressed on monocytes and on other myeloid cells but is not expressed on NK cells (13). FcγRIIa can also mediate ADP, complement, cytokine release, and other functions including ADCC (15, 208). The plasma samples in this cohort had the expected hierarchy of dimeric FcγR activity based on known affinities of antibodies for FcγRs (12, 13, 15, 208), with plasma demonstrating higher binding to the high affinity FcγRIIIaV158 than to the low affinity FcγRIIIaF158, and also showing greater binding to the high affinity FcγRIIaH131 compared to the low affinity FcγRIIaR131. Infant plasma binding to all four soluble dimeric FcγR variants were highly significantly correlated with each other, suggesting that these passively-acquired

plasma antibodies have the potential to mediate multiple effector functions. A growing number of studies investigating polyfunctionality of antibodies have shown that polyfunctionality is correlated with reduced disease progression (73), protection from infection in NHP (85), and was induced in RV144 trial vaccinees (215, 216), suggesting that polyfunctionality has an important role to play in HIV prevention and outcome.

The soluble dimeric FcγR ELISA has been shown to correlate well with cell-based ADCC assays and with IgG binding assays that simply measure the ability of the antibody to bind to the antigen regardless of the potential to mediate effector function (213, 214). Interestingly dimeric FcγR activity correlates more strongly with NK cell activity (as measured by different cell-based assays) compared to total IgG levels or total IgG binding (213, 214), suggesting that dimeric FcγR activity is not simply a proxy for binding antibody levels, but actually represents the potential to mediate effector function. Our results are consistent with this data. Here, there were similar but not identical correlational hierarchies and strengths between activities of the four soluble dimeric FcγR variants versus RFADCC activity compared to between dimeric FcγR activities versus total HIV-specific IgG. The correlational hierarchy of dimeric FcγR activity versus RFADCC activity from strongest correlation to weakest correlation with RFADCC activity was: IIIaV158 > IIaH131 > IIaR131 ~ IIIaF158. The correlation hierarchy of dimeric FcγR activity versus total HIV-specific IgG from strongest correlation to weakest correlation with total HIV-specific IgG was: IIIaV158 > IIaH131 ~ IIaR131 > IIIaF158. As these hierarchies are similar but not identical, these data suggest that the soluble dimeric FcγR ELISA is partially dependent on levels of IgG, but also measures activity distinct from pure antibody levels consistent with validation studies conducted by the developers of the assay (213, 214).

In our prior study of ADCC activity in the NBT cohort, IgG1, and not IgG3, was shown to mediate the RFADCC activity and be responsible for the association of passively-acquired ADCC with infant outcome (54). The data in the present study supported these findings as levels of IgG1 were significantly correlated with dimeric FcγRs IIIaV158, IIaH131, and IIaR131, while levels of IgG3 were not correlated with dimeric FcγR activity for any of the 4 variants tested.

The main goal of this study was to explore the biological mechanism responsible for the association of passively-acquired RFADCC activity with improved infant outcome (54). Neither maternal nor infant dimeric FcγR activity was associated with risk of MTCT, consistent with our previous ADCC study with this cohort which showed a weak and non-significant association between passively-acquired plasma ADCC and lower risk of infant infection in this cohort (54). The previous study did show a statistically significant association of passively-acquired ADCC in infants with improved infected infant survival, while there was no association of maternal plasma ADCC with infected infant survival (54). In this study, dimeric FcγR activity was not significantly associated with infected infant survival for either maternal plasma or infant plasma containing passively-acquired antibodies. However, dimeric FcγR activity of infant plasma did show non-significant associations with improved infected infant survival. Specifically infant plasma dimeric FcγRIIIaF158 activity and a combination of low affinity FcγR IIIaF158 and IIaR131 activities of infant plasma were associated with trends toward improved HIV-infected infant survival. The lack of a significant association in this study could be due to the different method used to measure Fc-effector function, as perhaps even though the dimeric FcγR ELISA is strongly correlated with RFADCC activity (this chapter and (214)), the ELISA does not fully recapitulate the biological process measured in the cell-based assay. The lack of association may also be limited by the fact that many of the samples had FcγR activity near the upper and below

the lower limit of detection, further compromising our sensitivity and reliable sample size for analyses that relied on binding as continuous variable. It should be noted that two HIV-infected infants who had plasma samples available for use in the prior study conducted by Milligan et al did not have plasma available to be run in the dimeric FcγR ELISA (54). One of these infants died. Therefore, the power of the current study is lower than that conducted by Milligan, which could explain why we saw similar trends to the previous correlate of improved outcome but did not observe any statistically significant findings.

This study had a number of limitations. It was limited by low power due to the lack of plasma availability for 5 plasma samples compared to the prior ADCC study with this cohort (54). Additionally, we focused on FcγRs IIIa and IIa in the soluble dimeric FcγR ELISA as an initial attempt to explore the biological mechanism being measured by the RFADCC assay and driving the association with improved infant outcome (54). While NK cells only express FcγRIIIa, monocytes express both FcγRs IIIa and IIa (13); therefore, it is difficult to discern whether NK cell activity, monocyte activity, or both play a role in the association of RFADCC activity with improved infant outcome (54). The current study has suggested that maternal and passively-acquired infant antibodies in this cohort have the potential to mediate multiple effector functions by activating multiple Fcγ receptors, and that dimeric FcγR IIa and IIIa activity may partially explain the association of passively-acquired ADCC with improved infant outcome observed in this cohort (54). However, due to the aforementioned limitations, further studies are necessary to before conclusions can be drawn.

Chapter V

Effect of ADCC on infant outcome in larger MTCT cohort

Introduction

Multiple studies have shown that ADCC activity is associated with improved infant outcome in the setting of MTCT of HIV-1. For instance, in a small cohort of high-risk mother-infant pairs from the NBT, breast milk ADCC activity was correlated with reduced risk of MTCT (53). Passively-acquired ADCC in infants and ADCC in maternal sera have been shown to correlate with trends or non-significant associations towards reduced risk of MTCT (54, 55, 138), but these associations did not reach statistical significance. Overall, the majority of MTCT studies to date have shown no correlation between plasma ADCC and risk of MTCT (56, 57, 119, 137, 152, 153); however, some of these studies were either unable to determine infant infection status prior to 15 months of age, making it difficult to know the mode of transmission and whether infection occurred prior to passively-acquired antibodies waning in the infant (56, 57), while other studies did not include breastfeeding transmissions (119, 153).

As discussed in chapter III, in the NBT passively-acquired ADCC in infant plasma was significantly associated with improved survival of infected infants ((54) and recapitulated in Chapter III Figure 3.4). Studies from other groups have also shown ADCC to be associated with improved infant outcome, such as reduced disease progression to AIDS or death among HIV-infected infants, although they had some of the limitations mentioned above regarding poorly defined timing of infection (55-57). These studies do collectively suggest that ADCC may be playing a beneficial role in MTCT, but studies with other cohorts with well-defined infection

times and samples from breastfeeding cohorts are warranted to determine whether these findings are reproducible, and to better understand the mechanism to inform future HIV treatment and prevention strategies.

Our prior ADCC study with samples from the NBT cohort which showed passively-acquired ADCC in infants, but not maternal plasma ADCC, to be significantly associated with improved infected infant survival and a trend towards reduced risk of MTCT was limited by low statistical power and the inclusion of mother-infant pairs where transmission happened after 6 months of age, by which time passively-acquired antibodies may have waned from infant circulation (54, 103). To further explore the trend associated with reduced risk of MTCT and investigate whether the significant association of passively-acquired ADCC with infected infant survival was reproducible across different cohorts, we measured passively-acquired ADCC activity in a larger cohort of infants from a second study, referred to as the CTL study (154-156). As this study was larger, it had greater statistical power, which we hoped would allow us to explore the trend towards reduced risk of MTCT observed with the NBT cohort. Importantly, in this study with the CTL cohort, we focused only on infants where transmissions were detected before 6 months of age, and all infants were breastfed for at least 6 months or until the time of transmission, which allowed us to examine passively-acquired antibody ADCC activity in relation to outcome.

Materials and Methods

Study Design and Plasma Samples

Samples were from the Cytotoxic T Lymphocyte (CTL) study which was conducted in Nairobi, Kenya from 1999 to 2002 (154-156). HIV-positive mothers were enrolled during the third trimester of pregnancy. Infant cord blood or neonatal plasma samples were collected near birth and periodically for 1 year. Infants who became HIV-infected were followed up for two years, or more in some cases, to determine length of survival. Mothers received a short course of zidovudine near delivery. 184 CTL infants (157 HEU and 27 HIV-infected) met criteria similar to the NBT ADCC cohort ((54) and Chapters II, III, IV): 1) infants were HIV-DNA and RNA negative at birth 2) HEU infants were HIV-negative for a minimum of 6 months and at each follow up timepoint; 3) an infant plasma or cord blood sample was available from the first week of life; 4) infants breastfed for a minimum of 6 months or until time of transmission; 5) among HIV-infected infants, detection of infection was after 1 week of age and prior to 6 months of age; 6) infants who received nevirapine at delivery were excluded (N=2). From this cohort of 184 infants, a random sample of 86 was selected to achieve a case-cohort design. 9 cases (HIV-infected infants) were randomly selected by this approach. The remaining 18 cases were added to the cohort. To account for the non-randomly selected cases being included in the cohort, we weighted the samples using the Borgan II Weights method (217): all cases were given a weight of 1, HEUs were given a weight of 2.039. The final cohort consisted of 27 HIV+ cases and 77 HEUs. Among the cohort, 36 infants had a plasma sample available, 17 had cord blood available, and 51 had both plasma and cord blood available. All plasma and cord blood samples were heat inactivated at 56 degrees Celsius for 1 hour.

The major differences between the CTL cohort compared to the NBT cohort were: 1) all transmissions in the CTL cohort were detected prior to 6 months of age, whereas in the NBT cohort, 7 of the 21 HIV+ infants had an estimated time of infection after 6 months of age; 2) sample size (77 HEUs and 27 HIV+ infants were in the CTL cohort; 51 HEUs and 21 HIV+ were infants in the NBT cohort); 3) study design (the CTL cohort utilized a case cohort design; the NBT cohort included all infants that met sample criteria).

Infant dried blood spots were tested for HIV infection by nested HIV DNA PCR (160). Infants testing positive for HIV DNA, were retrospectively tested for HIV RNA using a prototype Gen-Probe/Hologic HIV viral load assay that detects diverse subtypes from samples collected from previous timepoints (161). Estimated time of infection was defined as the first HIV positive test (156). The Kenyan Ministry of health gave permission for the Cytotoxic T Lymphocyte study to be conducted, and the Institutional Review Boards of the University of Nairobi, University of Washington, and the Fred Hutchinson Cancer Research Center approved the current study.

Rapid and fluorometric ADCC Assay (RFADCC Assay)

The rapid and fluorometric ADCC assay (RFADCC assay, (191)) was used to measure plasma and cord blood ADCC activity as described previously (54). Briefly, CEM-NkR cells (NIH AIDS Reagent Program, catalog #: 458, contributed by Dr. Peter Cresswell) were double stained with PKH26-cell membrane dye (Sigma Aldrich) and CFSE (Vybrant CFDA SE Cell Tracer Kit, Life Technologies) and coated with clade A/D BL035.W6M.ENV.C1 gp120 protein (Immune Tech) for 1 hour at RT. Cells were coated with 1.5ug gp120 per 100,000 cells. Coated target cells were washed and added at a concentration of 5000 cells/well to 96-well plates

containing 100ul of plasma or cord blood sample at a 1:5000 dilution or 100ng/ml of the monoclonal control Fi6v3 flu-specific antibody (produced by stably transfected 293F cells kindly provided by Jesse Bloom) and incubated for 15 minutes in the dark at RT to allow the plasma to bind to gp120. HIV-negative donor PBMCs (Bloodworks Northwest) were added at a 50:1 effector:target ratio to each well. The plates were incubated for 4 hours at 37 degrees C, and then fixed with 150ul of 1% paraformaldehyde in PBS (Affymetrix). 100ul of fixed cells were analyzed by flow cytometry using a Symphony flow cytometer (BD). PKH was detected in the PE channel and CFSE was detected in the FITC channel. ADCC was measured as the percent PE+, FITC- cells of total PE+ cells after subtracting out background (ADCC against uncoated target cells), which was set to 3-5% as analyzed by FlowJo (Treestar). ADCC was normalized to killing mediated by HIVIG (NIH AIDS reagent program, catalog #3957, contributed by NABI and National Heart Lung and Blood Institute (Dr. Luiz Barbosa)), which was set to 100%. Results are averaged from two biological replicates. Each biological replicate contained two technical replicates.

All cells were cultured in RPMI complete (RPMI (Gibco) supplemented with 10% FBS (Gibco), 1% PSF antibiotic-antimycotic (Life Techonologies), 1% 4.0mM Glutamax (Gibco)). All antibodies and plasma samples were diluted in RPMI complete.

Statistical Analysis

Statistical analyses were performed with (StataCorp, College Station, TX) and GraphPad Prism7 (GraphPad Software, Inc., San Diego, CA). All graphs were generated by Prism7. To determine whether ADCC activity affected risk of MTCT, ADCC of HEU vs HIV-infected (HIV+) infant plasma or cord blood was compared by a Mann-Whitney U test and logistic

regression analysis adjusted for maternal plasma viral load. The effect of passively-acquired ADCC on HIV+ infant survival was assessed by a Cox-proportional hazards model and a log-rank test comparing Kaplan-Meier curves of HIV+ infants with ADCC at/above vs. below the HIV+ infant cohort median as noted in the figure legends. Statistical significance was defined as $p<0.05$.

Results

ADCC activity measured in cord blood and neonatal plasma samples

To measure passively-acquired ADCC in the CTL cohort, we selected a cohort of 104 infants that had cord blood and/or neonatal plasma available from the first week of life. We chose this timepoint because passively-acquired maternal antibodies are at the highest levels in the infant near birth (54, 103, 104, 113) and because it was prior to detection of infection for all of the HIV+ infants. This allowed us to investigate the effect of pre-existing passively-acquired ADCC activity that is present in infants prior to breastfeeding HIV exposure and possible transmission. To be consistent with the methods from the NBT cohort, we diluted the CTL cohort samples 1:5000 for the RFADCC assay, which was the optimal dilution to achieve a dynamic range among the samples while avoiding the prozone effect in the NBT cohort, in which antibody activity decreases when antibody concentration is above a certain threshold (54, 218-220). Unlike the NBT cohort, which had a roughly equivalent distribution of plasma and cord blood samples between the HEU and HIV+ infants, there was unequal distribution of the availability of plasma and cord blood samples between the HEU and HIV+ infants in the CTL cohort (Table 5.1).

Table 5.1. Sample types available from infants in the CTL cohort.

CTL Cohort	Only Plasma Available	Only Cord Blood Available	Both Plasma and Cord Blood Available	Total Plasma Available	Total Cord Blood Available
HEU (N=77)	28	5	44	72 (93.5%)	49 (63.6%)
HIV+ (N=27)	8	12	7	15 (55.6%)	19 (70.4%)

Because this unequal distribution of sample type could confound our results and contribute to a false positive difference in ADCC activity between the HEU and HIV+ infants, we measured ADCC activity for both plasma and cord blood for the 51 infants that had both sample types available to determine whether we could analyze the data from the entire cohort (N=104 infants) together, which would maximize our statistical power, or whether we would need to stratify the analysis based on sample type (Figure 5.1). For the 51 infants that had paired plasma and cord blood samples, the cord blood samples had significantly higher ADCC than plasma samples at an equivalent plasma dilution (1:5000) (paired t test: mean difference = 15.2% ADCC, $p<0.0001$). Among these 51 infants, plasma ADCC was more evenly distributed across the dynamic range, whereas cord blood ADCC was negatively skewed (plasma skewness: -0.011, plasma kurtosis: -1.20; cord blood skewness -0.96, cord blood kurtosis: -0.052) (Figure 5.1). Since cord blood samples mediated greater ADCC than paired plasma samples, we stratified the cohort by sample type for all subsequent analyses.

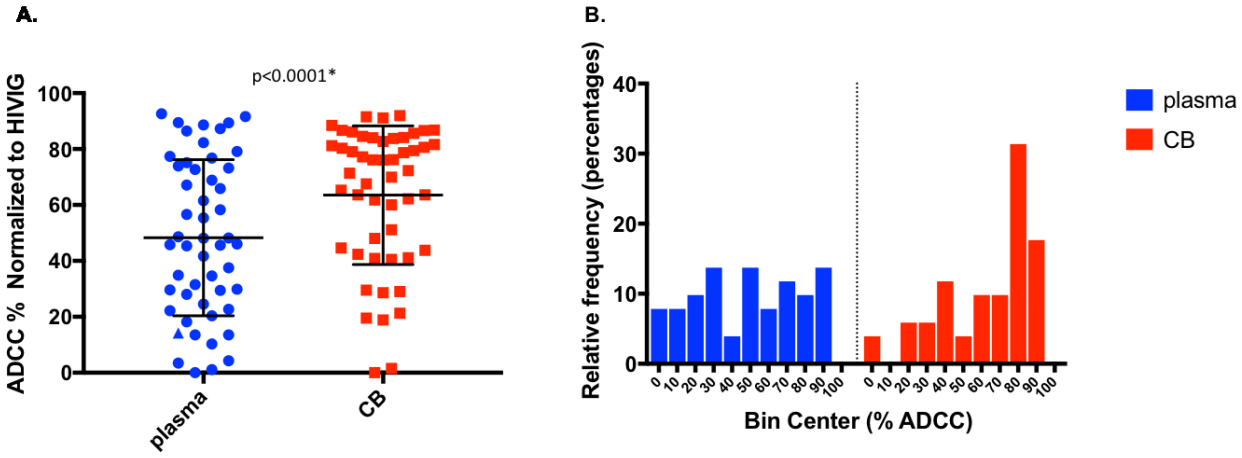


Figure 5.1. RFADCC activity in paired neonatal plasma and cord blood samples. The RFADCC assay was performed on paired neonatal plasma and cord blood samples from 51 infants in the CTL cohort. A) Percent ADCC normalized to HIVIG is shown. Plasma and cord blood ADCC were compared by a paired t test. Error bars represent mean +/-SD. Statistical significance was defined as $p<0.05$. B) Histogram of frequency distributions of plasma ADCC and cord blood ADCC from data in A. The x-axis displays % ADCC divided into bins 10% wide.

Effect of passively-acquired ADCC activity on risk of MTCT

To measure the association of passively-acquired ADCC with risk of MTCT, we measured ADCC activity from 104 infants (155 samples because as described above for 51 infants, we had both plasma and cord blood samples available) in the RFADCC assay and compared ADCC activity between 77 HEU and 27 HIV+ infants stratified by sample type (Figure 5.2). Because maternal viral load is a known risk factor for MTCT (101), we also performed a logistic regression analysis comparing passively-acquired ADCC between HEU and HIV+ infants adjusting for maternal plasma viral load (Figure 5.2). There was no association of passively-acquired ADCC with risk of MTCT by either analysis method for either sample type.

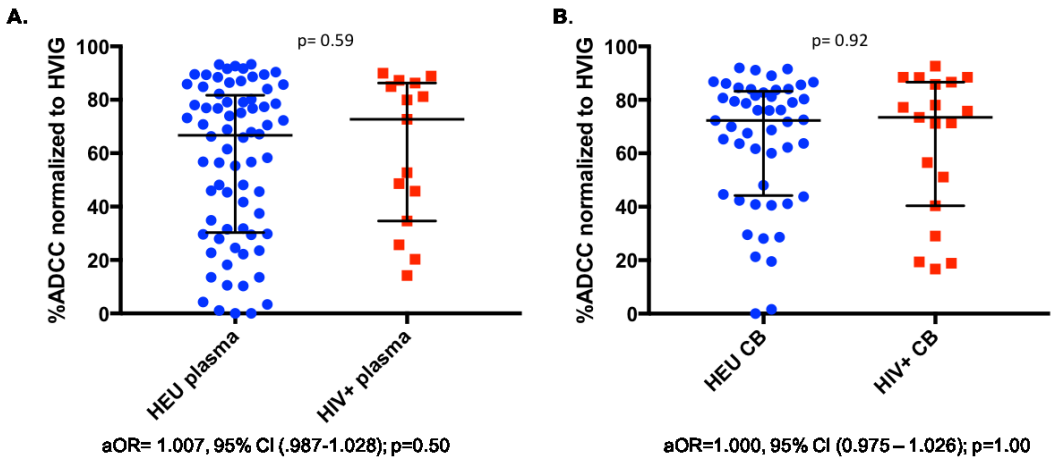


Figure 5.2. Association of RFADCC activity with MTCT in CTL cohort.
The RFADCC assay was performed on 87 neonatal plasma samples (A) and 68 cord blood samples (B) from 104 infants (77 HEU; 27 HIV+). Percent ADCC normalized to HIVIG is shown. ADCC between HEU and HIV+ infants was compared by a Mann-Whitney U test. Error bars represent median +IQR. Statistical significance was defined as $p<0.05$. The association of ADCC with odds of MTCT was measured by logistic regression adjusted for maternal viral load. Adjusted odds ratios (aOR), 95% confidence intervals (95% CI) and p-values are shown below the graphs.

Effect of overall ADCC on infant survival in this cohort

To measure the association of passively-acquired ADCC on infant survival in this cohort, we performed the same two types of survival analyses described previously for the NBT cohort (Figure 5.3) (Chapters III and IV, (54)). Briefly, we measured the association of passively-acquired ADCC with mortality risk for the infected infants (N=27) with a Cox-proportional Hazards model, in which ADCC activity is a continuous variable to determine whether or not there is change in mortality risk for each percentage increase of ADCC activity. We also determined if there was an association of high or low levels of passively-acquired ADCC with infected infant survival times by comparing Kaplan-Meier curves of infected infants with ADCC at/above the infected cohort median or below the cohort median by a log-rank test. Interestingly, passively-acquired ADCC in plasma was non-significantly associated with improved infected

infant survival time in the log-rank test (X^2= 2.26, p=0.13; N=15, 7 deaths), and showed a trend towards reduced mortality risk in the Cox-proportional hazards model (HR=0.976, p=0.09) (Figure 5.3A). The results with cord blood ADCC were strikingly different. Cord blood ADCC was associated with a trend toward reduced infected infant survival in the log-rank test (X^2 = 2.65, p=0.10; N=19, 8 deaths) and was not associated with infected infant mortality risk in the Cox-proportional Hazards model (HR=1.018, p=0.285) (Figure 5.3B).

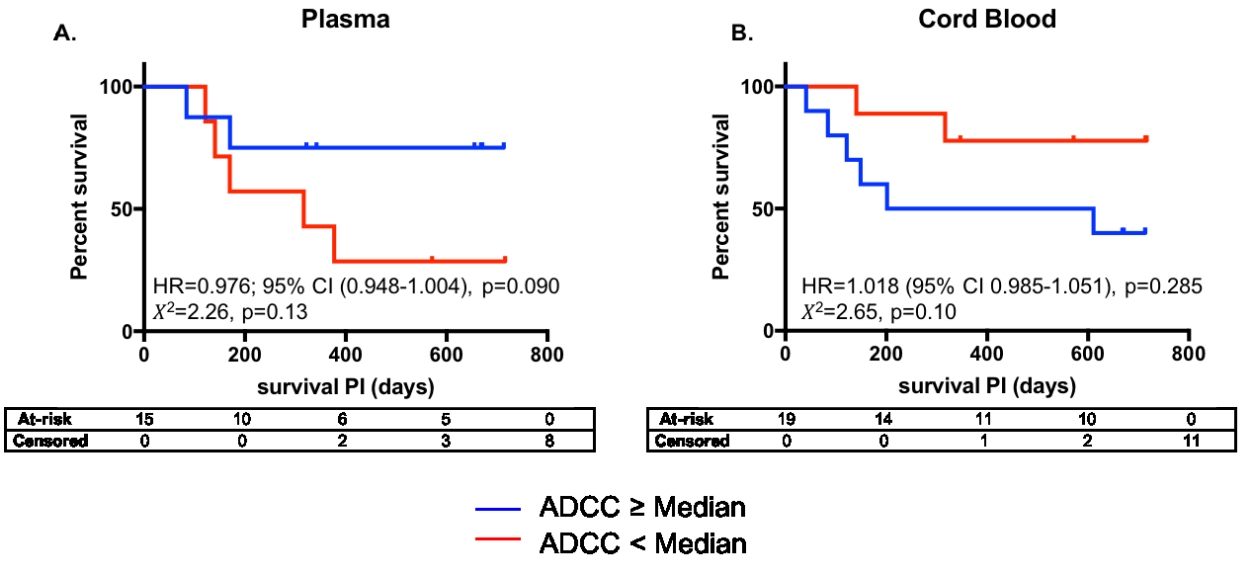


Figure 5.3. Association of RFADCC activity with HIV-infected infant survival in CTL cohort.
Kaplan-Meier survival curves between infants who had passively-acquired ADCC at/above the HIV+ infant cohort median (blue line) or below the HIV+ infant cohort median (red line) were compared by a log-rank test. X^2 values and p-values are shown. The x-axis shows days survival post infection (PI). The association of passively-acquired ADCC with risk of HIV+ infant mortality was measured in a Cox-proportional hazards model. Hazard ratios (HR), 95% confidence intervals (CI), and p-values are shown below the graphs. Statistical significance was defined as $p<0.05$ (*). Cumulative number of infants at-risk or censored by the end of each day on the x-axis is shown in the tables. Results from plasma samples are shown in A. Results from cord blood samples are shown in B.

Discussion

ADCC has been shown to correlate with reduced risk of infection, reduced disease progression, and improved clinical outcome in MTCT, chronic infection, and vaccination (52-67, 93, 194). In the NBT cohort, our lab previously showed that passively-acquired plasma ADCC in infants is associated with improved infected infant survival and a trend towards reduced risk of MTCT (54). We were particularly intrigued by the associations of passively-acquired ADCC in infants with improved infant outcome because the passively-acquired ADCC-mediating antibodies are present in infant circulation prior to breastfeeding exposure, and this correlate of improved outcome could provide valuable information for the use of antibodies in prevention of MTCT as well as prevention of horizontal transmission and vaccine design. However, the NBT was limited by low statistical power and included 7 transmissions which were estimated to have occurred after 6 months of age, by which time passively-acquired ADCC activity has waned from infant circulation and thus may have limited relevance to transmission risk at these later times (54, 103). Here, we aimed to replicate the study conducted by Milligan et al in a larger, more statistically powerful cohort where all transmissions were detected prior to 6 months of age. We selected a cohort from the CTL study that included 104 (77 HEU and 27 HIV+) infants compared to 72 (51 HEU and 21 HIV+) infants in the NBT cohort.

Because there was an unequal distribution of sample types between HEU and HIV+ infants in the CTL cohort, we directly compared ADCC from paired plasma and cord blood samples for 51 infants who had such samples available within our cohort. We found that cord blood samples mediated higher levels of ADCC for a given sample dilution compared to paired neonatal plasma. This was surprising as we had expected there to be no difference in the level of ADCC activity between cord blood and neonatal plasma, as we and others have used cord blood

and plasma samples interchangeably (54, 103, 152). This was even more unexpected because in the NBT cohort, plasma and cord blood mediated roughly equivalent levels of ADCC (data not shown). Additionally, cord blood is regularly used to draw labs to determine health status in newborns. For instance, two studies that directly compared blood composition between neonatal peripheral blood and cord blood found strong correlations between cord blood and neonatal peripheral blood and determined that cord blood could be an accurate substitute for peripheral blood (221, 222). In contrast, a recent study reported differences in immune composition between cord blood and neonatal plasma. This study showed differences in infant immune cell composition and plasma proteins between cord blood and neonatal plasma collected at birth and 1 week post-birth (223). Additionally, a number of studies have shown that cord blood can become contaminated by maternal blood, specifically by maternal blood cells (224, 225). Perhaps maternal blood contamination is contributing to the differences in cord blood compared to neonatal plasma in the CTL cohort. Taken together, these data suggest that further comparisons between cord blood and neonatal plasma may be warranted, and that these sample types should not be assumed to be biologically equivalent.

Because cord blood ADCC was significantly higher than paired plasma ADCC for this cohort, we stratified our results by sample type to avoid confounding the data by this sample difference. There was no effect of either plasma or cord blood passively-acquired ADCC with risk of MTCT, consistent with the lack of significant association with risk of infant infection in our prior NBT plasma ADCC study, as well as a number of other studies that also did not find an association of plasma ADCC with MTCT risk (54-57, 119, 137, 138, 152, 153). Taken together, these data suggest that ADCC alone is not a major correlate of reduced risk of MTCT. But a number of these studies did find plasma ADCC to be associated with a trends or non-significant

associations towards reduced risk of MTCT suggesting that ADCC may provide partial protection (plasma data in this chapter, (54, 55, 138)). Additionally, polyfunctional antibody responses consisting of multiple antibody effector functions, including ADCC, have been shown to coordinate and are associated with elite controller status (73).

Our previous NBT study showed that pre-existing passively-acquired ADCC in infants was associated with improved infected infant survival (54). We replicated the methods and survival analysis performed by Milligan et al for the CTL cohort in the present study (54). Consistent with the previous NBT cohort study, plasma ADCC was associated with improved infected infant survival in both types of survival analyses, although these associations did not reach statistical significance. However, cord blood did not show the same association, and actually showed a trend in the opposite direction in the log-rank analysis. Because of the conflicting results between sample types, we were unable to draw conclusions about the effect of passively-acquired ADCC on HIV-infected infant survival in the CTL cohort.

This study has a number of limitations. Due to the difference in cord blood and plasma ADCC described above, the sample strata resulted in lower statistical power for each sample type than intended for the entire cohort when we designed the study (each strata had approximately 15% less power than the entire cohort to detect a mean difference in ADCC activity of 11%, which was the mean difference reported in the NBT cohort (54)). Other caveats to this study are that we used the RFADCC assay with coated target cells, as discussed in Chapter III.

One notable limitation of the cord blood sample data was that cord blood ADCC was negatively skewed, with the majority of the cord blood samples mediating ADCC levels at the high end of the dynamic range, whereas plasma ADCC activity was well distributed. This

suggests that the 1:5000 dilution at which all samples were run, is optimal for plasma, but not for cord blood in the CTL cohort. Because many of the cord blood samples had ADCC activity at the high end of the dynamic range, it is possible that some of the cord blood samples were in the "prozone effect". The prozone effect is a common effect in a variety of immune assays including ELISAs and ADCC assays in which the measured activity increases with increasing antibody concentration, peaks, and then the measured activity decreases with further increases in antibody concentration (219, 226-228). The mechanism of the prozone effect is hypothesized to be caused by excess non-antigen-bound antibody competing for Fc receptor binding or the antibody concentration being so high that antigen-specific antibodies are only able to bind the antigen monovalently, rather than bivalently which is necessary to cross-link Fc receptors and trigger their signaling cascade (218, 220, 229). If some of the cord blood samples are indeed undergoing the prozone effect, then they would have artifactually low measured ADCC activity, which would explain why so many samples had ADCC at the high end of the dynamic range, and why the distribution of samples was different for cord blood and plasma. The cord blood samples with ADCC activity clustered at the high end of the dynamic range would be a combination of samples at their maximum activity and samples in the prozone area, which really have higher ADCC activity than what was measured. The paired cord blood and plasma samples from the CTL cohort will be run at multiple dilutions to find the optimal dilutions that maximize the dynamic range with an even distribution of samples while avoiding the prozone effect, but these pilot experiments have not been conducted at the time of this writing. It is highly likely that the cord blood and plasma samples will have different optimal dilutions, as suggested above.

If a number of the cord blood samples are in the prozone effect, it is possible that the when the dilution is optimized, the cord blood samples will also show an association with

improved infant outcome. However, until the dilutions are optimized, conclusions about the effect of passively-acquired ADCC on infant outcome in the CTL cohort reported in this chapter cannot be drawn. Additionally, the surprising difference in ADCC between cord blood and plasma samples warrants further investigation into the mechanism causing this difference. If cord blood is not representative of neonatal plasma for humoral immunity, future infant studies should take this into consideration when designing their study criteria.

Chapter VI

Conclusions and Future Directions

MTCT is a unique natural setting in which the infant has pre-existing HIV-specific antibodies in their circulation prior to breastfeeding HIV exposure, the timing and route of infection can be accurately determined, and the "source partner" is known, all of which are factors that are either not possible (pre-existing antibodies) or often unknown (infection timing and route, source partner) in the case of horizontal transmission. Therefore, MTCT is an ideal setting from which we can investigate correlates of reduced risk of transmission and improved disease progression. In this thesis, the aim was to identify humoral correlates of reduced risk of transmission or improved infant outcome in the setting of breastfeeding MTCT.

In this thesis we did not find any correlates of reduced risk of transmission (Figure 6.1).

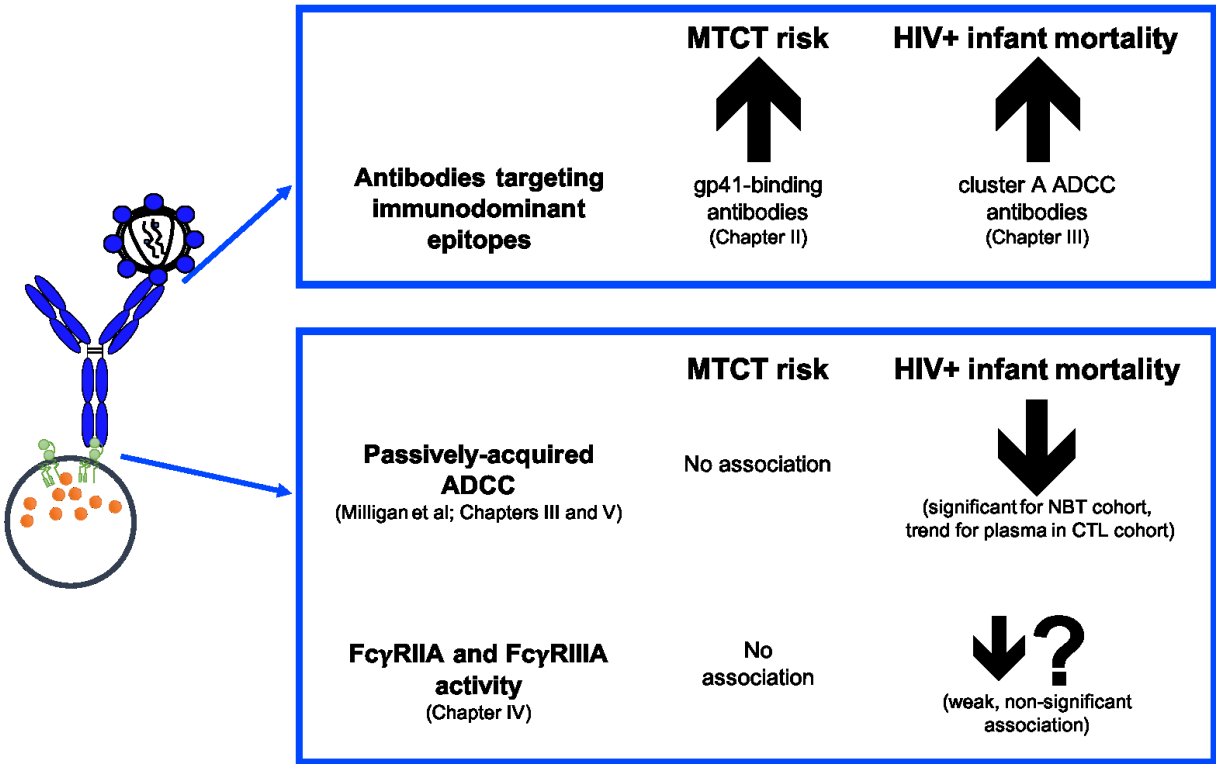


Figure 6.1. Summary schematic of thesis findings.
In this thesis, we found that both the antibody epitope, driven by the Fab and Fc-mediated effector functions are important in MTCT of HIV. Antibodies targeting immunodominant epitopes including the gp41 ectodomain (Chapter II) and cluster A on gp120 (Chapter III) were associated with worse infant outcome (top). Conversely, passively-acquired ADCC was associated with improved infant outcome, which may be mediated at least in part by FcγRs IIa and IIIa (bottom).

In Chapter II, we found that IgG binding to the gp41 ectodomain is a correlate of increased risk of MTCT (Figure 6.1, top). Similarly, in Chapter III, we found that ADCC-mediating antibodies targeting cluster A CD4i epitopes are associated with worse infected infant survival (Figure 6.1, top). In Chapters IV and V, we explored Fc effector functions in two separate cohorts and had some tantalizing results that require more exploration. In Chapter IV, we measured the effect of potential Fc effector activity, as measured by plasma binding to soluble dimeric FcγRs, on infant outcome. Plasma dimeric FcγR binding was not associated with risk of MTCT but was associated with a weak trend towards improved infected infant survival (Figure 6.1, bottom). In Chapter V, the results were difficult to interpret due to the surprising difference in ADCC activity between plasma and cord blood, but plasma ADCC was associated with a trend toward improved infected infant survival (Figure 6.1, bottom). The following chapter will discuss future directions and implications for HIV prevention.

Fine epitope mapping of antibodies in NBT cohort

In Chapter II, plasma IgG binding to the gp41 ectodomain was associated with increased odds of MTCT; however, this study was not designed to finely map the epitope(s) driving this association. The Overbaugh lab has used phage immunoprecipitation sequencing (Phip-seq) to map epitopes from monoclonal antibodies and recently adapted this method to map epitopes from polyclonal plasma samples (164). We plan to run plasma samples from the NBT cohort through our established Phip-seq protocol to more finely and comprehensively map epitopes of binding antibodies associated with decreased or increased risk of MTCT. Using this technique, we may be able to identify the exact epitope driving the association with increased odds of

MTCT, and if so, this information would be useful to guide future vaccine design away from this epitope.

Similarly, in Chapter III we did not observe any epitopes of ADCC-mediating antibodies that were associated with reduced risk of MTCT. Instead, ADCC-mediating antibodies targeting the cluster A CD4-inducible epitopes were associated with reduced infected infant survival. This study only measured ADCC activity targeting three epitopes, but ADCC activity was only detected against two epitopes. Thus, the majority of ADCC measured in the NBT cohort is targeting as yet unidentified epitopes. To more comprehensively identify epitopes of ADCC-mediating antibodies that may correlate with reduced risk of MTCT, we will use a B cell sorting approach similar to the approach we have used previously (41, 230). We have identified three potential non-transmitting candidate mothers from the NBT cohort who have: 1) high total ADCC activity as measured by the RFADCC assay; 2) high viral loads, and thus are at high risk to transmit HIV to their infants; 3) low A32-like ADCC and low C11-like ADCC. We will sort and culture all memory B cells from a PBMC sample from these women, screen the secreted antibodies for ADCC activity, and map the epitopes of the monoclonal ADCC-mediating antibodies. Because these candidates are high-risk women who did not transmit HIV to their infants, they may have protective ADCC-mediating antibodies in their repertoire, and because they have low cluster A-specific ADCC, they may have ADCC targeting a novel epitope.

Antibodies targeting immunodominant epitopes are associated with worse infant outcome

Multiple studies have shown that antibodies targeting immunodominant epitopes do not correlate with improved outcome, have been associated with worse outcome, and have been associated with antibody-dependent enhancement in some studies (60, 126, 127, 231, 232). Our results from Chapters II and III are consistent with this association. Plasma IgG binding to antigens containing the immunodominant region of gp41 were the most strongly associated with increased odds of MTCT. The cluster A CD4-inducible epitope region, especially the A32 epitope, is immunodominant as well (100, 173, 183, 185), and was also associated with worse outcome in this study. Many studies have investigated cluster A ADCC-mediating antibodies (37, 41, 43, 44, 100, 173-186), but this is the first study to our knowledge in humans to directly assess whether cluster A ADCC activity is associated with improved outcome. A number of studies have provided evidence to explain why cluster A ADCC-mediating antibodies may not be beneficial including: 1) cluster A ADCC-mediating antibodies do not mediate strong ADCC against wildtype virus-infected cells (37, 40, 174-177, 180, 181, 186, 199); and 2) cluster A ADCC-mediating antibodies primarily mediate ADCC against shed gp120 on uninfected bystander cells as measured by traditional ADCC assays (176, 177, 206). Taken together, these data provide further evidence that in the context of HIV infection, antibody responses targeting immunodominant epitopes in general are not beneficial and should be avoided in vaccine design. Studies with immunogens with immunodominant epitopes occluded, such as trimers, are underway (233).

Polyclonal and Polyfunctional Antibody Responses

Interestingly, ADCC responses from the NBT cohort with the most dominant A32-like activity, actually had weak total ADCC activity. One possible explanation for this phenomenon is that the strongest ADCC activity is actually polyclonal in nature. Additionally, there were strong correlations between dimeric FcγR binding between different FcγRs and RFADCC activity suggesting that these plasma antibodies may be able to coordinate polyfunctional responses. Polyfunctional responses and synergy between antibodies has started to become more appreciated and have been correlated with protection and improved outcome in both NHP and humans (73, 85, 184, 215, 216). Polyclonal responses have been largely understudied due to their complexity. Further studies exploring the polyfunctional nature and polyclonal nature of HIV antibodies may help identify beneficial and synergistic relationships which can be exploited in antibody-based therapies or vaccine design. Keeping this in mind, we have provided samples from this cohort to our collaborators Dr. Galit Alter and Dr. Andrez Finzi to measure effector function among the NBT cohort in infectious cell-based ADCC assays, phagocytosis assays, Fc binding assays detecting binding to other FcRs including FcγRI (activating receptor on monocytes/macrophages and other cells that can mediate a number of functions including phagocytosis) and FcγRIIb (an inhibitory receptor) (12, 13, 15). This comprehensive investigation of effector function in this cohort will allow us to gain a better understanding of the metric measured by the RFADCC assay and of the combination of effector functions in general that are important to MTCT risk and infant outcome in this unique breastfeeding cohort. This information will add to the increasing body of knowledge on the role of effector functions may play in HIV infection and disease progression, and the potential they have to be exploited as prevention and treatment strategies.

ADCC and Fc effector function

The data presented here are largely consistent with previous ADCC studies in the setting of natural infection (52-64, 66, 67, 74, 119, 137, 138, 152, 153, 194). Although there are caveats to consider described earlier, in Chapters IV and V, the results described here generally indicate that ADCC and Fc effector function is associated with improved disease progression (as indicated by improved infected infant survival), but not with risk of infection, consistent with the following studies (52, 54-64, 66, 67, 119, 137, 138, 152, 153). Passive vaccination studies also support these data. Many NHP studies show that passive transfer of non-neutralizing antibodies can reduce post-infection viremia and transmitted/founder variants, and that Fc-effector functions of neutralizing antibodies contribute to their protective effects (reviewed by Lewis et al, (77)), but passively-transferred non-neutralizing antibodies cannot protect from infection on their own. ADCC-mediating antibodies elicited through active vaccination have been shown to be correlated with protection from infection (83-85, 92, 93), but often only when certain other immune situations are true, such as low circulating IgA for the RV144 vaccine or low vaccine-elicited T cell responses (83, 93). These data combined with data from Milligan et al and other MTCT studies, vaccination studies, and EC cohorts all show the same general pattern: ADCC and Fc effector activity can reduce viral load and disease progression but need to coordinate with other immune responses to effectively prevent infection (52, 54-64, 66, 67, 73, 83, 85, 93). ADCC-mediating antibodies used as part of a polyfunctional antibody-based therapy and vaccine design aiming to elicit a combination of ADCC-mediating and neutralizing antibodies may prove to be effective.

Conclusion

By investigating multiple aspects of the humoral responses of the same cohort, we were able to gain a more complete picture of the types of antibody responses desired in a vaccine or antibody-based therapy. The correlative studies presented in this thesis have shown the importance of antibody specificity as well as effector function on risk of transmission and clinical outcome. Both aspects of the antibody must work in tandem to provide the strongest effect on outcome.

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VITA

Nicole Naiman was born and raised near Cleveland, Ohio. She majored in Biomedical Science at The Ohio State University,where she received her B.S. During college, Nicole became interested in research and worked in Dr. Stephen Kolb's lab where she studied motor neuron diseases. After college, Nicole was a post-baccalaureate fellow at the National Institutes of Health. There, she worked in Dr. Robert Yarchoan's lab and studied HIV protease inhibitors and Kaposis Sarcoma-Associated Herpesvirus. Nicole then joined the Medical Scientist Training Program and Molecular and Cellular Biology Program at the University of Washington. She rotated in Dr. Michael Lagunoff's lab and Dr. Julie Overbaugh's lab. Nicole joined Dr. Julie Overbaugh's lab and studied HIV-specific antibody responses in the context of mother-to-child transmission of HIV. After completing her PhD, Nicole plans to return to medical school and eventually become a physician-scientist with a focus on virology and infectious disease.