Humoral Immune Responses in HIV Mother-to-Child Transmission and Disease

Caitlin Milligan

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Reading Committee:
Julie Overbaugh, Chair
Grace John-Stewart
Barbra Richardson

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Abstract

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Caitlin Milligan

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

Although significant progress has been made in combating HIV/AIDS, a safe and effective vaccine is still of high priority. Considering that data from HIV vaccine trials are limited, information from other settings can help elucidate what types of immune responses provide protection. Mother-to-child transmission (MTCT) provides one setting in which to study the immune correlates of protection and is unique because infants born to HIV-infected women have HIV-specific antibodies at birth, which may provide protection. This thesis addresses the impact of humoral immune responses on MTCT, with particular focus on antibody-dependent cellular cytotoxicity (ADCC) and neutralizing antibodies (NAbs), which have both been described as potential immune correlates of protection.

First, we investigated the role of pre-existing HIV-specific ADCC in infants. We observed that ADCC activity was higher in uninfected infants than infected infants, but the difference was not statistically significant. In infected infants, however, higher pre-existing ADCC activity was associated with decreased mortality, suggesting a therapeutic benefit of ADCC antibodies.

As ADCC depends on Fc gamma receptors (FcγRs) on host effector cells, we next examined the influence of FcγR polymorphisms on MTCT. We observed no association

between infant or maternal FcγRIIa, or infant FcγRIIIa, polymorphisms and MTCT. Unexpectedly, maternal FcγRIIIa heterozygotes were at increased risk of transmission compared to homozygote mothers. This effect was predominately in the early breastfeeding period, but additional studies need to be done to confirm this association and its mechanism.

Finally we examined maternal autologous NAbs and their impact on MTCT. We observed that non-transmitting and transmitting mothers had similar levels of NAbs when tested against individual viruses. Similarly, there was not a significant difference in the number of neutralization resistant viruses in the two groups. These results suggest that maternal autologous NAbs do not significantly contribute to transmission risk during the early breastfeeding period.

Overall, these results highlight the roles of humoral immune responses in MTCT and suggest a potential protective influence of ADCC-mediating antibodies in HIV transmission and disease. Future studies should focus on characterizing ADCC antibody epitopes and determining how to best elicit ADCC responses by vaccination.

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ADCC: Antibody-Dependent Cellular Cytotoxicity

ADCVI: Antibody-Dependent Cell-Mediated Viral Inhibition

AIDS: Acquired Immunodeficiency Syndrome

ART: Antiretroviral Therapy

CRF: Circulating Recombinant Form

ELISA: Enzyme-Linked Immunosorbent Assay

Env: Envelope

EPT: End Point Titer

FcγR: Fc gamma Receptor

HIV: Human Immunodeficiency Virus

HWE: Hardy-Weinberg Equilibrium

IC50: Inhibitory Concentration (dilution) at which 50% of the input virus is neutralized

IgG: Immunoglobulin G

MFI: Median Fluorescence Intensity

MTCT: Mother-to-Child Transmission

NAb: Neutralizing Antibody

NK Cell: Natural Killer Cell

PBMC: Peripheral Blood Mononuclear Cell

RFADCC: Rapid Fluorometric Antibody-Dependent Cellular Cytotoxicity

SIV: Simian Immunodeficiency Virus

SNP: Single Nucleotide Polymorphism

VL: viral load

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Chapter I

Introduction

HIV Epidemiology

Since the discovery of human immunodeficiency virus (HIV) in 1983, there has been progress in combating the HIV/AIDS epidemic. Increasing knowledge of the virus, how it is transmitted, and how to treat the disease have resulted in declines in the number of new infections and rates of death due to AIDS and AIDS-related causes. In fact, since 2001 there has been a 38% decline in the total number of new infections, and in children there has been a 58% reduction (1). This decrease in pediatric infections is in large part due to increasing access to prenatal care and antiretrovirals for prevention of mother-to-child transmission (MTCT).

While these improvements are impressive, there are many barriers in eliminating the virus. This challenge becomes evident when considering the HIV care continuum in the United States. In the U.S., it is estimated that 82% of HIV-infected individuals have been diagnosed, but only 37% are retained in care and only 25% achieve viral suppression (2) (**Figure 1.1**). Viral suppression is a major goal of HIV treatment as it slows disease progression in the infected individual (3,4) and decreases the likelihood of transmitting the virus (5,6). These gaps in diagnosis, care, and viral suppression are even more profound in low-income areas that harbor the majority of infections. For example, in Sub-Saharan Africa, only 45% of people living with HIV know their status and globally only 24% of HIV-positive children are receiving treatment (1). Overall, it is estimated that there are 35 million people currently living HIV (7) and in 2013, 2.1 million people were newly infected, including 240,000 children (1). These numbers highlight the challenges in achieving viral suppression and eliminating virus transmission. Thus, development of an effective vaccine that prevents HIV-infection is essential for eliminating the

virus and bypassing the care continuum problems that continue to contribute to the spread of HIV.

Figure 1.1

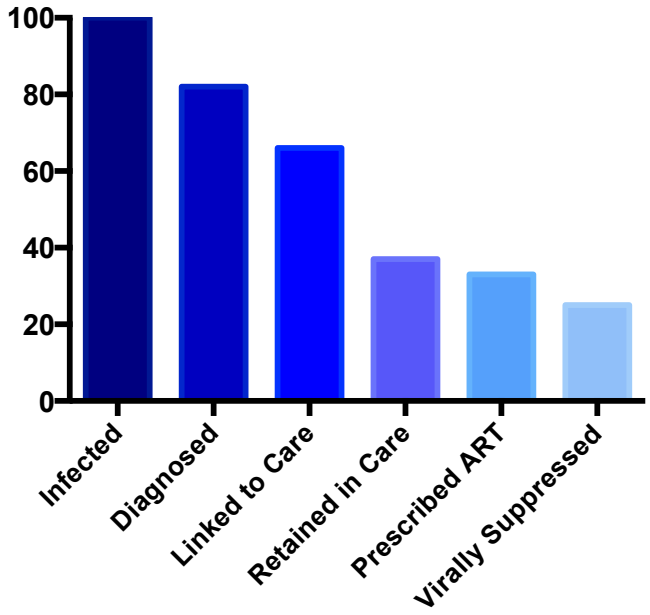


Figure 1.1 HIV Care Cascade in the United States
Figure adapted from (2). Percentage of HIV-infected individuals in the United States who have been diagnosed, linked to care, retained in care, prescribed antiretroviral therapy (ART), and virally suppressed.

HIV Diversity

One challenge to vaccine development is the great diversity of circulating HIV variants. The extent of this diversity is highlighted by comparing HIV to influenza, which we vaccinate against annually: It is estimated that the diversity of HIV sequences found within a single infected person at one point in time is roughly equivalent to the entire diversity of influenza sequences worldwide in a given year (8). This diversity in HIV arises mainly through the error-prone reverse transcription process and high rates of viral replication.

Reverse transcription is a hallmark of the *Retroviridae* family to which HIV belongs. During this process, DNA is generated from the single-stranded HIV RNA genome by reverse

transcriptase. Reverse transcriptase is a low-fidelity enzyme that lacks proofreading capability, resulting in an estimated 0.2-2 mutations per genome per cycle (9). In addition to this baseline mutation rate, reverse transcription can result in recombination events. Template switching between the two HIV RNA genomes that are packaged into each virion can occur resulting in additional mutations due to recombination (10).

High rates of viral replication and large numbers of infected individuals also contribute to HIV's total diversity. In total, it is estimated that over 78 million people have been infected by HIV (1) and within each individual, over a billion viruses are estimated to be made per day (11). Therefore, in order to prevent new HIV infections, an effective HIV vaccine must be able to target the sum of this large and diverse circulating virus population.

The extent of diversity that must be targeted by a vaccine is encapsulated by that observed in the HIV envelope, one of nine proteins encoded by the HIV genome. The major HIV pandemic, known as HIV-1 group M, is divided into multiple subtypes or clades based on envelope sequence. Circulating today are subtypes A,B,C,D,F,G,H,J,K and multiple circulating recombinant forms (CRFs). Within a given subtype, amino acid diversity varies by as much as 8-17%, while cross-clade variation is usually in the range of 17-35% (8).

HIV Envelope

As envelope is the major target of humoral immune responses elicited in vaccinated and naturally infected individuals (discussed below), the protein requires a more thorough discussion of its structure and function. The *env* gene encodes a gp160 protein that is translated in the endoplasmic reticulum and then cleaved by the host protease furin in the Golgi complex. This cleavage event splits the protein into the surface subunit gp120 and the transmembrane domain

gp41. Three gp120s and three gp41s combine as heterodimers to form the trimer found on the surface of virions (**Figure 1.2**). These trimers are responsible for binding to host cell receptors (CD4 and CCR5 or CXCR4) to initiate infection. Thus, the envelope protein is essential for infection and any host immune response or therapy that targets the protein has the potential to prevent infection.

Targeting the envelope protein, however, is not an easy feat. In addition to the genetic diversity of envelope discussed above, there is a low density of envelope trimers on the surface of virions (12,13), and the trimers are shielded by host glycans. These glycans (predominantly N-linked oligosaccharides) are added to the envelope protein by host machinery as translation occurs in the endoplasmic reticulum. Impressively, glycans can contribute up to 50% of the molecular weight of envelope (14), and they are largely unrecognizable by the host immune system, serving as a form of immune evasion.

Figure 1.2

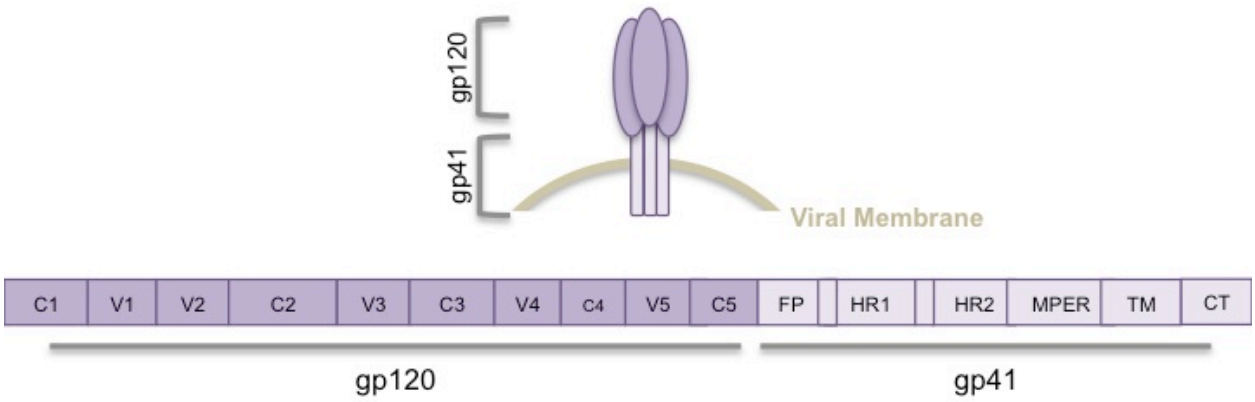


Figure 1.2 HIV Envelope
The HIV envelope consists of a trimer of gp120-gp41 heterodimers. gp120 (dark purple) is made up of five variable regions (V1-V5) and five conserved regions (C1-C5). gp41 (light purple) consists of the fusion peptide (FP), heptad repeats (HR1 & HR2), membrane proximal region (MPER), transmembrane domain (TM), and cytoplasmic tail (CT). Figure adapted from (15).

Antibodies and Vaccines

Envelope is an important HIV vaccine target as the protein is exposed on the surface of virions and is critical for infection. Generation of an envelope-specific antibody response is one major goal of HIV vaccine research, given that the majority of licensed vaccines against other viruses elicit antibodies that target a specific pathogen to prevent infection (16).

Antibodies, also known as immunoglobulins (Ig), are the major mediators of pathogen-specific humoral immunity. Their structure consists of two identical heavy chains and two identical light chains (**Figure 1.3**). These chains are connected by disulfide bonds, creating a Y-shaped molecule. The arms of the Y form the Fab (fragment, antigen binding) region, while the stalk is known as the Fc (fragment, crystallizable) region. The Fab region, as its name implies, recognizes foreign objects and binds to specific antigens. The Fc fragment, on the other hand, determines the antibody's class and effector function. There are five main classes (isotypes) of

antibodies: IgA, IgD, IgE, IgG, and IgM. IgG is the most abundant antibody in blood, and is the main focus of the work presented in this thesis.

Antibodies are made and secreted by B cells. The process of B cell maturation and antibody generation results in molecules that can respond to an incredibly diverse range of antigens. This diversity is created through affinity maturation processes including V(D)J recombination and somatic hypermutation. Isotype or class switching, which changes the constant portion of the antibody, also contributes to diversity in effector functions.

Antibodies can have many functions. The majority of viral vaccines work, in part, by neutralizing the pathogen (16). These neutralizing antibodies (NAbs) bind to viruses via the antigen-specific Fab region to prevent the pathogen from infecting host cells. However, antibodies that bind antigen can also act through their constant (Fc) portion by binding to Fc gamma receptors (FcγRs) found on the surface of host effector cells, such as natural killer (NK) cells, macrophages, and monocytes. Cross-linking of antigens and host FcγRs by antibodies triggers functions such as antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis to kill infected cells or prevent infection from spreading. These effector functions in relation to HIV infection will be discussed further below.

Figure 1.3

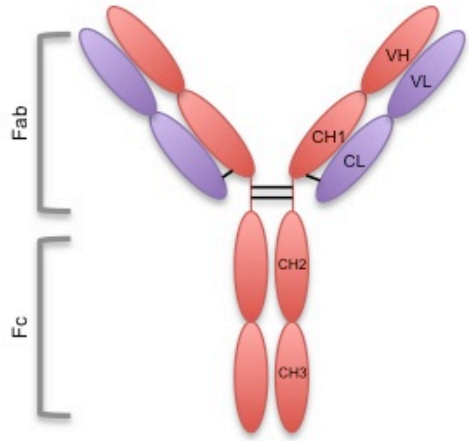


Figure 1.3 Antibody Structure
Structure of an IgG molecule. Each IgG is made up of two identical light chains (purple) and two heavy chains (red). The light chain is made up of a variable (VL) and constant (CL) region. The larger heavy chain consists of one variable region (VH) and three constant regions (CH1, CH2, CH3). Chains are covalently linked by disulfide bonds (black lines). The protein can be digested by the protease papain to produce two Fab (fragment, antigen binding) and one Fc (fragment, crystallizable) fragments.

HIV Vaccines

Unfortunately, generating an effective antibody response by an HIV vaccine has not been an easy task. To date, only one HIV vaccine trial has demonstrated any efficacy in humans. The RV144 vaccine trial was conducted in Thailand from 2003-2009 and consisted of a prime-boost vaccination regimen (17). In this regimen, individuals were first vaccinated with a recombinant canarypox vector (ALVAC-HIV) containing genes for envelope and other HIV proteins (*gag*, *pol*, and *nef*). Subsequently, individuals were boosted with injections of recombinant envelope gp120 (AIDSVAX B/E). This strategy aimed to induce both cellular and humoral immune responses. However, in hypothesis-generating analyses to determine the immune correlates of protection in RV144, neutralizing antibody titers did not correlate with protection from infection (18). The authors found, instead, that non-neutralizing antibodies that bound variable regions of envelope (V1/V2) were associated with protection, and HIV-specific IgA responses correlated

directly with the rate of infection (18). After primary analyses, the authors then did further exploratory analyses to better understand the observed protective effect. In these secondary analyses, the authors observed that IgG ADCC responses correlated with protection in vaccinated individuals with low plasma IgA.

Although RV144 was the first human vaccine trial to show some protection, it only had about 31% efficacy and this protection declined over time (17). Additionally, other major vaccine efficacy trials have not demonstrated any efficacy (Vax003 (19), Vax004 (20), HVTN 505 (21)) or have shown increased risk of acquisition in vaccinated individuals (Step Study (22)). Due to this limited success of HIV vaccine trials and because HIV-positive individuals do not clear the virus on their own during natural infection, we still do not understand what an optimal protective antibody response in the context of HIV looks like. Thus, garnering clues from other settings is important to help inform us about humoral immune correlates of protection for rational HIV vaccine design.

HIV Mother-to-Child Transmission

HIV mother-to-child transmission provides one setting in which to study humoral immune correlates of protection. While *in utero*, IgG is passively transferred from the mother to the fetus across the placenta. Thus, infants born to HIV-infected mothers have HIV-specific antibodies present in circulation at birth that may provide protection during virus exposure. This situation is similar to that of a vaccine, whereby HIV-specific antibodies are present prior to and at the time of virus exposure. Therefore, studying HIV-specific antibodies in infants who become infected and those who remain uninfected despite virus exposure may be useful in defining

immune responses important for preventing infection. This thesis focuses on antibody responses in MTCT, and thus a more thorough background of MTCT is warranted.

In the absence of any intervention, approximately 30-45% of infants born to HIV-infected mothers become infected (Reviewed in (23)). With antiretrovirals and other interventions such as alternate feeding and delivery mechanisms, the number of infants diagnosed with HIV has decreased. In 2013, there were 240,000 new infant diagnoses and the percentage of HIV-exposed infants that become infected is now around 15% (1).

Infant infection may occur while the infant is *in utero*, during delivery, or via breastfeeding. The majority of these infections occur across mucosal surfaces as infant oral, gastrointestinal, and respiratory mucosa are in contact with infected maternal fluids (cervicovaginal secretions, blood, and breast milk) during gestation, delivery, and early childhood. Breastfeeding infections account for approximately 40% of all infant infections (23,24).

There are many known risk factors for mother to child transmission, the majority of which center around exposure to maternal virus. Maternal plasma viral load is one of the strongest predictors of transmission; women with high viral loads are more likely to transmit the virus to their infants (Reviewed in (23)). Thus, antiretrovirals that reduce maternal viral load help prevent infant infections. Obstetric factors, such as prolonged labor and membrane rupture, which increase the infant's exposure to HIV-infected fluids, also are associated with risk of MTCT. This idea is supported by twin studies - the first twin, which often spends longer in the birth canal, is more likely to be infected than the second twin (25). Based on this risk factor, U.S. recommendations include Cesarean sections (C-sections) for women with viral loads ≥1000 copies/mL to reduce infant exposure to infected fluids in the birth canal (26). Additionally,

although breast milk provides important nutrients and protective factors such as antibodies, breastfeeding is associated with increased risk of infant infection (24). Thus, in places where there is access to clean water and formula, alternate feeding mechanisms are often recommended (27). However, in settings where access to safe formula is not the case, breastfeeding remains the norm.

As infection does not occur in every infant born to an HIV-infected mother despite ample virus exposure, and because infants receive HIV-specific antibodies from their mothers, MTCT is a unique setting in which to study if those antibodies provide some level of protection. Indeed, maternal antibodies have been implicated in infant protection from other viruses, including respiratory syncytial virus (28) and influenza (29), amongst others. Studying antibodies in the context of MTCT will give insight into what type of immune responses are protective in natural infection, and thus, what kind of responses should be elicited by an effective HIV vaccine. This thesis focuses on two specific antibody functions in mother-to-child transmission: ADCC and neutralization.

Antibody-Dependent Cellular Cytotoxicity in HIV Infection

The first immune mechanism addressed in this thesis is antibody-dependent cellular cytotoxicity. As mentioned above, ADCC was implicated as a protective factor in a secondary analysis of the RV144 vaccine trial (18). Thus, addressing whether or not ADCC is protective in other settings will inform if it is an approach that should be pursued in vaccine research.

ADCC results in the killing of infected cells via an Fc-mediated pathway (**Figure 1.4**). In ADCC, the Fab portion of an antibody binds to viral antigens present on the surface of infected cells and the Fc portion binds to FcγRs found on a variety of effector cells (including natural

killer cells, monocytes, and macrophages). This cross-linking results in the release of cytolytic granules (containing perforin and granzyme) and/or anti-viral cytokines and subsequent killing of the infected cell. ADCC may, therefore, prevent infection by killing cell-associated virus transmitted by the HIV-infected individual or by eliminating early newly infected cells in the exposed person before systemic spread occurs. As cell-associated virus has been suggested to play a major role in MTCT (Reviewed in (30)) and other settings (Reviewed in (31)), ADCC may be important for preventing infection.

Figure 1.4

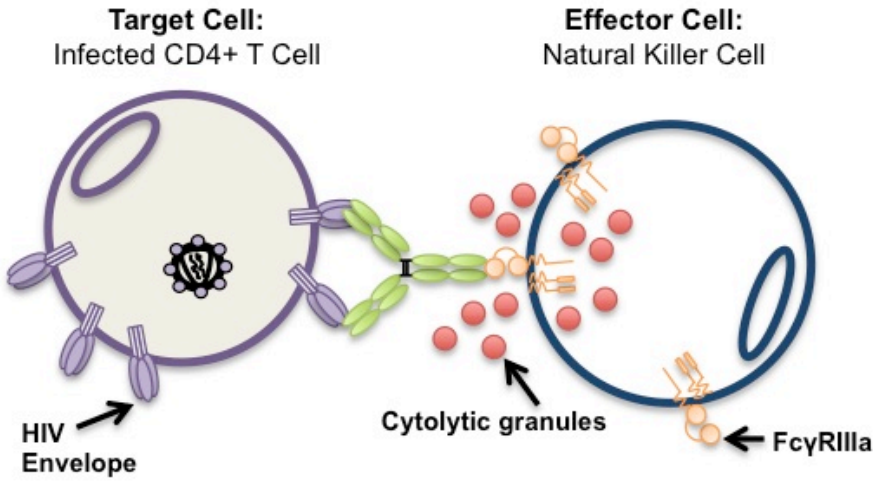


Figure 1.4 Antibody-Dependent Cellular Cytotoxicity
In ADCC, cross-linking of an infected target cell (left) and an effector cell (right) by an antibody (green) results in the release of cytolytic granules from the effector cell. These granules (red) contain enzymes such as perforin and granzyme and target the infected cell. This process results in death of the target cell and prevents further spread of infection.

In addition to the results from the RV144 vaccine trial, there is evidence that ADCC antibodies provide protection in other settings. Firstly, studies in macaques have suggested that vaccine-induced ADCC responses correlate with lower viral loads and/or delayed disease progression following simian immunodeficiency virus (SIV) challenge (32-37). In natural infection in humans, ADCC antibodies have been detected earlier than neutralizing antibodies

(**Figure 1.5**) and these *de novo* ADCC responses have been associated with lower viral loads and are higher in disease controllers than progressors (38-44). These settings, however, do not clearly demonstrate the role of antibodies present at the time of exposure (pre-existing antibodies) in humans and if ADCC is protective in such a setting.

Figure 1.5

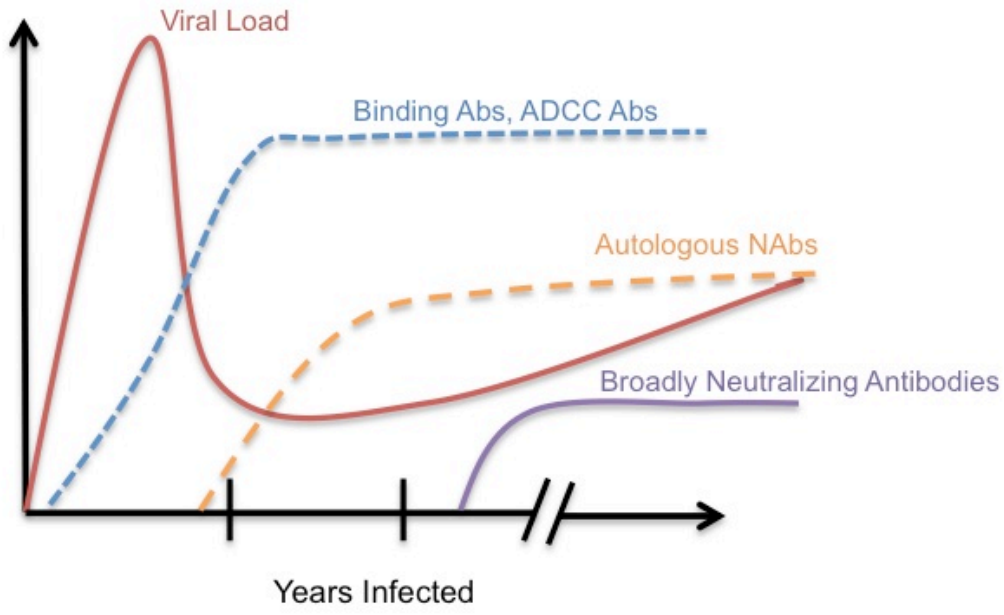


Figure 1.5 Antibody Responses in Natural HIV Infection
As viral load (red) peaks, the first HIV-specific antibodies arise (blue). These antibodies can bind to the virus and elicit ADCC activity but are not capable of neutralization. Neutralizing antibodies against an individual's own (autologous) virus arise afterwards (orange). Broadly neutralizing antibodies (purple) arise in a minority of individuals after more than 2 years of infection. Abs = antibodies, NAbs = neutralizing antibodies.

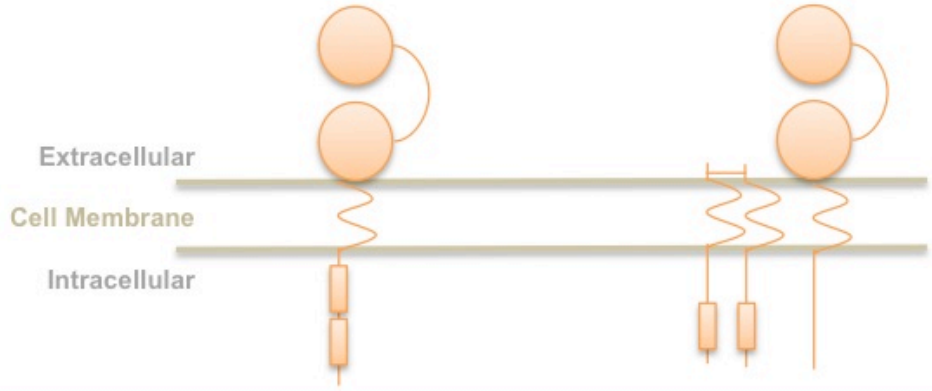
Mother-to-child transmission provides another setting in which to study if ADCC antibodies present at the time of exposure are protective. On the maternal side, ADCC activity was recently implicated in protection: mothers with higher ADCC levels in their breast milk had a lower risk of transmission via breastfeeding (45). In infants, the role of passively acquired (pre-existing) antibodies remains unclear. A number of older studies observed no association between

maternal and infant plasma ADCC and risk of infection (44,46-49). However, these early studies may have been limited by techniques available at the time including: 1) use of lab-adapted viruses that are not representative of transmitted strains; 2) infant infection status determined by ELISA at 15 months, and thus timing of infection and presence of passively-acquired antibody levels at the time of transmission could not be accurately determined; and 3) ADCC activity was measured at various ages in the infants up until 2 years, at which point passively acquired antibodies may not be relevant and *de novo* antibody responses may have been measured. With improvements in infant diagnosis/follow-up and ADCC methods, including ability to assay relevant viruses, we are now more aptly positioned to determine if pre-existing ADCC antibodies play a role in MTCT. Chapter II of this thesis will address the hypothesis that pre-existing ADCC-mediating antibodies protect infants from infection and/or disease progression.

Host FcγRs

ADCC activity is dependent on both antibodies and host FcγRs present on effector cells (**Figure 1.6**). Two FcγRs of particular interest are FcγRIIa (CD32a) and FcγRIIIa (CD16a). Single nucleotide polymorphisms (SNPs) in the genes encoding these receptors have been shown to change antibody binding affinity and, thereby alter effector function (including ADCC activity) (50-52). Accordingly, these receptor polymorphisms have been associated with altered susceptibility to different autoimmune (53), and infectious diseases (54,55), as well as responses to monoclonal antibody cancer treatment (56).

Figure 1.6



	FcγRIIa	FcγRIIIa
Cellular Expression	macrophages, monocytes, neutrophils, dendritic cells, T cells, eosinophils, basophils, platelets, placental endothelial cells	macrophages, monocytes, NK cells, dendritic cells, T cells
Alleles	H 131 (high affinity) R 131	V 158 (high affinity), F 158
IgG Affinity	IgG3 = IgG1 > IgG4 = IgG2	IgG3 = IgG1 >> IgG4 > IgG2

Figure 1.6 FcγRIIa and FcγRIIIa
Overview of FcγRIIa and FcγRIIIa. The structure of the receptors is shown at top. Both receptors consist of two extracellular domains (circles) that bind the Fc portion of antibodies and two intracellular immunoreceptor tyrosine-based activation motifs (ITAMs) (orange rectangles) that transmit signals inside the cell. Below the structures, the receptors' cellular expression, alleles, and IgG affinities are outlined. IgG affinity adapted from (52).

FcγRIIa is an activating receptor found on many cell types: monocytes, macrophages, neutrophils, and platelets amongst others. The receptor has a polymorphism (SNP rs1801274) at amino acid position 131 that can either encode a histidine (H) or an arginine (R) (57,58). The presence of a histidine (H) results in higher affinity binding to IgG and specifically, the presence of at least one H is required for efficient binding to IgG2 (52,58,59). Thus, effector cells homozygous for the histidine allele have higher ADCC and phagocytosis activity than cells homozygous for the low affinity arginine (50,60,61).

FcγRIIIa is another activating receptor found on a more narrow range of cell types: macrophages/monocytes, dendritic cells, and gamma-delta T cells. Additionally, and of note, FcγRIIIa is the major FcγR found on NK cells which are main mediators of ADCC activity. A SNP (rs396991) at amino acid position 158 encodes either a valine (V) or a phenylalanine (F) (62). Individuals with the VV genotype have increased affinity for IgG, and thus have higher ADCC activity (50,52,61,63).

Host genetics have been shown to alter susceptibility to infection and disease progression in natural infection with HIV. The most clear-cut evidence of such an effect is the CCR5Δ32 mutation which is a 32-base pair deletion resulting in a truncated and non-functional co-receptor (64). Individuals who are homozygous for this deletion are much less likely to become infected and heterozygotes have slower disease progression than individuals who are homozygous for the functional receptor (65,66). Human leukocyte antigen (HLA) alleles have also been clearly implicated in disease progression (Reviewed in (67)). But, while the functional consequences of FcγRIIa and FcγRIIIa polymorphisms have been clearly demonstrated *in vitro*, their impact on HIV acquisition and progression *in vivo* remains unclear (summarized in **Table 1.1**). In some populations the high affinity FcγRIIa and FcγRIIIa alleles have been shown to be protective, but in other populations the high affinity alleles appear to be detrimental.

As ADCC activity has been implicated as a protective factor in MTCT, the impact of FcγRIIa and FcγRIIIa genotypes in these populations is important to consider. In Chapter III of this thesis, I address the impact of FcγRIIa and FcγRIIIa polymorphisms in a MTCT cohort. Results from this study will give insight into whether or not host genotypes may impact therapeutic or vaccination mechanisms that rely on ADCC and other FcγR mediated activity.

Table 1.1 FcγRIIa and FcγrIIIa polymorphisms on HIV infection and progression

Summary of studies examining the impact of FcγRIIa and FcγRIIIa SNPs on HIV infection and progression. Results in red indicate studies that suggest the high affinity alleles (FcγRIIa H131 or FcγRIIIa V158) are associated with increased infection risk or progression; results in green indicate studies that suggest high affinity alleles are protective. (KS = Kaposi's sarcoma, HIV+ = HIV-positive, HIV- = HIV-negative MSM = Men who have sex with men, VL = viral load, ADCVI = antibody-dependent cell-mediated viral inhibition).

STUDY	POPULATION	Impact of FcγRIIa	Impact of FcγRIIIa	Other/Notes
Lehrnbecher 2000 (68)	250 mostly white HIV+ MSM United States	No impact	V158 associated with risk of KS	KS is a marker of HIV progression
Brouwer 2004 (69)	448 HIV+ mothers & their infants Kenya	HH associated with infant infection risk Maternal genotype not associated with transmission	Not tested	Infant FcγRIIa genotype not associated with mortality risk
Forthal 2007 (61)	231 HIV+, 299 HIV- from Vax004, majority white North America	HH genotype associated with higher ADCVI activity	VV genotype associated with higher ADCVI activity	ADCVI activity inversely associated with risk of infection in the vaccine trial
Forthal 2007 (60)	559 Male HIV+, mostly white United States	RR progressed more quickly to CD4 count<200 cells/mm^3 HH more likely to develop *Pneumocystis jiroveci* pneumonia	Not associated with HIV progression Higher risk of KS in V158 individuals	*Pneumocstis jiroveci* pneumonia is an AIDS defining illness
French 2010 (70)	HIV+ patients in a therapeutic HIV vaccine trial: 17 receiving vaccination, 6 placebo	Presence of H131 associated with lower RNA VL in presence of anti-p24 IgG2	Not tested	
Poonia 2010 (71)	59 HIV+ progressors, 43 HIV+ virus suppressors, 70 HIV- controls Majority African American United States	Not associated with HIV status	VV genotype more commonly found in HIV progressors than virus suppressors or healthy controls	
Deepe 2012 (72)	73 HIV+ Viral Controllers 100 HIV+ Non-Controllers United States	In Caucasian Americans FcγRIIa HH homozygotes, GM21 non-carriers were more likely to be viral controllers	In Caucasian American FcγRIIIa VV & VF, GM21 non-carriers were more likely to be viral controllers	This study examined epistatic interactions between Fc (GM) & FcγR genotypes & control of infection
Forthal 2012 (73)	1725 male subjects in Vax004 vaccine trial North America & Europe	Not associated with risk of HIV infection or vaccine efficacy	VV vaccinees in the lowest risk group had higher rate of infection than VF or FF vaccinees or VV placebo recipients	
Pandey 2013 (74)	777 males from the Step Trial North America, Caribbean, South America, Australia	Not associated with HIV acquisition	GM23-carriers and FcγRIIIa FF were more likely to acquire HIV infection.	This study examined epistatic interactions between Fc (GM) & FcγR genotypes and HIV acquisition
Li 2014 (75)	125 HIV+ (51 vaccine, 74 placebo); 205 HIV- vaccine recipients RV144 Vaccine Trial Thailand	Not associated with vaccine efficacy	Not associated with vaccine efficacy	FcγRIIc genotype associated with vaccine efficacy against HIV subtype CRF01_AE 169K
Weis 2015 (76)	253 HIV+ female sex workers Kenya	Not associated with set point VL, VL increase, CD4 decline, or disease progression	Not associated with set point VL, VL increase, CD4 decline, or disease progression	

Neutralizing Antibodies in HIV Infection

Although neutralizing antibodies were not implicated in the observed protection in the RV144 vaccine trial, they remain a major goal of an HIV vaccine. NAbs are the gold standard of sterilizing immunity; such antibodies can bind to and prevent free virus from infecting cells and they are implicated in the protection elicited by most licensed vaccines against other pathogens (16). Furthermore, studies from non-human primates have shown as proof-of-concept that neutralizing antibodies can block infection from occurring (77-82). In the majority of these studies, however, the challenge virus was known to be readily neutralized by the antibodies given to the animals and high doses of antibodies were used. In humans, neutralizing antibodies must be able to recognize a wide range of the diverse circulating strains and except for a few "broadly neutralizing" antibodies most HIV-specific antibodies cannot neutralize the majority of HIV variants. Even with broadly neutralizing antibodies, a combination of antibodies is likely needed to protect against all circulating viruses (83,84). The feasibility of a vaccine eliciting such broadly neutralizing antibodies at protective levels remains unclear.

In natural HIV infection, a minority of individuals elicit neutralizing antibodies capable of recognizing diverse HIV isolates. Individuals first develop antibodies that can neutralize their own (autologous) virus at a few months post infection (Reviewed in (85); **Figure 1.5**). After 2-3 years, in a subset of individuals (10-35%), antibodies develop that can neutralize diverse virus isolates (Reviewed in (86)). These antibodies are capable of recognizing viruses present in other individuals (heterologous). In an even smaller group of individuals (~1%), known as elite neutralizers, broadly neutralizing antibodies develop which are capable of recognizing the majority of circulating viruses (87). As evidenced by the limited numbers of elite neutralizers and the RV144 trial results, eliciting broadly neutralizing antibodies is not easily achieved

In mother-to-child transmission, there is evidence that neutralizing antibodies contribute to an observed genetic bottleneck. Infants are typically infected with a single viral variant and these variants have been shown to be more resistant to maternal plasma neutralization than maternal virus variants in a number of studies (88-91). Accordingly, the role of maternal NAbs in MTCT has been directly assayed, but results have varied (**Table 1.2**). Maternal neutralizing antibodies have been associated with protection from transmission in some studies (90-102), while no association has been observed in others (46,103-108). The differences in outcome observed in these studies may be due to methodological differences including the viruses used in the neutralization assays and/or timing of antibody sampling in the mothers. For example, many studies used lab adapted or heterologous viruses, which may not represent the viruses present in the mother (and to which the infant was exposed). With regard to sample timing, the most relevant time to analyze antibody responses is around the time of estimated transmission, however, studies have varied in the time at which they analyzed maternal antibodies. In Chapter IV, I will address the role of maternal NAbs from around the time of transmission against autologous viruses in MTCT.

Table 1.2 Maternal neutralizing antibody responses in HIV MTCT

Summary of studies examining the impact of maternal neutralizing antibodies on transmission risk. (TM = transmitting mother, NTM = non-transmitting mother, NAb = neutralizing antibodies, VL = viral load, HIV+ = HIV-positive, HIV- = HIV-negative). Results in green: Higher NAb responses associated with decreased transmission; results in red: lower NAb responses associated with decreased transmission; Results in black: no difference in maternal NAb responses for TM & NTM.

Study	Cohort	Viruses Tested	Infant Infection Timing	Timing of maternal viruses/plasma	Results
Ugen 1992 (94)	13 TM, 7 NTM U.S.	IIIB (lab adapted)	Not specified	Maternal sera from 3rd trimester of pregnancy	NTM had higher NAb activity than TM
Broliden 1993 (46)	12 TM, 23 NTM	IIIB, RF (lab adapted)	Not specified	Maternal sera from delivery	No difference in presence of NAbs in NTM & TM
Scarlatti 1993 (93)	10 TM, 10 NTM TM had lower CD4 counts Italy	Maternal PBMC co-culture	Not specified	Viruses & sera from within 4 months of delivery	NTM had NAbs against autologous virus more often than TM
Scarlatti 1993 (92)	10 TM, 10 NTM TM had lower CD4 counts Italy	Maternal PBMC co-culture MN, IIIB	Not specified	Viruses & sera from same visit; 13 from delivery, 7 w/in 4 months post delivery	NAbs against autologous virus were more frequent in NTM than TM, but not statistically significant NAbs against one heterologous isolate more common in NTM than TM but no difference against other 2 other isolates or MN or IIIB
Kliks 1994 (91)	6 TM, 12 NTM U.S.	Maternal PBMC co-culture	Not specified, at least 3 HIV+ at birth	Viruses +/- 4 months of delivery Plasma from +/- 2 months of delivery	NAbs against autologous virus were more frequent in NTM than TM ($p<0.07$)
Husson 1995 (103)	16 TM, 16 NTM matched on CD4, AZT use, mode of delivery U.S.	Maternal PBMC co-culture	*In utero*/peripartum No breastfeeding in cohort	Virus from delivery or as close to delivery as possible; Serum from delivery or 2nd/3rd trimester (N = 5)	Low autologous NAbs in NTM & TM No difference in NAb titers between TM & NTM
Mabondzo 1995 (48)	(1) 12 TM, 14 NTM (2) 6 TM, 13 NTM (3) 5 TM, 5 NTM France	(1) BaL (lab adapted) (2) DAS (primary isolate) (3) Maternal PBMC co-culture	Not specified	Serum collected at delivery	Neither heterologous nor autologous NAbs associated with transmission
Bal 1996 (106)	10 TM, 12 NTM U.S.	HIV-SB (heterologous virus from culture)	Not specified	Serum from at/near delivery	NAbs more frequently detected in TM than NTM, but not statistically significant
Mabondzo 1998 (105)	7 TM, 10 NTM France	Maternal PBMC co-culture	Not specified	Serum collected at delivery	No significant difference in NAb titers between TM & NTM
Hengel 1998 (104)	14 TM, 10 NTM Matched on CD4 U.S.	LAI, MN (lab adapted) Maternal PBMC co-culture	Not specified No breastfeeding in cohort	Virus & Sera from average of 0.5 (NTM) and 3.5 (TM) days post delivery	No difference in NAb titers against LAI/MN or Autologous virus
Lathey 1999 (95)	8 TM, 20 NTM TM had lower CD4s U.S.	Maternal PBMC co-culture	Not specified	Virus & plasma/serum from same visit close to delivery	Autologous NAbs more common in NTM than TM ($p = 0.04$)

Table 1.2 (continued)

Study	Cohort	Viruses Tested	Infant Infection Timing	Timing of maternal viruses/plasma	Results
Louisirirotchanakul 1999 (96)	(1) Variable # of TM/NTM (2)6 TM, 6NTM TM mom had higher VLs Thailand	(1)MN, Subtype E lab adapted virus (SL7/SupT1) (2) 4 primary isolates	Not specified No breastfeeding	Serum from delivery & postpartum (usually 6 months)	(1) No difference in NAbs measured at (7 TM, 16 NTM) /after (32 TM, 90 NTM) delivery against subtype E heterologous virus; trend in higher NAbs against MN after delivery (but not at delivery) in NTM than TM (20 TM, 66NTM) (p=0.06) (2) No difference in NAb titers against primary isolates
Bongertz 2001 (98)	(1) 20 TM, 96 NTM (2) 7 TM, 16 NTM ARVs given to some women Brazil	(1) MN (lab adapted) (2) Maternal PBMC co-culture	Not specified	Majority during pregnancy (10^{th}-36^{th} week); minority from after delivery (1-18 months postpartum)	Autologous & heterologous NAbs were comparable for NTM & TM NAbs against MN more common in NTM than TM (only in samples from pregnancy)
Bongertz 2002 (97)	12 TM, 31 NTM Brazil	MN (lab-adapted)	Not specified	Not specified	NTM had higher NAb titers against MN than TM
Guevara 2002 (109)	(1) 50 TM, 25 NTM (2) 37 TM, 21 NTM higher VLs in TM Zimbabwe	(1) MN (lab-adapted) (2) Z822 (subtype C)	Majority *in utero*, not specified for all	Serum from 36 weeks gestation	NAb titers against subtype C virus not associated with transmission; TM had higher NAb titers against MN than NTM
Barin 2006 (99)	28 TM, 62 NTM Thailand	Heterologous primary isolates: KON, FRO, GIL, MBA	*In utero* (N = 11) or intrapartum (N = 17) No breastfeeding	From enrollment during pregnancy, prior to AZT prophylaxis	Higher titer of NAbs to a MBA in NTM, in particular with regard to intrapartum transmission NAb titers against KON, FRO, & GIL were similar for TM & NTM
Dickover 2006 (90)	21 TM, 17 NTM U.S.	Maternal PBMC co-culture	14 *in utero*, 7 intrapartum No breastfeeding	Plasma & viruses from delivery	Autologous NAbs more common & higher titer in NTM than *in utero* TM No difference in NAbs between NTM & intrapartum TM
Kittinunvorakoon 2009 (107)	(1) 28 TM, 56 NTM (2) 24 TM, 24 NTM (3) 10 TM, 14 NTM Thailand	(1) MN (lab-adapted) (2) two maternal isolates (3) autologous maternal PBMC co-culture	10 *in utero*, 18 intrapartum No breastfeeding	Plasma from 36 weeks gestation	No significant difference in NAbs between TM & NTM for any virus tested
Samleerat 2009 (100)	45 TM, 45 NTM matched on VL & AZT duration Thailand	6 Heterologous primary isolates: FRO, BIG, CHA (Clade B) & C1712, LEA, MBA (CRF01_AE)	14 *in utero*, 29 intrapartum, 2 unknown	From enrollment, prior to AZT prophylaxis (4-11 weeks prior to delivery)	A lower risk of MTCT, especially intrapartum transmission, was associated with higher NAb titers against MBA No association between NAbs and transmission risk for any other virus
Russell 2011 (108)	46 TM, 50 NTM Malawi	Heterologous lab adapted viruses	23 *In utero*, 23 intrapartum	Plasma from delivery	No difference in neutralization activity or breadth between TM/NTM

Table 1.2 (continued)

Study	Cohort	Viruses Tested	Infant Infection Timing	Timing of maternal viruses/plasma	Results
Diomede 2012 (101)	16 TM, 24 NTM S. Africa	Lab-adapted viruses: SF162, IIIB & primary viruses: DU172, DU156	*In utero* (only examined HIV+ vs. HIV- at birth)	Plasma from enrollment during pregnancy/delivery	Higher NAbs in NT than NTM against DU172 & DU156 Statistical significance of NAbs against SF162 & IIIB for TM & NTM not reported, NAbs appeared higher against SF162 in TM than NTM
Chaillon 2012 (102)	57 TM, 57 NTM matched on location, date France	Panel of 10 heterologous primary isolates from various clades	16 *in utero*, 29 peripartum, 12 unknown	Plasma from delivery prior to AZT	No difference in NAb breadth between TM & NTM; No difference in NAb titers against individual viruses; except 1 case: Non-Subtype B infected NTM had higher NAb titers against FRO than TM
Baan 2013 (110)	7 TM, 4 NTM matched on VL, CD4 Rwanda	Envelope clones from plasma used to make env-pseudotyped virus (total N = 23)	4 *In utero*, 3 peripartum or breastfeeding	Env clones from delivery through W16 Plasma from 1 week before delivery & 16-18 weeks post delivery (2 time points tested)	Higher NAb responses in TM, especially *in utero* TM
Omenda 2013 (111)	14 TM, 46 NTM Kenya	Primary pseudotyped viruses: Q461.d1, Q842.d16, QD435.A4	Delivery/ breastfeeding	Plasma from delivery	No difference in NAb responses between TM & NTM (trend toward higher responses in TM)

The Nairobi Breastfeeding Clinical Trial

The studies presented in this thesis utilize samples from the Nairobi Breastfeeding Clinical Trial (24). The trial, conducted from 1992-1998 in Kenya, followed 425 HIV-infected women and their infants. Maternal blood and cervicovaginal samples were collected at maternal enrollment, during the third trimester of pregnancy. Subsequently, infant blood and maternal blood and breast milk samples were collected at delivery, 6 weeks, 14 weeks, 6 months, and every 3 months until the infant was two years of age. Infant infection was determined by HIV-specific PCR. In the original study, mothers were randomized to either formula feed or breastfeed and results from the trial showed that breastfeeding increases the risk of mother-to-child transmission, particularly in the early breastfeeding period (24).

In addition to the initial study results, the trial continues to provide valuable information about mother-to-child transmission risk factors and immune correlates of protection. The cohort is unique in that there are longitudinal samples from a large number of HIV-infected women and infants from before and after infection. Additionally, the use of antiretroviral treatment does not confound the results from these studies as the original trial was conducted prior to when antiretrovirals were provided as standard of care in Kenya.

Prior studies have demonstrated associations between both ADCC and neutralizing antibodies and risk of MTCT in this cohort. Firstly, mothers who transmitted the virus via breastfeeding tended to have lower ADCC activity in their breast milk than mothers who did not transmit (45). These results suggest that ADCC activity may be protective during the early breastfeeding period and Chapter II of this thesis will address the role of passively acquired antibodies in the infants. With regard to neutralizing antibodies, Wu *et al*. showed that early infant viruses tend to be more resistant to neutralization by maternal plasma than maternal virus

variants near the time of transmission (88). These results suggest that infant viruses may escape maternal neutralizing antibody responses. However, this study only examined transmitting mothers and whether or not non-transmitting mothers tend to have fewer neutralization-resistant viruses (and are therefore less likely to transmit) has not been examined in this cohort. Chapter IV of this thesis will address the question of autologous maternal neutralizing antibodies in MTCT.

Goals for this Thesis:

The overall goal for this thesis is to further explore the role of humoral and host immune responses in mother-to-child transmission. Studying the role that such responses play in natural infection will increase our understanding of effective immune responses against the virus and aid in rational vaccine design. In Chapter II, I will first explore the role of passively acquired pre-existing HIV-specific ADCC activity in infants born to HIV-infected mothers. As ADCC activity is dependent on host FcγRs, Chapter III will explore the impact of FcγR polymorphisms on MTCT. Next, Chapter IV will address the role of maternal neutralizing antibodies in MTCT by exploring the impact of autologous neutralizing antibodies and infection risk. Finally, in Chapter V, I will discuss the implications of these antibody responses in the larger HIV treatment and prevention fields.

Chapter II

Passively Acquired Antibody-Dependent Cellular Cytotoxicity Activity in HIV-Infected Infants is Associated with Reduced Mortality

The text in this chapter has been modified slightly from: Cell Host Microbe 2015 Apr 8; 17(4):500-6. doi: 10.1016/j.chom.2015.03.002
PMID: 25856755

INTRODUCTION

Rational design of an effective vaccine against HIV requires understanding the functional characteristics of antibodies capable of preventing virus transmission or providing a therapeutic benefit. One function of antibodies is antibody-dependent cellular cytotoxicity (ADCC), and HIV-specific ADCC activity has been suggested to provide a protective and/or therapeutic effect in multiple settings. Evidence for a therapeutic effect in humans comes from studies showing that *de novo* ADCC antibody responses are inversely associated with viral load and higher in viral controllers than progressors (Reviewed in (112)). However, human data on whether or not ADCC antibodies are protective if present at the time of exposure (pre-existing antibodies) are more limited. In the RV144 vaccine trial, vaccine-induced ADCC antibodies correlated with reduced infection risk in an exploratory analysis of individuals with low plasma IgA (18). Furthermore, ADCC activity in the index case has been associated with protection in the setting of mother-infant transmission. There, high maternal breast milk HIV-specific ADCC activity correlated with reduced risk of infant infection via breastfeeding (45). Additional support for the protective role of ADCC antibodies comes from studies in macaques that have shown vaccine-induced ADCC responses correlate with lower viral loads and/or delayed disease progression following simian immunodeficiency virus (SIV) challenge (reviewed in (112)). Collectively, these findings support the hypothesis that ADCC antibodies present at the time of HIV exposure

may have a role in protecting against HIV acquisition or modulating viral load in those who become infected. However, translating results from macaque studies and hypothesis-generating studies in humans to more definitive human studies is critical for determining the importance of pre-existing antibodies in protection

HIV mother-to-child transmission (MTCT) is a unique setting in which to examine the protective role of ADCC antibodies present at exposure because maternal IgG crosses the placenta during pregnancy. Thus, infants born to HIV-infected mothers have HIV-specific antibodies present in circulation at birth that may provide protection during virus exposure, particularly during breastfeeding. Several early studies of ADCC in MTCT showed no correlation of infant or maternal ADCC and infection risk (44,46-49). However, these studies may have been limited in their ability to detect a protective effect of ADCC antibodies based on techniques available, including 1) use of lab-adapted viruses that do not represent transmitted strains; 2) infant infection status was often determined by ELISA at 15 months, and thus, timing of infection (including *in utero* infections) could not be verified; and 3) infant ADCC activity was measured at various ages up until 2 years, at which point passively transferred antibodies may not be relevant and *de novo* responses may have been measured. With advances in infant diagnosis/follow-up and improvements in ADCC methods, we are now more aptly positioned to determine if pre-existing ADCC antibodies in HIV-exposed infants influence virus acquisition or disease progression.

In this study, we evaluated passively acquired ADCC antibody activity in plasma near the time of birth from infants born to HIV-infected mothers. We hypothesized that this pre-existing HIV-specific ADCC antibody activity in infants would provide a protective and therapeutic benefit to infants exposed to HIV via breastfeeding. We found that both ADCC activity and the

magnitude of IgG1 but not IgG3 antibody binding were significantly associated with a decreased risk of mortality in infants who became infected. These results suggest that pre-existing HIV-specific IgG1-mediated ADCC activity may provide a therapeutic benefit in individuals who become infected and is an important component to consider for an HIV vaccine.

MATERIALS & METHODS

Study Design

Plasma samples were from the Nairobi Breastfeeding Clinical Trial conducted in the mid-1990s, before antiretrovirals were used for prevention of MTCT (24). Infants were tested for HIV DNA at birth, 6 weeks, 14 weeks, and every 3 months until 2 years of age. For those infants who tested positive, samples prior to the first HIV DNA positive test were tested for HIV RNA to more precisely define infection timing. Time of infection was estimated as the midpoint between the last negative HIV DNA or RNA test and the first positive test. Infant samples were selected for study based on the following criteria: 1) HIV DNA and RNA negative at birth, 2) breastfed for ≥3 months, 3) remained HIV-negative for at least 6 months and throughout all follow-up if in the uninfected arm, and 4) availability of a plasma sample from the first week of life. Based on these criteria, 72 infants were included, 21 of who became infected during follow-up. Corresponding maternal plasma samples from the third trimester of pregnancy (n = 69) or delivery (n = 3) were also selected for testing. Longitudinal plasma samples were analyzed for six infants. These infants were chosen based on the availability of multiple samples prior to and post infection. The ethical review committee of the Kenyatta National Hospital Institutional Review Board, the Institutional Review Board of the University of Washington and the Institutional Review Board of the Fred Hutchinson Cancer Research Center gave permission to conduct the Nairobi Breastfeeding Clinical Trial.

Rapid Fluorometric Antibody-Dependent Cellular Cytotoxicity Assay

ADCC was measured using the rapid fluorometric ADCC (RFADCC) assay as previously described (45,113). Target cells, CEM-NKr T cells, were maintained in RPMI complete (RPMI + 10% FBS, 1% PSF, 1% L-glutamine). For the RFADCC assay, these target cells were double stained with a membrane (PKH) and cytosolic (CFSE) dye and then coated with a gp120 protein (BL035.W6M.Env.C1 [BL035]; Immune Technology Corp). Patient plasma samples were run at a 1:5,000 dilution, which detected a wide range of ADCC activity while avoiding a prozone effect. Samples were run in duplicate and in comparison studies, all samples were run with the same peripheral blood mononuclear cell (PBMC) donors to reduce donor variability. Infant samples were run three times in duplicate with two PBMC donors, and maternal samples were run two times in duplicate with one donor. Pooled IgG from HIV-positive individuals, HivIg (NIH AIDS Reagent Program), was used as a positive control. In a pilot study, four infant plasmas and two plasma pools (HivIg and pooled plasma from 30 chronically infected Kenyans [VA Pool]) were tested in duplicate against target cells coated with different gp120 antigens (BL035.W6M.Env.C1, MG505.W0M.Env.H3, MK184.W0M.Env.G3, Bal, WITO4160, YU2, CAP210.2.00, and Du422.1; Immune Technology Corp). In all studies, ADCC activity was defined as the percentage of membrane-labeled cells (PKH-positive) that were CFSE-negative after subtracting the level of killing in the media only wells (background). Final reports of ADCC activity were normalized to HivIg, which was set at 100%.

HIV-Specific Total IgG and IgG3 ELISAs

HIV-envelope-specific ELISAs were performed as previously described (45), with the following modifications. ELISA plates (Immulon 2HB plates) were coated with 25 ng/well (total IgG ELISA) or 50 ng/well (IgG3 ELISA) of BL035 gp120 in 0.1 $NaHCO_3$ and incubated overnight. The following day, plates were blocked with phosphate buffered saline (PBS) containing 10% dry milk and 0.05% Tween-20 for 1 hour. Plasma dilutions started at 1:25,000 (total IgG) or 1:50 (IgG3) and were titrated 2-fold. If IgG end point titer (EPT) was less than 1:25,000, 2-fold dilutions were conducted starting at 1:1,000. Samples were incubated for 1 hour at 37^{o}C. For total IgG, samples were then detected with goat anti-human IgG-HRP diluted 1:3,000. IgG3 samples were incubated with mouse anti-human IgG3-biotin (SouthernBiotech) at 2 mg/ml and then with streptavidin-HRP at 1 mg/ml. EPT was defined as the plasma reciprocal dilution at which the average OD value was greater than two times the average OD value of background measured against HIV-uninfected plasma. Samples were run in duplicate two times. If the sample did not reach EPT at the lowest dilution tested, the EPT was set as the midpoint between 0 and the lowest dilution tested.

Detection of IgG1 Cell-Surface Binding

To measure the magnitude of HIV-specific IgG1 binding to gp120-coated cells, the following protocol was adapted from Smalls-Mantey, *et al.* (114). CEM-NKr cells were coated with BL035 gp120 at 15 mg/1 million cells (the same concentration as used in the RFADCC assay). Next, 25,000 CEM-NKr cells were incubated with 1:1,000 patient plasma at room temperature for 30 minutes to allow antibody to bind to HIV-coated target cells. Samples were run in duplicate. Samples were washed twice with 2% FBS in PBS to remove unbound patient antibody and then

stained with mouse anti-human IgG1- Alexa-488 at a 1:1,000 dilution (SouthernBiotech). Samples were fixed using 1% paraformaldehyde and median fluorescence intensity (MFI) was quantified by flow cytometry.

Statistical Analysis

The statistical plan for infant and maternal ADCC antibody responses were established prior to data collection and based on literature suggesting ADCC activity may impact acquisition and clinical outcome, thus negating the need to adjust for multiple comparisons (115). Following the observed positive association between infant ADCC antibody activity and survival, experiments and analyses were conducted to further explore the mechanism and antibody subclass responsible for the effect. Independent groups were compared by two-sided Welch's t-test. Logistic regression analysis was used to compare independent groups and control for maternal viral load. Survival analyses were conducted using Cox-proportional hazards models and Kaplan-Meier estimates with log rank tests. Correlations were estimated by the Spearman rank method. ELISA and MFI data were $\log_2$ transformed, and viral load data were $\log_{10}$ transformed for all analyses. Statistical analyses were performed using STATA version 12.1 (College Station, TX).

RESULTS

Passively Acquired ADCC Antibody Activity and Infant Infection Risk

To investigate the impact of HIV-specific, ADCC-mediating passively acquired antibodies on infant infection and disease progression, we examined ADCC responses in 72 infants who were HIV RNA negative at birth and were continually exposed to HIV via breastfeeding. We focused on infants with a plasma sample from the first week of life because

passively acquired antibodies are the highest at this time and because the first weeks of life are when infants are at the highest risk of breastfeeding infection (24). Of the 72 infants who met the study criteria, 21 became infected and were detected as HIV positive at the following visits: week 2 (n = 1), week 6 (n = 10), week 14 (n = 1), month 6 (n = 1), and months 7–24 (n = 8).

To select a representative envelope antigen to measure infant ADCC activity against, we first tested eight HIV gp120s from diverse clades against six plasmas. This experiment suggested that although absolute values of ADCC activity varied, a similar pattern of ADCC antibody activity was observed across envelope antigens (**Figure 2.1A**). For example, plasma samples such as BK431 with high ADCC antibody activity against one gp120 also had high ADCC activity against the other gp120s. Conversely, plasma BL757 had the lowest ADCC activity with each of the 8 gp120s tested. We selected the BL035 gp120 because it was representative of the results with the different gp120s (**Figure 2.1B**) and because it was cloned from an early infant virus in the cohort.

Figure 2.1

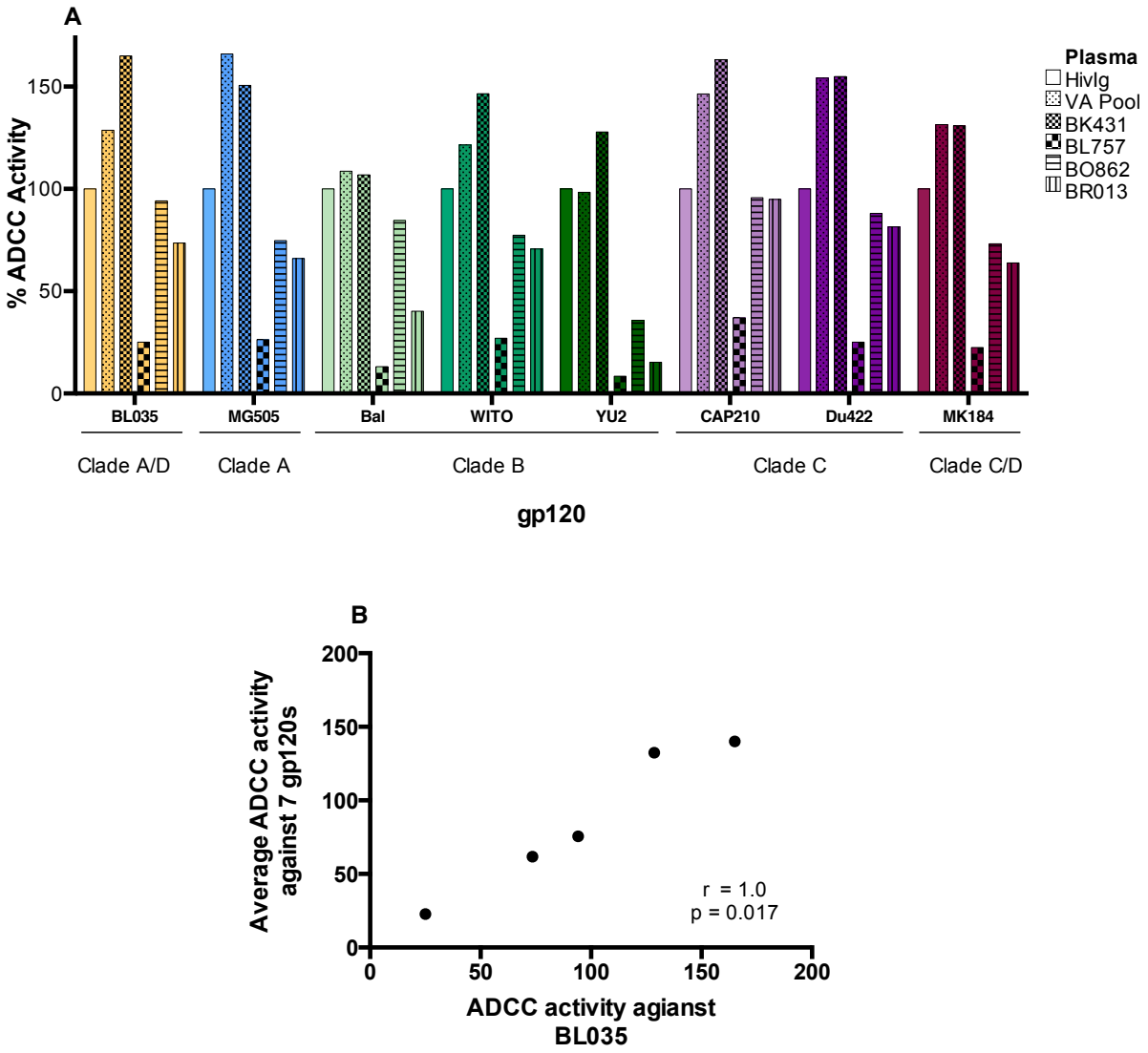


Figure 2.1 Plasma ADCC activity measured against multiple gp120s
(A) ADCC activity of 2 plasma pools (HivIg and VA Pool) and 4 infant plasma samples (BK431, BL757, BO862, BR013) measured against multiple envelope antigens (gp120s) from clade A/D (BL035.W6M.Env.C1 (BL035)), clade A (MG505.W0M.Env.H3 (MG505)), clade B (Bal, WITO4160 (WITO), YU2), clade C (CAP210.2.00 (CAP210), DU422.1 (DU422)), and clade C/D (MK184.W0M.Env.G3 (MK184)). Data represent the mean of one experiment run in duplicate. **(B)** ADCC activity against BL035 vs. ADCC activity against the mean of the 7 other gp120s tested.

We then measured the ADCC antibody activity of the 72 infant plasmas against BL035. Overall, uninfected infants had higher mean ADCC activity than infected infants (36.7% versus

26.3%; **Figure 2.2**); however, this association was not statistically significant (p = 0.12). In a logistic regression controlling for maternal viral load, a known risk factor for MTCT, there was not a significant association between infant ADCC activity and risk of infection (odds ratio [OR]: 0.99, 95% CI 0.97 to 1.01, p = 0.26).

Figure 2.2

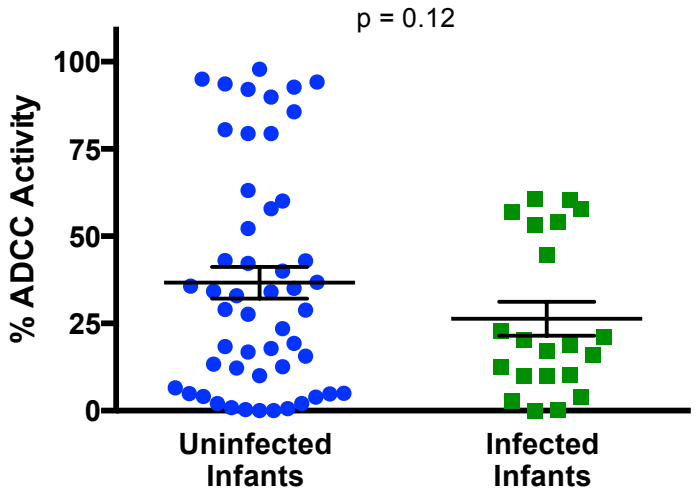


Figure 2.2 Infant ADCC antibody responses by infection status
Passively acquired infant ADCC responses are shown in relation to infection outcome for the 72 infants. Results are normalized to a positive control (HivIg) and data are represented as mean ± SEM

As passively acquired antibodies wane over time, we considered the possibility that ADCC antibody levels would be less relevant to infant exposures that occurred later in life. To address this hypothesis, we first examined the decay of passive ADCC antibody activity in nine uninfected infants. This analysis showed that ADCC levels declined to 31.6% of initial levels by 6 weeks and 4.0%, and 1.3% by 3.5 and 6 months, respectively (**Figure 2.3A**). This decay suggested that passive ADCC antibodies are likely most relevant to protection in the first few months of life. Therefore, we performed an analysis that focused on the role of ADCC antibodies during this early exposure period by restricting the analysis of ADCC activity and infant

outcome to infants infected early. When including only those infants infected within the first 6 weeks of life (n = 11), the mean ADCC antibody activity for uninfected infants remained higher than for infected infants, but the difference was not statistically significant (36.7% vs. 25.0%, p = 0.17; **Figure 2.3B**). While this result was not significant, with the small number of infected infants we only had 30% power to detect a 10% difference in the mean ADCC activity across the two groups. Interestingly, the infants who acquired infection had a bimodal distribution of ADCC antibodies, with 3 having levels slightly higher than the average for infants who remained uninfected and the remainder (n = 8) having very low levels. The numbers of cases were too small to attempt to define any correlates for these subgroups.

Figure 2.3

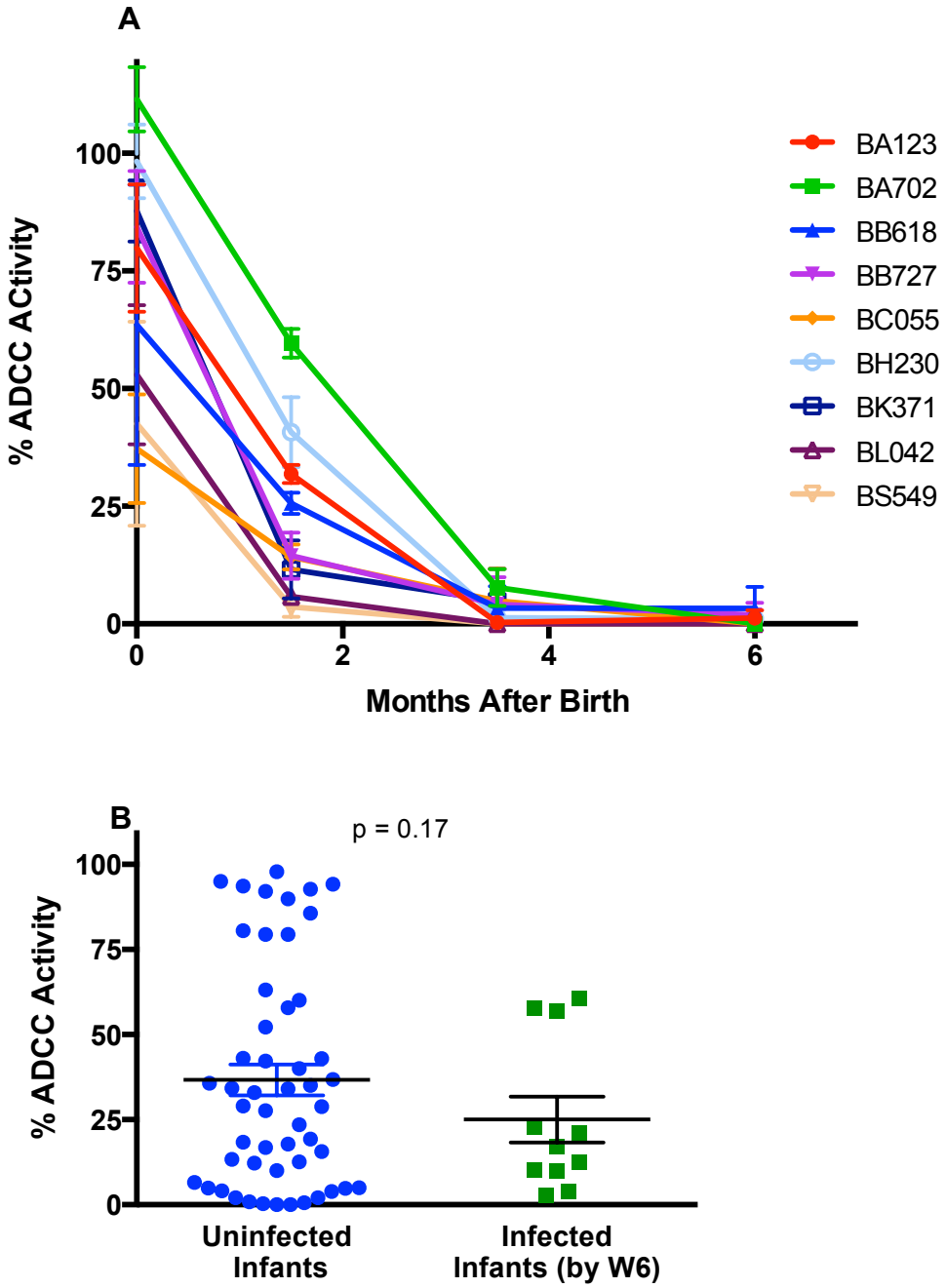


Figure 2.3 Decay of passively acquired ADCC activity in HIV-uninfected infants
(A) ADCC activity measured in longitudinal plasma samples from nine HIV-uninfected infants, with each infant shown as a line with the indicated symbol at the right. Data represent the mean ± SD of two independent experiments run in duplicate. **(B)** Passively acquired infant ADCC responses are shown in relation to infection outcome for a subset of infected infants that were first detected as HIV-infected by 6 weeks (W6) of age (during which time passively acquired antibodies have their highest activity). Results are normalized to a positive control (HivIg) and data are represented as mean ± SEM.

Passively Acquired ADCC Antibody Activity and Survival in HIV-Infected Infants

While sterilizing immunity is the gold standard measure of protection, pre-existing ADCC antibodies may also provide a therapeutic benefit in those who acquire HIV. Thus, as part of a pre-specified analysis plan, we examined time to mortality after infection to determine the impact of passively acquired ADCC antibodies on clinical outcome. In the 21 infected infants, there were eight deaths during follow-up (38%). In a Cox-proportional hazards model, each 10% increase in ADCC antibody activity was associated with a 49.1% reduction in risk of mortality ($p = 0.033$). Additionally, when comparing Kaplan-Meier survival functions for infected infants with ADCC greater than or equal to than the infected infant cohort median with those infants who had ADCC antibody activity less than the median, the survival curves were significantly different ($\chi^2 = 4.22$, $p = 0.04$; **Figure 2.4A**). Set point viral loads (116) were only available for a subset of the infected infants. Among this smaller group ($n = 10$), there was a trend for a negative association between pre-existing ADCC antibody activity and set point viral load ($r = - 0.59$, $p = 0.074$; **Figure 2.4B**).

Figure 2.4

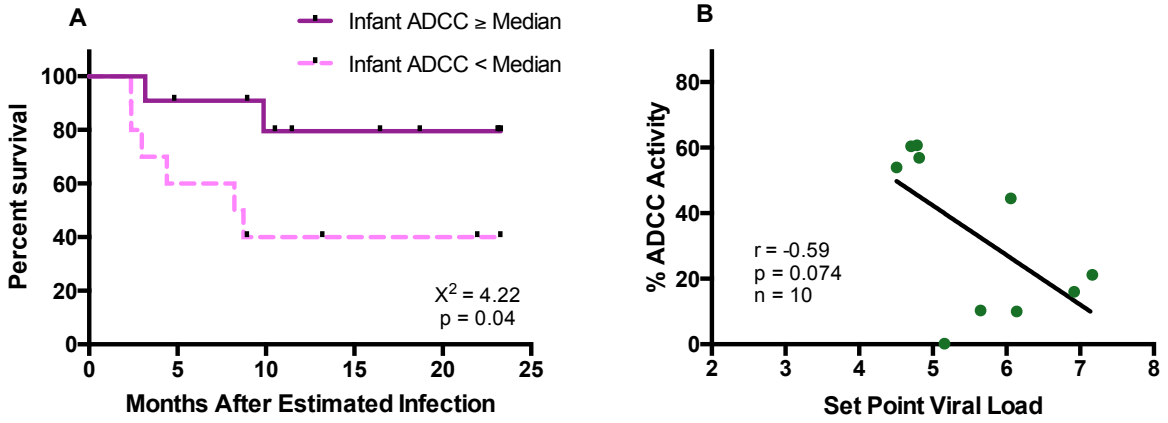


Figure 2.4 Passively acquired ADCC activity and disease progression in infected infants
(A) Kaplan-Meier estimates for infected infants with infant ADCC antibody activity ≥ the infected infant cohort median ADCC activity (solid line) and infected infants with ADCC antibody activity < median (dashed line).
(B) Correlation between pre-existing infant ADCC activity and set point viral load in infected infants. Line represents line of best fit.

Maternal ADCC Antibody Activity and Infant Infection Risk

In many published MTCT studies, maternal antibodies were measured in an attempt to define immune correlates of protection in infants. To address whether maternal antibody activity correlates with protection and whether maternal antibodies provide a similar measure of ADCC activity as passively acquired antibodies in the infants, ADCC activity in the 72 corresponding mothers was examined. Plasma from the third trimester or delivery was chosen based on the fact that the majority of passive antibody transfer occurs late in pregnancy and thus the maternal antibody repertoire during this time should most closely resemble the passively acquired antibodies present in infants at birth (reviewed in (117)). Maternal ADCC antibody activity correlated with infant responses ($r = 0.75$, $p < 0.0001$; **Figure 2.5A**) and maternal ADCC levels were higher than those in the infants, with a median fold-difference in ADCC activity of 1.7. However, unlike their infants, the mothers had nearly identical ADCC antibody levels of 44.2%

for transmitters and 42.1% for non-transmitters (p = 0.81; **Figure 2.5B**). This relationship remained non-significant when controlling for maternal viral load in a logistic regression analysis (OR: 1.00, 95% CI: 0.99 to 1.02, p = 0.76). Additionally, in contrast to infant samples, there was not a significant association between maternal ADCC activity and infected infant survival in a Cox-proportional hazards model (hazard ratio [HR]: 0.99, p = 0.30) or when comparing Kaplan-Meier estimates (**Figure 2.5C**), suggesting a unique role of passively acquired antibodies in the observed association with survival.

Figure 2.5

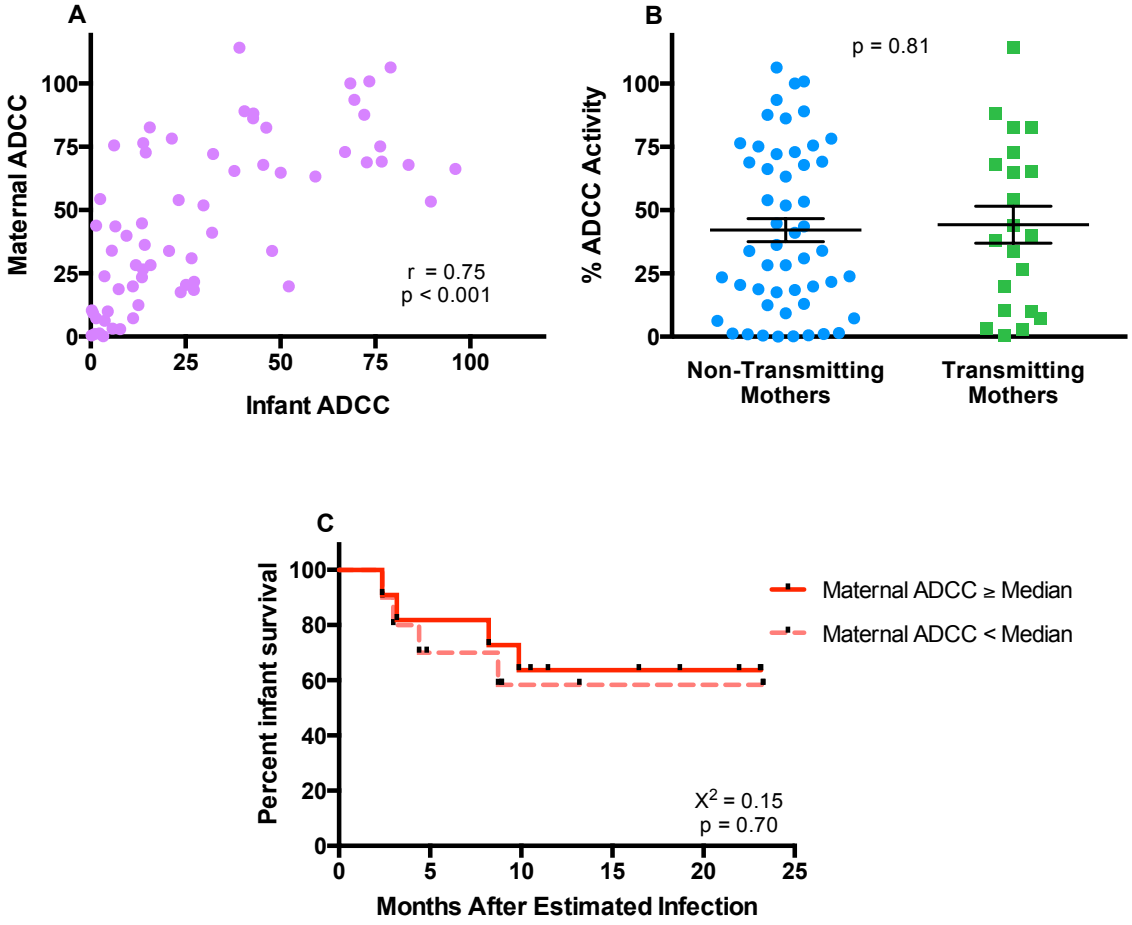


Figure 2.5 Maternal ADCC antibody responses
(A) Correlation between maternal and infant ADCC antibody responses.
(B) Maternal ADCC responses are shown in relation to infant infection outcome. Results are normalized to a positive control (HivIg) and data are represented as mean ± SEM.
(C) Kaplan- Meier estimates for infected infants with maternal ADCC antibody activity ≥ the maternal transmitter cohort median ADCC activity (solid line) and infected infants with maternal ADCC antibody activity < median (dashed line).

Passively Acquired HIV-Specific Neutralizing and Binding Antibodies in HIV-Exposed Infants

To determine whether the association between passively acquired ADCC activity and infected infant survival was unique to the ADCC antibody function, we also examined the

impact of neutralizing antibodies (NAbs) on infant infection and survival using available IC50 data for eight viruses representing four clades (118). In the 72 infants included in this study, NAb breadth and potency were not associated with infant infection status (**Figures 2.6A & 2.6B**), as observed with a larger group of infants in the prior study (118). There was a moderate, but significant, association between infant ADCC activity and NAb breadth ($r = 0.50$, $p < 0.0001$) and potency ($r = 0.57$, $p < 0.0001$) (**Figures 2.6C & 2.6D**). Nevertheless, in a Cox-proportional hazards model, there was not a significant association between passively acquired NAb breadth (HR: 0.86, $p = 0.35$) or potency (HR: 0.94, $p = 0.34$) and infected infant survival. Additionally, there was no correlation between neutralization IC50 and infected infant survival for each of the eight individual viruses (data not shown).

We also examined HIV-specific IgG binding titers (end point titers [EPTs]), which encompass neutralizing and non-neutralizing antibodies, including those that mediate ADCC activity. These titers correlated with ADCC antibody activity ($r = 0.59$, $p < 0.0001$) but similarly did not differ between infected and uninfected infants ($p = 0.94$) (**Figures 2.6E & 2.6F**). We observed a trend in association between these EPTs and infant survival (HR: 0.80, $p = 0.091$).

Figure 2.6

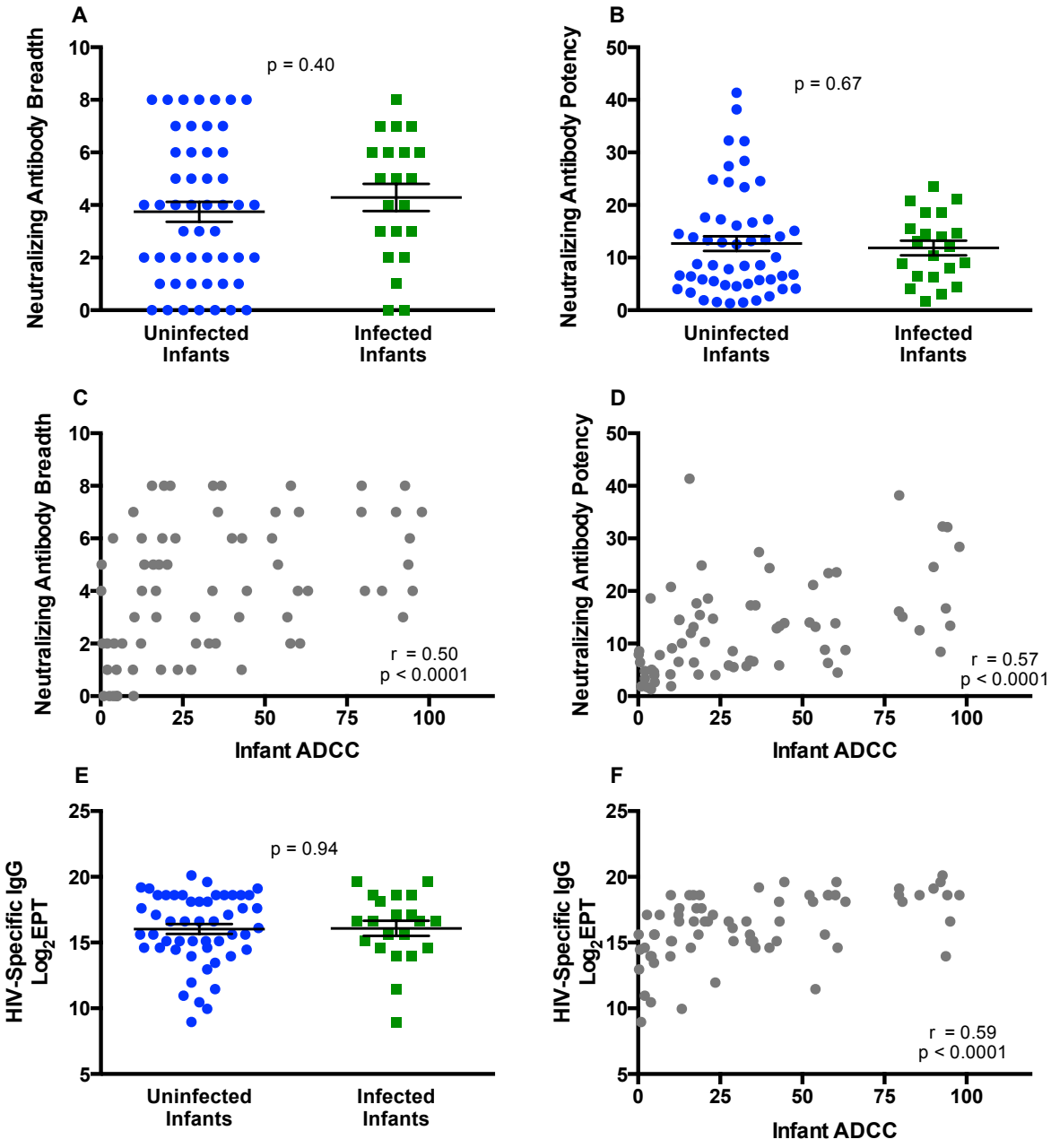


Figure 2.6 Neutralizing antibody responses and total HIV-specific binding titers in infants
Infant plasma-mediated neutralizing antibody breadth **(A)**, neutralizing antibody potency **(B)**, and HIV-specific IgG log_2EPT **(E)** in relation to infant infection status. Data represent mean ± SEM. Correlation between infant ADCC antibody activity and neutralizing antibody breadth **(C)**, neutralizing antibody potency **(D)**, and HIV-specific log_2EPT **(F)**.

Passively Acquired IgG1 and IgG3 in Infant ADCC

We next sought to understand whether a particular IgG subclass of passively transferred antibodies was associated with the survival benefit observed in infected infants. ADCC is reported to be predominately mediated by IgG1 and IgG3, and thus, we focused on these subclasses. Infant ADCC antibody activity was strongly correlated with IgG1 antibody binding, as measured by binding to cell surface gp120 ($r = 0.92$; $p < 0.0001$; **Figure 2.7A**). There was no difference in surface IgG1 antibody binding between infected (mean $\log_2$MFI: 9.51) and uninfected infants (mean $\log_2$MFI: 9.46) ($p = 0.82$; **Figure 2.7B**). In infected infants, however, there was a strong association between surface IgG1 binding and survival (HR: 0.24; $p = 0.005$), with higher $\log_2$MFI associated with increased survival in a Cox-proportional hazards model. Furthermore, comparing Kaplan-Meier survival functions for infected infants with a $\log_2$MFI greater than or equal to the cohort median with those infants who had $\log_2$MFI activity less than the cohort median, the survival curves were significantly different ($\chi^2 = 7.74$, $p = 0.005$; **Figure 2.7C**).

IgG3 binding was measured by ELISA because, similar to previous reports (114), we could not detect IgG3 surface binding by the flow-cytometry-based method. IgG1 surface binding MFI and gp120-specific ELISA binding titers correlated (**Figure 2.7D**), demonstrating that the assays provide similar measures. Overall, HIV-specific IgG3 was detected in 34 (47.22%) of infant samples. IgG3 did not correlate with infant ADCC activity ($r = -0.16$, $p = 0.18$; **Figure 2.7E**). Similarly, IgG3 binding was not associated with infant infection ($p = 0.58$; **Figure 2.7F**) or survival in infected infants (**Figure 2.7G**).

Figure 2.7

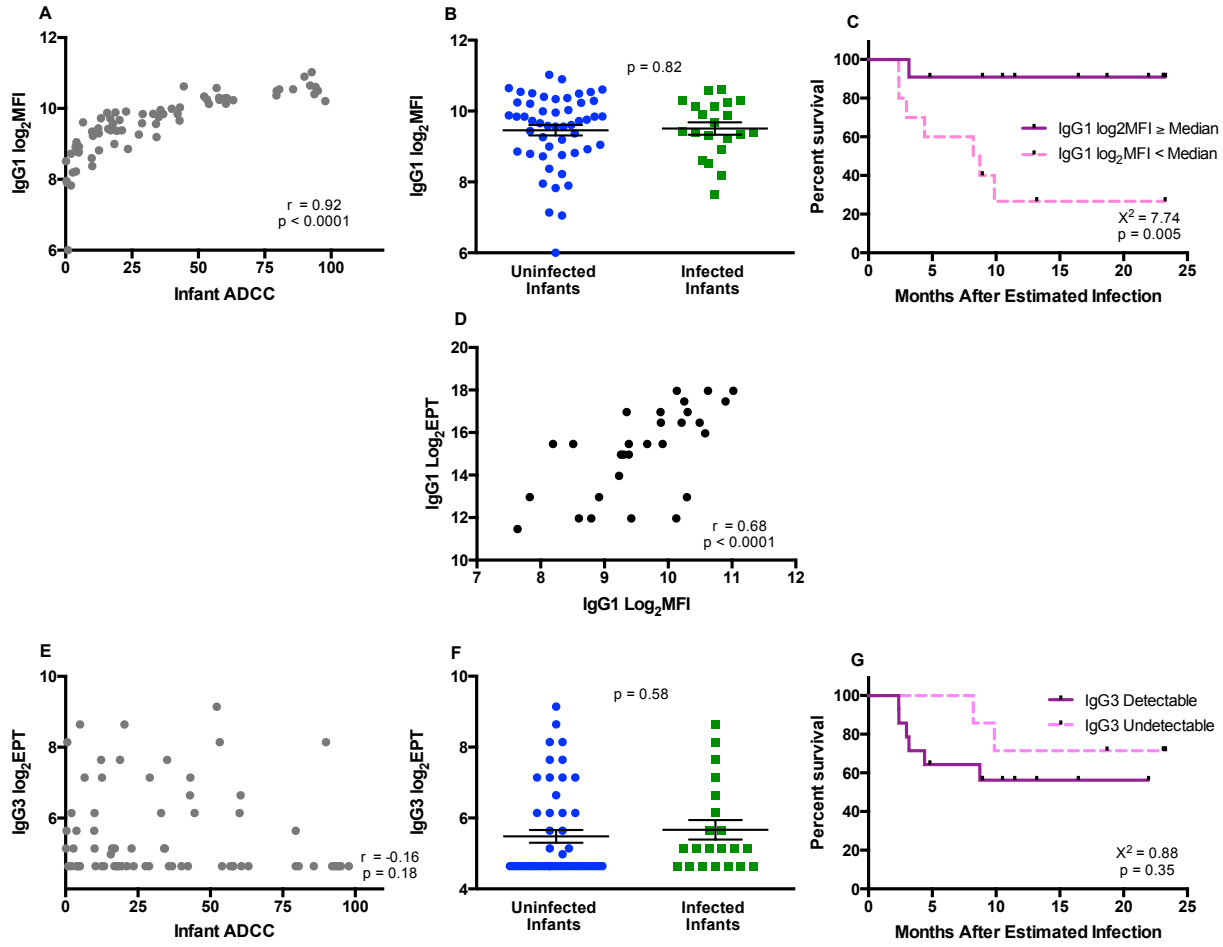


Figure 2.7 IgG1 and IgG3 HIV-specific antibody responses in infants
Correlation between infant ADCC antibody activity and IgG1 log_2MFI responses **(A)** or IgG3 log_2EPT **(E)**. IgG1 **(B)** or IgG3 **(F)** responses in relation to infant infection status; mean ± SEM are shown. Kaplan-Meier estimates for infected infants with IgG1 log_2MFI ≥ infected infant cohort median (solid line) and infected infants with IgG1 log_2MFI < cohort median (dashed line) **(C)** or IgG3 levels detectable at a 1:50 dilution (solid line) vs. undetectable (dashed line). Correlation between IgG1 log_2EPT and IgG1 log_2MFI **(D)**.

Kinetics of ADCC Antibody Responses in Infected Infants

In macaques, higher passive NAb levels have been linked to more robust *de novo* responses (119,120), raising the possibility that the passive ADCC activity in infants was a surrogate measure for *de novo* ADCC responses contributing to infection control. To test this possibility, longitudinal plasma samples from six infected infants in the study were tested for ADCC activity. The highest passively acquired ADCC antibody activity was detected within the

first week of life and declined to undetectable levels in all six infants prior to a subsequent increase in *de novo* ADCC activity after infection (**Figures 2.8A–2.8F**). *De novo* ADCC antibody activity could be detected as early as 2 months post-infection; however, responses appeared delayed in infants infected within 6 weeks of birth. In four cases, the maximum *de novo* ADCC activity was higher than that of the passively acquired activity measured at birth. There was no correlation between passively acquired ADCC antibody activity measured at birth and maximum *de novo* activity measured after infection ($r = -0.14$, $p = 0.79$; **Figure 2.8G**), suggesting that our finding showing an association with passive ADCC antibodies and infant outcome was not confounded by an association between passive and *de novo* responses.

Figure 2.8

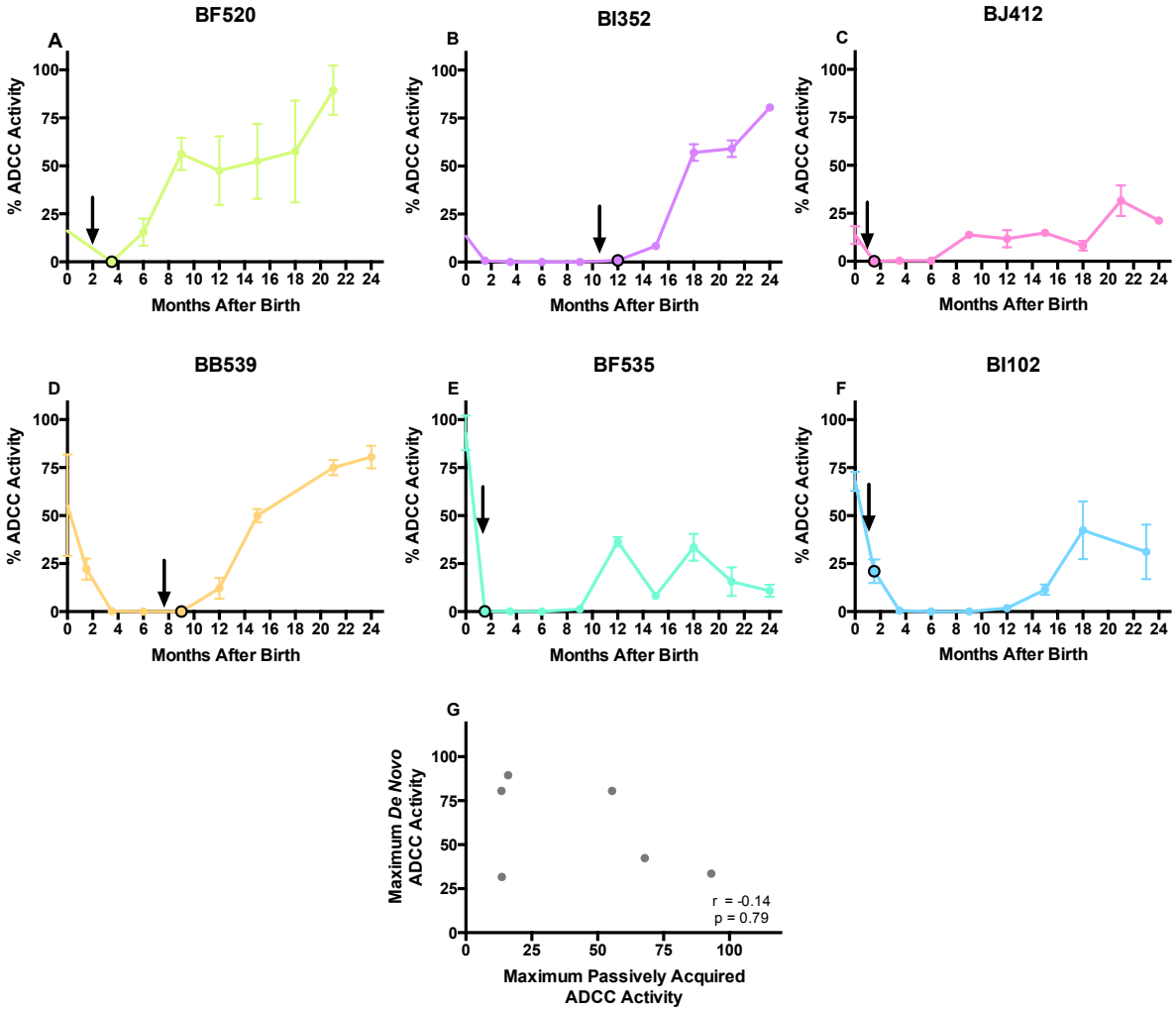


Figure 2.8 Kinetics of ADCC antibody responses in HIV-infected infants

(A-F) ADCC antibody activity in plasma in longitudinal samples from 6 HIV-infected infants. Three had passively acquired ADCC activity < infected infant cohort median (A,B,C), and three had activity > median (D,E,F). Black arrow indicates estimated time of infection defined as the midpoint between the last negative and first positive DNA test. Unfilled circular dot represents first HIV DNA positive test. Results represent mean ± SD for 2 independent experiments in duplicate. (**G**) Correlation between the passively acquired ADCC antibody response (measured in the first week of life) and the maximum *de novo* ADCC antibody activity measured after infection, for the six infants.

DISCUSSION

Defining the role of HIV-specific antibodies in protection in natural infection settings is important to inform rational vaccine design. Insights from animal models and studies of viral control in chronic HIV infections provide indirect support for ADCC antibodies in protection,

but these settings do not directly address the efficacy of antibodies present at the time of exposure in humans. In this study, we sought to understand whether pre-existing ADCC antibody activity provided protection from infection and/or disease progression in HIV-exposed infants. Here we report an association between pre-existing HIV-specific ADCC antibody activity and better clinical outcome in humans.

We found that uninfected infants had higher ADCC antibody activity than infected infants, however, this association was not statistically significant. Our power to detect a truly significant difference was limited by the small numbers of infected infants, particularly when considering only those infants infected in the first 6 weeks of life. Prior studies of similar or smaller size also did not detect a significant association between ADCC antibody activity and infant infection risk, although these studies were limited by the lack of plasma samples from the most relevant window for measuring protection, lack of relevant envelope antigens, and/or imprecise measures of infection timing (44,46,47,49). Thus, larger studies using well-timed samples tested against antigen representing circulating viruses are needed to clarify if higher pre-existing ADCC activity in infants is a significant correlate of protection from infection.

A correlate of protection was evident when examining clinical outcome. In infected infants, each 10% increase in ADCC activity was associated with a 49.1% reduction in the risk of death. Interestingly, two older studies also observed a positive association between ADCC antibody activity and clinical outcome in infected infants even though ADCC activity was measured at various ages (44,46). Due to this variation in sample timing, the contribution of *de novo* versus pre-existing ADCC antibodies in protection was unclear, and *de novo* responses may have been primarily measured in these older studies. In our results, *de novo* ADCC levels did not correlate with passively acquired activity, further supporting the role of pre-existing antibodies in

the survival benefit we observed. As binding titers measure a contribution of both non-neutralizing (including ADCC) and neutralizing antibodies, the role of ADCC antibodies in survival was also indirectly supported by the trend between IgG binding titers and survival. Additionally, the observation that passive NAbs were not associated with outcome suggests that the infant ADCC activity measured was not a surrogate for overall HIV-specific antibody activity but rather was specific for the ability of antibodies to mediate ADCC.

In addition to the positive association between pre-existing ADCC antibody activity and infected infant survival, we also observed a trend toward a negative correlation between infant ADCC and set point viral load. Unfortunately, our ability to detect a true association between ADCC and set point was limited by the sparse set point viral load data for the infected infants. Nevertheless, the observed trend supports the hypothesis that pre-existing antibodies may have acted to clear infected cells, thereby lowering set point viral loads and protecting against disease progression. This hypothesis is supported by the fact that infant set point viral loads are predictive of HIV disease progression (121,122) and is consistent with macaque vaccine studies showing pre-existing ADCC antibody activity is associated with lower viremia in macaques infected by virus challenge (reviewed in (112)). The association between pre-existing ADCC activity and viral control should be analyzed in future human vaccine studies to understand if ADCC provides a therapeutic effect in individuals who become infected.

Interestingly, our results suggest that IgG1, and not IgG3, was important for the ADCC activity and survival effect observed in this study. While IgG1 and IgG3 are major mediators of ADCC activity, recent data from RV144 suggested that vaccine-induced IgG3 was important for the ADCC activity and protective effect observed in that trial (123,124). In our study, IgG3 levels did not correlate with risk of infant infection or survival in infected infants. IgG1 levels,

however, did directly correlate with survival in infected infants. This difference between our study and the RV144 results highlights differences in antibodies elicited by natural infection versus the ALVAC/ AIDSVAX vaccination method and suggests that IgG1 ADCC-mediating antibodies could be an important component of an effective vaccine response.

De novo ADCC activity was detected as early as 2 months post-infection and 6 months of age in our study, which is earlier than has previously been reported for infant HIV-specific ADCC antibodies (49). Although numbers are small, there was evidence that responses were delayed in infants infected within 6 weeks of birth. A delay in *de novo* responses may be due to the detrimental impact of early HIV infection on the developing immune system (reviewed in (125)). While adult ADCC antibody responses have been suggested to peak at approximately 6 months post-infection (126), infant *de novo* responses continued to increase for over a year post-infection in three infants. These ADCC levels exceeded those acquired by passive transfer, and this difference from adults may be due to the higher peak viral loads and thus increased antigenic stimulation observed in infants (116).

Unexpectedly, transmitting and non-transmitting mothers had nearly identical ADCC levels, and in those mothers who transmitted, maternal ADCC antibody levels were not predictive of infant survival. This difference between maternal and infant ADCC activity may be partially explained by the timing of maternal sampling. We sampled maternal plasma primarily from the third trimester of pregnancy as the majority of passive antibody transfer occurs during this time. However, as reports suggest that the majority of passive transfer occurs within the last few weeks of pregnancy (117), samples from earlier in the third trimester may not be representative of the infant antibody repertoire at birth, particularly if there is variation in gestational age. This variation in the time when the infant was born in relation to when the

maternal antibodies were sampled may have allowed us to discriminate the effect of maternal versus passive antibodies in protection. A more intriguing possibility is that the differences between maternal and infant ADCC are due to differences in passive transfer of antibodies to the infants. IgG subclass and the glycosylation profile of antibodies impact ADCC activity and have also been suggested to be differentially transferred across the placenta (127-132), suggesting that maternal ADCC antibody levels may not always be indicative of infant repertoires. In any case, these results have important implications given that prior studies often focused on the relationship between maternal antibody responses, rather than infant responses, and protection. The observed differences in infant and maternal responses suggest that maternal samples may not be an accurate surrogate measure of passively acquired responses in infants for some antibody functions such as ADCC.

In summary, we show that pre-existing HIV-specific ADCC antibody activity is associated with survival in HIV-infected infants. This association was detected when measuring infant passive antibody levels but not maternal antibodies. Infected infant survival was also associated with HIV-specific IgG1, and not IgG3, levels and was not linked to *de novo* ADCC activity. These data support a role for developing vaccines that are designed to elicit ADCC-mediating IgG1 antibodies.

Chapter III

The Role of FcγRIIa and FcγRIIIa Genotypes in HIV Mother-to-Child Transmission

INTRODUCTION

Recent data from human and macaque studies suggest that non-neutralizing HIV-specific antibodies may play a role in protection from infection and/or disease progression (Reviewed in (112)). In particular, functions mediated through the Fc (constant) portion of the antibody, such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cell-mediated viral inhibition (ADCVI), have been implicated in protection from infection and disease progression in multiple settings.

These Fc-mediated antibody functions depend on host Fc gamma receptors (FcγRs) that bind to the Fc portion of antibodies. The cross-linking of infected cells or antigen and effector cells bearing FcγRs by antibodies results in Fc-mediated activity and clearance of the infected cells. Single nucleotide polymorphisms (SNPs) in FcγRs have been shown to affect IgG binding affinity, and thus, alter the ability of an individual's effector cells to facilitate Fc-mediated antibody functions, including ADCC (50-52,59-61).

Two FcγRs of particular interest are FcγRIIa (CD32a) and FcγRIIIa (CD16a). FcγRIIa is one of the most common FcRs and is found on the surface of macrophages, monocytes, and dendritic cells in addition to other innate immune cells. The RNA for this receptor encodes either a histidine (H) or arginine (R) at codon 131 (57,58). The H allele is associated with higher affinity binding to IgG, especially IgG2 (52,58,59). FcγRIIIa is less widely expressed than FcγRIIa, but is found on monocytes, macrophages, some T-cell subsets. Additionally, FcγRIIIa is the major FcγR found on natural killer (NK) cells, a key mediator of ADCC activity. FcγRIIIa

encodes either a valine (V) or phenylalanine (F) at codon 158, and the V form is associated with higher affinity binding to IgG (52,62,63). Cells expressing receptors with higher affinity alleles (FcγRIIa-H131 and FcγRIIIa-V158) have been shown to mediate increased ADCC and other Fc-mediated effector functions compared to cells expressing their lower affinity counterparts (50,51,61). Studies examining the impact of FcγRIIa and FcγRIIIa polymorphisms on HIV acquisition and progression, however, have produced variable results (60,61,68-76).

FcγR genotype may be particularly relevant to mother-to-child transmission (MTCT) because ADCC activity has been implicated in both maternal transmission risk as well the disease course in infants who acquire HIV (45,133). With regards to MTCT, FcγRIIa polymorphisms have been previously examined in one study (69). This study found that infants homozygous for the high affinity FcγRIIa allele (H131) had an increased risk of infection, suggesting an antibody-dependent enhancement of infection. The study, however, did not examine the impact of FcγRIIIa genotype on infection risk and this gene is particularly relevant because FcγRIIIa is the main FcγR on natural killer cells, which are major mediators of ADCC.

This study addresses the impact of FcγRIIa and FcγRIIIa polymorphisms on mother-to-child transmission in a historical cohort in which ADCC has been previously described as a correlate of protection (45,133). In this Chapter, we show that infant genotypes are not associated with HIV infection or disease progression. In mothers, FcγRIIa genotype was not associated with transmission, but there was some evidence of FcγRIIIa genotype impacting early breastfeeding transmissions.

MATERIALS AND METHODS

Study Design

Samples from the Nairobi Breastfeeding Clinical Trial (1992-1998) (24), which was originally conducted to determine the impact of formula feeding vs. breastfeeding on infant infection risk and mortality, were selected for study. At the time of the original trial, antiretrovirals were not the standard of care in Kenya, and thus no mothers or infants received treatment for prevention of MTCT. The original study included 425 mother and infant pairs that were monitored for trial endpoints and samples for this study were selected based on availability of HIV infection outcome and sample availability (N = 379). Infants were tested for HIV DNA at birth, 6 weeks, 14 weeks, and every 3 months until two years of age. For those infants who tested positive, samples prior to the first HIV DNA positive test were screened for HIV RNA to more precisely define infection timing. Time of infection was estimated as the midpoint between the last negative HIV DNA or RNA test and the first positive test. Regular sampling allowed for accurate estimation of infection timing as well as disease progression in those infants who became infected.

Maternal RNA plasma viral loads from enrollment during pregnancy (N = 362) were used for calculations (24). If maternal viral load from pregnancy was not available (N = 17), the first available viral load after delivery was used. Breast milk RNA viral loads were available for 265 women (134). The earliest breast milk viral load available was used for each woman; the majority were from the first 6 weeks after delivery (N = 244), the remaining were from week 7 to month 7 after delivery (N = 21) Infant set point viral load was defined as the first viral load available 4 to 12 months post estimated infection (116). For viral loads less than the assay cutoff, the viral load was set at the midpoint between 0 and the cutoff.

In addition to viral load data, data on pregnancy and delivery were available for the mothers and infants. These data included: maternal gravidity, delivery type (vaginal vs. Cesarean section), membrane rupture during delivery, labor duration (hours), maternal CD4 count, infant birth weight, and infant prematurity. Infant prematurity was defined as delivery at <37 weeks gestation and birth weights <2500 grams were considered low.

FcγR Genotyping

DNA extracted from blood (peripheral blood mononuclear cells (PBMCs) or filter paper), breast milk, or cervical/vaginal secretion samples was available for most moms and infants (135-138). If DNA was not already available, it was extracted from plasma samples (31 mothers, 9 infants) using the Qiagen DNEasy kit (Qiagen) or filter paper (6 infants) using the Qiagen QIAamp DNA Mini Kit (Qiagen) per manufacturer's protocol.

FcγRIIa and FcγRIIIa genotype was determined using the Taqman SNP Genotyping assays C_9077561_20 and C_25815666_10, respectively (Life Technologies, Carlsbad, CA). Samples were run on an ABI Prism 7700 Sequence Detection System (Perkin-Elmer). Included in each run of samples was control PBMC DNA from the Coriell Cell Repository (Camden, NJ). Each run contained at least one control of each genotype at multiple concentrations (1ng, 10ng, or 50ng), which represents the range of DNA concentrations recommended by the manufacturer.

Control DNA sequence was verified by Sanger sequencing. Specifically, FcγRIIa and FcγRIIIa gene fragments were amplified using the following primers: FcγRIIa forward (FcgR2A.F): TTGGGATCTATCCTTACAAC, FcγRIIa reverse (FcgR2A.R): CCTACTTGTTGGTCAATACT, FcγRIIIa forward (FcgR3A.F3): CACCGTGGGTGTGATTAGC, FcγRIIIa reverse (FcgR3A.R2):

CCAAAAGCCACACTCAAAGAC. Cycling parameters were: 5 minutes 94°C, 30 cycles of 1 minute 94°C, 1 minute 47°C (FcγRIIa) or 57°C (FcγRIIIa), 1 minute 72°C, followed by 8 minutes at 72°C and a 4°C hold. PCR products were visualized by gel electrophoresis and then sequenced (Big Dye; Applied Biosystems) for genotype using the FcgR2A.F or FcgR3A.R2 primers. This sequencing protocol and the TaqMan assays were validated using a subset of 57 DNA samples that had been previously genotyped by Dr. Donald Forthal's laboratory at UC Irvine. With the 57 DNA samples, there was 100% concordance for both FcγRIIa and FcγRIIIa genotypes when comparing results from the two labs.

The control DNAs and their genotypes verified by sequencing and Taqman SNP assays are shown in **Table 3.1**. Of note, the observed FcγRIIIa genotypes for samples from the Coriell cell repository did not match the genotypes reported by the repository. However, we were confident in our genotyping based on the fact that: 1) Our TaqMan and Sanger sequencing genotyping results agreed and 2) We validated our TaqMan and Sanger sequencing assays with samples independently genotyped in another laboratory. All FcγRIIa genotypes matched those reported by the repository.

Table 3.1 Control DNA genotypes verified by TaqMan and Sanger sequencing
FcγRIIa and FcγRIIIa genotypes for Coriell Cell Repository DNA are reported below. Of note, the genotypes we observed for FcγRIIIa differed from that reported by the repository (shown in parentheses). Catalog ID is from the Coriell Cell Repository.

Sample (Catalog ID)	**FcγRIIa Genotype**	**FcγRIIIa Genotype** (reported genotype)
NA26 (NA18526)	H/H	F/F (V/V)
NA32 (NA18532)	H/R	F/F (V/F)
NA40 (NA18540)	R/R	F/F (V/F)
NA49 (NA18949)	H/H	F/F (F/F)
NA50 (NA18550)	H/R	V/F (V/V)
NA58 (NA18558)	H/H	V/F (V/V)
NA70 (NA18570)	R/R	V/V (V/V)

Statistical Analyses

Chi-squared tests for categorical variables and t-tests with Welch's correction for comparisons of means were used to determine which cohort characteristics were associated with HIV infection/transmission. The associations between FcγRIIa and FcγRIIIa genotypes and infection/transmission were first analyzed using chi-squared tests for independence. Further analyses used logistic regression controlling for appropriate covariates to determine the association between FcγR genotype and infection risk. The association between maternal viral load and genotype was analyzed by linear regression. Kaplan-Meier estimates with log-ranks tests and Cox-proportional hazards models were used to determine the association between genotype and time to infection/time to infant mortality. A Pearson's chi-squared test was used to determine if SNPs were in Hardy-Weinberg Equilibrium and also to determine linkage disequilibrium. Maternal and infant viral loads were $\log_{10}$ transformed for all analyses.

RESULTS

Study Population Characteristics

In this study, 379 moms and their corresponding infants from the Nairobi Breastfeeding Clinical Trial (24) were genotyped for FcγRIIa and FcγRIIIa. Overall, there were 87 infant infections (**Table 3.2**). Mothers who transmitted the virus to their infants had higher plasma viral loads (4.96 vs. 4.47 $\log_{10}$ copies/mL, $p < 0.0001$), lower CD4 counts (360 cells/mm^3 vs. 447 cells/mm^3, $p = 0.0002$), and were more likely to be in the breastfeeding arm of the original study (64.4% vs. 45.2%, $p = 0.002$). Higher breast milk viral loads were also associated with transmission (3.13 $\log_{10}$ copies/mL vs. 2.76 $\log_{10}$ copies/mL, $p = 0.004$). In this cohort (which included *in utero*, delivery and breastfeeding transmissions), maternal age, gravidity, delivery type (vaginal vs. Cesarean section), prolonged membrane rupture (≥4 hours), labor induction, and labor duration were not associated with transmission risk. HIV-infected infants were more likely to be premature (12.7% vs. 4.6%, $p = 0.029$) and there were more deaths during follow-up in infected infants than uninfected infants (44.8% vs. 10.3%, $p < 0.0001$). Infected infants had an average set point viral load of 5.85 $\log_{10}$ copies/mL. These characteristics are similar to those found in the larger Nairobi Breastfeeding Clinical Trial cohort (24,139).

Table 3.2 Infant and maternal cohort characteristics

Data are represented as number (percentage) or mean (standard deviation). NA = not applicable. P-values are from chi-squared tests of categorical variables and t-tests with Welch's correction for comparisons of means.

	HIV Infected Infants	HIV Uninfected Infants	p-value
Number	87	292	
Premature (<37 weeks)	7/55 (12.7%)	9/196 (4.6%)	0.029
Low birth weight (<2500g)	8/83 (9.64%)	16/267 (5.99%)	0.25
Death during 2 year follow-up	39/87 (44.8%)	30/292 (10.3%)	<0.0001
Mean plasma set point viral load (log_{10} copies/mL)	5.85 (0.86)	NA	

	HIV Transmitting Mothers	HIV Non-Transmitting Mothers	
Number	87	292	
Mean age	23.66 (4.09)	23.96 (4.37)	0.56
Mean plasma RNA viral load (log_{10} copies/mL)	4.96 (0.65)	4.47 (0.83)	<0.0001
Mean CD4 count (cells/mm^3)	360 (171)	447 (224)	0.0002
Mean gravidity	2.48 (1.61)	2.31 (1.39)	0.37
Vaginal delivery	77/87 (88.5%)	264/286 (92.3%)	0.27
Prolonged membrane rupture (≥ 4 hours)	35/86 (40.7%)	92/279 (33.0%)	0.19
Mean labor duration	10.18 (5.86)	10.95 (7.38)	0.32
Induced labor	7/87 (8.1%)	18/286 (6.3%)	0.57
Breastfeeding arm of original trial	56/87 (64.4%)	132/292 (45.2%)	0.002
Mean breast milk RNA viral load (log_{10} copies/mL)	3.13 (0.90)	2.76 (0.81)	0.004

FcγRIIa and FcγRIIIa Genotypes

Of the 379 infants genotyped for FcγRIIa, 88 (23.2%) were homozygous for the high affinity allele (H/H), 178 (47.0%) were heterozygous (H/R), and 113 (29.8%) were homozygous for the low affinity allele (R/R) (**Figure 3.1**). Mothers had similar distributions of FcγRIIa alleles: 88 (23.2%) H/H, 174 (45.9%) H/R, and 117 (30.9%) R/R. For the FcγRIIIa genotype, 41 (10.8%) infants were homozygous for the high affinity allele (V/V), 173 (45.6%) were heterozygous (V/F), and 165 (43.5%) were homozygous for the low affinity allele (F/F). Mothers also had similar distributions of FcγRIIIa alleles: 44 (11.6%) V/V, 152 (40.1%) V/F, and 183

(48.3%) F/F. These FcγRIIa and FcγRIIIa genotype distributions are similar to what has been reported in other populations (60,68,69,71,76)

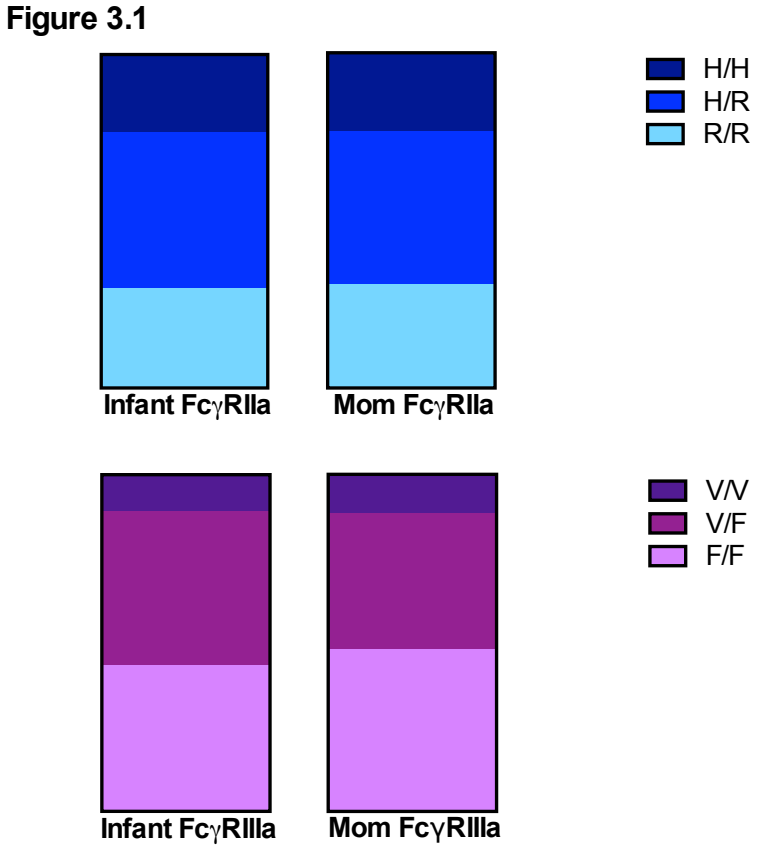


Figure 3.1 Infant and maternal FcγR genotype distributions
Distributions of infant (left) and maternal (right) FcγRIIa (top, blue) and FcγRIIIa (bottom, purple) genotypes. Colors represent different genotypes, indicated at right. Darker colors represent high affinity alleles.

As all subsequent analyses assume that the FcγRIIa and FcγRIIIa SNPs are free from selective pressures and are independent, we next tested for Hardy-Weinberg Equilibrium (HWE) and linkage disequilibrium. Combined data from mothers and infants indicates that the FcγRIIa and FcγRIIIa genotypes do not differ significantly from expected frequencies, suggesting the genotypes are in HWE (**Table 3.3**). While there was some evidence that FcγRIIa genotype was not in HWE ($\chi^2 = 3.35$, $p = 0.07$), using combined data from related individuals (i.e. mothers and

infants) can increase the type I error rate (140). Furthermore, separate analyses of FcγRIIa genotype distributions in mothers and infants, suggests that the SNP is under HWE (**Table 3.3**). With regard to linkage disequilibrium, in a chi-squared test for independence, the two genes did exhibit the potential for linked inheritance: $\chi^2 = 11.36$, $p = 0.02$ (**Table 3.4**). These data show some evidence of linkage disequilibrium and suggest that higher affinity alleles tend to be more likely to be inherited together. This evidence of potential linkage disequilibrium between FcγRIIa and FcγRIIIa is consistent with reports from other cohorts (76,141).

Table 3.3 FcγRIIa and FcγRIIIa SNP tests for Hardy-Weinberg Equilibrium

SNP	Pearson χ^2	p-value
FcγRIIa		
combined	3.35	0.07
infants	1.21	0.27
mothers	2.21	0.14
FcγRIIIa		
combined	0.48	0.49
infants	0.19	0.66
mothers	2.03	0.16

Table 3.4 Test for linkage disequilibrium between FcγRIIa and FcγRIIIa SNPs
Data are represented as observed frequency (expected frequency) for each haplotype. Genotype data for mothers and infants are combined.

	FcγRIIIa Genotype		
FcγRIIa Genotype	**V/V**	**V/F**	**F/F**
H/H	27 (19.7)	85 (75.5)	64 (80.8)
H/R	37 (39.5)	151 (150.9)	164 (161.6)
R/R	21 (25.8)	89 (98.6)	120 (105.6)

$\chi^2 = 11.36$, $p = 0.02$

FcγRIIa Genotypes and HIV Risk

The association between infant/maternal genotype and infection/transmission was analyzed by a chi-squared test for independence for each SNP. Infant FcγRIIa genotype was not associated with infection status ($\chi^2 = 1.23$, p = 0.54; **Table 3.5**). Similarly, maternal FcγRIIa genotype was not associated with transmission ($\chi^2 = 0.90$, p = 0.64).

One previous study of FcγRIIa genotypes in mother-to-child transmission in Kenya found a positive association between infant FcγRIIa genotypes and infant infection risk (69). Specifically, this study by Brouwer *et al*. was of similar sample size and found that infants who were homozygous for the high affinity allele (H/H) had an increased risk of perinatal infection (defined as infection within the first four months). This relationship remained significant when controlling for factors associated with infant infection risk in their cohort (gravidity, birth weight, placental malaria, maternal viral load, and duration of labor). Although we did not observe an association between infant FcγRIIa genotype and overall infection risk, we decided to restrict our analyses to only those infants infected within the first four months to determine if the genotype impacts perinatal infection in the time interval studied by Brouwer *et al*. Of the 87 infected infants, 62 were infected within the first 4 months of life (71.3%). Infants infected after 4 months of life were considered "uninfected" per the methodology of the previous study. Thus, comparing those infants infected within the first four months to those infected after 4 months/those who remained uninfected, the high affinity FcγRIIa genotype (H/H) was not associated with infection risk (p = 0.61; **Table 3.6**). Similarly when controlling for risk factors (maternal viral load, gravidity, labor duration, and infant birth weight) in a multivariate logistic regression, the relationship between infant FcγRIIa genotype and infection status remained non-significant (p=0.70).

In the previous study by Brouwer *et al*., only women who were asymptomatic for HIV infection were included, and thus, the viral loads in that study were lower than viral loads in the Nairobi Breastfeeding Clinical Trial cohort. In the Brouwer *et al*., the median viral load for transmitting mothers was reported to be 3.89 $\log_{10}$ copies/mL, while for non-transmitting mothers it was 3.01 $\log_{10}$ copies/mL (69). In the mothers included in this study, median viral loads were over a log higher: 4.97 $\log_{10}$ copies/mL for transmitting women and 4.56 $\log_{10}$ copies/mL non-transmitting mothers. It is possible that infant FcγRIIa may only impact transmissions that occur at low viral loads. To address this possibility, we restricted our analyses to women with viral loads less than the median viral load (4.68 $\log_{10}$ copies/mL) in our cohort. In this subset (N=189), the median maternal plasma viral load was 4.10 $\log_{10}$ copies/mL and there were 14 infected infants. Similar to results with the overall cohort, in infants whose mothers had low viral loads, the high affinity infant FcγRIIa genotype (H/H) was not associated with infection (OR 0.84; 95% CI: 0.16 to 4.39; p = 0.84).

Table 3.5 Maternal and infant genotypes by transmission/infection status
Data represent percentage of infants or mothers with indicated genotype.

		HIV Infected/ Transmitter (N=87)	HIV Uninfected/ Non-Transmitter (N=292)	χ^2	p-value
Infant FcγRIIa Genotype					
	H/H	27.59	21.92		
	H/R	43.68	47.95	1.23	0.54
	R/R	28.74	30.14		
Maternal FcγRIIa Genotype					
	H/H	26.44	22.26		
	H/R	45.98	45.89	0.90	0.64
	R/R	27.59	31.85		
Infant FcγRIIIa Genotype					
	V/V	10.34	10.96		
	V/F	49.43	44.52	0.66	0.72
	F/F	40.23	44.52		
Maternal FcγRIIIa Genotype					
	V/V	8.05	12.67		
	V/F	50.57	36.99	5.44	0.07
	F/F	41.38	50.34		

Table 3.6 Comparison of Brouwer *et al.* and Nairobi Breastfeeding Clinical Trial FcγRIIa Results

NBT = Nairobi Breastfeeding Trial; Brouwer Analysis results from (69).

	Univariate Analysis			Multivariate Analysis*		
Infant Genotype	**Odds Ratio**	**95% CI**	**p-value**	**Odds Ratio**	**95% CI**	**p-value**
NBT Analysis						
H/H	1.19	0.61-2.33	0.61	1.15	0.56-2.38	0.70
H/R	1 (Reference)			1 (Reference)		
R/R	1.02	0.53-1.94	0.96	1.09	0.54-2.19	0.81
Brouwer Analysis						
H/H	1.78	1.05-3.03	0.034	2.22	1.23-4.02	0.009
H/R	1 (Reference)			1 (Reference)		
R/R	0.56	0.29-1.09	0.086	0.67	0.33-1.37	0.276

*NBT and Brouwer multivariate analyses adjusted for gravidity, infant birth weight, maternal viral load, and duration of labor. The Brouwer analysis also adjusted for placental malaria.

FcγRIIIa Genotypes and HIV Risk

With regard to FcγRIIIa, infant genotype was not associated with infection status (χ^2 = 0.66, p = 0.72; **Table 3.5**). There was, however, a trend for an association between maternal FcγRIIIa genotype and transmission (χ^2 = 5.44, p = 0.07; **Table 3.5**). Unexpectedly, heterozygote mothers appeared to be at greatest risk of transmission. When dichotomizing mothers into FcγRIIIa heterozygotes (V/F) and homozygotes (V/V or F/F), in a Cox-proportional hazards model, heterozygotes had a 64.5% increased risk of transmission compared to homozygotes (p = 0.02). Furthermore, in a logistic regression analysis controlling for risk factors associated with maternal transmission (maternal plasma viral load, breastfeeding, and infant prematurity) the heterozygote genotype (V/F) was significantly associated with increased odds of infant infection compared to FcγRIIIa low affinity homozygotes (F/F) (OR: 2.17, 95% CI: 1.11 to 4.24; p=0.024). When comparing mothers with at least one high affinity allele (V/F or V/V) to those mothers with only the low affinity allele (F/F), there was not a statistically significant association between genotype and transmission (χ^2 = 2.16, p = 0.14), suggesting that the

presence of the high affinity allele alone was not associated with transmission. Infant FcγRIIIa heterozygotes were not at an increased risk of infection compared to homozygotes in a Cox-proportional hazards model (HR: 1.18, p = 0.44)

We next examined time to infant infection using Kaplan-Meier analyses to determine if maternal FcγRIIIa genotype was associated with a particular transmission mechanism. In a log-rank test, there was a trend in association between maternal FcγRIIIa genotype and time to infection in infants ($\chi^2 = 5.89$, p=0.053). The Kaplan-Meier curves suggest the majority of the excess transmission risk associated with heterozygotes occurs during the peripartum period, with most infections occurring in the first 2-3 months (**Figure 3.2A**). During this time, infections may be due to delivery or early breastfeeding. To address these two possibilities, we dichotomized the mothers into those who breastfed (N=188) and those who formula fed (N=191). The excess peripartum transmission risk in heterozygotes was observed in the breastfeeding mothers but not the formula feeding mothers (**Figure 3.2B,C**) The effect in breastfeeding mothers was not statistically significant by log-rank analyses ($\chi^2 = 4.14$, p=0.13), but may have been limited by power. However, in a Cox-proportional hazards model, a trend in heterozygote breastfeeding mothers having increased risk of transmission compared to homozygotes was observed (HR: 1.64, p = 0.064).

As FcγR genotypes influence antibody effector functions (e.g. ADCC and ADCVI) that may modulate viral load (Reviewed in (112,142,143)), and as maternal breast milk viral load is a major risk factor for transmission (134), we hypothesized that FcγRIIIa genotype impacts breast milk viral loads in mothers. Maternal FcγRIIIa heterozygotes (V/F; 2.73 $\log_{10}$ copies/mL) did have slightly higher median breast milk viral loads than V/V (2.61 $\log_{10}$ copies/mL) and F/F

(2.54 $\log_{10}$ copies/mL) homozygotes, however, in a linear regression analysis, maternal FcγRIIIa genotype was not associated with breast milk viral load ($p = 0.37$; **Figure 3.2D**).

Unlike maternal FcγRIIIa genotype, maternal FcγRIIa and infant FcγRIIa and FcγRIIIa genotypes were not associated with time to estimated infection (**Figure 3.3 A-C**).

Figure 3.2

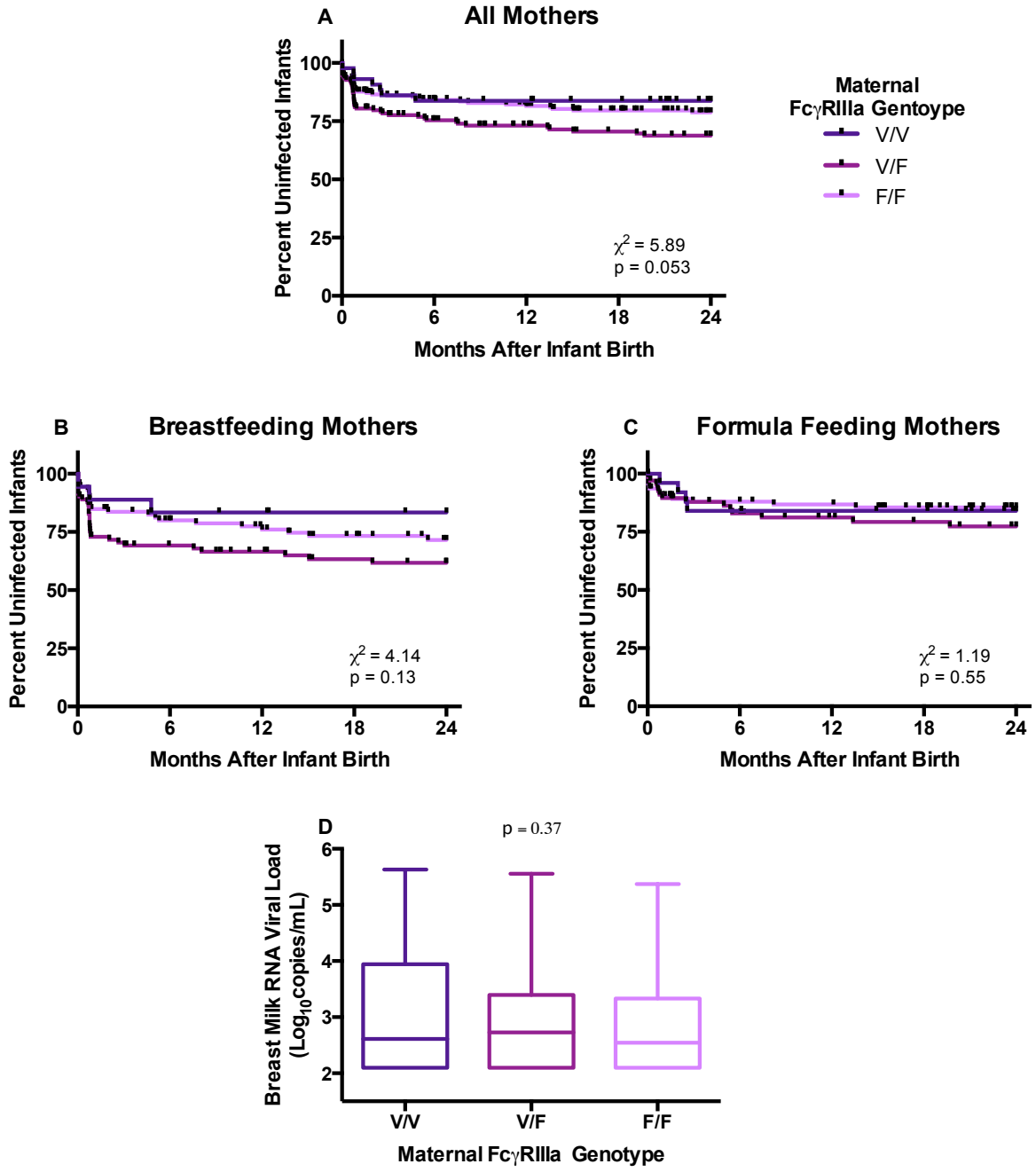


Figure 3.2 Impact of maternal FcγRIIIa genotype on time to transmission and breast milk viral loads

Kaplan-Meier estimates for time to infant infection by maternal FcγRIIIa genotype for all mothers (A), breastfeeding mothers (B), and formula feeding mothers (C). Breast milk viral load by maternal FcγRIIIa genotype (D).

Figure 3.3

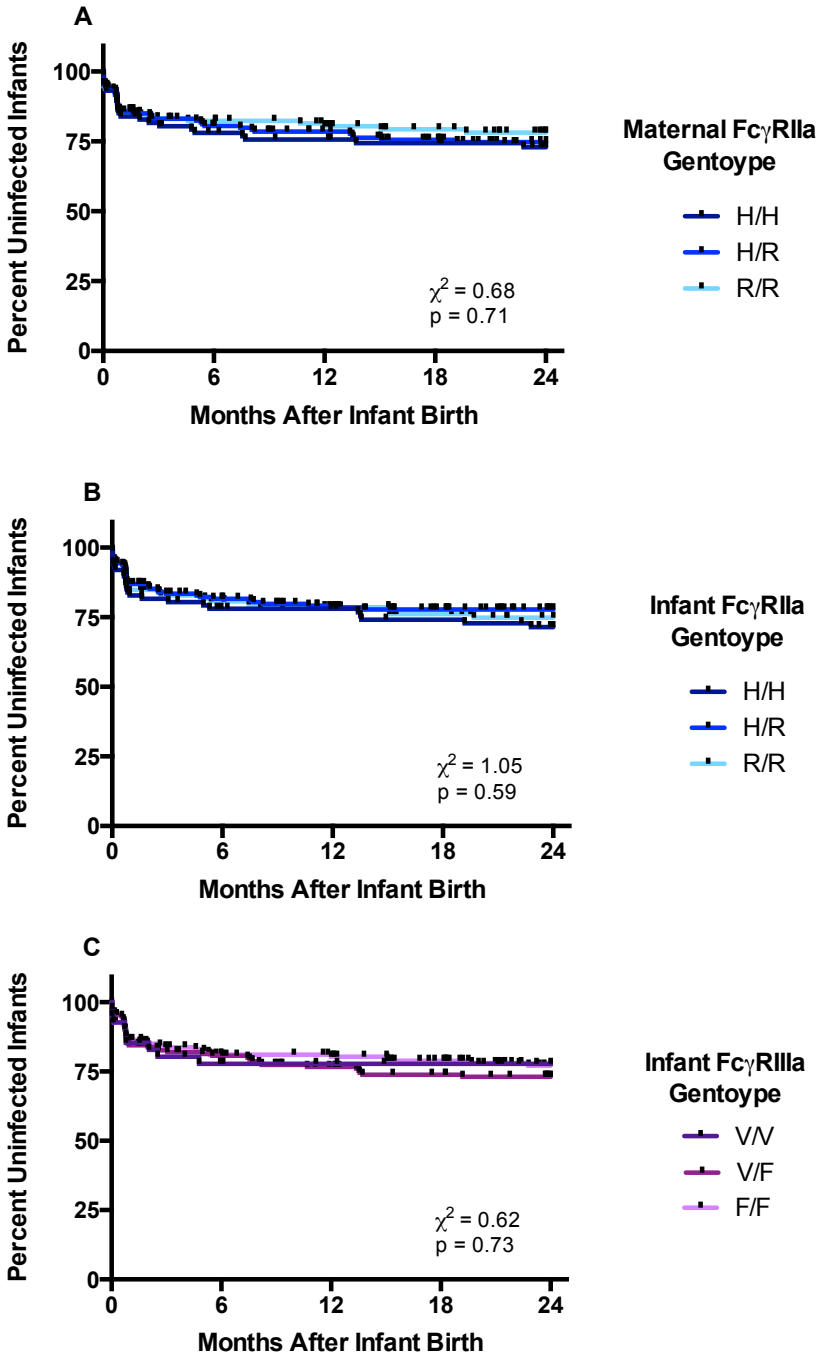


Figure 3.3 Impact of maternal FcγRIIa and Infant FcγRIIa and FcγRIIIa genotypes on time to infant infection

Kaplan-Meier estimates for time to infant infection by maternal FcγRIIa (A), infant FcγRIIa (B) or infant FcγRIIIa (C) genotype.

FcγRIIa and FcγRIIIa Genotypes on Infant Disease Progression

Although infant FcγR genotypes were not associated with infant infection risk, genotypes may alter the course of disease in infected infants. As passively acquired Fc-mediated ADCC activity has been associated with increased survival in HIV-infected infants in this cohort (133), we hypothesized that high affinity alleles (FcγRIIa H131 and FcγRIIIa V158) would be associated with slower disease progression. We examined both set point viral load and time to mortality after estimated infection as measures of disease progression in the cohort. Set point viral loads were available for 49 (56.3%) infected infants (116). In these infants, set point viral load was not associated with either FcγRIIa ($p = 0.35$) or FcγRIIIa ($p = 0.70$) genotypes (**Figure 3.4 A,B**). In the 87 infected infants, there were 39 deaths (44.8%) during the two-year follow-up. FcγRIIa ($\chi^2 = 3.68$, $p=0.16$) and FcγRIIIa ($\chi^2 = 0.10$,$p=0.95$) infant genotypes were not associated with mortality after estimated infection in log-rank analyses of Kaplan-Meier survival estimates (**Figure 3.4 C,D**). When comparing infant FcγRIIa heterozygotes (H/R) to homozygotes (H/H and R/R), there was a trend in survival in infected infants: heterozygote infants had reduced survival ($\chi^2 = 3.67$, $p = 0.06$).

Figure 3.4

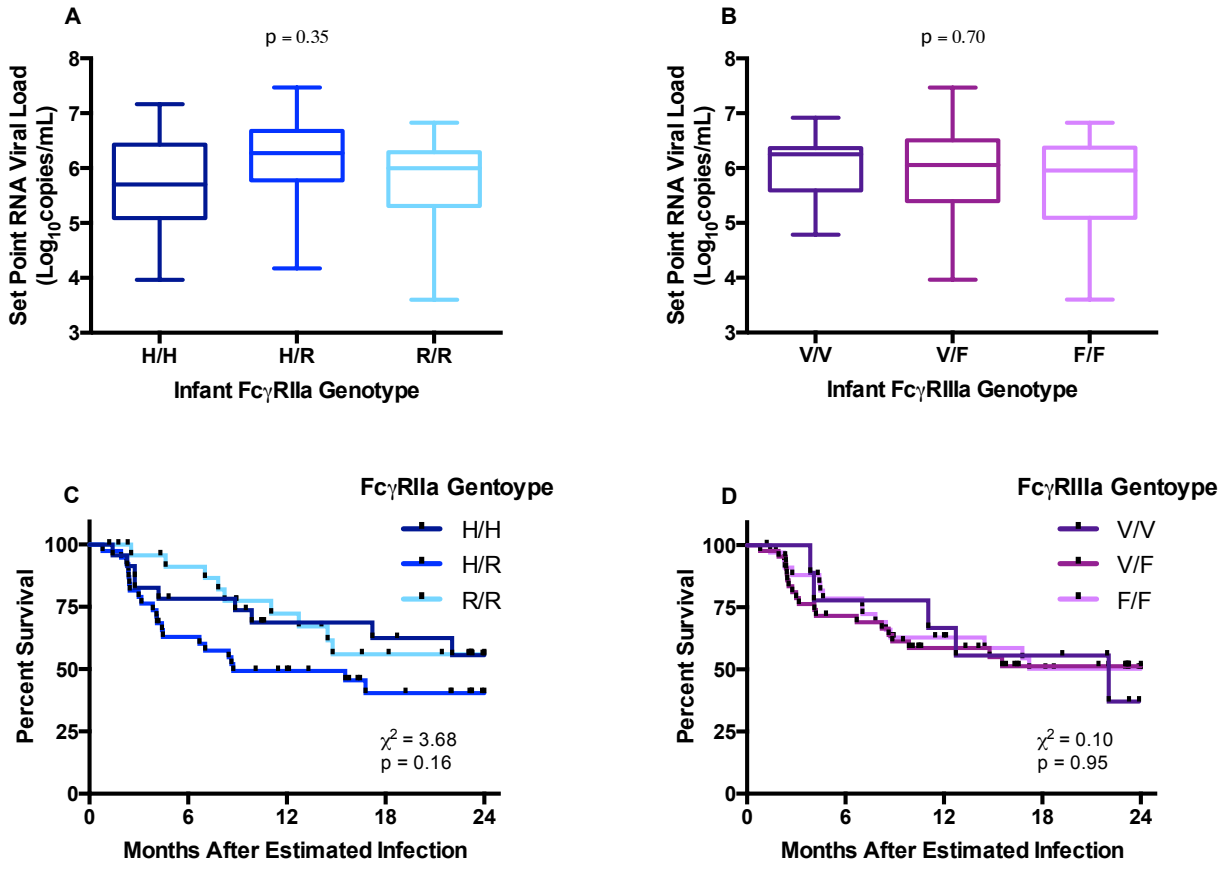


Figure 3.4 FcγR genotypes and disease progression in infected infants
Infant set point viral load by infant FcγRIIa (A) and FcγRIIIa (B) genotypes. Kaplan-Meier estimates for survival after estimated infection for by infant FcγRIIa (C) and FcγRIIIa (D) genotypes.

FcγR Genotypes and HIV Status in Infants with Known Durations of Follow-Up

As some infants in the cohort were lost to follow-up prior to two years of age, the results of maternal and infant FcγR genotypes and infection/transmission may have been affected by infants who were misclassified as uninfected but may have died or been lost to follow-up prior to diagnosis. To address this possibility, we limited our analyses to only those infants with known infection or who were uninfected at 12 months of age (i.e. to rule out infants that were lost to follow-up or died prior to 12 months who may have been infected). With these restrictions, 329 mother/infant pairs were included in the analysis (50 pairs excluded). We then examined the

association between maternal and infant genotypes and transmission/infection status by chi-squared analyses. Similar to results for the overall cohort, infant FcγRIIa, infant FcγRIIIa, and maternal FcγRIIa genotypes were not associated with infection or transmission (**Table 3.7).** Maternal FcγRIIIa genotype was associated with transmission in this subset ($\chi^2 = 7.27$, $p = 0.03$), supporting the trend observed in the overall cohort.

We also further limited the analyses to only those infants with known infection or who were followed for the entire 24-month study period. In this cohort of 274 mother/infants pairs, no genotype was associated with transmission or infection. Of note, the maternal FcγRIIIa genotype was no longer associated with transmission ($\chi^2 = 3.51$, $p = 0.17$), but we may have had limited power to detect an association as 105 mothers were excluded in this analysis.

Table 3.7 Maternal and infant genotypes and transmission/infection status in infants with known follow-up.

Association between genotype and infection/transmission status. This analysis was restricted to HIV-negative infants with follow-up data until at least 12 months of age. Data represent percentage of infants or mothers with genotype indicated.

		HIV Infected/ Transmitter (N=87)	HIV Uninfected/ Non-Transmitter (N=242)	χ^2	p-value
Infant FcγRIIa Genotype					
	H/H	27.59	23.14		
	H/R	43.68	49.17	0.95	0.62
	R/R	28.74	27.69		
Maternal FcvRIIa Genotype					
	H/H	26.44	24.38		
	H/R	45.98	42.98	0.76	0.68
	R/R	27.59	32.64		
Infant FcγRIIIa Genotype					
	V/V	10.34	10.74		
	V/F	49.43	43.39	0.99	0.61
	F/F	40.23	45.87		
Maternal FcγRIIIa Genotype					
	V/V	8.05	14.05		
	V/F	50.57	34.71	7.27	0.03
	F/F	41.38	51.24		

DISCUSSION

In this study, we examined FcγRIIa and FcγRIIIa genotypes in a cohort in which Fc-mediated antibody responses in the mother and infant have been shown to be predictive of transmission risk and infected infant survival (45,133). Overall, infant FcγRIIa and FcγRIIIa genotypes were not associated with infant infection or disease progression, indicating that these polymorphisms may not be important predictors of HIV outcome. In mothers, FcγRIIa genotype was not associated with transmission, but FcγRIIIa heterozygotes had an increased risk of virus transmission.

Our finding that infant FcγRIIa genotype does not impact infant infection risk differs from one previous study of MTCT in Kenya that observed an increased risk of infection in infants with the high affinity FcγRIIa genotype (69). This older study by Brouwer *et al*. was of similar sample size but only considered perinatal infections detected within the first 4 months of life. Restricting our analyses to a similar time point, we did not observe a significant correlation between infant FcγRIIa genotype and infection risk. Another difference between the two studies is that in Brouwer *et al*., mothers were only included if they had no underlying chronic diseases and were asymptomatic for HIV-infection, but no such restrictions were made in the Nairobi Breastfeeding Clinical Trial. Accordingly, mothers in Brouwer *et al*. had median viral loads almost a log lower than the mothers in this study, and thus were presumably a subset of women who were at lower risk for transmission. It is possible that FcγRIIa effects are most pronounced in settings of low risk, i.e. mothers of lower viral load, however, when only considering mothers with viral loads below the median in our cohort, infant FcγRIIa still did not correlate with infection. Overall, the differences in results between the two studies are not readily explained

and further studies in MTCT cohorts are needed to clarify the impact of FcγRIIa genotype on infant infection risk.

Infant genotype was also not associated with infected infant disease progression. We measured disease progression using both set point viral load and mortality after estimated infection, which have both been previously used as markers of infant disease progression (121,122,133). These results support conclusions from Brouwer *et al.* who also did not observe an association between FcγRIIa genotype and infant mortality (69). Overall, these data suggest that FcγRIIa and FcγRIIIa genotypes like do not substantially contribute to disease progression in HIV-infected infants.

The observation that infant FcγRIIa and FcγRIIIa genotypes do not influence infant infection or disease progression has important implications for treatment and therapy. Given that Fc-mediated ADCC activity has been suggested to provide protective and/or therapeutic benefits (Reviewed in (112)), our results suggest that prevention or treatment outcomes of infants passively immunized with ADCC-mediating antibodies may be independent of FcγR genotype. Similarly, protection mediated by vaccines that elicit ADCC antibodies may not be dependent on FcγRIIa and FcγRIIIa genotypes. However, the results from this study do not rule out the possibility that other host FcγR SNPs/copy number variations or host Fc genotypes may impact infant infection risk and disease progression. For example, results from the RV144 vaccine trial, which showed evidence of protection in vaccinated adults, suggest that a FcγRIIc polymorphism, which has unclear functional significance, was associated with protection (75).

In mothers, FcγRIIa genotype was not associated with transmission, but there was evidence that FcγRIIIa genotype was associated with transmission risk. Unexpectedly, FcγRIIIa heterozygotes (V/F) had increased risk of transmission compared to homozygotes (V/V or F/F).

In particular, the increased risk in heterozygotes appeared to be during the peripartum period, especially in breastfeeding mothers - indicating that early breastfeeding transmissions may be impacted by FcγRIIIa genotype. The biological mechanism for how this heterozygote disadvantage may occur, however, is unclear. *In vitro* data suggest that heterozygotes bind antibodies and have ADCC activity that is either equal to high affinity homozygotes (V/V), or intermediate between high affinity (V/V) and low affinity (F/F) homozygotes (50,51,61,144). Additionally, in our analyses, maternal FcγRIIIa genotype, was not associated with breast milk viral load, a major risk factor for breastfeeding transmission during the early breastfeeding period (24,134). Thus, the observed heterozygote disadvantage does not have a clear biological mechanism to explain the association between genotype and transmission risk. As the significance of this heterozygote disadvantage was modest, it is important that the results be verified in a larger cohort before efforts are made to define the mechanism of this effect.

This study was the first to examine the impact of both FcγRIIa and FcγRIIIa genotypes in HIV-infected mothers and their infants. Importantly, these receptor genotypes were studied in a population in which Fc-mediated ADCC activity has been shown to impact infant outcome (45,133). Overall, the results from this study suggest that infant FcγR genotypes do not impact infant infection or disease progression. In mothers there was some evidence that FcγRIIIa genotypes may impact transmission risk in the early breastfeeding period, however, more work will be needed to confirm this association and explore potential mechanisms.

Chapter IV

Maternal Autologous Neutralizing Antibodies in HIV Mother-to-Child Transmission

INTRODUCTION

The development of an effective HIV-specific neutralizing antibody response remains a major goal of HIV-vaccine research. As a proof-of-concept, neutralizing antibodies have been shown to protect non-human primates against simian immunodeficiency virus (SIV) challenge (77,78,80-82). In the majority of these studies, however, the passively administered antibodies were known to neutralize the challenge virus, and high levels of antibodies were used. In humans, where HIV antigenic diversity is extensive, neutralizing antibodies have not been clearly shown to prevent infection. In fact, in the RV144 vaccine trial, the only vaccine trial to date to show some level of protective efficacy, low levels of neutralizing antibodies were elicited and did not correlate with protection (18).

Mother-to-child transmission provides another setting in which to examine the humoral immune correlates of protection as infants receive antibodies from their mothers while *in utero*. Because HIV-infected mothers and their infants are followed closely during the pre- and post-natal periods, timing of infant infection can be accurately determined and relevant immune correlates around the time of transmission in the mother and infant can be studied. In the absence of any intervention, only about 30-45% of infants become infected despite ample virus exposure, including postpartum through breastfeeding, suggesting there may be a protective factor on the maternal or infant side (reviewed in (23)).

Several studies have suggested that neutralizing antibodies contribute to the genetic bottleneck observed in MTCT – viruses that are transmitted to infants tend to be more neutralization resistant (less neutralization sensitive) to maternal antibodies than maternal virus

variants from the same time (88-91). These results suggest that infant viruses have escaped the maternal neutralizing antibody response. However, results from other studies did not see a difference in neutralization sensitivity between infant and maternal virus variants (103,104,108,145), in some cases, potentially reflecting their limited study size.

Similarly, conflicting results exist as to whether or not a strong neutralizing antibody response in mothers is protective. A number of studies have observed that non-transmitting mothers have higher or more frequent neutralizing antibody responses than transmitting mothers, suggesting a protective effect (90-102). However, other studies have observed no association between neutralizing antibodies and infection risk (46,98,103-108), and some have observed an increased risk of transmission associated with higher neutralizing antibody responses (109,110). The variation in these results may be due to differences in methodology including the viruses tested (primary vs. lab-adapted; heterologous vs. autologous), timing of sampling (before or after transmission), and availability of key clinical data (timing of infant infection and maternal viral load).

Only one study to date has directly addressed the question of whether or not non-transmitting mothers tend to have fewer neutralization-resistant viruses to maternal plasma than transmitting mothers, a situation that could reduce the risk of transmitting. In the study Baan *et al.* cloned individual virus variants from transmitting and non-transmitting mothers and tested these viruses against autologous maternal plasma (110). The authors found that transmitting mothers tended to have higher autologous neutralizing antibody responses than non-transmitting mothers. The study, however, examined maternal viruses from at or after the time infant infection was detected in 4 of the 7 transmitting mothers. Similarly, the plasma samples tested in all mothers were from visits either before or after the time the envelope (virus) variants were

cloned. As antibodies and viruses evolve over the course of infection, these viruses and plasma samples may not be representative of the maternal viruses and immune responses present around the time of transmission. A more relevant study would be to compare viruses and plasma from the same time-point around the time that transmission is estimated to have occurred.

In this study, we examine the role of maternal neutralizing antibodies against contemporaneous maternal virus in transmitting and non-transmitting mothers with high viral loads. We focus on women whose infants were HIV-negative at birth and chose maternal samples from near the time of transmission (just prior to when infant infection was detected) for transmitting women and similar time points in non-transmitting women. Overall, there was not a statistically significant difference in maternal autologous neutralizing antibody responses in non-transmitting and transmitting mothers. Similarly, there was also not a significant difference in the fraction of viruses that were resistant to neutralization, suggesting that these immune responses do not contribute significantly to protection from transmission during the early breastfeeding period.

MATERIALS AND METHODS

Study Design

Samples for this study were from the Nairobi Breastfeeding Clinical Trial, which was originally conducted to determine the impact of formula feeding vs. breastfeeding on infection and mortality in infants born to HIV-infected mothers (24). For this study, women were included based on the following criteria: 1) high plasma viral load >4.6 $\log_{10}$ copies/mL (the overall cohort median (139)) ; 2) breastfed ≥ 3 months; 3) infant was HIV DNA negative at birth; and 4)

maternal sample available near the time of transmission (or a similar time point in the non-transmitting mothers). Based on these criteria, 10 non-transmitting mothers and 9 transmitting mothers were chosen for the study. One transmitting mother (ML035) with a viral load slightly lower than the cohort median (4.26 $\log_{10}$ copies/mL) was also included, for a total of 10 transmitting mothers.

Envelope Cloning

Full-length (gp160) envelope clones from peripheral blood mononuclear cell (PBMC) DNA were available from previous studies(88,146). Four envelope clones from mother MM596 were obtained using the previous protocols from already isolated PBMC DNA (88,146). Full-length gp160 envelope clones were each ligated into a pCI-neo mammalian expression vector (Invitrogen) for use in pseudovirus assays.

Pseudovirus Generation

HIV envelope genes were tested for functionality as pseudoviruses in a TZM-bl single cycle assay. To generate pseudoviruses capable of one round of infection, plasmids containing the maternal HIV envelopes were co-transfected with a second plasmid containing a subtype A env-deficient HIV genome (Q23Δenv) (147,148). Plasmids were mixed in a 1:2 (env to HIV) ratio and added to $2x10^6$ 293T cells in a T-75 flask. Cells were plated 24 hours prior to transfection. For each transfection, 4 μg of total plasmid DNA was mixed with 12 μL of Fugene-6 (Roche). The DNA-Fugene mixture was incubated for 15 minutes prior to adding the mixture to cells. Cells were then incubated at 37°C for 48-72 hours prior to pseudovirus harvest. Pseudoviruses were harvested by removing transfection supernatant and filtering using a 0.2 μM

filter (Millipore). If not used immediately after harvest, psuedoviruses were aliquoted and stored at -80°C for subsequent use.

Pseudoviruses were then screened for function and titered by infecting TZM-bl cells in DMEM complete (DMEM + 10% fetal bovine serum + L-glutamine and PSF) in the presence of 10 μg/ml DEAE-dextran. Forty-eight hours post infection, cells were stained and fixed. Titer was determined by counting β-galactosidase positive blue cells. Functional pseudoviruses giving low titers were remade in a larger volume (transfection of 2 T-75s), filtered, and then concentrated using 100K Amicon Ultra Centrifugal Filter Units (Millipore). Concentrated pseudovirus was resuspended in 1 mL total volume to give higher-titer pseudovirus stocks. Pseudoviruses requiring concentration were the following: MC046.P35M.Env.B1, MF535.W0M.Env.A4, MF535.W0M.Env.B1, MF535.W0M.Env.D1, and MM834.P32M.Env.F7.

Neutralization Assays

Functional pseudoviruses were tested in neutralization assays against contemporaneous autologous maternal plasma. Serial dilutions of heat inactivated maternal plasma in a total volume of 25 μL were incubated with 500 infectious pseudovirus particles (as determined by the TZM-bl titer) in an equal volume. Maternal plasma dilutions started at a dilution of 1:50. The plasma-virus mixture was incubated at 37°C for 60 minutes. Subsequently, TZM-bl cells ($1x10^4$ in 100 μL DMEM) were added to each well in the 96-well plate. Forty-eight hours post infection beta-galactosidase levels were measured using the Galacto-light system (Applied Biosystems). Percent neutralization was calculated as the percent reduction of beta-galactosidase activity compared to virus without any patient plasma. Each virus was run twice in duplicate with the corresponding maternal plasma. Additionally, each maternal plasma sample was tested against

SIV to ensure background neutralization was below the limit of detection. The reciprocal plasma dilution that resulted in 50% inhibition of virus infection (IC50) was determined from a dose-response curve. Samples were all run in duplicate two times. If IC50s from the two runs differed by more than 2-fold, a third assay was run (in duplicate) and results were averaged. Neutralization IC50s less than the lowest dilution tested (1:50) were set at the midpoint between 0 and the dilution (25). Viruses that did not display 50% inhibition at the highest dilution tested (1:1600) were assigned an IC50 of the highest dilution (1600). Neutralization assays were done in conjunction with Vrasha Chohan.

Phylogenetic Analysis

Full-length maternal envelope sequences were aligned used MacClade version 4.01. Alignments were manually edited to remove variable regions that could not be unambiguously aligned. Neighbor-joining phylogenetic trees using pairwise distance were made using PAUP* 4.0b10. Reference sequences for subtypes A, C, and K were accessed from the Los Alamos National Laboratory HIV database (http://www.hiv.lanl.gov) and the unrelated subtype K sequence was used as an outgroup. Virus subtypes, based on full-length envelope sequences, were determined by the phylogenetic trees and the NCBI Genotyping tool (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi). For each mother, alignments of the five envelope clones were made to determine percent maximum pairwise distance in PAUP.

Statistical Analyses

Clinical characteristics (viral load, CD4 count, duration of breastfeeding) of transmitting and non-transmitting mothers were compared by Mann-Whitney U tests. Percent maximum pairwise distance of envelope clones for transmitting and non-transmitting mothers were also compared by a Mann-Whitney U test. For analyses, IC50 values were $\log_2$ transformed. The $\log_2$IC50 of non-transmitting and transmitting mothers was compared using a logistic regression with clustered standard errors to account for intra-woman correlation. A two-tailed t-test with Welch's correction was used to compare the proportion of neutralization-resistant viruses in transmitting and non-transmitting women.

RESULTS

Cohort Characteristics

Ten non-transmitting and 10 transmitting mothers were chosen from the Nairobi Breastfeeding Clinical Trial for this study (**Table 4.1**). Mothers were chosen if they had a high viral load and breastfed for at least 3 months, and were therefore at risk of transmission. To accurately estimate the timing of infection, only women whose infants were HIV DNA negative at birth were included. For transmitting women, PBMC samples for envelope cloning were chosen just prior to the time that the infant was first detected as positive. Plasma samples from the same time point, which is around the time of transmission, were also used and similar time points were chosen for non-transmitting mothers.

The twenty women had a median viral load of 5.07 $\log_{10}$copies/mL, a median CD4 count of 360 cells/mm^3, and a median duration of breastfeeding of 13.5 months. Viral loads did not differ between non-transmitting women and transmitting women (5.09 $\log_{10}$copies/mL vs. 5.06

log_{10}copies/mL, p = 0.88). Duration of breastfeeding (15.5 months vs. 8.79 months; p = 0.36) and CD4 count (360 vs. 342.5; p = 0.93) also did not differ between non-transmitting and transmitting mothers. Of the 10 transmitting mothers, all infants were HIV DNA-negative at birth and first detected as HIV-infected at either 6 weeks (N = 9) or 14 weeks (N = 1) of age. For 9 of the infected infants, RNA samples were also available from birth and were negative for HIV RNA.

Table 4.1 Characteristics of mothers included in the study

Characteristics of the non-transmitting (light gray) and transmitting (dark gray) women. Maternal viral loads are from same visit as envelope cloning except where indicated in parentheses. BF = breastfeeding; VL = viral load; W = week after delivery; P = week in pregnancy; M = month after delivery; NA = not applicable.

Maternal ID Number	Infant's last negative HIV DNA result	Infant's first positive HIV DNA result	Duration of BF in months	CD4 Count (cell/mm^3)	Maternal log_{10} plasma VL	Env Virus Subtype	Env cloning & plasma neutralization time point	% Maximum pairwise distance
MA411	M24	NA	19.5	416	5.13	A	P33	4.86
MB807	M24	NA	14.5	217	4.78 (W6)	A	P32	3.30
MF074	M24	NA	12.5	NA	5.01	A	W3	1.12
MF600	M24	NA	16.5	344	5.49	C	W2	1.38
MG540	M24	NA	11.5	285	5.06	A	P32	2.58
ML156	M9	NA	11.23	410	5	A/D	P32	1.98
MM471	M24	NA	22.5	360	5.26	A	P32	3.20
MM834	M24	NA	18.5	633	5.11	D	P32	0.94
MO862	M24	NA	10.5	134	4.86	A	P33	5.97
MP199	M24	NA	18.5	389	5.22 (P38)	A	W0	5.12
MB549	W0	W6	3.47	411	6.25	A	P30	1.24
MC046	W0	W6	3	255	5.05	A	P35	2.55
MF403	W0	W6	29.5	213	5.07	A	W0	0.87
MF520	W1	W14	27.5	511	5.8	A	P32	1.41
MF535	W1	W6	19.5	690	5.53	A/D	W0	6.22
MJ412	W0	W6	22.5	293	4.98	A/C/D	P32	5.88
MJ613	W0	W6	9.67	104	4.7	A	P32	5.28
MK184	W0	W6	3.1	568	4.85	D	P34	3.03
ML035	W0	W6	7.9	249	4.26	A/D	P32	3.65
MM596	W0	W6	3.5	392	5.65	A	W0	3.66

Envelope Clones

From each mother, full-length envelope genes were cloned from samples collected just prior to when infant infection was first detected (in transmitting mothers) and similar time points in non-transmitting mothers. Five functional full-length gp160 envelope clones were obtained per woman and are shown in neighbor-joining trees for non-transmitting (**Figure 4.1A**) and transmitting (**Figure 4.2B**) mothers. For each mother, the HIV envelope sequence diversity was calculated using maximum pairwise distance (**Table 4.1**). The maximum pairwise distance ranged from 0.87% to 6.22%. There was no difference in median maximum pairwise distance between non-transmitting and transmitting women (2.89% vs. 3.34%, p = 0.65).

Maternal virus subtypes were determined from full-length envelope sequences. Seven non-transmitting mothers and 6 transmitting mothers were infected with subtype A viruses (13/20 mothers; 65%). The remaining mothers were infected with subtype C (N=1) subtype D (N = 2) or recombinant forms (N = 4).

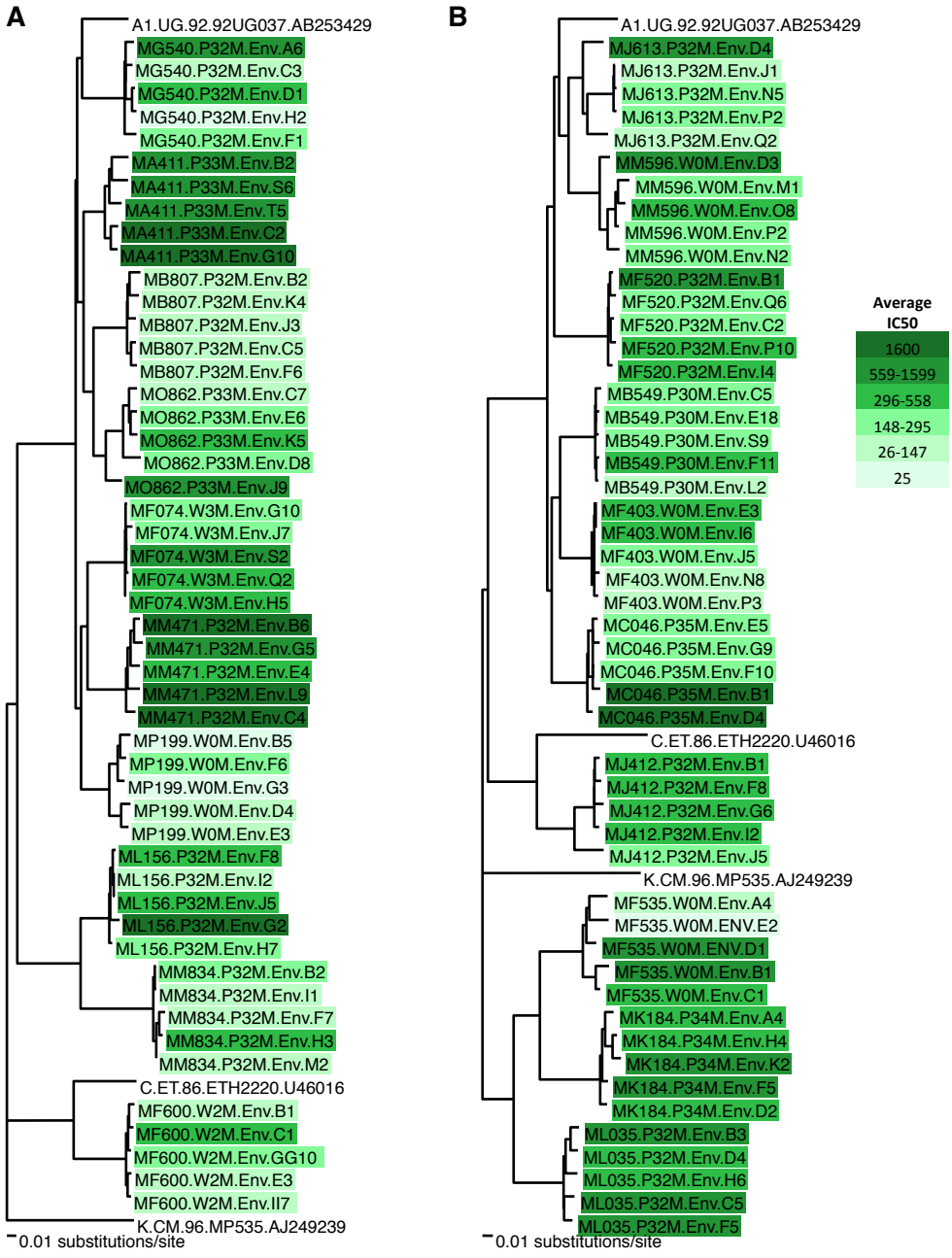


Figure 4.1 Neighbor-joining trees of maternal HIV envelope genes

Envelope clones from non-transmitting mothers (A) and transmitting mothers (B). Each envelope sequence is identified by the maternal ID number followed by the timing of isolation and envelope ID. P = pregnancy week, W = week after delivery. Samples are color-coded based on average IC50 (legend at right) when tested against contemporaneous maternal plasma. Reference sequences are not colored.

Neutralization Assay Results

To determine the impact of maternal autologous neutralizing antibodies near the time of transmission on transmission risk, the envelope clones were tested against contemporaneous plasma in a TZM-bl neutralization assay. Transmitting and non-transmitting maternal viruses displayed a range of neutralization sensitivities (IC50 Range 25 - 1600; **Figure 4.1; Table 4.2**). When tested against autologous plasma at a 1:50 starting dilution, non-transmitting and transmitting mothers had a similar number of neutralization-resistant viruses (6% vs. 2% p = 0.31). Overall, the median IC50 for non-transmitting mothers was 243 (IQR: 95, 594), which was lower than the median IC50 for transmitting mothers of 317 (IQR: 202, 521). In a logistic regression analysis with clustered standard errors to account for intra-woman correlation, there was no difference in autologous neutralizing antibody responses between transmitting and non-transmitting women (Odds Ratio: 1.25, p = 0.30; **Figure 4.2**).

Table 4.2 Neutralizing antibody IC50s for maternal envelope clones against contemporaneous autologous plasma

IC50s are the average of 2 or 3 separate experiments. Average log_2 IC50 calculated by taking the log_2 of each experiment and then averaging. IC50 values are colored with darker colors representing more neutralization sensitive variants.

Non-Transmitting Mothers

Maternal ID Number & Visit Cloned From	Env Variant	Average IC50	Average Log_2 IC50
MA411 P33	B2	594	9.02
	C2	1600	10.64
	G10	1600	10.64
	S6	667	9.06
	T5	1163	10.15
MB807 P32	B2	87	6.42
	C5	35	5.03
	F6	38	5.09
	J3	120	6.90
	K4	37	5.07
MF074 W3	G10	245	7.93
	H5	495	8.92
	J7	189	7.50
	Q2	398	8.63
	S2	660	9.32
MF600 W2	B1	103	6.69
	C1	366	8.49
	E3	75	6.23
	GG10	247	7.93
	II7	87	6.44
MG540 P32	A6	714	9.29
	C3	121	6.91
	D1	322	8.33
	F1	161	7.32
	H2	25	4.64
ML156 P32	F8	332	8.29
	G2	1600	10.64
	H7	270	8.01
	I2	114	6.83
	J5	334	8.33
MM471 P32	B6	1600	10.64
	C4	1600	10.64
	E4	360	8.48
	G5	806	9.58
	L9	1600	10.64
MM834 P32	B2	163	7.34
	F7	147	7.20
	H3	398	8.52
	I1	95	6.56
	M2	107	6.68
MO862 P33	C7	88	6.46
	D8	242	7.52
	E6	234	7.71
	J9	1118	10.09
	K5	374	8.43
MP199 W0	B5	25	4.64
	D4	77	6.27
	E3	83	6.37
	F6	148	7.15
	G3	25	4.64

Transmitting Mothers

Maternal ID Number & Visit Cloned From	Env Variant	Average IC50	Average Log_2 IC50
MB549 P30	C5	231	7.83
	E18	189	7.56
	F11	445	8.80
	I2	147	7.13
	S9	196	7.60
MC046 P35	B1	1600	7.58
	D4	1600	7.29
	E5	202	8.13
	F10	165	10.64
	G9	293	10.64
MF403 W0	E3	471	8.88
	I6	357	8.47
	J5	269	8.06
	N8	61	5.92
	P3	59	5.89
MF520 P32	B1	1259	10.24
	C2	226	7.57
	I4	296	8.21
	P10	389	8.60
	Q6	282	8.07
MF535 W0	A4	76	6.20
	B1	1530	10.58
	C1	361	8.42
	D1	974	9.90
	E2	25	4.64
MJ412 P32	B1	418	8.44
	F8	354	8.42
	G6	489	8.86
	I2	299	8.14
	J5	265	8.00
MJ613 P32	D4	645	9.33
	J1	72	6.16
	N5	243	7.92
	P2	190	7.57
	Q2	137	7.09
MK184 P34	A4	521	9.01
	D2	457	8.76
	F5	1174	10.12
	H4	334	7.98
	K2	1258	10.15
ML035 P32	B3	766	9.57
	C5	638	9.29
	D4	400	8.60
	F5	712	9.40
	H6	490	8.93
MM596 W0	D3	1405	10.44
	M1	241	7.88
	N12	193	7.56
	O8	301	8.20
	P2	209	7.69

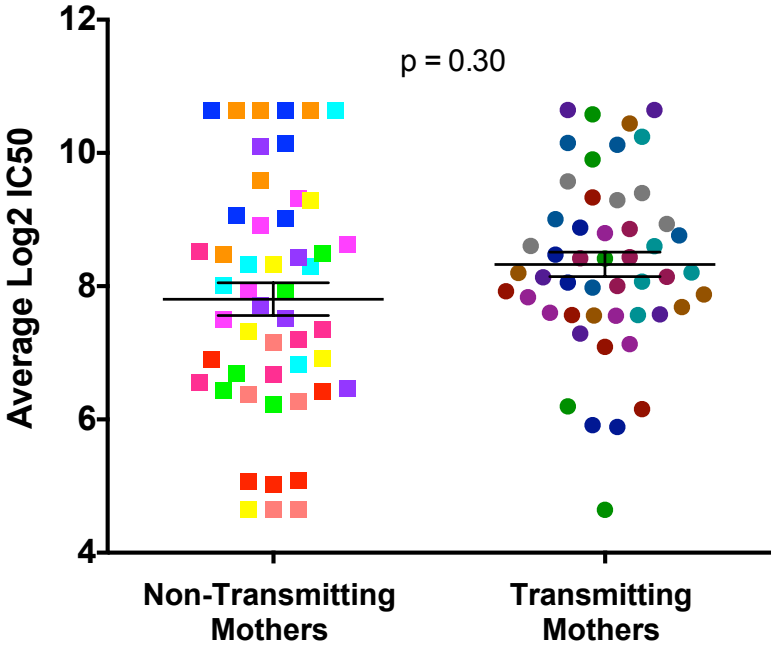


Figure 4.2 Autologous neutralizing antibody responses for non-transmitting and transmitting mothers.

Average Log_2IC50s for non-transmitting and transmitting mothers. Squares/dots are color-coded by mother with each square or dot representing the IC50 for an individual envelope variant tested against contemporaneous autologous plasma.

DISCUSSION

In this study we compared the autologous neutralizing antibody response against maternal envelope variants for transmitting and non-transmitting mothers. We specifically focused on antibody and envelope variants from the same time point, just prior to when HIV DNA and RNA was first detected in infected infants to ensure that the measured responses were the most relevant neutralizing antibody responses around the time of transmission. Importantly, we also included only breastfeeding mothers with high viral loads whose infants were HIV-negative at birth. By restricting our analyses to these mothers, we focused on the women at highest risk of transmission for whom we could accurately estimate the timing of transmission, and thus, pinpoint which samples were most relevant to the immune responses around the time of

transmission. Overall, we observed that there was not a statistically significant difference in maternal autologous neutralizing antibody responses between transmitting and non-transmitting mothers.

In total, 100 functional envelope variants were isolated from the 20 women (5 per woman). These envelope clones displayed a range of neutralization sensitivities against contemporaneous autologous plasma. Transmitting women had slightly higher IC50s (more neutralization sensitive viruses) than non-transmitting mothers, however, this difference was not statistically significant. These data suggest that autologous neutralizing antibodies around the time of transmission are not associated with transmission risk, however, with only 10 women in each group, our power to detect a true difference may have been limited. Similar to our results, a number of older studies showed no difference in autologous neutralizing antibody titers and risk of transmission (46,103-108). However, these results are different than those obtained from other studies with similar numbers of mothers which showed higher levels of neutralizing antibodies in non-transmitting mothers (90-102). Most of these studies utilized virus populations derived from short-term culture or lab-adapted viruses, rather than individually testing dominant virus variants, which might explain the difference in results. In particular, lab-adapted viruses may not be representative of the maternal viruses to which the infant was exposed, and culturing virus may alter the overall virus population.

Given that a number of studies (including one from the Nairobi Breastfeeding Clinical Trial) have suggested that neutralizing antibodies contribute to the genetic bottleneck observed in MTCT (88-91), we originally hypothesized that non-transmitting mothers would have more neutralization sensitive virus variants and fewer neutralization resistant variants than transmitting mothers. However, we observed similar numbers of neutralization-resistant viruses (when tested

against plasma at a 1:50 dilution) in both transmitters and non-transmitters. Although not statistically significant, opposite of our hypothesis, we observed slightly higher IC50s (more neutralization-sensitive viruses) and fewer neutralization-resistant viruses in the transmitting women than non-transmitting women. These results are similar to the one other smaller study (7 women, 23 viruses) that also examined individual viral variants from transmitting and non-transmitting women and observed significantly higher neutralizing antibody responses in transmitting mothers (110). As discussed above, these results utilizing individual virus variants differ from the majority of studies using lab-adapted or cultured virus populations, which have observed some protective effect of neutralizing antibodies. Thus, the results from these studies warrant further research into the role of autologous maternal and infant antibody responses against individual maternal virus variants to which the infant was exposed.

Overall, we present here the largest study of autologous neutralizing antibody responses in transmitting and non-transmitting mothers against individual maternal virus variants, to date. Our results show that transmitting and non-transmitting mothers harbor a mixture of mostly neutralization-sensitive viruses and have similar overall neutralization IC50s. Remarkably, even women at high risk of transmission (based on high viral load) had relatively neutralization resistant viruses and yet did not transmit the virus to their infants. These results suggest that lack of virus transmission in the early breastfeeding period is not simply due to an absence of maternal neutralization escape variants, and likely includes multiple factors, perhaps including selective pressure to transmit escape variants when other conditions are favorable for infection.

Chapter V

Conclusions and Implications for Future HIV Vaccine and Treatment Research

Our ability to create an effective vaccine against HIV is hampered, in part, by our lack of understanding of what a protective immune response looks like. As data from human vaccine trials are limited, garnering information from other settings is essential. Mother-to-child transmission provides one setting in which to study immune correlates of protection and is unique because infants have HIV-specific antibodies prior to, and at the time of, virus exposure. Additionally, with regular follow-up of HIV infected pregnant women and their infants, immune responses can be studied to understand what aspects of immunity correlate with protection during the relevant window near transmission.

This thesis addressed the impact of humoral immune responses in HIV mother-to-child transmission. In Chapter II we observed that ADCC activity was higher in uninfected infants than infected infants, however, this difference was not statistically significant. In infected infants, however, higher pre-existing ADCC activity was associated with decreased mortality, suggesting a therapeutic effect of ADCC antibodies. In Chapter III, we analyzed the impact of infant and maternal FcγRIIa and FcγRIIIa genotypes on MTCT in the same cohort. Infant genotypes were not associated with infection risk or disease progression, suggesting that prevention or treatment regimens that rely upon Fc-mediated antibody activity may not be impacted by host FcγR genotypes. Maternal FcγRIIIa genotype, however, was associated with transmission – mothers who were heterozygote for the SNP had an increased risk of infection. The significance of this association was modest, and thus, future studies will be needed to clarify the association as well as its potential mechanism. Finally, in Chapter IV, we examined the impact of maternal

neutralizing antibodies against maternal virus variants in transmitting and non-transmitting mothers. We observed no significant difference in autologous neutralizing responses in these two groups, suggesting that maternal Nabs did not contribute to the genetic bottleneck that has been observed in the cohort (88). Overall, these studies provide insight into the impact of neutralizing and Fc-mediated antibody functions in mother-to-child transmission. Their impact on the broader HIV prevention and treatment field will be discussed below, along with future avenues of research to build upon the results presented here.

Infant ADCC Activity: Remaining Questions

In Chapter II, we examined the ADCC activity of passively acquired antibodies in HIV-exposed infants. This study was one of the first to show a therapeutic effect of ADCC antibodies present at the time of exposure in humans and raises many new questions about ADCC in infants. Firstly, as ADCC depends on effector cell activity, it is important to gain a better understanding of the role of infant natural killer cells in such a response. One recent study suggests that NK cells from HIV infected infants tend to have lower activation and are less efficient at blocking HIV replication *ex vivo* than those from infants who remain uninfected (149). Thus, infected infants in our cohort may have had both lower ADCC antibody activity and NK cells that were less capable of mediating ADCC. This hypothesis could be directly tested by analyzing infant NK cells for markers of activation by flow cytometry and by using the cells in the RFADCC assay to measure ADCC activity. While such an experiment may be difficult to do with the relatively old and limited samples from the Nairobi Breastfeeding Clinical Trial, ADCC and NK cell activity/markers could also be studied in other cohorts to gain a more general understanding of infant responses.

Data from this thesis can also be utilized to examine the role of autologous ADCC responses in MTCT. Chapter II tested infant plasma against a heterologous virus using effector cells that were not genotyped. Such an approach was amenable to testing a large cohort and is often used for population-based studies, however, examining autologous ADCC responses in a subset of infants will give us better insight into the role of such responses in MTCT. Autologous ADCC responses can be examined by incubating infant plasma samples with cells infected with maternal virus (to which the infant was exposed), and then adding effector cells bearing the infant's FcγRIIa and FcγRIIIa genotypes. One approach would be to utilize the maternal envelope genes from Chapter IV of this thesis. These envelopes could be cloned into a plasmid containing a full-length env-deficient virus and then used to infect target cells for the RFADCC assay. Effector cells bearing the infant's FcγRIIa and FcγRIIIa genotypes (as determined in Chapter III of the thesis) would then be added to the HIV-infected cells and patient antibody. Comparing autologous ADCC responses in infected and uninfected infants will provide insight into infant responses in the context of maternal viruses. Additionally, comparing the results of such a study to those observed in Chapter II will help clarify the relevance of measuring ADCC activity against heterologous viruses.

ADCC Activity and Protection: Vaccine Implications

Although not statistically significant, we observed an inverse association between passively acquired, pre-existing, ADCC activity and infant infection risk. The small number of infected infants may have limited our results and, therefore, future studies should be conducted to determine if ADCC activity is protective in HIV-exposed infants. Such a result would bolster the results from the RV144 trial which suggested that ADCC activity was protective in

vaccinated individuals (18), and the results from mothers in our cohort which observed that breast milk ADCC activity was protective (45).

This protective effect of ADCC activity also suggests that future research should aim to better characterize ADCC-mediating antibodies and how to elicit such antibodies by a vaccine. Over the years, a number of broadly neutralizing antibodies have been isolated and characterized (reviewed in (150)), and similar studies should be conducted with ADCC antibodies from individuals who elicit strong ADCC responses. It would be particularly relevant to isolate and characterize ADCC antibodies from infants or mothers from the Nairobi Breastfeeding Clinical Trial, as ADCC has been implicated in protection in this cohort (45). Clarifying the epitopes targeted by broadly active ADCC antibodies will enable us to rationally design immunogens to elicit responses to these epitopes.

How to elicit effective ADCC responses is another area of research that should be pursued. One method by which to address this question is to compare vaccination methods and subsequent antibody responses elicited. Two recent studies have compared antibody responses from the RV144 trial and the Vax003 trial (123,124). The Vax003 trial had the same AIDSVAX B/E protein-based regimen, but different from RV144, did not include a ALVAC prime and showed no protection (19). Comparison of these two trials suggested that protection in the RV144 trial was associated with high ADCC-mediating IgG3 responses and low IgG4 responses (123,124). Interestingly, while IgG3 was implicated in protection in the RV144 trial, results from Chapter II of this thesis suggest that IgG1 ADCC antibodies may also be protective. Thus, more studies are needed to clarify the impact of antibody subclass on ADCC-mediated protection. Comparing vaccines that produce strong ADCC responses and those that do not will help to

clarify the best vaccination regimen, including immunogens, vectors, and adjuvants, for inducing ADCC responses.

The protective effect of ADCC antibodies observed in humans may also help elucidate the role of cell-associated virus in transmission and highlights the need for non-human primate models that use cell-associated virus for challenge. As ADCC targets and kills infected cells, ADCC antibodies that prevent infection must either act on transmitted infected cells or on early newly infected cells prior to systemic spread and establishment of a viral reservoir. In non-human primates, passive immunization with non-neutralizing antibodies has been associated with reduced viral loads but no study has definitively shown complete block of acquisition with these antibodies (Reviewed in (112)). The majority of these non-human primate studies utilized cell-free virus for challenge, which means that ADCC activity could only act after infection of the host cells occurred. If ADCC in humans primarily works by blocking cell-associated virus transmitted by the index person, an effect may not be observed in non-human primates. Indeed, cell-associated virus transmission has been implicated in mother-to-child transmission (Reviewed in (30)) and sexual transmission (Reviewed in (31)). Future non-human primate studies using cell-associated virus for challenge may help to clarify the impact of cell-associated virus as well as ADCC on protection in that setting. Similarly, it would be interesting to examine the levels of cell-associated virus in individuals in the Nairobi Breastfeeding Clinical Trial cohort with high and low ADCC activity to see if ADCC activity impacts cell-associated virus levels.

Passive Immunization with ADCC-Mediating Antibodies

In Chapter II, we observed that passively acquired ADCC activity provided a protective effect in infants who became infected. Additionally, there was a trend in a negative association between ADCC activity and set point viral load, suggesting that ADCC activity present at the time of exposure may have cleared early infected virus, thus lowering viral load. Future work should be done to clarify the mechanism behind the observed association between ADCC and survival.

One way to more thoroughly examine the mechanism and protective effect of ADCC antibodies is to test ADCC antibodies in a passive immunization study in infants. Passive immunization has been shown to be protective in MTCT for other viruses including cytomegalovirus (151) and Hepatitis B (152). With regard to HIV, two early studies of passive administration of HIV immunoglobulin for prevention of MTCT showed no protective effect, but the tested sera was selected based on antibodies to p24 and not neutralizing or ADCC activity (153,154). A passive immunization study with HIV-specific ADCC-mediating antibodies would provide insight into the role of these antibodies in MTCT. Such a study would first require the isolation and characterization of effective ADCC antibodies (described above), as well as safety and pharmacokinetic tests in non-human primates and adults. HIV-specific ADCC-mediating antibodies could then be administered to HIV-infected women or their infants at therapeutic levels. Infants should then be followed to determine infection status and disease progression. With regular sampling in these infants, we could determine if ADCC antibodies prevent infection or if they slow disease progression in infants who become infected. Further follow-up could determine the antibody's impact on viral reservoir size, peak viral load, and set point viral load.

In addition to passive immunization in uninfected individuals, passive administration of ADCC-mediating antibodies may have a therapeutic effect in already infected persons. Higher ADCC activity has been associated with slower disease progression in a number of human studies and a recent study has shown that passive administration of broadly neutralizing antibodies is safe and is effective at reducing viral loads (155). Therefore, administration of ADCC antibodies may have a similar effect in individuals and provides another avenue for HIV treatment research.

Neutralizing Antibodies in Mother-to-Child Transmission

In addition to ADCC antibody activity, this thesis also examined neutralizing antibodies in HIV-infected mothers. In Chapter IV, we observed no difference in neutralizing antibody titers or numbers of neutralization-resistant viruses in transmitting and non-transmitting mothers. These results suggest that maternal neutralizing antibody titers do not contribute to risk of transmission or the genetic bottleneck observed in MTCT. However, as infant viruses have been shown to be more resistant to neutralization than maternal viruses from around transmission in this cohort (88), it would be interesting to examine the impact of infant passively acquired neutralizing antibodies against the maternal virus. Infant plasma neutralization activity was tested in this cohort against heterologous viruses in one previous study (118). This study observed no association between heterologous neutralizing antibody activity and risk of infection. However, responses measured against maternal viruses to which the infants were exposed may be a more relevant measure of the infants' antibody responses around the time of transmission. Thus, a study examining infant antibodies against maternal virus may help to clarify the role of neutralizing antibodies in MTCT.

Conclusions

Although significant progress has been made in reducing the number of new HIV infections worldwide, a safe and effective vaccine is still of high priority. As data from HIV vaccine trials are limited, information from other settings can help elucidate what sort of immune responses are protective. In this thesis, we examined the impact of humoral immune responses on mother-to-child transmission. Overall, we observed a therapeutic effect of ADCC antibody activity in HIV-infected infants, saw no impact of FcγR genotypes on MTCT risk, and found that non-transmitting and transmitting mothers had similar levels of neutralizing antibodies against autologous virus. These results highlight the need for increasing research into ADCC antibody mechanisms and epitopes, how to elicit such antibodies, and the potential for passive immunization with ADCC-mediating antibodies.

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