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Investigating the role of antibody-dependent cellular cytotoxicity in the prevention of mother-to-child-transmission of HIV-1 through breastfeeding

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INVESTIGATING THE ROLE OF ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY IN THE PREVENTION OF MOTHER-TO-CHILD-TRANSMISSION OF HIV-1 THROUGH BREASTFEEDING

by

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DEDICATION

I would like to dedicate this work to my supportive parents and my loving fiancé, Tom.

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INVESTIGATING THE ROLE OF ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY IN THE PREVENTION OF MOTHER-TO-CHILD-TRANSMISSION OF HIV-1 THROUGH BREASTFEEDING

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ABSTRACT

Immune correlates of protection against Human Immunodeficiency Virus Type 1 (HIV-1) acquisition in humans remain unknown. The role of antibodies in providing protection, either through neutralization to prevent virions from infecting cells or Fragment crystallizable (Fc) effector functions to help clear already infected cells, are poorly understood. Previous work fails to support neutralizing antibodies (nAbs) as an in vivo protective correlate, therefore we sought to investigate whether the Fc effector function, antibody-dependent cellular cytotoxicity (ADCC), was protective. Mother-to-child-transmission (MTCT) cohorts provide a model of passive immunization and viral challenge, because infants acquire antibodies from their mother throughout gestation and breastfeeding and are consistently exposed to HIV. We hypothesized that infants of non-transmitting mothers would be protected due to higher ADCC responses compared to transmitting mother-infant pairs. To address our hypothesis, we developed a cell line susceptible to viruses incorporating HIV envelopes (Envs) isolated from maternal plasma for use in a luciferase-based ADCC assay. Serum samples came from the control arm of the Breastfeeding, Antiretroviral, and Nutrition (BAN) cohort in Malawi where HIV-

infected mothers that transmitted (TM) infection to their infants were matched to two non-transmitting (NTM) mothers on parameters known to be important in MTCT, including maternal age, viral load, absolute CD4 count, and time to sample collection. All transmission events occurred during breastfeeding and the samples assessed in this study came from a timepoint prior to documented transmission. HIV-exposed but uninfected (HEU) infants had significantly higher levels of ADCC responses against their corresponding mother's variants compared to HIV-exposed infected (HEI) infants. Additionally, higher ADCC was associated with reduced morbidity and mortality in infected infants up to 1 year after birth. Although nAbs were previously determined to not play a role in the prevention of MTCT in this cohort, our data suggests that neutralization and ADCC are independent antibody responses that may act in conjunction to prevent transmission. In order to utilize these protective antibodies in a preventative HIV-1 vaccine, the antibodies must recognize a range of Envs to be effective globally. ADCC breadth and potency ($ADCC_{BP}$) was determined against a panel of heterologous globally diverse variants, unrelated to the infected mother's Envs. No significant difference in $ADCC_{BP}$ was observed between TM and NTM mother-infant pairs. However, we identified samples with elite $ADCC_{BP}$, specified as the top 5% of samples tested, and future HIV-1 vaccines may want to elicit the types of antibodies present in these samples. In summary, maternally acquired ADCC correlates with protection against HIV-1 transmission to exposed infants and is associated with lower infant morbidity up to 1 year after birth. Further characterization of antibodies with elite $ADCC_{BP}$ may be helpful in developing an effective vaccine.

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LIST OF ABBREVIATIONS

ADCC ……Antibody-dependent Cellular Cytotoxicity

$ADCC_{AUC}$.....ADCC Area Under the Curve

$ADCC_{BP}$ ..... ADCC Breadth and Potency

ADCP ……...Antibody-dependent Cellular Phagocytosis

ADCVI ……...Antibody-dependent Cell-mediated Viral Inhibition

AIDS ..... Acquired immune deficiency syndrome

AMP..... Ampicillin

ARVs.....Antiretrovirals

ART.....Antiretroviral therapy

AUC .....Area under the curve

AZT.....azidothymidine

BAN .....Breastfeeding, Antiretroviral, and Nutrition Study

Beta-gal .....Beta-galactosidase

BnAbs ..... Broadly neutralizing antibodies

BM IgG ..... IgG isolated from Breast Milk

BMS .....Breast milk Supernatant

BP..... breadth and potency

BU ..... Boston University

CCR5..... C-C chemokine receptor type 5

CD4.....Cluster of differentiation 4

CD4bs .....Cluster of differentiation 4 binding site

CD4mc ..... Cluster of differentiation 4-mimetic compound

CD16 ..... Cluster of differentiation 16

CDC ..... Center for Disease Control

CDC ..... Complement Dependent Cytotoxicity

cDNA ..... Complementary DNA

CH ..... Constant Region of Heavy Chain

CHAVI ..... Center for HIV/AIDS Vaccine Immunology

CI ..... Confidence Interval

CMV ..... Cytomegalovirus

CRF ..... Circulating Recombinant Forms

CsA ..... Cyclosporin A

CSM ..... Complete Supplement Mixture

CXCR4 ..... C-X-C chemokine receptor type 4

ddH_2O ..... Double distilled water

DEAE ..... Diethylaminoethyl Cellulose

DMEM ..... Dulbecco's modified Eagle medium

DNA ..... Deoxyribonucleic acid

DNAse I ..... Deoxyribonuclease I

dNTP ..... Deoxynucleotides

DTT ..... Dithiothreitol

E. coli ..... Escherichia coli

EDTA ..... Ethylenediaminetetraacetic acid

ELISA ..... Enzyme-linked immunosorbent assays

Env ..... Envelope glycoprotein

Envs..... Envelopes

Fab..... Fragments of Antigen Binding

FBS ..... Fetal bovine serum

Fc..... Fragment Crystallizable

FcRn ..... Neonatal Fc-Receptor

FcγR ..... Fragment Crystallizable Gamma Receptor

FDA..... Food and Drug Administration

Fig ..... Figure

FOA..... Fluoroorotic acid

g..... Gram

Gag..... Group-specific antigen

GFP ..... Green Fluorescent Protein

GI ..... Gastrointestinal

Gp..... Glycoprotein

H_2O ..... Water

HAART..... Highly active antiretroviral therapy

HCl..... Hydrochloric acid

HEI..... HIV-exposed Infected

HEU ..... HIV-exposed Uninfected

HIV-1 ..... Human immunodeficiency virus-type 1

HIVIG ..... Anti-HIV Immunoglobulin

HPTN .....The HIV Prevention Trials Network

HR ..... Hazard Ratio

Hr ..... hour

HTLV-III..... Human T lymphotropic virus-III

ID .....Identification

Ig .....Immunoglobulin

IL-2 ..... Interlukin-2

IN .....Integrase

K.....Thousand

kg..... Kilogram

L .....Liter

LAV .....Lymphadenopathy-associated virus

LB ..... Lysogeny broth

Leu .....Leucine

LiAc .....Lithium acetate

LN .....Lymph nodes

LTR..... Long terminal repeat

Luc .....Luciferase

mAb..... Monoclonal Antibody

MFI ..... Mean Fluorescence Intensity

mg ..... Milligram

$MgSO_4$ ..... Magnesium sulphate

MHC I ..... Major Histocompatibility Complex I

MHC II ..... Major Histocompatibility Complex II

mins ..... Minutes

mL ..... Milliliter

mm ..... Millimeter

mM ..... Millimole

MMWR ..... Morbidity and Mortality Weekly Report

MOI ..... Multiplicity of Infection

MPER ..... Membrane-Proximal External Region

mRNA ..... Messenger Ribonucleic Acid

MSM ..... Men Who Have Sex with Men

MTCT ..... Mother-to-child-transmission

nAbs ..... Neutralizing antibodies

NaCl ..... Sodium chloride

NaOH ..... Sodium hydroxide

Nef ..... Negative Regulatory Factor

$Neut_{AUC}$ ..... Neutralization Area Under the Curve

$Neut_{BP}$ ..... Neutralization Breadth and Potency

ng ..... Nanogram

NIAID ..... National Institute of Allergy and Infectious Diseases

NIH ..... National Institute of Health

nAb..... neutralizing antibody

NK..... Natural Killer

NKr .....Natural Killer-resistant

nnAb..... non-neutralizing antibody

NPC.....Nuclear Pore Complex

NTM.....Non-Transmitting Mother

NTRI .....Nucleoside Reverse Transcriptase Inhibitors

OD..... Optical density

OR..... Odds Ratio

p..... Probability value

p-value..... Probability value

PEG .....Polyethylene Glycol

PEI..... Polyethylenimine

PBMCs.....Peripheral blood mononuclear cells

PBS ..... Phosphate buffered saline

PCP ..... *Pneumocystis carinii* pneumonia

PCR..... Polymerase chain reaction

pH.....Potential of hydrogen

PHA..... Phytohemagglutinin

PID .....Patient Identification Number

PMTCT .....Prevention of Mother-to-Child Transmission

Pol .....Polymerase protein

PP ..... Postpartum

PrEP .....Pre-exposure prophylaxis

R5..... C-C chemokine receptor type 5

Rev .....Regulator of expression of virion proteins

RFADCC.....Rapid Fluorometric Antibody-Mediated Cytotoxicity

RLU..... Relative light units

RM .....Rhesus Macaques

RNA .....Ribonucleic acid

RNAse H..... Ribonuclease H

rpm .....Revolutions per minute

RMPI..... Roswell Park Memorial Institute medium

RRE..... Rev Response Element

RT .....Room temperature

RT ..... Reverse transcriptase

s..... Seconds

SAE.....Serious Adverse Event

SGA..... Single genome amplification

SHIV .....Simian-human immunodeficiency virus

SIV ..... Simian immunodeficiency virus

S.O.C.....Super Optimal broth with Catabolite repression

SSIII RT. ..... SuperScript III Reverse Transcriptase

STD .....Standard Deviation

TAE..... Buffer with Tris base, acetic acid, and EDTA

TAR.....Trans-activating response element

Tat .....Trans-activating regulatory protein

TE..... Buffer with Tris and EDTA

T/F .....Transmitted/Founder

TM..... Transmitting Mother

U.....Units

UNAIDS ..... Joint United Nations Program on HIV/AIDS

US ..... United States

V1/V2.....Variable loop 1 and 2

V3.....Third variable loop

Vif .....Viral infectivity factor

VL ..... Viral load

Vpr ..... Viral Protein R

Vpu.....Viral Protein U

VSVG..... Vesicular Stomatitis Virus G

WHO .....World Health Organization

x.....Times

X4..... C-X-C chemokine receptor type 4

Xg..... G-force

YEPD .....Yeast Extract Peptone Dextrose

YNB ..... Yeast Nitrogen Base

ZDV-3TC.....Zidovudine and Lamivudine Combination Therapy

μg .....Microgram

μl ..... Microliter

μM.....Micromole

°C .....Degrees Celsius

%CV..... Percent Coefficient of Variation

CHAPTER I: INTRODUCTION

Human Immunodeficiency Virus Type 1

History

On June 5th, 1981 the Center for Disease Control (CDC) published an article in the Morbidity and Mortality Weekly Report (MMWR) describing five cases of *Pneumocystis carinii* pneumonia (PCP) in previously healthy young, white, gay men in Los Angeles, California. This was the first report of what is now known as Acquired Immune Deficiency Syndrome (AIDS) (CDC, 1981), however this term was not used by the CDC until over a year later in 1982. With cases of AIDS on the rise, researchers started to believe this disease was due to an infectious agent. Around the same time (1983/1984) two laboratories, Dr. Fançoise Barré-Sinoussi in Luc Montangier's laboratory at the Pasteur Institute in France and Dr. Robert Gallo at the National Cancer Institute in the United States of America, isolated virus from people living with AIDS. Although the labs had different nomenclature for the virus, Lymphadenopathy-associated virus (LAV) (Barré-Sinoussi et al., 1983) and Human T lymphotropic virus (HTLV)-III (R. Gallo et al., 1984), they were both referring to the same causative agent that was eventually named Human Immunodeficiency Virus (HIV) in 1986. HIV was determined to be spread by sexual contact, blood, and from mother to child (Curran et al., 1985; A. S. Fauci et al., 1984). It was also found that HIV caused AIDS by selectively infecting and inhibiting cells of the immune system (AS, 1984; Bowen et al., 1985; Anthony S. Fauci et al., 1985). Specifically, HIV binds the CD4 receptor expressed on the surface of a subset of T lymphocytes, depleting these cells that are critical for the

control of pathogen recognition, B cell and $CD8^+$ T cell function, as well as cytokine production that drive specific immune responses (Dalgleish et al., 1984; McDougal et al., 1986; Swain et al., 2012). By depleting CD4+ T cells, HIV results in systemic immune failure and immunodeficiency. A $CD4^+$ T cell count <200 was determined to be a clinical indicator of AIDS (CDC, 1992).

Since the initially discovery of HIV-1, much work has been done to identify its origin. Through phylogenetic tracing, researchers determined that HIV-1 likely arose from a transmission event involving a strain of simian immunodeficiency virus (SIV), SIVcpz, from chimpanzees in Cameroon (Sharp & Hahn, 2011). Although not discovered until the 1980s, one study showed that the HIV-1 pandemic (Group M) started as early as 1920s, in the Democratic Republic of Congo (Faria et al., 2014).

Epidemiology

While the rate of both new cases and HIV mortality have steadily declined since their peak in 1999 and 2006 respectively, there is still a long way to go to end the HIV epidemic (Frank et al., 2019). According to a report by UNAIDS, as of the end of 2019, 38 million people worldwide were living with HIV, 1.8 million being children under the age of 15 years (UNAIDS, 2020a). In 2017, there were almost 140,000 new infections reported in children under the age of one, a substantial number when considering the success of interventions that prevent mother-to-child-transmission (MTCT) (Frank et al., 2019). HIV-exposed uninfected infants have an increased risk of mortality compared to unexposed infants, with recent studies suggesting decreased T cell function and heightened inflammation may be responsible (Dirajlal-Fargo et al., 2019; Jalbert et al.,

2019) The epicenter of the HIV epidemic is in Africa, where almost 26 million people are infected, accounting for about two-thirds of the global cases (Giovanetti et al., 2020). Although treatment and prevention has drastically improved, there were still 1.7 million new infections in 2019, and only 25.4 million of the 38 million living with HIV had access to antiretroviral therapy (ART), highlighting the need to improve treatment distribution (UNAIDS, 2020a).

The landscape of the HIV epidemic is vastly different in the United States compared to Sub-Saharan Africa. In the United States, there were close to 38,000 new HIV diagnoses in 2018, predominantly among men who have sex with men (MSM). Additionally, Black/African Americans and Hispanics/Latinx populations continue to be disproportionally affected, making up over 50% of the US infections (CDC, 2020). However, in Sub-Saharan Africa women of child-bearing age make up the predominate number of cases, and HIV transmission is primarily through heterosexual contact (Giovanetti et al., 2020; Kharsany & Karim, 2016). Furthermore, there were 730,000 new infections reported in eastern and southern Africa in 2019. The higher infection rate seen in Africa is due to HIV being endemic in the population as well as limited access to healthcare and mediations (UNAIDS, 2020a). There is significant variability in treatment access between African countries, with as low as 25% of adults accessing treatments in some countries (Kharsany & Karim, 2016). Yet, testing in Sub-Saharan Africa has dramatically improved since 2000 when only 5.7% of people living with HIV knew their status, increasing to 84% in 2020 (Giguère et al., 2021).

Socio-economic status also plays a large role in clinical outcomes of HIV-infected individuals. One meta-analysis study showed that people of low socio-economic status had a greater than 50% higher risk of dying from HIV compared to people of high socio-economic status in Sub-Saharan Africa (Probst et al., 2016). This is also the case in the U.S. where the greatest burden of disease is seen in those people with the lowest socio-economic status (Pellowski et al., 2013). These socio-economic disparities pose a large barrier to ending the HIV epidemic. Interventions and treatments are ineffective if they cannot be reliably distributed everywhere. This is one of the leading reasons an effective vaccine is necessary to close this gap and end this epidemic.

Replication Cycle and Treatment

There are seven stages in the HIV replication cycle (Illustration 1). HIV first binds target cells expressing the cluster of differentiation 4 (CD4) receptor to enter cells expressing this receptor (Dalgleish et al., 1984; McDougal et al., 1986). HIV binding induces reorganization of CD4 on the cell surface, increasing local concentrations (Yuan et al., 2021). The HIV envelope (*env)* gene is responsible for encoding the 160 kD glycoprotein product, gp160, which is then cleaved into gp120 and gp41. Gp120 and gp41 remain non-covalently linked forming a single subunit of the trimer that coats the HIV virion and is essential for entry into target cells (Arrildt et al., 2012). The HIV envelope glycoprotein (Env) binds the CD4 receptor on the target cell through an interaction with gp120, causing a conformational change that exposes binding sites for the chemokine co-receptors, most commonly CXCR4 or CCR5 (Henderson & Hope, 2006; Wilen et al., 2012). Co-receptor binding leads gp41 pre-hairpin formation (Wang et

al., 2020), dissociation of gp120, and gp41 insertion of the fusion peptide into the target membrane, forming a fusion pore (S. A. Gallo et al., 2003). Finally, the six-helix bundle is formed and there is subsequent fusion of the virus and host cell membrane (Tran et al., 2012). HIV preferentially targets CD4+ T cells due to the high propensity of CD4

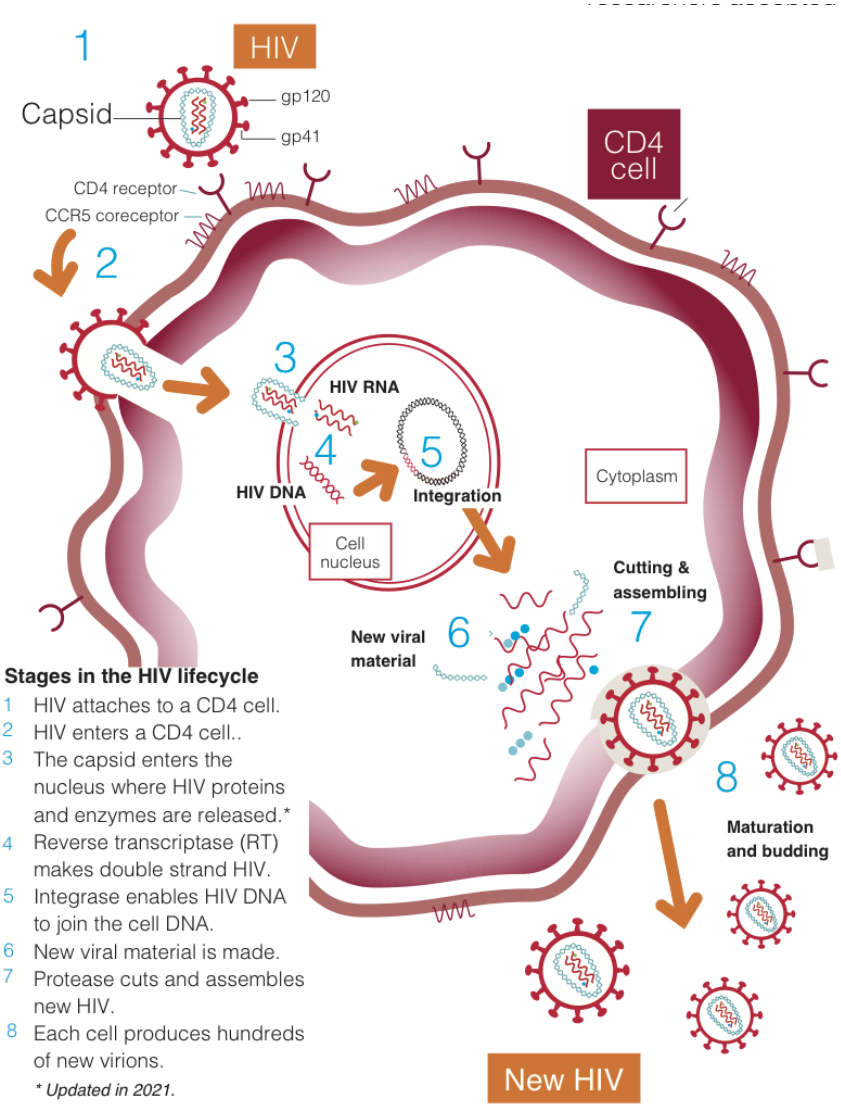

Illustration 1. HIV Replication Cycle. Schematic depicting HIV-1 attachment, fusion, reverse transcription, integration, budding, and maturation. Image adapted from (i-base.info, 2021).

expressed on the surface of the cells (B. Lee et al., 1999). However, HIV has been shown to also infect macrophages, as well as microglia cells in the central nervous system, which express low levels of CD4 along with the CCR5 co-receptor (Schnell et al., 2011; Sturdevant et al., 2015).

After membrane fusion, the HIV capsid is released into the cytoplasm and trafficked to the nucleus (Christensen et al., 2020). The HIV capsid contains necessary replication factors, including 2 copies of the viral genome, nucleocapsid protein (NC), reverse transcriptase (RT), and integrase (IN) (Christensen et al., 2020). Recent work has shown that cone shaped HIV-1 capsid docks to the nuclear pore complex (NPC) and is able to fit through the NPC central channel for transport into the nucleus (Zila et al., 2021). There is evidence to support that the capsid uncoats after exiting the NPC central channel, releasing its contents into the nucleoplasm (Zila et al., 2021). Although unclear as to the exact timing of this process in relation to uncoating, viral RNA is reverse transcribed into cDNA by HIV reverse transcriptase (RT) (Hu & Hughes, 2012). The RT has two enzymatic functions, one is a DNA polymerase that can copy a RNA or DNA template, and an RNase H to degrade RNA if part of an RNA-DNA duplex (Hu & Hughes, 2012). The RT lacks a proofreading mechanism which contributes to the high mutation rate of HIV and the vast genetic diversity of this virus (Hu & Hughes, 2012).

This viral cDNA integrates into the host genome, using HIV integrase (IN), where it becomes a part of the host's genetic material (Craigie & Bushman, 2012) establishing a lifelong infection. HIV favors integration into actively transcribed genes, although more research is necessary to understand the roles of integration sites in transcription and

conversely, latency, where HIV is transcriptionally silent (Bedwell & Engelman, 2021; Schroder et al., 2002).

In order to replicate, HIV uses host cell machinery for transcription of its genes. Initially, transcribed mRNA is spliced at multiple sites to yield a fully spliced mRNA encoding regulatory proteins Tat (transcriptional transactivator), Rev, and Nef (Barboric & Matija Peterlin, 2005; Cullen, 1995). These fully spliced mRNA are able to be directly exported to the cytoplasm where they can be translated (Truman et al., 2020). Once Tat reaches a critical concentration, it is able to initiate RNA Pol II elongation through the initial binding of the RNA stem-loop transactivation-responsive element (TAR), enhancing proviral transcription (Mori & Valente, 2020). Later gene transcripts, coding for Gag, Pol, and Env, as well as auxiliary genes Vpr, Vpu, and Vif are unspliced or single spliced mRNA and are retained in the nucleus (Truman et al., 2020). The export of these transcripts to the cytoplasm depends upon Rev accumulating in the nucleus and binding to the Rev responsive element (RRE) in the *env* gene (Felber et al., 1989; Malim et al., 1989, 1990). Once exported to the cytoplasm, these late gene products can then be translated into viral proteins. Conversely, instead of replicating, HIV can go into a phase of latency, where the integrated provirus is transcriptionally silent, allowing the virus to go unnoticed by the immune system (Siliciano & Greene, 2011). Latency is the major barrier to curing HIV and why people need to adhere to treatment for the rest of their lives, or risk viral rebound (Siliciano & Greene, 2011). Much work is being done in the HIV cure field to either reverse latency, the shock-and-kill approach, or force cells into a

permanent latency, the block-and-lock approach (Mori & Valente, 2020), although these are lofty goals that have yet to be achieved.

After translation, Gag as well as the GagPol polyprotein, containing the protease, RT, and IN, gets trafficked to the plasma membrane along with two copies of HIV RNA genome (Freed, 2015). At the plasma membrane, the viral components assemble into an immature virion which bud from the cell (Freed, 2015). HIV protease then cleaves the long protein chains in order to make a mature infectious virus (Kleinpeter & Freed, 2020). HIV encodes for several accessory viral proteins that facilitate infection, replication, and pathogenesis by targeting host proteins that restrict or limit HIV infection. For example, Nef downregulates expression of CD4, MHC I, and MHC II negatively impacting the host's ability to recognize infected cells (Collins et al., 1998; Stumptner-Cuvelette et al., 2001). Another example pertains to the host protein tetherin, which tethers virions at the surface of the cell, exposing them for recognition and clearance from the immune system as well as making virion release inefficient. HIV Vpu downregulates tetherin helping the virus escape immune recognition, making it harder to clear the infection (Alvarez et al., 2014; Arias et al., 2014).

To complicate things more, HIV spreads through the body as either cell-free or cell-associated virus (Sagar, 2014). Cell-free virus is found in the plasma, whereas cell-associated virus has yet to bud from the infected cell and can result in cell-to-cell transmission at sights of cell-to-cell contact (Showa et al., 2019). Although the impact of cell-associated virus on transmission has been understudied, it is suggested that cell-to-cell transmission is more efficient than cell-free transmission (Showa et al., 2019). Cell-

free virus is easily measured by looking at HIV RNA levels and cell-associated virus is typically measured by HIV proviral DNA (Milligan & Overbaugh, 2014). However, measuring HIV DNA does not directly quantify infectious virus, as a large proportion of HIV DNA is actually defective (Ho et al., 2013). Correlations between HIV RNA and HIV DNA viral loads have made it challenging to distinguish transmission risk from cell-free or cell-associated virus (Milligan & Overbaugh, 2014). While both cell-free and cell-associated virus have been detected in breast milk and can contribute to MTCT of HIV, cohort studies suggest cell-associated virus may play a larger role (Milligan & Overbaugh, 2014).

In 1987, the Food and Drug Administration (FDA) approved azidothymidine (AZT) for the treatment of AIDS, giving those infected hope. This drug is a nucleoside analog reverse-transcriptase inhibitor (NRTI), which can block reverse transcription because the drug is mistaken for thymidine and incorporated into the nascent viral DNA (Hu & Hughes, 2012). In 1995, the FDA approved the first protease inhibitor, Saquinavir, which was the start of the era of highly active antiretroviral therapy (HAART). This drug class targets the HIV protease, inhibiting the cleavage of polypeptide chains, and preventing the virion from maturing (Tompa et al., 2021). With multidrug therapies, HIV/AIDS was now no longer a death sentence. In 2007, Raltegravir was approved, a new class of drugs called integrase inhibitors, which prevents HIV from inserting its DNA into the host genome. In 2012 the FDA approved Truvada, a cocktail of emtricitabine and tenofovir, two RT inhibitors, for use as Pre-exposure prophylaxis (PrEP) for high risk individuals, contributing to decreasing numbers of new cases seen

yearly. Today, over 30 HIV medications are available, half of which are RT inhibitors, and many people only require one daily pill to control their infection (Tompa et al., 2021). Although great progress has been made since the first report of this disease 40 years ago, these drugs are not cures. Notably, there is still no effective preventive HIV vaccine, which will be important for eradicating HIV.

Access to highly effective treatments has resulted in a growing number of people in the US who are virally suppressed. Previous investigations demonstrating that low plasma viral load correlated with decreased transmission transmissibility prompted clinical trials to investigate the impact of treating chronically infected individuals to prevent virus spread (J. K. L. Gunn et al., 2021; Quinn et al., 2000). In 2011, the HIV Prevention Trials Network (HPTN) study 052 provided the first evidence of treatment as prevention, and subsequent studies provided more evidence (Eisinger et al., 2019). Later, the PARTNER study recruited both serodifferent heterosexual and gay couples in the first phase (PARTNER1) and recruited only serodifferent gay couples in the second phase (PARTNER2). Results found that the risk of HIV transmission, for both heterosexual couples and gay couples, when viral load is suppressed (plasma HIV-1 RNA <200 copies/mL) is effectively zero (Rodger et al., 2019). This data supports the message of the Undetectable = Untransmissible campaign (U = U), launched in 2016 by the Prevention Access Campaign, and has led to endorsements by the CDC as well as hundreds of other organizations (Eisinger et al., 2019). U = U helps to reduce the stigma of HIV and encourage people living with HIV to continue with testing and treatment,

however more work is being done to learn about sustained viral suppression and risk of viral rebound (Buchbinder & Liu, 2019; J. K. L. Gunn et al., 2021; Min et al., 2020).

Transmission and Pathogenesis

HIV can be transmitted through blood and bodily fluids and it has been estimated to take approximately 7-21 days for virus to be detectable after the first cell has been infected (Shaw & Hunter, 2012). In general, blood transfusion poses the highest risk with an estimated 9250 transmission per 10,000 exposures, followed by MTCT at 2260 infection per 10,000 exposures (Baggaley et al., 2006; Patel et al., 2014; Sperling et al., 1996). Risks from sexual exposure varies with receptive anal intercourse posing the largest risk at 138 in 10,000 exposures and oral sex posing low risk (Boily et al., 2009; Patel et al., 2014). Furthermore, injection drug use with needle sharing poses a risk of 63 in 10,000 exposures (Hudgens et al., 2002). The most common route of HIV transmission in the world is through sexual contact (Moir et al., 2011). Besides the route of exposure, transmission risks also vary depending on a multitude of other factors, including the, viral level in the infectious source, use of antiretroviral therapy (ART) or PrEP, and male circumcision (Auvert et al., 2005; Hughes et al., 2012; Patel et al., 2014; Sagar, 2014).

For most transmission events, HIV must first cross the mucosal epithelium, to access CD4+ cells (Shattock & Moore, 2003). In adults, this would constitute the reproductive tract, whereas in infants oral transmission from ingesting infected breast milk would be the result of virus crossing the mucosal epithelium of the gastrointestinal tract (Xu et al., 2013). The exact mechanism of how HIV crosses the mucosal epithelium remains unclear, although it has been suggested that this could occur by direct infection

or transcytosis through epithelial cells, uptake by Langerhans cells, or passage through a breach in the epithelial barrier (Shattock & Moore, 2003). Similarly, it is unclear where oral transmission occurs in infants, but one study in rhesus macaques suggests the oral mucosa, tonsils, upper GI tract, and stomach may be potential targets for SIV (Heron & Elahi, 2017). Across most mucosal barriers, there is access to an abundance of target cells expressing CD4, allowing HIV to establish infection and spread.

HIV starts off as an acute infection that progresses into a chronic infection. Within two to four weeks after transmission, infected individuals can begin to develop flu-like symptoms, signaling the acute phase of infection (Moir et al., 2011). In this acute phase, HIV is replicating rapidly, usually peaking around three to four weeks after exposure before settling at a viral set point (Moir et al., 2011). CD4+ T cells are rapidly declining during this time. The chronic phase of infection is notably marked by immune activation and patients taking ART can stay in this phase for decades (Moir et al., 2011). If left untreated, patients will eventually develop Acquired Immunodeficiency Syndrome (AIDS). The current clinical definition of AIDS is defined as all HIV-infected adults with a CD4+ T cell count of <200 cells/μL, a CD4+ T cell percentage of total lymphocytes <14%, or a diagnosis of an AIDS-defining condition, such as tuberculosis, pneumonia, or cancer (Schneider et al., 2008). With advancements in antiretroviral therapies, very few individuals in the United States will progress to AIDS, which is why most refer to HIV as a persistent chronic infection. People living with HIV, being treated with ART, have life expectancies very similar to uninfected adults. However, there is a high prevalence of co-morbidities such as cardiovascular disease, hepatic diseases, cancers, and osteoporosis

(Lerner et al., 2020) which impact the quality of life for the aging population of people living with HIV. While thought to be a result of chronic immune activation, the mechanisms involved in the associated systemic diseases have yet to be elucidated.

HIV Envelope, Diversity, and Subtypes

The lack of proofreading activity in the reverse transcriptase generates the mutational landscape of HIV-1 (Hu & Hughes, 2012). Furthermore, immune pressure from primarily neutralizing antibodies drives Env mutations resulting in a multitude of variants. These genetic variants are separated into three groups based on phylogenetic analysis: major (M), outlier (O), and nonmajor and nonoutlier (N) (Taylor et al., 2008). More recently, Group P was discovered, but this variant has only been identified in a two people (Plantier et al., 2009; Vallari et al., 2011). In fact, a 2018 analysis suggests this rare group may evolve to a dead-end infection (Alessandri-Gradt et al., 2018). The majority of the world's infections fall within Group M, which is further broken into subtypes or clades, identified as A-K, that are approximately the same genetic distance from one another (Hemelaar, 2012; Taylor et al., 2008). In addition to being genetically distanced, subtypes are often geographically separated (Illustration 2). Subtype B is the most studied subtype and is predominant in North America (Buonaguro et al., 2007). However, Subtype C infections make up 47% of all new HIV-1 infections worldwide and is the predominate subtype in Sub-Saharan Africa (Hemelaar et al., 2006). Most of the genetic diversity in the HIV genome occurs in the envelope (Env) region, with 35% amino acid differences between subtypes and 20% within a subtype, impacting the Env protein composition of the virion (Korber et al., 2001; R. M. Lynch et al., 2009). This

large genetic diversity, is one of the major barriers to developing a globally effective HIV-1 vaccine (Madhavi et al., 2014).

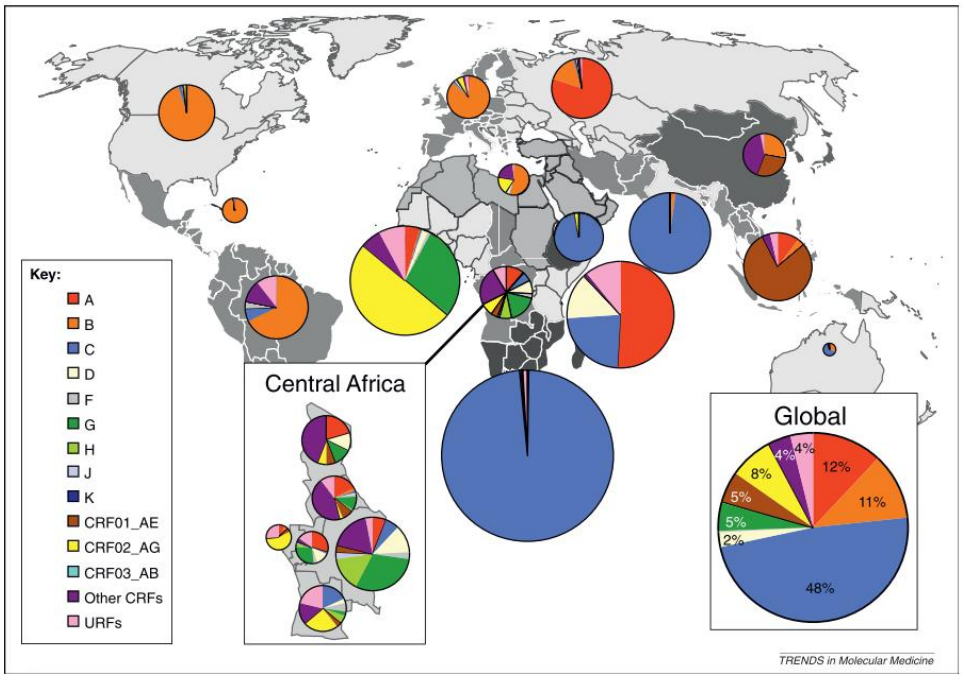

Illustration 2. Global Distribution of HIV-1 subtypes and recombinants. Pie charts depict distribution of subtypes for various areas globally. Adapted from (Hemelaar et al., 2012) with permission from Elsevier.

HIV naturally has low Env density, on average 14 Env spikes per virion (Schiller & Chackerian, 2014), making it more challenging to effectively use antibodies to target HIV Env. Additionally, HIV has evolved in numerous ways to avoid immune recognition. Env glycosylation as well as variation in sequence and conformation all contribute to immune evasion (Wang et al., 2020). Changes in the glycan shield allow HIV-1 to escape neutralization responses without impairing binding to necessary receptors (Wei et al., 2003). Env has multiple conformations, starting in the closed Stage

1 conformation and moving to the open Stage 3 conformation. An intermediate conformation, Stage 2 occurs during CD4 binding. Neutralizing antibodies tend to target Env in the closed confirmation, whereas it is suggested that antibodies capable of inducing antibody-dependent cellular cytotoxicity (ADCC), another function of antibodies, bind to epitopes that are hidden until Env transitions to the open confirmation. Small molecules, such as small CD4 mimetics (CD4ms), can facilitate this conformational change and expose these masked epitopes on the Env spike, making an HIV-1-infected cell susceptible to ADCC (Alsahafi et al., 2019). Antibodies targeting Env can cause immunological pressure that drives HIV to mutate in order to escape detection (Overbaugh & Morris, 2012). However, a recent study has suggested that these escape mutations may expose new epitopes rendering the virus susceptible to ADCC (Mielke et al., 2019). Yet, the host humoral response is always one step behind the virus, as it is common for the Env to have mutated to escape the contemporaneous neutralizing antibodies. The Env protein can be separated into two segments, gp41, which contains the cytoplasmic domain, and gp120, which is on the outside of the virion and contains constant and variable regions (Arrildt et al., 2012). Most of these escape mutations occur in the variable regions, V1/V2 loops and V3 loops, were binding of nAbs drives escape (Illustration 3).

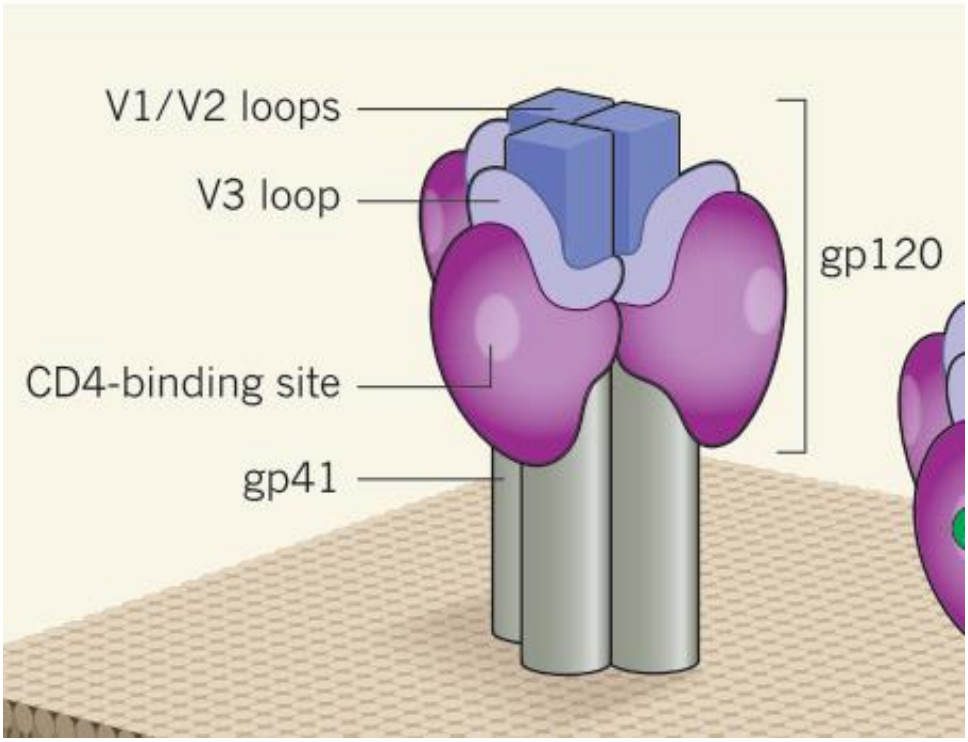

Illustration 3. HIV Envelope (Env) protein displayed on viral membrane. HIV Env is made up three molecules of gp120, the surface glycoprotein, and three molecules of gp41, the transmembrane glycoprotein. Gp120 contains variable V1/V2 and V3 loops, and a binding site for CD4. These sites are common targets of HIV-specific neutralizing antibodies. Adapted from (Clapham and Lu, 2011) with permission from SpringerNature.

Mother-to-child-transmission and breastfeeding

HIV can be transmitted from an HIV-infected mother to her child during pregnancy, labor and delivery, and breastfeeding (Lehman & Farquhar, 2007). This is known as mother-to-child-transmission (MTCT), or vertical transmission, and accounts for the majority of infections in children under the age of 14 years (AVERT, 2020). With adherence to ART, the risk of transmission is less than 5% (WHO, 2018). However, in the absence of medication, there are different risks associated at each stage: pregnancy

(5-10%), labor and delivery (10-20%), and breastfeeding (10-20%) (Lehman & Farquhar, 2007). Overall, the risk of transmission is 15-45% depending on the presence and duration of breastfeeding (Connor et al., 1994; De Cock et al., 2000; John-Stewart et al., 2004; John & Kreiss, 1996; Thorne & Newell, 2003). Although there is high risk associated with breastfeeding, the WHO still recommends exclusive breastfeeding for 6 months for women living in areas without a clean source of water, because the benefits of breastfeeding are greater than the risk of HIV-1 transmission (WHO, 2016).

Infants are highly susceptible to infection because their immune system is not functionally developed at birth (Kollmann et al., 2017). Neonates predominately make IgM antibodies, which are poor at recognizing pathogens for immune responses (Zola, 1997). Therefore, infants rely on maternal antibodies, namely IgGs, that are transferred through the placenta during pregnancy (Pasetti et al., 2020). IgG transport across the placenta relies on an interaction with Fc neonatal receptor (FcRn), which aids in the translocation of these antibodies (Palmeira et al., 2012; Roopenian & Akilesh, 2007). Additionally, human breast milk is full of factors that can impact infants' health. The benefits of exclusive breastfeeding may potentially outweigh the risks in resource poor areas, because intermittent breastfeeding can cause disruptions in the infant's intestines, and it is hypothesized that this could act as an entry point for HIV-1 (Henrick et al., 2017). Although IgG is the predominant immunoglobulin in plasma, secretory IgA makes up the majority of immunoglobulins in breast milk (Jackson & Nazar, 2006). In addition to antibodies, breast milk contains immune cells such as leukocytes, levels which vary over time (Henrick et al., 2017; Witkowska-Zimny & Kaminska-El-Hassan, 2017). Early

colostrum contains approximately 5 x 10^6 cells per mL, 10% of which are lymphocytes, including T cells, B cells, and natural killer (NK) cells (Jackson & Nazar, 2006). Evidence from animal studies suggest these cells can survive passage through the infant's gastrointestinal system where they are absorbed and can influence the infant's immune response (Jackson & Nazar, 2006). Beyond antibodies and immune cells, other factors such as fats and sugars can influence infant immunity by shaping the gut microbiome or acting as innate factors protecting against pathogens (Doare et al., 2018; Lis-Kuberka & Orczyk-Pawiłowicz, 2019; Plaza-Díaz et al., 2018). These passively acquired antibodies from the mother can help protect infants from pathogens until the infant's own immune system matures to make their own antibodies and mount effective immune responses (Tobin & Aldrovandi, 2013). Maternal antibodies are present in infants and begin to wane at around 6-12 months, just around the time of *de novo* antibody production (Niewiesk, 2014).

The majority of *in utero* transmission events occur during the third trimester (Lewis et al., 1990), likely when HIV-1 crosses the placenta (Chandwani et al., 1991; Maury et al., 1990; Menu et al., 1999). Transmission during labor and delivery accounts for approximately two-thirds of non-breastfed infants acquiring HIV-1, from exposure to infected maternal blood and genital secretions as the infant passes through the birth canal (De Cock et al., 2000). As a result, caesarean section has been used when available and is recommended when mothers have a plasma viral load >1000 copies/mL (Group, 1999). Breastfeeding accounts for a large majority of MTCT cases due to the high exposure to virus over a period of up to two years (Lehman & Farquhar, 2007). Unsurprisingly, viral

titers in breast milk and duration of breastfeeding are the most important determinants of transmission risk (Mofenson & McIntyre, 2000). Additionally, inflammation from mastitis has been associated with increased virus levels in the breastmilk, resulting in increased transmission risk (John et al., 2001). However, it is still unclear why more than half of infants born to infected mothers remain uninfected even in the face of this high virus exposure.

Antibodies and HIV-1

After HIV-1 infection, all infected individuals develop anti-HIV-1 antibodies over the course of the disease (Overbaugh & Morris, 2012). Immunoglobulins, or antibodies, consist of two heavy and two light chains, making up the Fab region, and CH domains which comprise the Fc portion (Schroeder & Cavacini, 2010). The Fab region is responsible for the recognition of antigen whereas the Fc region determines the isotype and subclass of the antibody (Schroeder & Cavacini, 2010). The Fc fragment can also mediate effector functions by interacting with Fc receptors on effector cells (Schroeder & Cavacini, 2010). Plasma B cells first produce IgM antibodies. After antigen exposure, IgM antibodies that can undergo class switching and somatic hypermutation to gain further specificity become highly specific for the targeted epitope (Mouquet, 2014). During the class switching process, IgM changes to IgD, IgG, IgA, and IgE (Tomaras & Haynes, 2009). IgG is the predominant immunoglobulin isotype found in the body, and can be further categorized into four subclasses: IgG1, IgG2, IgG3, and IgG4 (Vidarsson et al., 2014). These IgG subclasses were numbered based on the abundance found in the blood, with IgG1 being most abundant (Schroeder & Cavacini, 2010). The effector

mechanisms able to be induced vary based on IgG subclass. For example, IgG1 and IgG3 are often generated in response to viral infection, as they induce robust effector responses (Vidarsson et al., 2014). After undergoing class switching and somatic hypermutation plasma B cells, the cells that produce antibodies, will undergo affinity maturation in the germinal center to fine turn their specificity and increase their affinity for the antigen (Kepler & Wiehe, 2017). However, mutated HIV-1 can escape from these newly generated antibodies (Moore, 2017). In this way, HIV-1 and the antibody response are in an arms race (Moore, 2017). Additionally, the creation of these antibodies, specifically neutralizing antibodies, puts immunological pressure on the virus leading to the selection of specific variants (Bonsignori et al., 2017). This is another reason as to why a person's immune system is unable to clear the virus and is always one step behind. However, antibodies would prove effective if they were one step ahead of the virus. Researchers are using this tactic for vaccine development and passive immunizations, and some animal studies have shown protection when broadly neutralizing antibodies (bnAbs) are present prior to infection (B. M. Gunn & Alter, 2016).

There are two general categories of antibodies: neutralizing and non-neutralizing. Neutralization is an immune mechanism where Fab region of antibodies bind to the virion Env, preventing the virus from entering the host cell (Overbaugh & Morris, 2012). Eliciting neutralizing antibodies has become a major focus in vaccine development (Hraber et al., 2014). The exposure of immunogenic epitopes can vary across different subtypes of HIV-1 and can impact the levels of neutralization susceptibility of a virus (DeCamp et al., 2014). Several regions of the HIV-1 Env are highly susceptible to

neutralizing antibodies, including the CD4bs, the gp120 V1V2 glycan region, the V3 glycan region, and the membrane proximal external region (MPER) (Bonsignori et al., 2017). The most commonly targeted sites are those with high variability, such as V1V2 glycan region, which are variable regions in gp120. An uncommon subset of neutralizing antibodies, bnAbs, have widely been considered the gold standard when trying to develop effective vaccines, because these antibodies can bind a wide breadth of HIV-1 epitopes, spanning different clades, and neutralize the virus (Wibmer et al., 2015). A small number of people will naturally develop bnAbs, but these antibodies are not effective in controlling the virus in that infected person (Mouquet, 2014). Additionally, there are non-neutralizing antibodies that can potentially provide protection through mediating Fc-effector functions (Zolla-Pazner, 2016). These functions are induced after the antibody binds to a viral protein with their Fab region, leaving the Fc region accessible to engage with an Fc receptor (Forthal & Moog, 2009). There are a variety of Fc receptors found on immune cells, and these receptors can induce various immune mechanisms after binding to Fc regions on antibodies (B. M. Gunn & Alter, 2016). These functions include ADCC, antibody-dependent cellular virus inhibition (ADCVI), antibody-dependent cellular phagocytosis (ADCP), and complement dependent cytotoxicity (CDC) (Boesch et al., 2015). These functions may play some role in the control of infection, but the majority of vaccine research had been solely focused on neutralizing antibodies, and more specifically bnAbs.

To date, there has only been one vaccine clinical trial that demonstrated moderate efficacy. The RV144 Thai Vaccine Efficacy Trail was a double-blind, placebo-controlled

efficacy trial which evaluated four priming injections of the ALVAC-HIV vaccine plus two booster injections of the AIDSVAX B/E vaccine (Rerks-Ngarm et al., 2009). The trial enrolled 16,402 healthy men and women in Thailand who were, primarily at risk for transmission from heterosexual activity (Rerks-Ngarm et al., 2009). HIV-1 status was checked at the end of the 6-month vaccination series and participants were followed for 3 years with status checks every 6 months (Rerks-Ngarm et al., 2009). The vaccine was determined to have 31.2% efficacy. Vaccination, however, did not affect viral load or CD4+ T cell count decline in individuals who subsequently acquired HIV-1 (Rerks-Ngarm et al., 2009). A pre-planned correlation analysis of this study determined that the efficacy was not associated with cytotoxic T lymphocyte activation or the presence of broadly neutralizing antibodies (Haynes et al., 2012). Instead, non-neutralizing antibodies (nnAbs) capable of inducing ADCC and low IgA levels were implicated as a correlate of protection (Haynes et al., 2012). Interest in ADCC-inducing antibodies has grown as a result of the RV144 trial.

In 2013, another trial HVTN 097 (ClinicalTrials.gov NCT02109354) set out to evaluate whether this same protection seen in the RV144 Trial could be elicited in South Africans (Gray et al., 2019). One hundred participants were enrolled in this trial and T cell responses, as well as humoral responses, were compared to outcomes from RV144 (Gray et al., 2019). In both trials, participants generated cross-clade antibody responses and V1V2-specific IgG antibodies were significantly higher in the HVTN 097 participants as compared to the RV144 participants (Gray et al., 2019). Additionally, ADCC response rate was higher in HVTN 097 than in RV144, however the magnitude of

the ADCC responses were similar among responders in both trials (Gray et al., 2019). Although promising, the larger follow up study, HVTN 702 (ClincialTrials.gov NCT02968849) proved to be a failure, as the trial was stopped early in February 2020 because the intervention arm was unlikely to demonstrate lower HIV acquisition (UNAIDS, 2020b).

ADCC and protection against acquisition

ADCC is induced when an antibody binds to viral envelope glycoprotein expressed on the surface of infected cells via the Fab region (Su et al., 2019). The Fc portion of the bound antibody can crosslink with Fc receptors on various immune cells, including monocytes, macrophages, neutrophils, dendritic cells, and natural killer (NK) cells (Boesch et al., 2015). NK cells have been found to be the predominant mediators of ADCC, and therefore are the most commonly used effector cells in ADCC assays (Scully & Alter, 2016). Crosslinking between the antibody Fc portion and the Fc receptor (FcR) on NK cells induces the release of granzymes and perforin to induce the killing of infected cells (Hildreth et al., 1999; Su et al., 2019). ADCC has mainly been attributed to engagement of the FcγRIIIa, present on NK cells, by IgG1 and IgG3 isotypes (Holl et al., 2009). Furthermore, ADCC responses can be detected early during acute HIV infection (Chung et al., 2011).

Although ADCC was positively implicated in the RV144 trial, there is still controversy surrounding the role of this immune mechanism in preventing infection. The controversy primarily stems from the nonconfirmatory animal data. One study found simian immunodeficiency virus (SIV)-specific ADCC responses in infected macaques

correlated with delayed progression to AIDS (Banks et al., 2002). Another tested a pentavalent vaccine in macaques and found that improved ADCC responses, particularly those mediated by NK cells, resulted in increased protection from simian/human immunodeficiency virus (SHIV) acquisition (Bradley et al., 2017). An additional study found passive infusion of a human monoclonal antibody that binds the integrin binding site in the envelope (Env) variable loop 2 (V2) region, similar to epitopes of interest in the RV144 trial, impeded viral seeding after mucosal SHIV challenge (Hessell et al., 2018). Yet another study looked at the development of NK-mediated ADCC responses over time in SIVmac251-infected rhesus monkeys and was able to detect ADCC activity as early as 3 weeks post infection (Sun et al., 2011). However, other groups have been unable to detect ADCC-mediating antibody responses in rhesus macaques (Nguyen et al., 2018). Furthermore, various studies have shown passive infusion ADCC mediating antibodies fail to prevent virus acquisition in virus challenged animals (Astronomo et al., 2016; Burton et al., 2011; Dugast et al., 2014; Moog et al., 2014; Santra et al., 2015).

ADCC may also play a role in HIV disease progression (Forthal & Finzi, 2018). Elite controllers are a special group of individuals that are able to control their HIV infection without the use of ART. In one study, elite controllers were found to have higher levels of anti-HIV Env antibody responses that could mediate antibody dependent functions compared to viremic patients (Lambotte et al., 2009). A more recent study went on to show elite controllers with undetectable viral load had higher ADCC responses compared to viral controllers with low levels of virus, suggesting ADCC may be associated the ability to naturally control infection (Kant et al., 2020). Furthermore,

groups have shown inverse correlation with serum ADCC responses and disease progression (Baum et al., 1996). Indeed, this association between higher ADCC activity and improved survival can be seen in other viral infections such as cytomegalovirus (CMV) (Forthal et al., 1999). Moreover, a recent publication provided evidence to support that ADCC responses are important for controlling plasma viral load and contributes to slowing disease progression (Talathi et al., 2020). Discrepancies between human correlate analyses and animal studies has led some groups to investigate ADCC-inducing antibodies in MTCT of HIV-1.

Examining the role of ADCC in transmission in a MTCT cohort is ideal because there is a relatively low rate of transmission, 10-20%, seen in breastfeeding, suggesting a natural protective mechanism (Aldrovandi & Kuhn, 2010). Furthermore, using this model eliminates any doubt about exposure because infants are exposed to virus from their infected mothers during gestation as well as in the breast milk (Tobin & Aldrovandi, 2013). Additionally, infants acquire maternal antibodies, so it is known that antibodies are present (Fouda et al., 2011). Finally, samples are available from infected source (mother) and the exposed partner. A previous study using a MTCT model, showed that infants who were highly exposed but uninfected (HEU) had higher levels of ADCC responses in their plasma compared to infected infants, although this difference was not statistically significant (Milligan et al., 2015). Additionally, in those infants who did acquire the infection, those with higher ADCC responses had better clinical outcomes, suggesting ADCC may slow disease progression (Milligan et al., 2015). Notably, no

study has investigated responses against maternal isolates of HIV-1, which are the exposure strains the antibodies need to be protective against.

Scientific proposal and hypothesis

The following work aims to use the MTCT model to understand the role of ADCC-inducing antibodies in the prevention of HIV-1 acquisition and disease progression. We developed a new cell line for use in a luciferase-based ADCC assay, allowing us to investigate ADCC responses against circulating maternal HIV-1 variants. We hypothesized that non-transmitting mothers and their uninfected infants would have higher ADCC responses compared to transmitting mothers and their infants, suggesting ADCC is a correlate of protection in MTCT. Therefore, we assessed autologous ADCC responses from plasma and breast milk from mother-infant pairs where transmission did or did not occur. Additionally, we were interested in assessing the breadth and potency of the antibodies in these samples which was done using a panel of viruses that incorporated representative heterologous HIV Envs. This study is the first to investigate ADCC responses against maternal variants of HIV-1. In addition to clarifying the role of ADCC in preventing transmission, it provides preliminary data for further investigation of highly broad and potent ADCC-inducing antibodies, which will be useful for future vaccine design. The following aims were proposed to test our hypothesis:

Aim 1: Develop a new cell line to be used in a luciferase based ADCC assay.

There are a variety of ADCC assays currently being used in different studies. These assays can be divided into infection-based and non-infection-based assays. Non-infection-based assays often coat target cells with gp120-protein, which can cause issues with bystander killing. Additionally, coating cells presents the Env protein in a non-physiological manner, most likely in an open as opposed to closed confirmation. Infection-based models often use lab strain variants to infect targets for their assessments. These target cells, however, lack susceptibility to infection from clinical variants, limiting the questions that can be addressed. This aim, addressed in Chapter Three, details the development of a new cell line, MT4-CCR5-Luc that allows us to push past previous limitations of the current assays.

Aim 2: Assess the role of ADCC-inducing antibodies in the prevention of mother-to-child-transmission of HIV-1.

Relatively few infants born to HIV-infected mothers will acquire the infection, even in the absence of ART. In addition to being exposed to high levels of virus, infants also passively acquire antibodies from their mothers, leading us to believe antibodies play a role in reducing the transmission risk. Previous work from our lab assessed neutralization responses in this cohort and found it not to be a correlate of reduced transmission, leading us to assess an Fc effector function of antibodies. In Chapter Four, autologous ADCC responses will be assessed against autologous virus that is circulating in infected mothers. Data will be used to compare transmitting and non-transmitting mothers and their respective infants. We hypothesize that non-transmitting mothers and

highly exposed uninfected (HEU) infants will have higher ADCC responses than transmitting mothers and infants who acquired infection. Furthermore, we hypothesize that high ADCC responses will correlate with better outcomes in infected infants. This aim will clarify the role that ADCC has in prevention of transmission as well as in disease progression.

Aim 3: Identify broad and potent ADCC-inducing antibodies.

Similar to broadly neutralizing antibodies, there is a range of breadth and potency of ADCC-inducing antibodies, and highly broad and potent antibodies would be useful for vaccine development. Similar methodology from Aim 2 will be used to investigate breadth and potency of ADCC antibodies. A panel of 12 reference viruses, representative of over 200 Envs, will be used to determine how many viruses the antibodies respond to (breadth) and the magnitude of the response (potency). This aim, detailed in Chapter Five, will identify samples with high breadth and potency. These preliminary results will provide a starting point for further characterization of specific antibodies present in these samples that would be useful for future vaccine development.

CHAPTER TWO: MATERIALS AND METHODS

Breastfeeding, Antiretroviral, and Nutrition Study

Study information and subjects

Samples used for the work in this dissertation were acquired from the control arm of the Breastfeeding, Antiretroviral, and Nutrition (BAN) Study. We had access to maternal plasma, infant plasma, and breast milk. The BAN study was a clinical trial which began in 2004 that's objectives notably included determining the benefit and safety of giving antiretroviral (ARV) medications to infants or their mothers to prevent HIV transmission during breastfeeding (ClinicalTrials.gov number NCT00164736). As such, all mother-infant pairs examined in this study were treated peripartum with a single dose of oral nevirapine followed by zidovudine-lamivudine (ZDV-3TC) for seven days postpartum, as was standard care at the time. In order to be enrolled in the trial, infants needed to have a negative HIV DNA-PCR at birth and 14 days postpartum to rule out infection during pregnancy or labor and delivery. All mothers exclusively breastfed their infants, and samples were regularly collected for 48 weeks (Chasela et al., 2010a). Infants were considered to have acquired HIV through breast milk when they received their first positive HIV DNA-PCR test. Samples used in this study were obtained prior to the documented transmission event. Infants that acquired infection and their corresponding transmitting mother were then matched to two mother-infant pairs where transmission did not occur. Matching considered maternal viral load, maternal CD4+ T-cell count, as well as days postpartum to sample collection (Table 2.1).

Table 2.1 Demographics for HIV-1 transmitting and non-transmitting mother-infant pairs[a]

Demographic parameters[b]	**TM/HEI**	**NTM/HEU**
Mothers	TMs	NTMs
Age, years	26 (18-36)	26 (17-36)
Time PP Sample Collection, days	43 (1-256)	42.50 (12-297)
CD4 cells/mm^3	336 (210-1,092)	339 (240-1,145)
Log_{10} plasma VL	4.78 (3.32-5.80)	4.67 (1.59-5.99)
Infants	HEI infants	HEU infants
1st day detected as HIV^+	83 (42-293)	
Birth weight, kg	2.95 (2.30-4.00)	3.0 (2.3-3.7)
% female	28.6	38.1
% with SAE ≥ Grade 4 or death	38.1	14.3

[a]TM, transmitting mothers; NTM, non-transmitting mothers; HEI, HIV-exposed infected; HEU, HIV-exposed uninfected; PP, postpartum; SAE, serious adverse event; VL, viral load
[b]Mean values are shown with ranges given in parentheses

Cell lines

PM1 (Lusso et al., 1995), 293T (Graham & Smiley, 1977), and TZM-bl (Platt et al., 2009) cells were obtained from the NIH AIDS Reagent Program. CEM.NKr-CCR5-sLuc (subsequently referred to as CEM.NKr-CCR5-Luc) and the NK cell line, $CD16^+$KHYG-1, were generously provided by Dr. David Evans (Alpert et al., 2012). MT4-CCR5-GFP cells were generously provided by Dr. Paul Bieniasz (Kane et al., 2016).

A Tat-inducible luciferase reporter gene was introduced into PM1 and MT4-CCR5-GFP cells using the following protocol. Briefly, 293T cells were co-transfected with: 1) 7.5μg of a plasmid LNSX-Luc-1 (generously provided by Dr. David Evans), which has a luciferase gene in 5' position to a SIV long terminal repeat; 2) 6.75μg of plasmid pCL-10A1 (generously provided by Dr. Suryarahm Gummuluru), which expresses packaging proteins for murine leukemia virus (MuLV); and 3) 0.75μg of a

vesicular stomatitis virus protein G (VSV-G) expression vector (obtained from NIH AIDS Reagent Program), after incubating for 30 minutes in the presence of 45μL of Fugene. The virus was collected 48 hours later. Approximately $1X10^6$ PM1 and MT4-CCR5-GFP cells were exposed to concentrated viral supernatant in the presence of 20μg/mL diethylaminoethyl cellulose (DEAE) for 4 hours at 37°C. Cells were washed and plated in RPMI supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100U/mL of penicillin, and 100 μg/mL of streptomycin (RMPI complete) for 72 hours. Infected cells were selected by incubating cells in complete RPMI supplemented with 0.8μg/mL G418 (Sigma). The resulting cells were named PM1-Luc and MT4-CCR5-Luc. The PM1-Luc cells were a clonal cell line, but we were unsuccessful in generating a clonal line for MT4-CCR5-Luc cells. However, the parent MT4-CCR5-GFP line was already a clonal cell line, therefore we are confident our G418 selection was sufficient, resulting in only cells transduced with the Luciferase reporter. New cell cultures were periodically thawed to ensure optimal luciferase expression.

PM1-Luc cells were further transduced with a CCR5 expression plasmid. Briefly, 293T cells were co-transfected with: 1) 7.5μg of a CCR5 expression plasmid pCX4pur-synCCR5 (generously provided by Dr. Heinrich Gottlinger); 2) 6.75μg of pCL-10A1; and 3) 0.75μg of a VSV-G expression vector. Approximately $1X10^6$ PM1-Luc cells were exposed to concentrated virus supernatant in the presence of 20 μg/mL diethylaminoethyl cellulose (DEAE) for 4h at 37ºC. Cells were washed and plated in RPMI complete, and infected cells were selected by incubating cells in complete RPMI

supplemented with 0.8μg/mL G418 and 1 μg/ml puromycin. The subsequent cell line was named PM1-CCR5-Luc.

The NK cell line, $CD16^{+}$KHYG-1, was maintained at a density of 5 X 10^5 cells per mL in RPMI medium (Invitrogen) consisting of 10% fetal bovine serum (Invitrogen), 25 mM HEPES (Invitrogen), 2 mM L-glutamine (Invitrogen), 0.1 mg/ml Primocin (InvivoGen), 1 μg/ml cyclosporine (CsA)(Sigma), and 10U/mL interleukin-2 (IL-2)(NIH AIDS Reagent Program (Lahm & Stein, 1985).

Human epithelial kidney HEK293T cells and TZM-bl cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% FBS (Invitrogen), 2mM L-glutamine (Invitrogen), 100 U/ml penicillin (Invitrogen), and 100 μg/ml streptomycin (Invitrogen).

Virus Construction

RNA isolation

HIV-1 RNA was isolated from 140 μL of plasma using the QIAamp viral RNA Mini kit (Qiagen, 52904), following the manufacturer's protocol.

cDNA synthesis

cDNA was synthesized using PCR methodology. A Mastermix consisting of 5.25 μL/tube H_2O, 3.00 μL/tube dNTP mix (10 mM each), and 0.75 μL/tube OFM19 primer 5' GCACTCAAGGCAAGCTTTATTGAGGCTTA (20 μM stock) was first made. Then, 9 μL of Mastermix and 30 μL of RNA template was added to each PCR tube, for a total volume of 39 μL. Tubes were placed in a 65°C heat block for 3-5 minutes. Samples were

removed, and then place on ice for 1 minute. A second Mastermix was made containing 12.00 µL/tube of 5x Buffer (5x stock), 3.00 µL/tube DTT (100 mM stock), 3.00 µL/tube RNaseOUT (40 u/µL stock), 3.00 µL/tube Superscript III Reverse Transcriptase (SSIII RT) (200 u/µL stock). 21 µL of Mastermix was then added to each PCR tube for a total volume of 60 µL. After mixing well, tubes were incubated for 1 hour at 50°C, before increasing the temperature to 55°C for an additional hour. The temperature was increased again to 70°C for 15 minutes in order to inactivate SSIII RT. Finally, 3 µL of RNase H was added to each tube and incubated at 37°C for 20 minutes before the cDNA was ready for storage at -80°C or used in subsequent PCR steps.

Real-time PCR and Single Genome Amplification

First Round Amplification Reaction:

Each PCR reaction contained 15.3 µL/reaction dH20, 2.0 µL/reaction 10x buffer, 0.8 µL/reaction $MgSO_4$ (50 mM stock), 0.4 µL/reaction dNTPs (10 mM stock each), 0.1 µL/reaction Taq (High Fidelity Platinum) (final concentration of 0.025 units/ µL), 0.2 µL/reaction OFM19 5’ GCACTCAAGGCAAGCTTTATTGAGGCTTA (20 µM stock), and 0.2 µL/reaction VIF1 5’ GGGTTTATTACAGGGACAGCAGAG (20 µM stock). The PCR reaction components had a total volume of 19 µL in the PCR tube and then 1 µL of cDNA was added. Negative wells had 20 µL of the reaction mix, to keep the volume consistent across tubes. Tubes were placed in the thermal cycler and run with the following parameters: 2 min at 94°C, 35 cycles of [15 sec at 94°C, 30 sec at 55°C, 4 min at 68°C], 10-20 min at 68°C, hold at 4°C.

Second Round Amplification Reaction:

Each PCR reaction contained 15.3 µL/reaction dH20, 2.0 µL/reaction 10x buffer, 0.8 µL/reaction $MgSO_4$ (50 mM stock), 0.4 µL/reaction dNTPs (10 mM stock each), 0.1 µL/reaction Taq (High Fidelity Platinum) (final concentration of 0.025 units/ µL), 0.2 µL/reaction envA 5' GGCTTAGGCATCTCCTATGGCAGGAAGAA (20 µM stock), and 0.2 µL/reaction envN 5' CTGCCAATCAGGGAAGTAGCCTTGTGT (20 µM stock). The PCR reaction components had a total volume of 19 µL in the PCR tube and then 1 µL of the first round PCR was added and mixed well. Negative wells had 20 µL of the reaction mix, to keep the volume consistent across tubes. Tubes were placed in the thermal cycler and run with the following parameters: 2 min at 94°C, 45 cycles of [15 sec at 94°C, 30 sec at 55°C, 4 min at 68°C], 10-20 min at 68°C, hold at 4°C. SGA products were sized on a 1% agarose gel in TAE buffer.

Sequencing

Prior to sequencing all PCR products, either SGA plates yielding less than 30% PCR positive or bulk PCR products, were purified using ExoSap IT (Affymetrix, 78201). Products were sent for sequencing with primers (Table 2.2).

Table 2.2 Primers for Env sequencing.

Primer	Sequence
E00F 24	TAGAAAGAGCAGAAGACAGTGGCAATGA
E35R 2116	GGTGAGTATCCCTGCCTAACTCTATT
E110F 778	CTGTTAAATGGCAGTCTAGCAGAA
E115R 1127	AGAAAAATTCCCCTCCACAATTAA
E145R 757	CAGCAGTTGAGTTGATACTACTGG
E170F 1573	AGCAGGAAGCACTATGGG
E220F 2024	TATCAAAATGGCTGTGGTATATAA
E250F	GGAGGCTTGATAGGTTTAAGAATA

Yeast gap repair and plasmid rescue

A Mastermix containing 260 µL PEG, 36 µL LiAc, and 10 µL Salmon Sperm per sample was first made. Then, yeast was thawed in 37°C for 30 sec and spun down for 2 min at 1200rpm. 6 µL of PCR insert, 2 µL (3:1 ratio) of Vector (NL4-3 ltr), and 46 µL ddH_20 was added to the tube of yeast. After, 306 µL of the Mastermix was added and the tubes were incubated at 42°C for 45 min. Tubes were spun for 2 min at 12000rpm to remove liquid and then resuspend with 200 µL TE or less. Finally, the resuspended yeast was plated on -Leu+FOA plates and we waited about 4-6 days to see colonies. The yeast culture was centrifuged for 1 min at 13000rpm in a 2ml screw-top tube to remove supernatant, and the pellet was resuspended in 200 µL Yeast Lysis buffer. 200 µL of Acid Washed Baked beads (0.45mm diameter) and 200 µL of Phenol/chloroform were added to the tube (under fume hood). Tubes were vortexed at top speed for 2 min to mix before centrifuging for 10 min at 13000rpm. The top aqueous layer was transferred to a new 1.5 mL tube, and the phenol/chloroform-containing tubes and tips were safely discarded in a designated waste container. The final volume was determined in the new tube and 1/10 volume of 8M Lithium chloride and 2.5 times volume of 100% ethanol were added. Tubes were incubated for 20 min at -80°C then centrifuged at top speed (rpm) for 20 min at 4°C. Supernatant was discarded and the tubes were set aside to air dry. Pellets were resuspended in 100 µL of ddH_2O or TE.

Bacterial transformation

Prior to starting protocol, an ice bucket was prepared and cuvettes were placed in -20°C to chill. 2-10 µL of Yeast DNA was transferred to 50 µL of lab grown Top10

electrocompetent *E. coli* cells, and then the total volume was transfer to the chilled cuvette. The cuvette was wiped and then electrocuted. 100-150 μL of S.O.C. medium was added to the cuvette and the total volume was transferred to a 5 mL falcon tube. Tubes were incubated at 30°C for one hour. During incubation, LB plates were warmed upside down in the incubator. Cultures were transferred to the plates and spread with beads under a flame. Plates were incubated overnight at 30°C and checked for colonies the next morning. One colony was picked and transferred to 2 mL of LB AMP media and shaken overnight at 30°C. Qiaprep Miniprep kits (Qiagen, 27106) were used, following manufacturer's protocol, to rescue the plasmid.

293T Transfection

One day prior to transfection, 2 x 10^6 293T cells were seeded in 12 mL adding 2 mL per well in a 6-well plate. One the day of the transfection, 1-3 μg/μL plasmid, 1 μg/μL helper plasmid, 94 μL DMEM, and 9 μL polyethylenimine (PEI) were added to tubes. Tubes were vortexed well and then incubated for 15-30 min at room temperature. Tubes were added to each well and incubated for 48 hrs at 37°C. Superntant was collected and filtered through a 0.45 μM filter (Fisher, SLHV033RB). Aliquots were made, labeled, and frozen in -80°C.

TZM-Bl Titration

TZM-Bl titration assay was used to titer viral supernatants. TZM-bl cells were suspended at 1 x 10^6 cells in 10 mL DMEM plus 10 μL of 20 mg/mL DEAE-Dextran (Fisher, ICN19513310). 100 μL DMEM complete was added to each well of a 96 well black

Costar plate (Fisher, 07-200-588). 100 μL of 293T viral supernatant was added to row A and a 2-fold serial dilution was performed to row G. NL43 was used as a positive control, while wells absent of virus were used as a negative control. 100 μL of prepared TZM-bl cells were then added to each well. All conditions were tested in triplicate. The plate was incubated at 37°C for 48 hrs prior to assessing relative light units (RLU) using the beta-galactosidase assay (described below). RLU readings from the negative control wells were subtracting from all wells, and then the three replicate values were averaged.

Beta-galactosidase assay

The beta-galactosidase (beta-gal) gene in the TZM-bl cells is driven by a Tat-promoter. Therefore, beta-gal will be produced upon HIV-1 infection and can be measured using Galacto-Light Plus Systems (Invitrogen, T1009), giving RLU values as an indicator of infection. After the 48 hr incubation for the TZM-bl titration assay, supernatants were removed and the plate was washed 1x with PBS. After removal of PBS, 30 μL of lysis buffer (provided in kit) was added to each well and the plate shook at RT for 10 mins. During this incubation, the substrate needs to be diluted 1:100 using the provided diluent. 70 μL of diluted substrate was then added into each well and the plate shook again at RT for 1 hr. Finally, 100 μL of accelerator II (provided in kit) was added to each well and RLU was immediately measured.

CD4 isolation and infection

CD4 cells were isolated from healthy tonsils. First, tonsil tissue was washed in cold PBS and cut into small pieces. Tissue was transferred to a 15 mL conical tube with 10 mL of

RMPI complete, 1.2 U/mL Dispase II, and 100 U/mL DNAse I. After incubating at 4°C for 30 minutes, tissue was strained through a 70-micron strainer and washed 2X with PBS. A Ficoll Plaque density gradient was used to collect PBMC by centrifuging for 30 minutes at 1500rpm without breaks. PBMC layer was collected and washed 2X with PBS. Trypan blue was used to check viability before isolating CD4 cells. CD4 cells were isolated using EasySep Human CD4 Negative Selection Kit (StemCell). Isolated CD4 cells were then activated by incubating at 37°C for 4 days in RPMI complete with 20 U/mL IL-2 and 2 μg/mL PHA. After incubating, cells were washed 2X with PBS to remove PHA and incubated another 48 hours in RPMI complete with 20 U/mL IL-2 prior to infection. After incubating for 48 hours, cells were ready for infection. Cells were counted and 2 x 10^6 CD4 cells were spun down. The cell pellet was resuspended in 200 μL of RPMI in a 12-well plate. Plated cells were then infected with 300 μL of 293T virus incubating for 4 hours at 37°C. After, cells were washed with 2X in PBS and resuspended in 1 mL RPMI + 20 U/mL IL-2. Infected cells were then incubated at 37°C for 4 days, after which supernatant was removed and saved. Supernatant was again gathered on day 7 post-infection and the virus was titered.

Global Panel Virus Synthesis

Global Panel Viruses

A previous study determined 12 Envs to be representative of over 200 different Envs, therefore this panel represents the global diversity of HIV-1 (DeCamp et al., 2014). Expression plasmids for these 12 env cassettes were acquired from the NIH AIDS Reagent Program (Table 2.3) and inserted into an NL4-3 plasmid. Replication competent

viruses were then generated as described above. We were only able to generate viruses incorporating 10 of the 12 Envs proposed by deCamp *et al.*

Table 2.3 Reference strains for global panel.

Reference strain	Subtype	Catalogue number
TRO11	B	11023
25710	C	11505
398F1	A	12652
CNE8	CRF01_AE	12653
BJOX2000	CRF07_BC	12655
X1632	G	12656
CE1176	C	12657
246F3	AC	12658
CH119	CRF07_BC	12659
CNE55	CRF01_AE	12661

Breast milk delipidation and IgG isolation

Delipidation

Breast milk samples were centrifuged for at 4°C for 10 minutes at 5000 rpm in a 2 mL Eppendorf tube. Three distinct layers formed with cells pelleting to the bottom of the tube. The lipid layer and breast milk supernatant (BMS) layers were then transferred to a Filterfuge tube (Corning, CLS8160) and centrifuged at 25°C for 20 minutes at 9000 rpm in order to delipidate the BMS. Filtered BMS is stored at -80°C.

IgG Isolation

IgG was isolated from 50 µL of BMS using the Melon Gel IgG Spin Purification Kit (ThermoScientific, 45206), following the manufacturer's protocol. IgG isolation resulted

in a 10-fold dilution relative to the breast milk supernatant, which was accounted for when setting up assays by adding ten times the amount of diluted IgG.

Neutralization assay and analysis

The TZM-bl neutralization assay was used to test the neutralization sensitivity of various virus and antibody combinations. TZM-bl cells were exposed to virus in the presence of a 1:50 concentration of plasma. Plasma samples were tested in triplicate. All plasma was heat inactivated for 1 hr at 56°C to remove complement. Beta-galactosidase activity was measured to quantify the reduction in viral infection in the presence as compared to the absence of plasma. Neutralization was calculated using the following equation:

$$y = 1 - \frac{RLUt - RLUc}{RLUv - RLUc}$$

Where RLU_t refers to the RLU for the test wells (cells + virus + antibody), RLU_c refers to control wells (cells only), and RLU_v refers to virus control wells (virus + cells without antibody).

Antibody dependent cellular cytotoxicity assay and analysis

Approximately 5 X 10^5 target cells were re-suspended in 500 μL RPMI complete in each well of a 24-well plate. Plated cells, exposed to the virus of interest, were centrifuged for 1.5 hours at 1200 X *g* at room temperature and then incubated for 30 minutes at 37°C. Cells were washed twice with phosphate buffered saline (PBS) and cultured in RPMI complete at 37°C. Infections were monitored for luciferase expression over the next 7 days. Infected cells were used in the ADCC assay after relative light units (RLU) were approximately 10-fold higher compared to uninfected controls. Approximately 1X10^4 target cells were plated in 96-well plate (Corning #3610) and exposed to serially diluted

or a fixed amount of antibody or plasma for 20 minutes at 37°C. Post incubation, NK cell line, $CD16^{+}$KHYG-1, were added to each well at a target cell to effector cell ratio of 1:5. Co-cultures were incubated for 4 hours when using PM1-CCR5-Luc cells or 24 hours when using MT4-CCR5-Luc cells and then examined for luciferase expression using Bright Glo Luciferase Assay System (Promega E2620) per manufacturer's instructions. Of note, percent ADCC when using MT4-CCR5-Luc cells can be estimated anywhere between 8 and 24 hours, although the latter allows for a more convenient timeframe. When CEM.NKr-CCR5-Luc cells were used as targets, a 1:10 target cell to effector cell ratio was used and luciferase expression was examined after 4 hours of incubation. Uninfected cells in the presence of NK cells served as the negative background control, and RLU values from these wells were subtracted from each sample well. The positive control reference wells lacked antibodies but contained infected cells with NK effector cells. ADCC activity was estimated as the percent luciferase expression difference observed in the sample of interest as compared to that observed in positive control wells. An in-depth explanation of the optimization and validation of this assay can be found in Chapter 3 of this dissertation.

Human Isotyping Multiplex

All maternal plasma, infant plasma, and breast milk supernatant were tested using a Bio-Plex Pro Human Isotyping Assays Kit (Bio-Rad, 171A3100M) to quantify IgG1, IgG2, IgG3, IgG4, IgA, and IgM per manufacturer's protocol. Samples were tested after a 1:40,000 dilution with isotyping diluent (provided in kit). Data was acquired on the MAGPIX through access from Boston University's Analytical Core.

Statistical analyses

All normally and not normally distributed unpaired comparisons used an unpaired t test with Welch's correction and Mann-Whitney U test, respectively. For statistical comparisons in Chapter 4, Env-ADCC$_{AUC}$ was divided into two categories (≥ 0.01 and < 0.01) based on the demarcation in the data. Proportions were compared using Fischer's exact test. For paired analysis, the Wilcoxon matched-rank test was used after averaging values from the paired two non-transmitting controls. All correlations were assessed using Spearman statistic. Multivariate logistic regression analysis was conducted with the transmission status as the dependent variable. Predictors included ADCC$_{AUC}$ or Env ADCC$_{AUC}$ (categorical group) or ADCC$_{AUC}$ + Neut$_{AUC}$, maternal plasma virus level, absolute CD4 count, and days from birth to sample collection. Clinical adverse events were graded by the BAN study investigators prior to our sample evaluations and according to toxicity tables from the Division of AIDS at the National Institute of Allergy and Infectious Diseases (NIAID). For the Kaplan Meier event curves and log-rank (Mantel-Cox) analysis, ADCC$_{AUC}$ were dichotomized as high (ADCC$_{AUC}$ $\geq$ cohort median) versus low (ADCC$_{AUC}$ $<$ cohort median). This analysis was stratified by HIV status of the infant. Cox proportional hazard models were not used because of the limited number of grade 4 or greater SAE in the cohort. Similar analyses were performed in Chapter 5. Heat maps were generated using the Los Alamos HIV sequence database heatmap tool (www.hiv.lanl.gov). Hierarchical clustering with Eucleadian distance method were used to generate all heat maps and bootstraps were generated using the Los

Alamos tool. Statistical analysis was done using R version 4.0.3 and GraphPad Prism (Version 8.0). All p-values are based on two-sided tests.

Lab grown competent cells

Competent Top 10 E. coli cells

A 5 mL culture of Top 10 *E. coli* was grown in LB broth lacking ampicillin overnight at 30°C. The 5 mL culture was then split: 2.5 mL into 1 L and 1.25 mL into 2 – 500 mL LB broth flasks. These flasks were placed on a shaker at 30°C for about 4 hours to allow for growth. The OD was checked and when OD≤0.400, bacteria was ready for extraction. The bacterial cultures were centrifuged at 6000 rpm for 10 min at 4°C, then washed 3x with autoclaved ddH_2O for 5 min. Top 10 was resuspended in 2.5 mL of 30% glycerol, aliquoted in 50 μL, and stored at -80°C.

Competent yeast cells

A 5 mL culture of yeast was grown in YEPD media overnight at 30 °C. Approximately 2 mL of the 5 mL culture was added to a 250 mL flask containing 200 mL of YEPD media and 50 mL dextrose. The culture shook overnight at 30°C. The next day, about 1 mL of the 250 mL culture was added to a large flask containing 500 mL of YEPD media and 125 mL dextrose. Flasks were cultured and shook at 30°C for until OD_{600} was between 0.4 and 0.6. To harvest, the culture was centrifuged at 3000xg for 5 minutes at 4°C, and washed twice with ddH_2O. The pellet was resuspended in approximately 2 mL sterile 30% glycerol. 100 μL was aliquoted into 0.5 mL tubes and stored at -80°C.

Buffers and reagents

LB media

10 g Tryptone, 10 g NaCl, and 5 g Yeast extract were added to 1 L ddH_2O. After mixing, LB media was autoclaved for 30 mins, and 100 µg/mL ampicillin was added when cooled.

LB Amp plates

40 g LB Agar was added to 1 L ddH_2O, mixed, and autoclaved. After cooling to 55°C, 100 µg/mL ampicillin was added and plates were poured after mixing well.

-Leu media

6.7 g Yeast nitrogen base, 20 g Dextrose, and 0.69 g -Leu CSM media were added to 1 L ddH_2O, and autoclaved after mixing.

-Leu + FOA plates

20 g Agar, 6.7 g YNB with ammonium sulfate, and 0.69 g -Leu CSM media were added to 500 mL ddH_2O, and autoclaved after mixing. 20 g Dextrose and 1 g 5-FOA were added to 350 mL ddH_2O. The volume was adjusted to 500 mL, mixed, and filtered. Both solutions were mixed, plates poured, and stored in the dark.

50x TAE buffer

242 g Tris base, 57.1 mL glacial acetic acid, and 100 mL 0.5M EDTA (pH 8.0) were added to 600 mL of ddH_2O. Buffer was mixed and the volume was adjusted to 1 L. For electrophoresis, buffer was diluted to 1x.

Yeast Lysis buffer

10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, 1% SDS, and 2% Tritox X-100 were added to 1 L ddH_2O.

YEPD media

10 g Yeast extract and 20 g Peptone were added to 800 mL ddH_2O, mixed, and autoclaved. 20 g Dextrose was added to 200 mL ddH_2O and filtered. After everything had cooled, both solutions were mixed together.

10x TE buffer

100 mL of 1M Tris-Cl and 20 mL of 0.5M EDTA (pH 8.0) were added to 880 mL of ddH_2O. Buffer was mixed well and autoclaved. For use in yeast gap repair, buffer was diluted to 1x.

CHAPTER III: A NEW CELL LINE FOR ASSESSING HIV-1 ANTIBODY DEPENDENT CELLULAR CYTOTOXICITY AGAINST A BROAD RANGE OF VARIANTS

Disclaimer: Portions of this dissertation were included in or adapted from:

A new cell line for assessing HIV-1 antibody dependent cellular cytotoxicity against a broad range of variants. Thomas, Allison S., Ghulam-Smith, M., Olson, Alex, Coote, Caroyln, Gonzales, Oscar, Sagar, Manish. Journal of Immunological Methods 480 (2020). Doi: 10.1016/j.jim.2020.112766.

Background

While nAb responses have been thoroughly examined in various patient samples and among monoclonal antibodies (mAbs), ADCC assessments have been hindered due to assay limitations. Estimating nAb responses has been greatly facilitated by the development of the TZM-bl assay. In this assay, neutralization is estimated by the relative proportion of TZM-bl reporter cells that are infected in the presence as compared to the absence of either plasma or an antibody of interest (Montefiori, 2004). Importantly, the TZM-bl assay is highly versatile because the target cell line is universally susceptible to diverse HIV variants. In addition, the level of infection can be easily monitored by measuring reporter gene expression, through either luciferase or beta-galactosidase readout. These reporters have large linear ranges, and this lends reliability to the TZM-bl

assay. A similar method to measure ADCC would be of great utility for future studies to decipher the role of ADCC during initial infection and disease progression.

While nAb assays measure the ability of an antibody to prevent infection of a naïve susceptible cell, ADCC methods estimate killing of virus bearing cells. ADCC depends on both the antigen binding fragment (Fab) and the fragment crystallizable (Fc) portion of an antibody. The antibody Fab binds to an epitope present on infected cells, such as the HIV-1 envelope glycoprotein (Env), and the Fc interacts with the Fc receptor (FcR) on immune cells, such as monocytes, macrophages, neutrophils, dendritic cells, and natural killer (NK) cells (Boesch et al., 2015). NK cells have been found to be the predominant mediators of ADCC, and as a result, they are commonly used as effector cells in ADCC assays (Scully & Alter, 2016). ADCC can be estimated by co-culturing HIV-1 infected primary cells with autologous immune cells in the presence as compared to the absence of antibody. This methodology, however, is labor intensive and highly susceptible to primary cell variability. Numerous techniques have been used to overcome some of the hurdles inherent to primary cell-based assays. For instance, the challenge of having Env present on the surface of target cells is often accomplished by pulsing cells with HIV-1 Env monomer, gp120 (Mabuka et al., 2012; Milligan et al., 2015; Orlandi et al., 2016). These non-infection-based assays are problematic for a number of reasons. First, accessible Env epitopes and Env density are likely different among productively infected cells as compared to gp120 pulsed target cells. Second, these assays are not amenable to high throughput testing against a large variety of HIV-1 isolates, such as the different transmitted-founder (T/F) strains, because it requires the generation of the

gp120 protein. Finally, pulsing cells with gp120 often leads to killing of both cells with attached Env and bystander cells (Richard et al., 2018). These potential shortcomings may account for observed differences in ADCC assessments from cell infection-based assays as compared to methods that use gp120 pulsed target cells (Bredow et al., 2016).

Alpert *et al.* (Alpert et al., 2012) developed an infection-based ADCC assay that uses CEM.NKr-CCR5-Luc as targets and the NK cell line $CD16^{+}$KHYG-1 as effectors to overcome issues associated with both primary cell-based and gp120-using methods (Bruel et al., 2017; Richard et al., 2018). The CEM.NKr-CCR5-Luc cells endogenously express the CD4 and CXCR4 receptors, and they have been engineered to have high levels of CCR5 receptors (Trkola et al., 1999). HIV requires a combination of these receptors to enter cells and initiate infection (Choe et al., 1996; Deng et al., 1997; Doranz et al., 1996). Furthermore, the CEM.NKr-CCR5-Luc cells express luciferase after HIV-1 infection, which aids in estimating infected cell levels (Spenlehauer et al., 2001). The NK cell line KHYG-1 was also engineered to express FcγRIIIa (CD16), termed $CD16^{+}$KHYG-1 (Alpert et al., 2012; Yagita et al., 2000). CD16 interaction with an antibody's Fc portion is necessary for NK cell-mediated ADCC, and this cell line was shown to express CD16 at levels similar to what has been demonstrated on primary NKs (Alpert et al., 2012; Bournazos & Ravetch, 2017; Nimmerjahn & Ravetch, 2008). In this ADCC assay, infected target cells are incubated with an antibody or plasma and then co-cultured with $CD16^{+}$KHYG-1. ADCC activity is estimated as the decrease in luciferase expression in the presence as compared to the absence of antibody.

Although the Alpert *et al.* assay has become a standard in the field, the CEM.NKr-CCR5-Luc cells often do not support replication of diverse HIV-1 variants. Alpert *et al.* used spinoculation to "overcome the relative resistance of CEM.NKr-CCR5 cells to infection" (Alpert et al., 2012). A recent publication, and our studies, demonstrate relatively poor reporter gene expression in the CEM.NKr-CCR5-Luc cells after exposure to various HIV-1 variants (Li & Chen, 2019). This has limited the ability to assess ADCC responses against a broad range of primary strains, such as those isolated from acutely infected individuals (transmitted-founder (T/F)) or the diverse variants present in chronically infected individuals.

We sought to overcome these issues with the CEM.NKr-CCR5-Luc cells by engineering novel lymphocyte cell lines that are both resistant to NK cell-mediated killing in the absence of antibody and more susceptible to a diverse range of HIV-1. The development of a new cell line to use as target cells in the Alpert *et al.* luciferase based ADCC assay will allow one to estimate ADCC breadth and potency in a high-throughput manner against diverse and clinically relevant variants, which will be useful for future prevention and virus remission efforts.

Results

3.1 Generation of two new target cell lines for ADCC assessments.

We are specifically interested in examining ADCC responses against HIV-1 variants of clinical significance, such as T/F strains and the swarm encountered by infants that ingest breast milk from their infected mothers. We first proposed to employ the well-established Alpert *et al.* infection-based, luciferase ADCC assay that uses CEM.NKr-

CCR5-Luc as targets (Alpert et al., 2012). We were unable to use this assay, as designed, because the infected target cells failed to generate significantly higher luciferase levels as compared to the uninfected target cells after exposure to a T/F or a swarm containing maternal envelopes (Fig. 3.1). A large luciferase increase was observed among cells exposed to a lab-adapted CXCR4-using strain, NL4-3, using various different protocols. Targets, exposed to T/F (REJO) or maternal envelope virus (1431), however, failed to generate significantly higher relative luminescence units as compared to uninfected cells regardless of the infection protocols, such as spinoculation and use of anions (Fig. 3.1A), the multiplicity of infection (MOI) (Fig. 3.1B), or the days post-infection (Fig. 3.1C). Others have also reported problems with reporter gene expression in the CEM.NKr-CCR5-Luc cell line (Li & Chen, 2019; Spenlehauer et al., 2001). Thus, we needed to

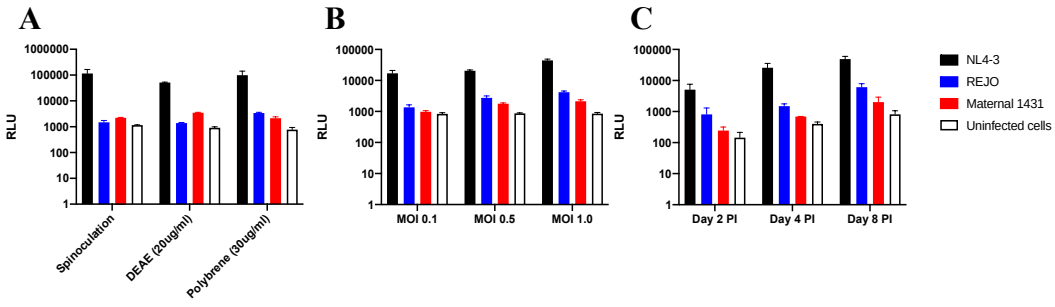

Figure 3.1. CEM.NKr-CCR5-Luc fail to produce adequate luciferase levels after exposure to strains of interest. Representative examples of relative luminescence units (y-axis) after CEM.NKr-CCR5-Luc cells were exposed to virus using different protocols (A), varying multiplicity of infection (MOI) (B), and sampled at different days post infection (PI) (C). Each bar represents the mean and standard error mean from three replicates for NL4-3 (black bar), REJO (blue bar), swarm with maternal envelopes 1431 (red bar), and uninfected cells (white bar).

modify the existing infection-based ADCC assay to examine responses against strains of high interest.

Two new cell lines were generated to overcome this issue. CD4+ T cell lines, MT4-CCR5-GFP and PM1, were chosen because of documented ability to support replication of multiple R5 variants (Kane et al., 2016; Li & Chen, 2019; Lusso et al., 1995; Rihn et al., 2017). We sought to develop an assay that could be used with viruses harboring diverse primary Envs and where infected cell killing could be estimated in an efficient manner. Thus, PM1 and MT4-CCR5-GFP were transduced with a plasmid harboring a SIV long terminal repeat (LTR) dependent firefly luciferase. Cell clones, subsequently named PM1-Luc and MT4-CCR5-Luc, were chosen that demonstrated low background and high luciferase expression after infection with a CCR5-tropic virus. The PM1-Luc cells were further transduced with a CCR5 expression construct to enhance susceptibility to R5 strains, and then a single limiting dilution clone was selected to generate PM1-CCR5-Luc cells. CCR5 expression was assessed in all cell lines by flow cytometry after staining cells with an α-CCR5 antibody. Following transfection of the

Table 3.1. Cell lines and CCR5 Expression.

	CCR5 Expression	
Cell Lines	**Percentage***	**MFI**
CEM.NKr-CCR5-Luc	59.1	72.5
PM1	62.0	38.3
PM1-CCR5-Luc	100.0	3908.7
MT4-CCR5-Luc	93.3	1.49E4

*Percentage of cells that are CCR5+
Mean fluorescent intensity (MFI) of CCR5 expression on individual cells

PM1 T-cell line with a CCR5 expression construct, the percentage of cells expressing CCR5 increased from 62% to 100%.

3.2 MT4-CCR5-Luc cells are resistant to CD16^{+}KHYG-1 cell killing in the absence of antibodies.

Previous ADCC assays specifically used the CEM.NKr cells because they are resistant to NK cell mediated lysis in the absence of antibody (Howell et al., 1985). PM1 and MT4 cells have not been selected for NK cell resistance, and thus, NK cell resistance was also assessed in the new PM1-CCR5-Luc and MT4-CCR5-Luc cell lines. Infected PM1-CCR5-Luc and MT4-CCR5-Luc were incubated with the NK cell line CD16^{+}KHYG-1. CD16^{+}KHYG-1 has been previously shown to induce CD16-dependent ADCC in the presence of appropriate antibodies (Alpert et al., 2012; Bredow et al., 2016). Percent ADCC was estimated by the decrease in luciferase levels in the presence relative to the absence of antibody. As opposed to the published resistance of CEM.NKr-CCR5-Luc to NK cells (Alpert et al., 2012), the PM1-CCR5-Luc were more susceptible to background lysis in the absence of antibody (Fig. 3.2A). In contrast, MT4-CCR5-Luc were resistant to CD16^{+}KHYG-1 cell killing in the absence of antibody (Fig. 3.2B). Importantly, all wells contained effector cells, thus any background lysis would be present in all wells. Percent ADCC can be estimated by both newly generated cell lines, although MT4-CCR5-Luc cells may be more ideal due to their natural resistance to the CD16^{+}KHYG-1 cell line used in this assay.

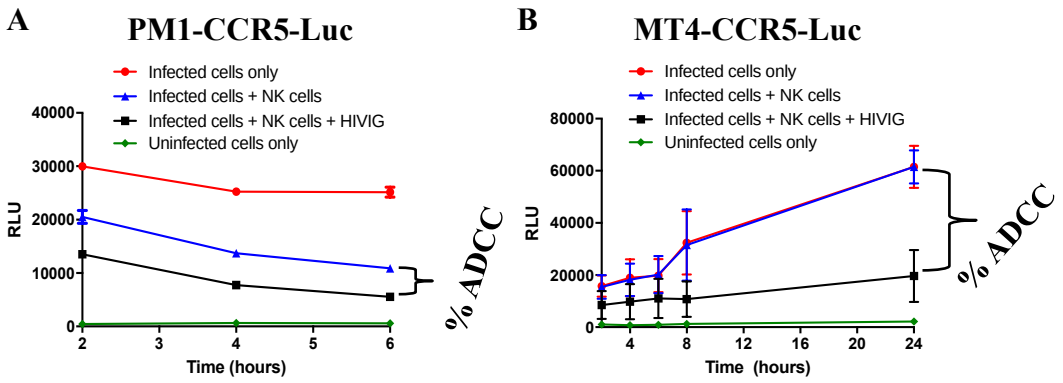

Figure 3.2. MT4-CCR5-Luc, but not PM1-CCR5-Luc, cells are resistant to CD16^{+}KHYG-1 cell killing in the absence of antibodies. Relative light units (y-axis) over time (x-axis) in NL43-infected PM1-CCR5-Luc (A) and MT4-CCR5-Luc (B) cells without antibody or NK cells (red circle), with NK cells but no antibody (blue triangle), and with NK cells and 500 μg/mL of HIVIG (black square). ADCC is determined as the percent difference as graphed. Uninfected cells (green diamond) represents background RLU reading. Data from 2 independent experiments with 3 replicates each. Bars shows standard error mean (SEM).

3.3 PM1-CCR5-Luc, MT4-CCR5-Luc, and CEM.NKr-CCR5-Luc cells yield similar ADCC estimates.

All cell lines were infected with the same virus and incubated with the same antibody preparation to assess if the three cell lines yielded similar percent ADCC estimates under identical conditions. After infection with an X4 variant (NL4-3), percent ADCC was similar for the known NK cell resistant cell line, CEM.NKr-CCR5-Luc, as well as PM1-CCR5-Luc and MT4-CCR5-Luc over a range of antibody concentrations (Fig. 3.3). The calculated AUC did not differ between CEM.NKr-CCR5-Luc (AUC = 0.579), MT4-CCR5-Luc cells (AUC = 0.614) and PM1-CCR5-Luc cells (AUC = 0.560) ($p > 0.05$ for all pairwise comparisons using Wilcoxon matched-pair test at each HIVIG dilution). The MT4-CCR5-Luc ($r^2 = 0.977$), PM1-CCR5-Luc ($r^2 = 0.911$), and CEM.NKr-CCR5-Luc

(r^2 = 0.903) also showed similar goodness of fit to a linear curve. The MT4-CCR5-Luc cell line, however, displayed a significantly lower coefficient of variation (%CV) across all six HIVIG concentrations as compared to the CEM.NKr-CCR5-Luc cell line (p = 0.031, Wilcoxon matched-pair test) but not PM1-CCR5-Luc (p = 0.156, Wilcoxon matched-pair test).

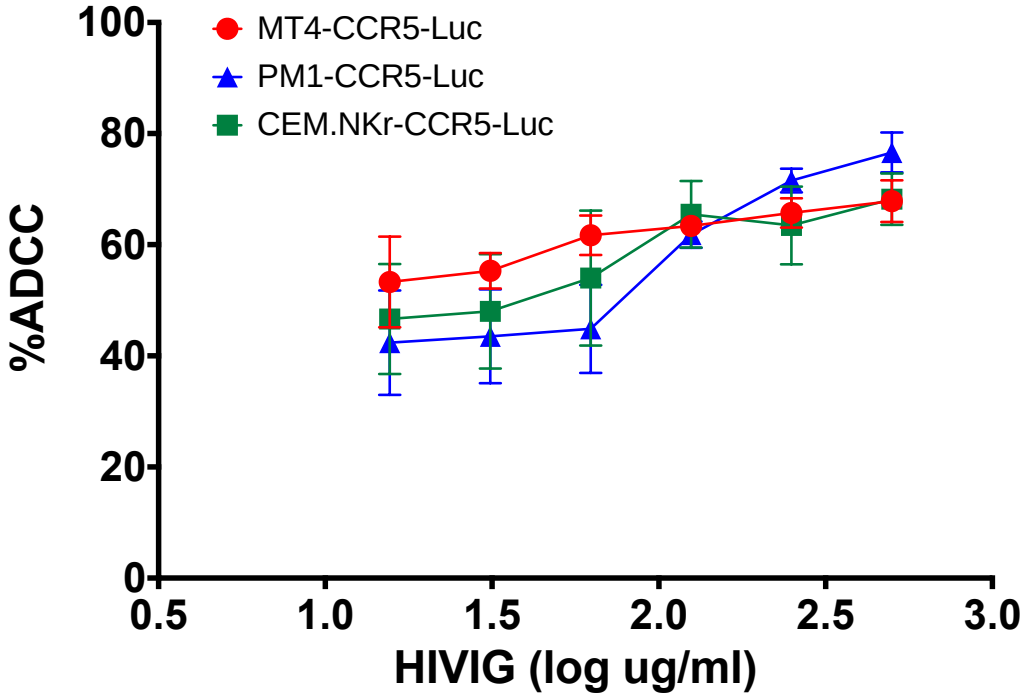

Figure 3.3. PM1-CCR5-Luc, MT4-CCR5-Luc, and CEM.NKr-CCR5-Luc cells yield similar ADCC estimates. Percent ADCC (y-axis) observed against exclusively CXCR4-using virus NL4-3 infected MT4-CCR5-Luc (red), PM1-CCR5-Luc (blue), and CEM.NKr-CCR5-Luc (green) cells with serially diluted HIVIG (x-axis). Bars show SEM from 5 independent experiments with each condition tested in triplicate.

3.4 MT4-CCR5-Luc as compared to PM1-CCR5-Luc and CEM.NKr-CCR5-Luc demonstrates susceptibility to the widest range of HIV-1 variants.

The original description, a subsequent study, and our preliminary data showed that CEM.NKr-CCR5-Luc cells failed to yield high luciferase expression when exposed to a number of different R5 variants (Li & Chen, 2019; Spenlehauer et al., 2001) (Fig. 3.4). We compared the breadth of luciferase expression among CEM.NKr-CCR5-Luc, PM1-CCR5-Luc, and MT4-CCR5-Luc cells to establish if one cell line would be better for estimating ADCC against a large panel of viruses. Luciferase expression was examined after target cells were exposed to T/F strains and recombinant viruses containing Envs from a global reference panel or those isolated from chronically infected mothers that were part of a breastfeeding transmission study (DeCamp et al., 2014; Ghulam-Smith et al., 2017; Keele et al., 2008; Salazar-Gonzalez et al., 2009). Envs in this global reference panel are representative of a larger set of 219 variants. Neutralization against Envs in this global reference panel has been effectively used to estimate the neutralization spectrum of infected and vaccinated individuals. We have previously examined the capacity of infected mothers' and exposed infants' plasma to neutralize strains incorporating maternal Envs as a correlate of protection during mother-to-child-transmission. ADCC against these and other primary Envs can be examined in future studies if target cells yield acceptable reporter gene expression after virus exposure. Equal amounts of virus were used to infect each cell line by spinoculation at 1200 X *g* for 1.5 hours in the absence of any anions. Comparisons were made between cell lines using matched pairwise comparisons across all the different HIV-1 variants. Fold luciferase induction

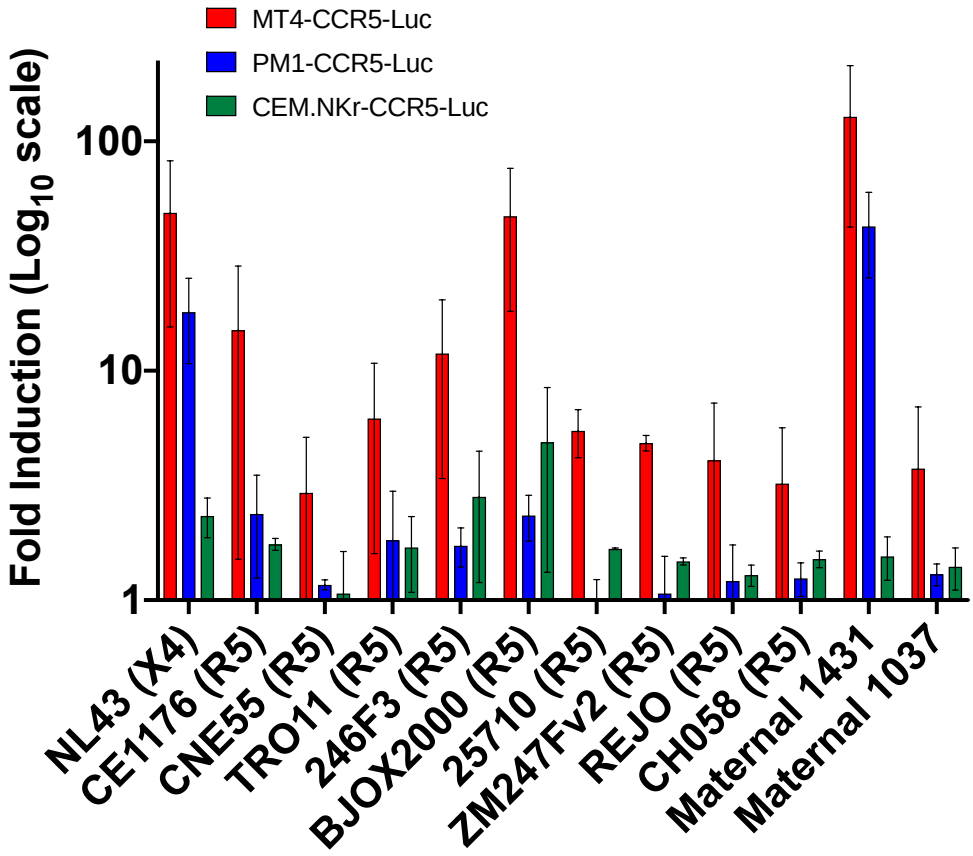

Figure 3.4. PM1-CCR5-Luc and MT4-CCR5-Luc as compared to CEM.NKr-CCR5-Luc support replication of diverse HIV-1 variants. Relative light unit (RLU) fold induction 4 days post infection in MT4-CCR5-Luc (red), PM1-CCR5-Luc (blue), and CEM.NKr-CCR5-Luc cells (green) infected with various viruses compared to uninfected cells. Viruses and co-receptor usage are indicated at the bottom. Bars represent standard error of the mean (SEM) from minimum of 2 independent experiments.

was significantly greater in the MT4-CCR5-Luc as compared to PM1-CCR5-Luc or CEM.NKr-CCR5-Luc cells ($p < 0.001$ for both comparisons, Wilcoxon matched-pair test). However, fold luciferase induction did not differ significantly between PM1-CCR5-Luc and CEM.NKr-CCR5-Luc cells ($p = 0.850$, Wilcoxon matched-pair test). MT4-CCR5-Luc cells were chosen as the ADCC target cell line for further validation because they may be amenable to estimating ADCC against a greater range of HIV-1 variants.

3.5 HIV-1 Env and antibody ADCC specificity against infected MT4-CCR5-Luc cells.

We next determined the specificity of the observed ADCC activity in the MT4-CCR5-Luc cells using two independent methods. First, HIVIG mediated ADCC was assessed against MT4-CCR5-Luc cells infected with a VSV-G pseudotyped virus that did not express the HIV-1 Env after infection. Average ADCC of 10.990% (standard deviation (STD) 4.053, n = 3 independent experiments) was observed against these infected cells. Second, ADCC activity in HIV-1 seronegative plasma was assessed against NL4-3 infected MT4-CCR5-Luc cells. HIV-1 seronegative plasma showed a mean ADCC of 8.356 (STD 6.905, n = 6 independent experiments) against the NL4-3 infected MT4-CCR5-Luc cells. A combined average plus approximately 2.5 standard deviations of the ADCC observed with HIV-1 seronegative plasma and against infected cells that did not express HIV-1 Env (25%) was used as a threshold for ADCC specificity. Any ADCC estimate below 25% was deemed as HIV-1 antibody and HIV-1 Env independent cytotoxicity. This threshold was plotted on the subsequent ADCC graphs to clearly demonstrate positive ADCC activity.

3.6 ADCC estimates based on reduction in luciferase expression are similar to those obtained using flow cytometry.

The MT4-CCR5-Luc cells displayed exceptionally high luciferase after virus exposure as compared to the other two cell lines, and thus, flow cytometry was further used to validate the use of luciferase reduction in MT4-CCR5-Luc cells as an estimate for percent ADCC. ADCC was concurrently estimated by luciferase reduction and the loss of

live HIV+ target cells after co-culture with CD16^{+}KHYG-1 cells in the absence as compared to the presence of HIVIG. Zombie UV staining was used to distinguish live from dead target cells (Fig. 3.5A), and the tat-inducible GFP was used to differentiate infected from uninfected cells (Fig. 3.5A – 3.5C). At a single HIVIG dilution, similar ADCC estimates were obtained with the two methods against NL4-3 infected MT4-CCR5-Luc cells (p = 0.25, Wilcoxon matched-pair test) (Fig. 3.5D). As a further test, HIVIG ADCC was also concurrently estimated using both flow cytometry and luciferase decrease against HIV-VSV-G pseudovirus infected MT4-CCR5-Luc cells that did not

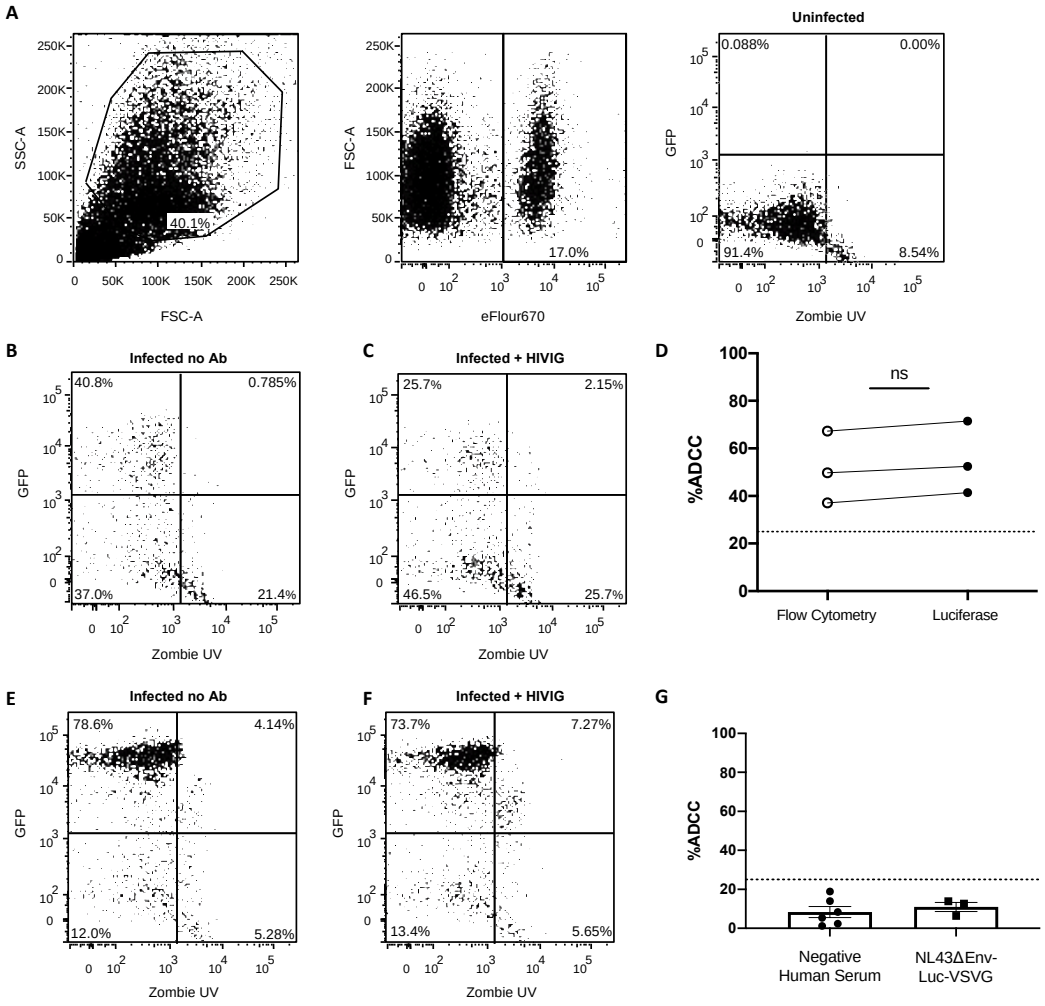

Figure 3.5. Proposed ADCC assay has been validated via flow cytometry. MT4-CCR5-Luc cells were stained with eFluor670 prior to use in assay. (A) Gating strategy is shown for the condition with uninfected MT4-CCR5-Luc cells and NK cells, which represents background signal. The final panel shows live GFP+ target cells (Q1). (B) NL43-infected MT4-CCR5-Luc cells and NK cells (no antibody added). (C) NL43-infected MT4-CCR5-Luc cells, NK cells, and HIVIG. (D) Percent ADCC calculated using luciferase readout did not differ from percent ADCC calculated as the change in percentage of live GFP+ target cells (Q1) in the flow cytometry analysis ($p = 0.25$, Wilcoxon matched-pair test). (E) NL43-ΔEnv-Luc-VSVG-infected MT4-CCR5-Luc cells and NK cells (no antibody added). (F) NL43-ΔEnv-Luc-VSVG-infected MT4-CCR5-Luc cells, NK cells, and HIV Ig. (G) Nonspecific cytotoxicity was determined by taking the average plus two standard deviations of ADCC induced by HIV negative human serum as well as ADCC against NL43-ΔEnv-Luc-VSVG-infected MT4-CCR5-Luc cells (21.17%, $n = 9$) and threshold was set at 25%.

express HIV-1 Env (Fig. 3.5E and 3.5F). Flow cytometry as compared to luciferase reduction yielded a not statistically significant lower ADCC estimate against cells that do not express HIV-1 Env ($p = 0.25$, Wilcoxon matched-pair test) (Fig. 3.5G). Importantly, these negative control estimates were below the pre-defined threshold (25%) for positive ADCC. This suggests that our reduction in reporter gene expression is a valid way to estimate percent ADCC in a high throughput manner.

3.7 ADCC estimates using MT4-CCR5-Luc cells are not biased from plasma contents.

A large number of individual's plasma samples have been screened for neutralization breadth and potency using the TZM-bl assay, and bnAbs were engineered from the "elite neutralizers" cells (Landais & Moore, 2018; Simek et al., 2009). Similarly, future studies may screen individual's plasma for ADCC breadth and potency to identify those that harbor "elite ADCC" activity, however, it is unclear as to whether plasma contents other than IgG skew ADCC estimates obtained from MT4-CCR5-Luc cells. IgG was isolated

from HIV-1 positive plasma using Melon G columns, and ADCC estimates were compared among the isolated IgG and the original plasma sample. The amounts of isolated IgG used was estimated as the same as that present in a dilution of the original plasma sample. There was no significant difference between isolated IgG and whole plasma ($p = 0.563$, Wilcoxon matched-pair test) (Fig. 3.6A). Additionally, 500 µg/mL HIVIG and 500 µg/mL HIVIG spiked into HIV-1 negative heat inactivated plasma yielded similar ADCC activity against NL4-3 infected MT4-CCR5-Luc cells (mean 56.7% and 58.9%, respectively) (Fig. 3.6B). These observations suggest ADCC estimates from using MT4-CCR5-Luc cells as targets are not skewed by plasma contents aside from IgG, the variable of interest. Thus, future studies may be conducted with plasma as opposed to isolated IgG, which decreases both time and effort.

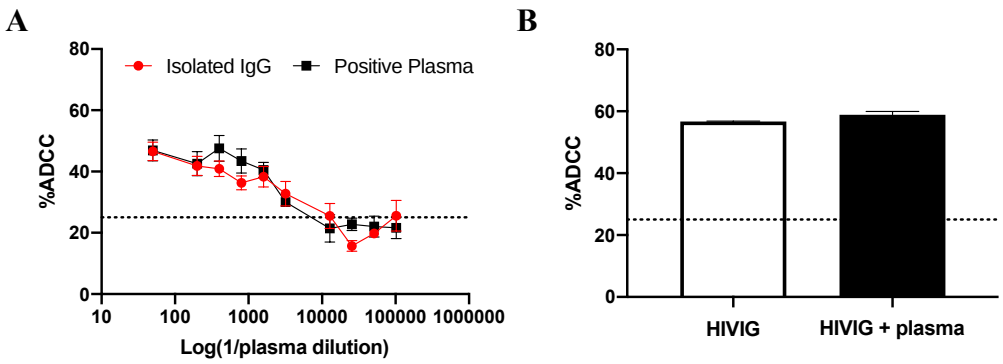

Figure 3.6. Plasma contents, besides IgG, do not impact ADCC estimates. Percent ADCC (y-axis) observed against NL4-3 infected MT4-CCR5-Luc cells with plasma from an antiretroviral naïve individual versus IgG isolated from that plasma (top dilution 1:50) (A) and 500 µg/mL HIVIG versus 500 µg/mL HIVIG spiked into heat inactivated HIV-1 seronegative plasma (B). The bars show SEM and values are from 2-3 independent experiments with each condition done in triplicate. Dashed line at y = 25 denotes threshold for nonspecific cytotoxicity.

3.8 MT4-CCR5-Luc cells can be used to estimate ADCC against viruses with primary Envs of interest.

We have previously examined the plasma neutralization capacity of maternal infant dyads against HIV-1 variant's circulating in the infected mother (Ghulam-Smith et al., 2017). Plasma ADCC capacity against exposure strains has not been examined in these mother infant dyads primarily because previous assays used CEM.NKr-CCR5-Luc cells, and these cells failed to express adequate reporter levels after infection (Fig. 3.1 and 3.4). We assessed ADCC present in the maternal and infant samples against these primary exposure strains using our new cell line (Fig. 3.7A). All maternal and only one

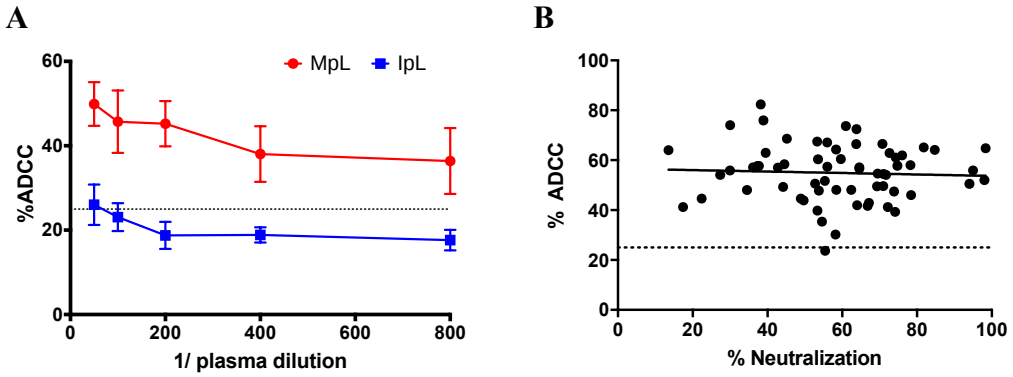

Figure 3.7. MT4-CCR5-Luc cells can be used to estimate ADCC against viruses with primary Envs of interest. (A) MT4-CCR5-Luc cells were infected with viruses representing the circulating strains present in an infected mother. Percent ADCC (y-axis) was determined after incubating infected cells with NK cells and a serial dilution (x-axis) of either maternal plasma (MpL) or infant plasma (IpL). Top dilution was 1:50 of plasma. Bars show SEM from three independent experiments with each condition tested in triplicate. (B) Heterologous ADCC responses of maternal plasma against BJOX2000-infected cells (y-axis) were plotted against neutralization responses (x-axis) from the same samples against the same virus. Dashed line at y = 25 denotes threshold for nonspecific cytotoxicity.

infant plasma dilution yielded ADCC estimates above the non-specific threshold implying that maternal but not the infant plasma harbored ADCC activity.

We have also previously examined the ability of maternal and infant plasma to block viruses incorporating global reference Envs to estimate neutralization breadth and potency (Ghulam-Smith et al., 2017). ADCC against viruses with global panel Envs has not been examined before because CEM.NKr-CCR5-Luc cells often fail to yield adequate luciferase levels after exposure to viruses incorporating global panel reference Envs (Fig. 3.4). We used our assay to examine ADCC responses from 63 maternal plasma samples against a virus with one of the reference global panel Env, BJOX2000 (DeCamp et al., 2014). Interestingly, plasma ADCC activity did not correlate with neutralization capacity against BJOX2000 among these 63 maternal samples ($p = 0.872$, $r = 0.021$, Spearman's rank test) (Fig. 3.7B), suggesting neutralization and ADCC are not always correlated, similar to what has been suggested in previous publications (Bredow et al., 2016; Smalls-Mantey et al., 2012; von Bredow et al., 2019). All but one maternal sample demonstrated ADCC activity against BJOX2000 above the non-specific threshold.

Discussion

We have developed a new cell line, MT4-CCR5-Luc to quantify HIV-1 ADCC using a high-throughput assay, originally developed by Alpert *et al.* (Alpert et al., 2012). There is great interest in examining antibody dependent Fc-mediated effector cell lysis because presence of ADCC antibodies have been deemed a correlate of protection in human vaccine studies (Lambotte et al., 2009; Madhavi et al., 2017; Scott-algara et al., 2003; Wren et al., 2013). Despite having a high-throughput assay available to detect

ADCC activity (Alpert et al., 2012), the field is still unable to evaluate a large number of samples against relevant virus strains, such as those circulating in the transmission source (Montefiori, 2004), due to issues with reporter gene expression in CEM.NKr-CCR5-Luc cells (Li & Chen, 2019; Spenlehauer et al., 2001). We have engineered and validated the use of a new cell line in an ADCC protocol that overcomes this hurdle. Use of this new cell line in our assay will allow investigators to estimate ADCC breadth and potency among antibodies and plasma samples by examining activity against diverse Envs, such as those in the global reference panel (DeCamp et al., 2014). Thus, this new method satisfies an area of need within the HIV antibody field.

The cornerstone of our assay is a target cell line capable of infection with a diverse range of CCR5-using viruses, which make up the majority of early stage infectious variants. The current standard target cell line, CEM.NKr-CCR5-Luc, did not yield adequate reporter expression over background for a large number of HIV-1 variants, limiting the ability to calculate ADCC capacity as a reduction in luciferase levels. Previous reports and communication with other investigators have reported similar difficulties, supporting the need to develop a new cell line for ADCC studies (Li & Chen, 2019; Spenlehauer et al., 2001). To do this, we first chose to use human cells of lymphoid origin because these are most similar to primary CD4+ T-cells, which are responsible for >99% of HIV-1 replication in vivo (Perelson et al., 1996). Next, we chose and engineered cell lines that express CD4, CCR5, and CXCR4 receptors to permit replication of HIV-1 strains with diverse phenotypes. Finally, we introduced a tat-responsive luciferase reporter for rapid, convenient end-point assessment (Trkola et al., 1999). Similar

considerations were made during the engineering of the CEM.NKr-CCR5-Luc cell line, yet *in vitro* analysis revealed sub-optimal CCR5 expression and low infection rates with a number of R5 strains (Spenlehauer et al., 2001) (Table 3.1, Figure 3.1 and 3.4). The new MT4-CCR5-Luc cell line showed significantly higher reporter gene expression after HIV-1 exposure as compared to CEM.NKr-CCR5-Luc and PM1-CCR5-Luc (Fig. 3.4). In addition, MT4-CCR5-Luc expressed a greater level of CCR5 receptors, suggesting that increased luciferase may be related to enhanced infection. CCR5 receptor levels, however, do not entirely explain the observed differences in reporter gene expression after virus exposure. For instance, MT4-CCR5-Luc showed significantly greater luciferase expression compared to PM1-CCR5-Luc even though percent of cells with CCR5 was not vastly different (Table 3.1). Furthermore, PM1-CCR5-Luc and CEM.NKr-CCR5-Luc did not have significant luciferase expression differences after virus exposure even though cell surface CCR5 percentage and mean fluorescence intensity were markedly different. Other factors, such as the density of the CD4 receptor and presence of attachment factors, may influence the observed differences. Our studies did not formally evaluate if CEM.NKr-CCR5-Luc cells fail to become infected after virus exposure, or if these cells induce less reporter gene expression after infection.

Natural killer (NK) cells expressing the low-affinity IgG receptor FcγRIIIa (CD16) are the primary effectors of ADCC *in vivo* (Scully & Alter, 2016). We use cells derived from the immortalized cell line KHYG-1, as effector cells in this assay (Yagita et al., 2000). The KHYG-1 parent line was transduced with CD16, yielding $CD16^{+}$KHYG-1 cells, and the new cell line was shown to express similar levels of CD16 as primary NK

cells (Alpert et al., 2012). Furthermore, the use of cell lines for both targets and effectors reduces the time and resources needed for primary cell isolation and removes donor to donor variability.

CEM.NKr-CCR5 cells were engineered to be resistant to NK cells. NK cells can often kill T lymphocytes in the absence of antibody, which may skew ADCC estimates (Howell et al., 1985). We found MT4-CCR5-Luc to be naturally resistant to the $CD16^{+}$KHYG-1 NK cell line (Fig. 3.2B), whereas PM1-CCR5-Luc cells showed susceptibility to NK cell killing in the absence of antibodies (Fig. 3.2A). Although these new cell lines had differing NK susceptibility, ADCC estimates were similar, suggesting that ADCC estimates can still be assessed using targets lacking inherent NK-resistance (Fig. 3.3). ADCC estimations, however, will require relatively high reporter gene expression when using cells that are sensitive as opposed to resistant to NK cell killing in the absence of antibody. In our assay, we also estimated a threshold level that represents HIV-1 specific ADCC activity. Estimates below this threshold were deemed as non-specific cytotoxicity against HIV-1 infected cells that was not due to HIV-1 Env specific antibodies.

It is possible that excessively high reporter gene expression in the MT4-CCR5-Luc cells after virus exposure may yield skewed ADCC estimates that are based on decreases in luciferase. We confirmed that decreased luciferase reporter expression accurately represents cell killing using flow cytometry to estimate ADCC (Fig. 3.5). Our results indicate that ADCC calculated using our luciferase assay is analogous to calculating ADCC by the more tedious flow cytometric technique. To overcome the poor

susceptibility of cell lines, ADCC assays will often pulse target cells with gp120. The Env monomer, however, can have both receptor dependent and non-specific binding, resulting in overestimation of ADCC responses due to bystander killing (Richard et al., 2018). Congruity between the luciferase and flow cytometry-based results suggest that our cell-infection based assay is specifically measuring the elimination of infected virus producing cells.

Our primary motivation in developing a new ADCC assay was to assess whether ADCC is a correlate of protection in mother-to-child-transmission (MTCT) of HIV-1. We have previously shown that nAbs are not a correlate of protection in a cohort of 63 mother-infant pairs where transmission either did or did not occur during breastfeeding in the absence of antiretroviral therapy (Ghulam-Smith et al., 2017). Although previous studies have implied that ADCC-inducing antibodies protect against HIV transmission (Haynes et al., 2012; Mabuka et al., 2012; Permar et al., 2015), studies have not specifically examined ADCC activity against exposure strains, like those present in the infected mother. We first wanted to confirm that any ADCC activity we saw from plasma samples could be attributed to only the antibodies and not additional factors present in plasma. Our results suggest that only IgG is contributing to ADCC responses observed in plasma (Fig. 3.6), allowing us to confidently use plasma for high throughput analysis rather than having to first isolate IgG from subject samples.

We detected plasma-induced ADCC against viruses incorporating Envs isolated from a chronically infected mother (Fig. 3.7A). Importantly, uninfected infants are exposed to these Envs, and thus future studies using our assay will allow us to address if

ADCC is a correlate of protection in MTCT of HIV-1. Furthermore, we have shown that ADCC does not always correlate with neutralization, against BJOX2000 (Fig. 3.7B), supporting the need to test ADCC activity in cohorts which may not have shown protective neutralizing responses (Bredow et al., 2016; Smalls-Mantey et al., 2012; von Bredow et al., 2019) .

Widespread assessment of ADCC activity in large cohorts against panels of HIV-1 variants has been limited due to the lack of a reliable, high throughput assay. Examining ADCC activity against a broad range of Envs may identify plasma samples that have “elite ADCC” capacity. Antibodies with the ability to mediate broad and potent cellular cytotoxicity may be isolated from these samples in the future. The use of our assay will facilitate these types of studies, which will aid vaccine development and antibody-based treatment efforts.

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CHAPTER IV: AUTOLOGOUS ADCC RESPONSES CORRELATE WITH PREVENTION OF MTCT AND BETTER INFECTED INFANT OUTCOMES

Disclaimer: Portions of this dissertation were included in or adapted from:

Pre-existing infant antibody-dependent cellular cytotoxicity associates with reduced HIV-1 acquisition and lower morbidity. Thomas, Allison S., Moreau, Yvetane, Jiang, Wenqing, Isaac, John E., Ewing, Alexander, White, Laura F., Kourtis, Athena P., Sagar, Manish. [*Under review*].

Background

It is imperative to identify immune factors that can decrease HIV-1 transmission in humans. The recent finding that passive infusion of large quantities of a broadly neutralizing antibody (bnAb) did not demonstrate a significant decrease in subsequent HIV-1 acquisition further highlights this need (Corey et al., 2021). In this regard, examining mother-to-child-transmission (MTCT) cohorts can be useful because infants acquire HIV-1 at a lower frequency than may be expected, especially considering the long duration of viral exposure in-utero and during breastfeeding. This risk of HIV-1 MTCT has been primarily associated with higher maternal plasma viral load and lower absolute CD4 counts (Group, 1999). In the absence of antiretroviral treatment (ART), transmission risk during the breastfeeding period is approximately 10-20% depending on duration, suggesting natural immune mechanisms may protect against acquisition (Aldrovandi & Kuhn, 2010). Infants passively acquire antibodies throughout gestation, as well as during

breastfeeding (Fouda et al., 2011; Palmeira et al., 2012; Pasetti et al., 2020). Therefore, humoral immunity may protect infants against HIV-1 acquisition. Studies from our group and others, however, have shown that pre-existing broad and potent neutralizing antibody (nAb) responses do not associate with a lower risk of HIV-1 acquisition in highly exposed infants (Ghulam-Smith et al., 2017; J. B. Lynch et al., 2011), although some investigations have suggested otherwise (Martinez et al., 2017; Scarlatti et al., 1993; Wu et al., 2006). The goal of this study was to investigate the impact of another aspect of the humoral response on HIV-1 MTCT, namely antibody-dependent cellular cytotoxicity (ADCC).

While nAbs can prevent viruses from infecting their targets, ADCC can clear infected cells. The ability to eliminate HIV-infected cells is important because the infectious source, such as breast milk, contains both cell free virus and infected cells (Hoffman et al., 2003). ADCC is induced when the Fab region of an antibody binds to HIV-1 envelope glycoprotein (Env) expressed on the surface of infected cells. The Fc portion of the bound antibody can then interact with Fc receptors (FcRs) on various immune cells, such as (FcγRIII), CD16, on natural killer (NK) cells (Boesch et al., 2015). This Fc-FcR bridge induces the killing of the infected cell. ADCC may be especially important in preventing transmission and improving outcomes after infection because it can potentially eliminate both breast milk cells that contain infectious HIV-1 and infant cells that become productively infected after exposure.

ADCC has been a major focus of study after being associated with the modest protection observed in the RV144 HIV-1 vaccine trial (Haynes et al., 2012). Previous investigations, however, have often yielded conflicting conclusions about the role of

ADCC in preventing transmission and in providing a therapeutic benefit (Su et al., 2019). HIV-specific ADCC activity present in breast milk supernatants was associated with lower MTCT via breast feeding (Mabuka et al., 2012). Furthermore, passively transferred ADCC activity in HIV-infected infants was associated with improved infant survival (Milligan et al., 2015). Previous studies, however, have not examined ADCC against the viruses circulating in infected mothers. This is important because assessing the responses that exist prior to transmission against the maternal variants is most analogous to understanding how pre-existing antibodies, possibly induced by a vaccine, may prevent infection from the exposure strains and improve outcomes.

Previous investigations were not able to examine ADCC against cells infected with maternal strains primarily because of assay limitations. In this study, we investigated ADCC activity present in maternal and infant plasma, as well as breast milk, against strains circulating in the chronically infected mother using a novel infection-based ADCC killing assay (Thomas et al., 2020). We found that the ADCC was higher in infants that did not acquire HIV-1 compared to the infants that became infected. Furthermore, higher ADCC was associated with lower infant morbidity and mortality after HIV-1 infection. Our observations suggest that eliciting ADCC against the exposure strains provide both protection against transmission and a therapeutic benefit in setting where infected infants cannot get ART.

Results

4.1 Infant ADCC responses are associated with lower transmission.

We examined ADCC responses in plasma and breast milk samples from mother-infant pairs in the control arm of the Breastfeeding, Antiretroviral, and Nutrition (BAN) study (Chasela et al., 2010b). BAN assessed HIV-1 breast milk transmission and infant outcomes when ART was provided either to the mother or the infant compared to the control arm where ART was given for a maximum of 7 days after birth. We examined responses in a sample collected before the first infant HIV-1 PCR positive result to assess antibodies pre-existing prior to the transmission event. Every transmitting mother-infant pair was matched to two dyads with no documented transmission based on maternal virus level, absolute CD4 count, the mother's age, and interval of time after birth. We examined ADCC against virus stocks incorporating Envs isolated from 16 transmitting mothers (TMs) and 26 non-transmitting mothers (NTMs). Demographics were not significantly different among the mothers and infants in these two groups (Table 4.1).

Table 4.1. Demographics for HIV-1 transmitting and non-transmitting mother-infant pairs included in Chapter 4[a]

Demographic parameters[b]	TM/HEI	NTM/HEU
Mothers - Number	16	26
Age, years	26 (19-37)	26 (20-33)
Time PP Sample Collection, days	78 (12-260)	74 (1-256)
CD4 cells/mm^3	352 (210-595)	372 (258-677)
Log_{10} plasma VL	4.80 (3.70-5.70)	4.82 (3.12-5.99)
Infants - Number	15[c]	26
1st day detected as HIV$^+$	114 (42-293)	
Birth weight, kg	3.0 (2.5-4.0)	3.0 (2.3-3.7)
Female (%)	6 (40.0)	10 (38.5)
SAE ≥ Grade 4 or death (%)	5 (33.3)	6 (23.1)

[a]TM, transmitting mothers; NTM, non-transmitting mothers; HEI, HIV-exposed infected; HEU, HIV-exposed uninfected; PP, postpartum; SAE, serious adverse event; VL, viral load

[b]Mean values are shown with ranges given in parentheses

[c]ADCC could not be examined in 1 infant plasma because of insufficient sample (infant ID 99)

ADCC was estimated using a previously validated infected cell killing assay (Thomas et al., 2020). Briefly, a reporter cell line with trans-activator of transcription (Tat)-responsive luciferase expression was infected with virus stocks incorporating maternal Envs. NK cell line mediated decrease in luciferase expression in the presence as compared to the absence of serially diluted plasma was used to estimate ADCC. ADCC activity was compared using an area under the killing curve (AUC) estimate (Yu et al., 2012). AUC ranged from 0, signifying no ADCC, to 1, representing 100% ADCC at all tested concentrations (Appendix 1). Overall, HIV exposed uninfected (HEU) (mean 0.16, range 0.02-0.45) as compared to HIV exposed infected (HEI) (mean 0.10, range 0.00-0.25, $p = 0.05$, Fig. 4.1A) infants had higher $ADCC_{AUC}$ against the cells infected with their corresponding mother's variants. Multivariable logistic regression analysis demonstrated a trend that the odds of infection was around 60% lower with a 0.1 unit increase in pre-existing infant $ADCC_{AUC}$ (odds ratio (OR) 0.41, 95% confidence interval (CI) 0.16 – 1.07, $p = 0.07$) after accounting for maternal plasma virus level, maternal absolute CD4 count and duration between birth and sample collection.

In our previous work, we set a conservative ceiling that up to 25% of the HIV-1 infected cells could be killed via NK cell mediated cytotoxicity directed at non-Env antigens (Thomas et al., 2020). Env-specific $ADCC_{AUC}$ was estimated by subtracting this 25% threshold from the percent killing observed at all the different plasma dilutions. After this subtraction, majority of Env-specific $ADCC_{AUC}$ values were zero (Fig. 4.1B). For statistical comparisons, Env-specific $ADCC_{AUC}$ values were categorized as above or below 0.01 based on demarcation visually evident in the distribution. A greater proportion of HEU

(11 of 26) as compared to HEI (1 of 15, p = 0.03) had Env-specific $ADCC_{AUC}$ value above 0.01. In multivariable logistic regression analysis, infant Env-specific $ADCC_{AUC}$ above 0.01 was associated with around 98% lower odds of transmission (OR 0.02, 95% CI 1.0 X 10^{-3} – 0.62, p = 0.02) after adjusting for baseline maternal characteristics.

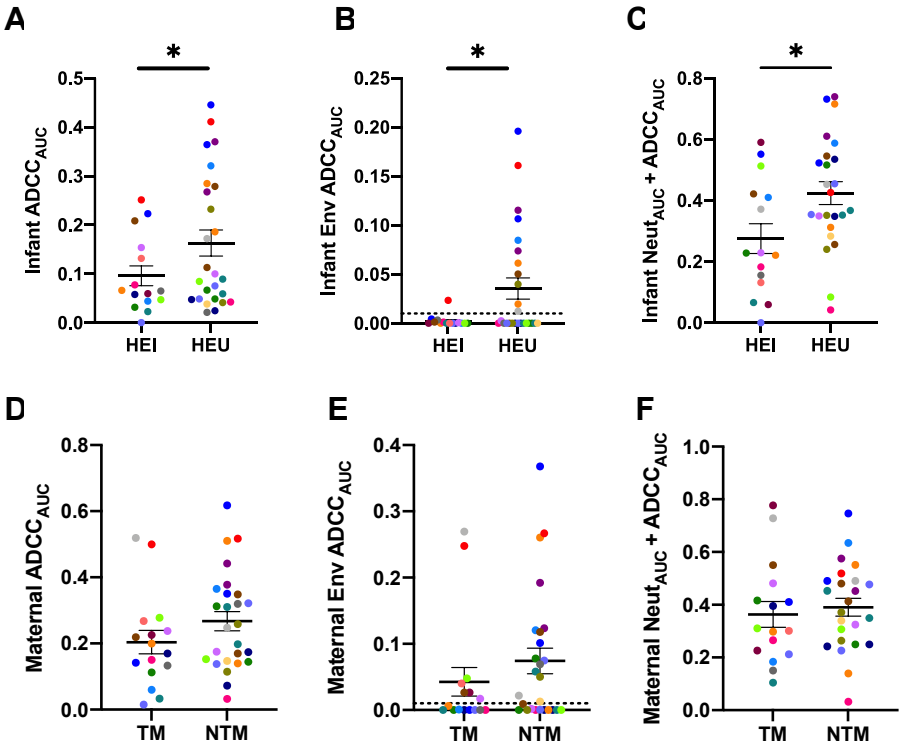

Figure 4.1. Infant and maternal ADCC and neutralization plus ADCC responses. HIV exposed infected (HEI) and HIV exposed uninfected (HEU) infants (A-C) and transmitting (TM) and non-transmitting (NTM) mothers (D-F) $ADCC_{AUC}$ (A&D), Env $ADCC_{AUC}$ (B&E), and $Neut_{AUC}$ + $ADCC_{AUC}$ (C&F) are shown in relation to transmission outcome. Colors signify matched pairs. Bars show mean and standard error. Dotted line in (B&E) shows Env $ADCC_{AUC}$ levels above and below 0.01 level. Group comparisons were done using Welch's t-test, Fischer's exact test, and/or multivariable logistic regression. * denotes $p \leq 0.05$ with one or both of these statistical tests.

We also hypothesized that combined neutralization and ADCC responses contribute to protection against transmission. We previously measured neutralization AUC ($Neut_{AUC}$), based on the same 0 to 1 scale in the infant and maternal plasma against the same maternal virus strains (Ghulam-Smith et al., 2017). An additive measure ($Neut_{AUC}$ + $ADCC_{AUC}$) was significantly higher in HEU (mean 0.42, range 0.04-0.74) as compared to HEI infants (mean 0.28, range 0.00-0.59, $p = 0.02$, Fig. 4.1C). Multivariate logistic regression analysis demonstrated that the odds of infection was around 40% lower with a 0.1 unit increase in pre-existing infant $Neut_{AUC}$ + $ADCC_{AUC}$ (OR 0.64, 95% CI 0.42 – 0.97, $p = 0.04$, Fig. 4.1C) after accounting for maternal characteristics.

NTMs as compared to TMs also had higher ADCC (Fig. 4.1D), Env-specific ADCC (Fig. 4.1E), and $Neut_{AUC}$ + $ADCC_{AUC}$ (Fig. 4.1F) although these differences were not significantly different in magnitude or in predicting the odds of transmission. In contrast to a previous study (Mabuka et al., 2012), TM and NTM's $ADCC_{AUC}$ from breast milk supernatant (Fig. 4.2A) and from breast milk isolated IgG (Fig. 4.2B) was not different in magnitude and did not associate with odds of transmission. Similar to our previous reports (Ghulam-Smith et al., 2017; Registre et al., 2019), AUC estimates were directly correlated to the outcome of interest observed at the highest tested plasma/antibody dilution ($p < 0.0001$, $\rho > 0.91$ in all cases, Fig. 4.3).

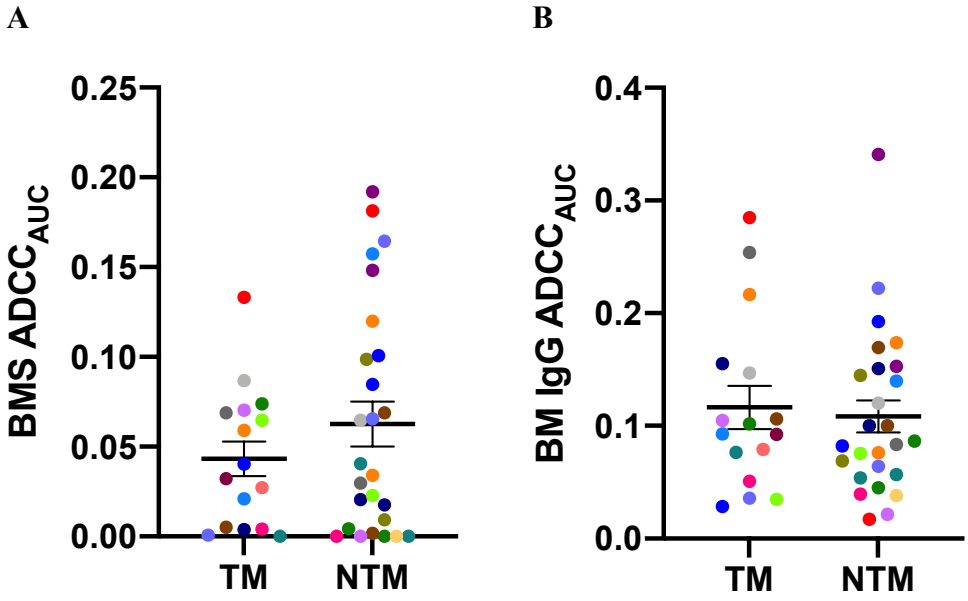

Figure 4.2. ADCC responses in breast milk supernatant (BMS) and IgG isolated from breast milk (BM IgG) against autologous strains. BMS (A) and BM IgG (B) from transmitting (TM) and non-transmitting (NTM) mothers $ADCC_{AUC}$. Colors signify matched pairs. Each dot represents a unique individual. Bars show mean and standard error.

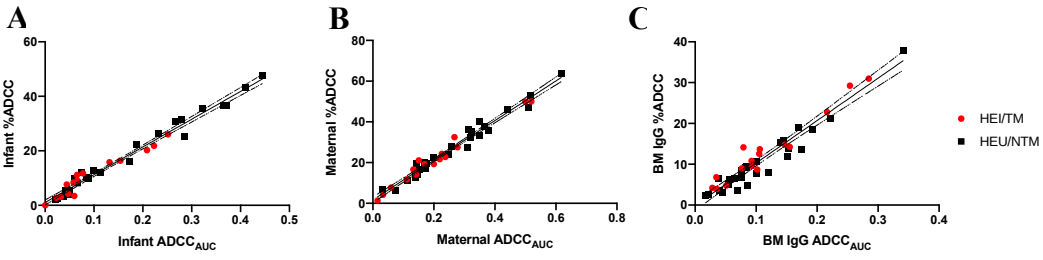

Figure 4.3. Area under the curve (AUC) and Percent ADCC. ADCC measurements using area under the curve ($ADCC_{AUC}$) were compared to measurements of percent ADCC at the top dilution tested (1:50) in infant plasma (A), maternal plasma (B), and BM IgG (C). The red and black dots indicate TMs/ HEI and NTMs/ HEU infants respectively. Correlations were assessed using Spearman's statistic. Line indicates linear regression fit with 95% confidence interval.

4.2 High infant ADCC responses in infected infants associates with better outcomes.

ADCC activity may also impact the outcomes of infants who eventually become infected (Milligan et al., 2015). As part of a pre-specified analysis, HEI and HEU infants were divided into 2 groups, those with less than and those with greater than or equal to median ADCC activity, group division as previously described (Ghulam-Smith et al., 2017; Milligan et al., 2015). In the absence of ART, a serious adverse event (SAE) ≥ grade 4 occurred in 5 and 6 of the 15 HEI and 26 HEU infants included in our analysis, respectively (Table 4.2). The time to a grade 4 or greater SAE or death occurred earlier among the HEI infants with low as compared to high ADCC (log rank hazard ratio 9.2, 95% CI 1.4 to 62.0, p = 0.01, Fig. 4.4A). Probability of incurring a SAE ≥ grade 4 or death was not different among the HEU infants that had low as compared to high ADCC (Fig. 4.4B). The probability of a SAE ≥ grade 4 or death was not significantly different among the HEI (Fig.

Table 4.2. Serious Adverse Events (SAE) ≥4 or death in BAN cohort infants examined in Chapter 4.

Patient ID	If infected, days PP to infant first HIV+ DNA PCR	Days to SAE ≥4 or death	SAE type	SAE grade
99	55	331	Meningitis	4
146	83	354	Gastroenteritis	5
170	127	306	Gastroenteritis	5
804	129	169	Death	5
878	56	221	Death	5
1459	127	253	Pneumonia	5
317	--	351	Anemia	4
355	--	352	Anemia	4
1225	--	133	Anemia	4
1289	--	283	Death	5
1379	--	218	Pneumonia	4
1666	--	113	Death	5

*Patient ID 99 was not examined for infant ADCC due to insufficient sample

4.4C) and HEU infants (Fig. 4.4D) that had high as compared to low $Neut_{AUC}$ + $ADCC_{AUC}$. Thus ADCC, but not a combination of ADCC and nAb, responses against the exposure strains associates with lower morbidity up to 1 year after birth in the infected infants.

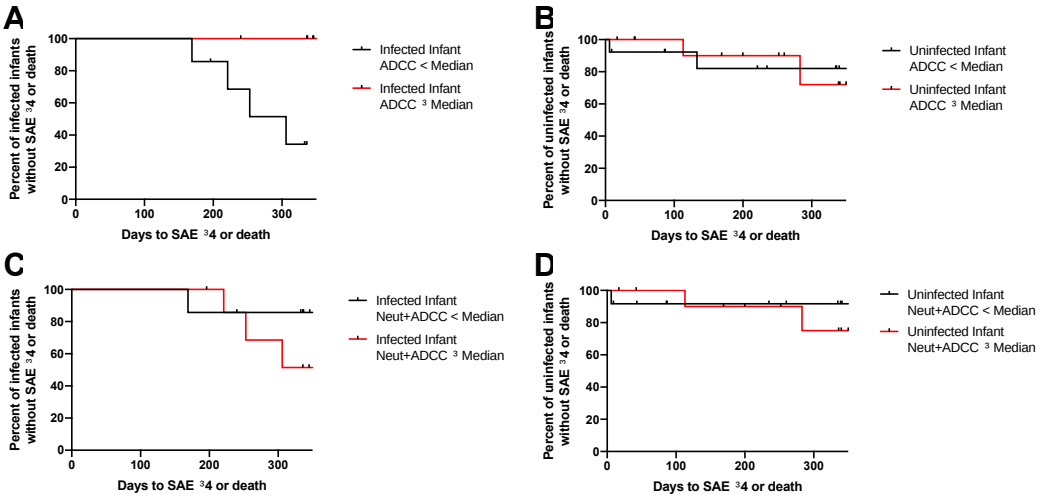

Figure 4.4. Morbidity associated with pre-existing humoral responses. Kaplan-Meir curves estimating time (days) to a grade 4 or greater serious adverse event (SAE) or death for HIV exposed infected (HEI) (A&C) and HIV exposed uninfected (HEU) infants (B&D) with $ADCC_{AUC}$ (A&B) and $Neut_{AUC}$ + $ADCC_{AUC}$ (C&D) greater than or equal to the cohort median (red) or less than the cohort median (black). Tick marks denote right censoring.

4.3 ADCC activity does not correlate with neutralization responses but is possibly affected by the levels of IgA.

We previously demonstrated that pre-existing neutralization responses against the maternal strains did not correlate with HIV-1 acquisition or infant outcomes in this cohort (Ghulam-Smith et al., 2017). Autologous ADCC and neutralization responses were not correlated in infant plasma ($p = 0.70$, $\rho = 0.06$, Fig. 4.5A) or maternal plasma ($p = 0.97$, ρ

= -0.001, Fig. 4.5B). The absence of any association suggests that neutralization and ADCC against maternal strains are independent activities.

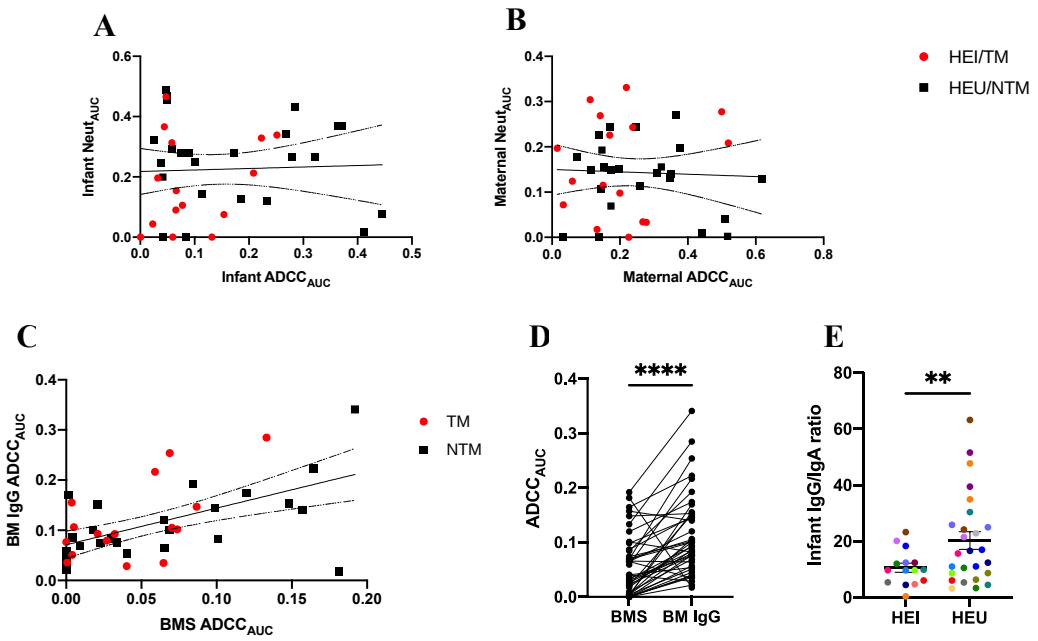

Figure 4.5. Plasma and breast milk ADCC relative to neutralization and immunoglobulin isotypes. $ADCC_{AUC}$ correlation with $Neut_{AUC}$ among infants (A) and mothers (B). ADCC from breast milk (BM) isolated IgG relative to breast milk supernatant (BMS) (C&D). IgG to IgA ratio among infants (E). The red and black dots indicate TMs/ HEI and NTMs/ HEU infants respectively, and colors in (E) signify matched pairs. Correlations were assessed using Spearman's statistic. Line indicates linear regression fit with 95% confidence interval. Group comparisons were done using matched pairs Wilcoxon rank-sum (D) or Welch's t-test (E). ** and **** denote $p < 0.01$ and $p < 0.0001$ respectively.

ADCC association with HIV-1 acquisition and infant outcomes also does not merely reflect higher immunoglobulin levels. Total IgG trended higher in the HEI as compared to HEU infant plasma (p = 0.06, Fig.4.6A). There was no difference in total IgG levels in TM and NTM plasma (p = 0.72, Fig. 4.6B), or the breast milk (p = 0.78, Fig.

4.6C). Furthermore, total IgG magnitude did not correlate with ADCC activity in infant plasma ($p = 0.82$, $\rho = 0.04$, Fig. 4.6D), maternal plasma ($p = 0.56$, $\rho = -0.09$, Fig. 4.6E), or breast milk IgG ($p = 0.88$, $\rho = -0.02$, Fig. 4.6F).

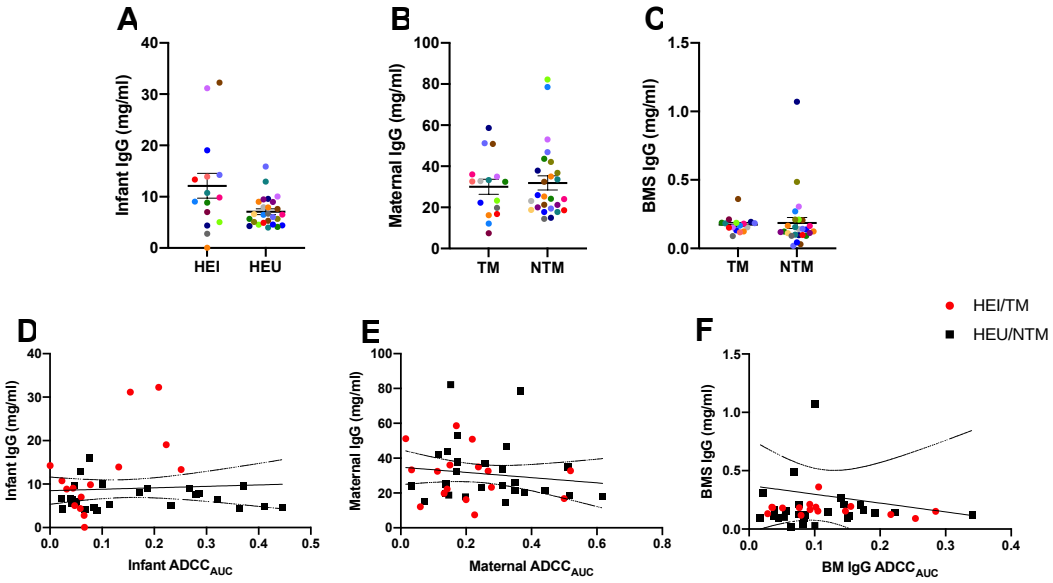

Figure 4.6. Immunoglobulin differences and relation to ADCC. Total IgG levels (A-C) and comparison to $ADCC_{AUC}$ (D-F) in infants' plasma (A&D), mothers' plasma (B&E) and mothers' breast milk supernatant (C&F). The red and black dots indicate TMs/ HEI and NTMs/ HEU infants respectively, and colors in (A-C) signify matched pairs. Correlations were assessed using Spearman's statistic. Line indicates linear regression fit with 95% confidence interval.

ADCC from breast milk supernatant and breast milk IgG was highly correlated ($p < 0.001$, $\rho = 0.51$, Fig. 4.5C). Interestingly, breast milk IgG consistently yielded higher ADCC relative to breast milk supernatant ($p < 0.0001$, Fig. 4.5D). In contrast, we previously demonstrated that ADCC was not significantly different in plasma as compared to IgG isolated from plasma (Thomas et al., 2020). Breast milk supernatant as compared to

plasma contains high levels of IgA (Jackson & Nazar, 2006), and this may be inhibiting IgG mediated ADCC. The HEU infants that had higher ADCC as a group (Fig. 4.1) also had significantly higher IgG/IgA ratios as compared to HEI infants ($p = 0.01$, Fig. 4.5E). NTMs as compared to TMs also had non-significant higher IgG to IgA ratio in plasma (Fig. 4.7A) but not in breast milk (Fig. 4.7B). Collectively, these results support that high IgA levels interfere with IgG mediated ADCC (Haynes et al., 2012; Ruiz et al., 2016).

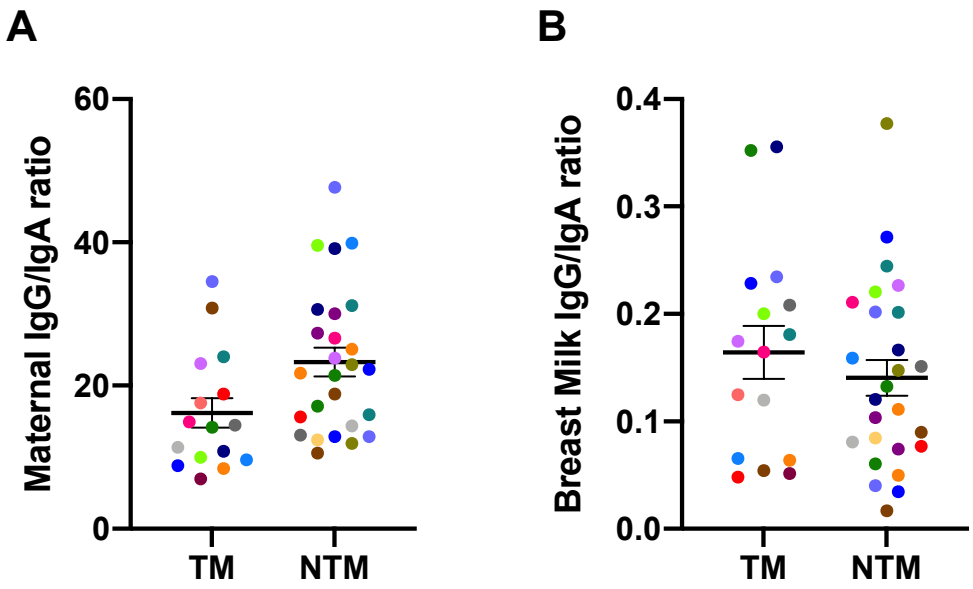

Figure 4.7. Immunoglobulin IgG/IgA ratios. IgG to IgA ratio in maternal plasma (A) and breast milk supernatant (B). Colors signify matched pairs. Each dot represents a unique individual. Bars show mean and standard error.

4.4 Infants acquire ADCC-inducing antibodies from their mothers and are not affected by the mother's duration of disease.

Infants passively acquire maternal antibodies throughout gestation, and these maternal antibodies wane over the course of 6 to 12 months (Niewiesk, 2014). Maternal

and infant ADCC responses were highly correlated ($p < 0.0001$, $\rho = 0.73$, Fig. 4.8A) and infant responses negatively correlated with the days postpartum to sample collection ($p < 0.0001$, $\rho = -0.60$, Fig. 4.8B). These results suggest that the ADCC responses are from the passive transfer of antibodies from the mother, rather than de-novo production in the child.

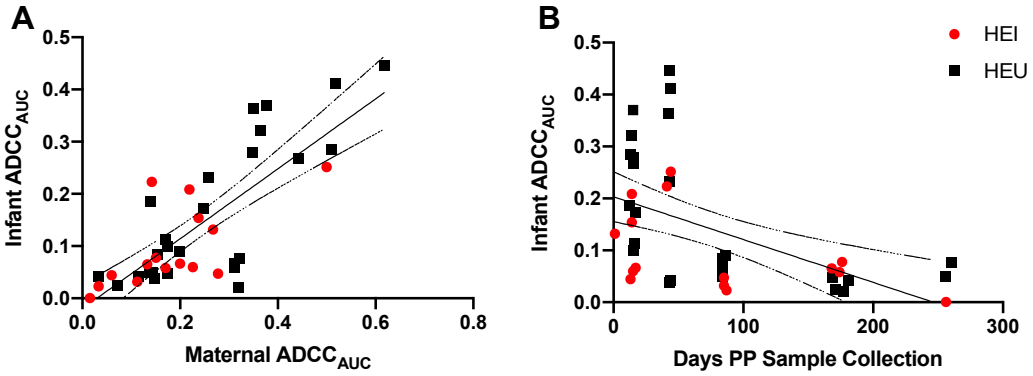

Figure 4.8. Infant and Maternal $ADCC_{AUC}$ and duration after birth. Correlation between infant and maternal $ADCC_{AUC}$ (A) and Infant $ADCC_{AUC}$ relative to days after birth (B). The red and black dots indicate TMs/ HEI and NTMs/ HEU infants respectively. Correlations were assessed using Spearman's statistic. Line indicates linear regression fit with 95% confidence interval.

In general, nAb responses are higher among individuals with high plasma virus levels and lower CD4 counts (Rusert et al., 2016). In our cohort, maternal ADCC was not associated with maternal viral load ($p = 0.83$, $\rho = -0.03$, Fig. 4.9A) or CD4 count ($p = 0.31$, $\rho = 0.16$, Fig. 4.9C). Similarly, infant ADCC was not associated with maternal viral load ($p = 0.57$, $\rho = 0.09$, Fig. 4.9B) or CD4 count ($p = 0.48$, $\rho = 0.11$, Fig. 4.9D). These results

imply that the duration of HIV-1 disease, as estimated by virus levels and number of CD4 cells, does not impact ADCC capacity in the mothers or the infants.

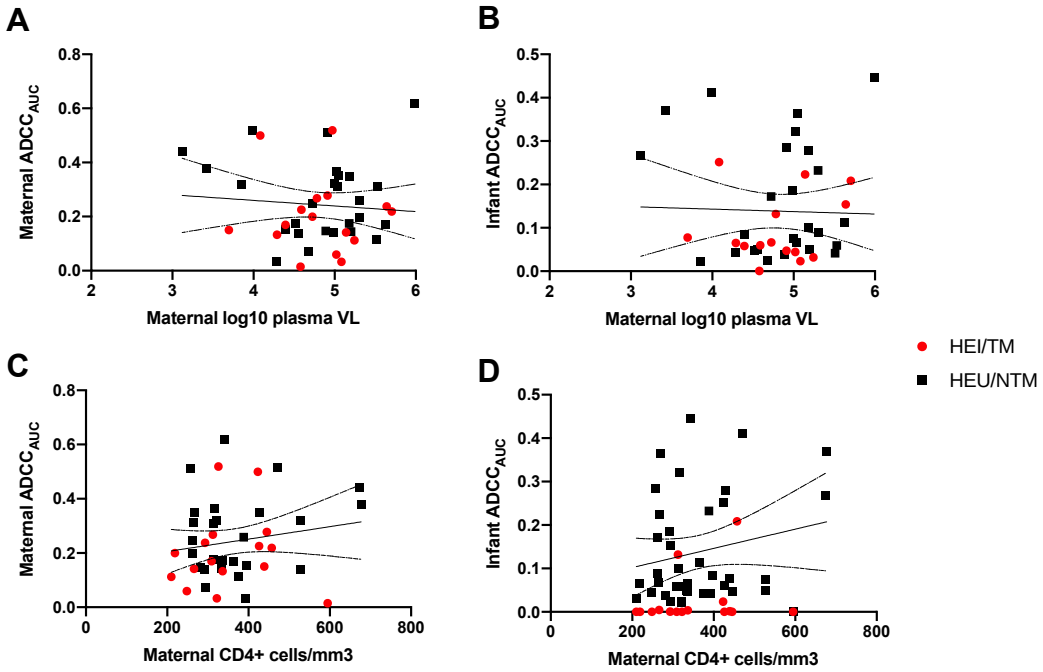

Figure 4.9. Maternal viral load and CD4$^+$ T cell counts do not impact ADCC responses. Maternal ADCC$_{AUC}$ (A&C) and infant ADCC$_{AUC}$ (B&D) were compared to maternal log_{10} plasma viral load (A&B) and CD4$^+$ cells/mm^3 (C&D). The red and black dots indicate TMs/ HEI and NTMs/ HEU infants respectively. Correlations were assessed using Spearman's statistic. Line indicates linear regression fit with 95% confidence interval.

Discussion

Efforts to develop an effective HIV-1 vaccine have aimed to elicit antibodies that can prevent infection. Passive transfusion investigations in animal models have clearly demonstrated that nAbs, especially bnAbs, prevent infection against cell-free virus challenge, demonstrating the protective potential of antibodies (Julg & Barouch, 2021).

Recent results from clinical trials and prior MTCT cohorts, however, have not confirmed these findings, possibly because humans are exposed to neutralization resistant viruses and infectious sources that contain both infected cells and cell-free virus (Corey et al., 2021; Thomas et al., 2018). Identifying the characteristics of the pre-existing immune response that limit acquisition in humans remains vital for future vaccine efforts.

MTCT cohorts are an ideal model to examine the protective role of antibodies because infants passively acquire maternal antibodies before and while they are consistently exposed to the virus. Results from previous MTCT studies are conflicting concerning the role of ADCC in limiting virus acquisition (Hompe et al., 2019; Mabuka et al., 2012; Milligan et al., 2015; Naiman et al., 2019). Differences in assays used to assess ADCC, as well as the number and source of the samples examined, have likely contributed to the discrepancy in results. In this study, using a breastfeeding MTCT cohort, we found that pre-existing infant ADCC responses are associated with both decreased transmission and lower HIV-1 infected infant morbidity. In contrast to previous studies, we examined ADCC responses against variants encountered by naïve infants with pre-existing antibodies. Furthermore, we estimated ADCC using an assay that quantified the elimination of infected targets only and not bystander cells (Alpert et al., 2012; Thomas et al., 2020). Infection-based assays that quantify infected cell killing have been deemed to have fewer artifacts compared to other methods that use gp120-coated targets or surrogate markers for effector cell activity (Forthal & Finzi, 2018; Richard et al., 2018). Furthermore, our methodology for generating replication competent viruses incorporating the Envs of interest recapitulates the major strains present in a sample from a chronically infected

individual (Chatziandreou et al., 2012). Thus, our studies are directly relevant for understanding how pre-existing antibodies prevent viruses present in the infectious source from establishing an infection and subsequent morbidity.

Animal studies have clearly demonstrated that nAbs prevent transmission more efficiently in the presence of ADCC and other antibody mediated functionalities (Bournazos et al., 2014; Halper-Stromberg et al., 2014; Hessell et al., 2007; Lu et al., 2016). Similarly, we found that infants that remained uninfected as compared to those that acquired HIV-1 had a higher magnitude of combined neutralization and ADCC against their maternal strains. Some investigations showed an association between transmission and binding antibodies (Mabuka et al., 2012; Martinez et al., 2017; Permar et al., 2015), and this may be a proxy for neutralization and ADCC against maternal strains. Other human studies also suggest that antibody functionalities, besides neutralization alone, are important for preventing virus acquisition (Ackerman et al., 2012; Haynes et al., 2012; Jefferis, 2012; Rerks-Ngarm et al., 2009; Ruiz et al., 2017; Tomaras et al., 2013). Neutralization alone may not protect against acquisition because maternal viruses mutate to escape this selection pressure (Bonsignori et al., 2017; Dickover et al., 2006; Kumar et al., 2018; Martinez et al., 2020; Wu et al., 2006), but these changes may impart susceptibility to pre-existing antibodies in other ways. For instance, one previous study suggested that neutralization pressure on the virus renders viruses ADCC susceptible (Mielke et al., 2019). Although our findings imply that combination of neutralization and ADCC are important in preventing transmission, other antibody mediated functions, not examined in our study, may contribute to protection. Importantly, antibodies that can

provide some modicum of neutralization along with ADCC, and possibly other effector functions, may be easier to elicit with a future vaccine strategy as compared to those strategies that focus solely on generating bnAb.

Similar to a previous study, we found that pre-existing infant ADCC was associated with better outcomes after HIV-1 infection, although the prior investigation used different methodology and had different cohort sample characteristics (Milligan et al., 2015). HEU infants presumably do not have any HIV-1 infected cells, and thus pre-existing HIV-1 directed ADCC failed to show any association with subsequent morbidity. In infected infants, high ADCC activity may eliminate infected cells, lowering virus levels, and preserve the nascent immune system. Both animal studies and an observed positive association between cervicovaginal ADCC levels and genital HIV-1 RNA loads in infected women support this potential mechanism (Himes et al., 2018; Nag et al., 2004; Ng et al., 2010). However, to date, this hypothesis has not been directly tested by quantifying the number of infected cells harboring infectious virus. Interestingly, although infant $Neut_{AUC}$ + $ADCC_{AUC}$ was correlated with transmission, it was not associated with infected infant outcomes, as was seen with ADCC alone. NAbs may act as a first line of defense and ADCC may clear cells infected with neutralization escape variants. This dual block may prevent the establishment of a systemic infection. However, after an infant becomes infected, nAbs may not further modulate the subsequent disease course because of the presence of mostly neutralization resistant viruses.

In our study, the ADCC associations with transmission and outcomes were not reflective of total IgG concentrations. Of note, we did not measure HIV-specific IgG

isotype levels using ELISA-based methods because we were interested in responses against maternal exposure variants and not unrelated strains captured by consensus proteins. Therefore, it is possible that the magnitude of HIV-specific total IgG varies between groups and may contribute to the observed outcome differences. We also observed significant difference in IgG/IgA ratios between HEI and HEU infants and in ADCC from breast milk IgG as compared to supernatant, which supports previous arguments that IgA may interfere with ADCC (Haynes et al., 2012; Ruiz et al., 2016). We primarily found significant ADCC and immunoglobulin differences in HEI as compared to HEU plasma and less so in TM as compared to NTM plasma and breast milk. This supports the notion that the preferential transfer of antibody with specific characteristics from the mother to the baby are likely important for preventing HIV-1 transmission and infant health (Martinez et al., 2019). Overall, our observations indicate that the quality, not quantity, of the antibodies is important in inducing robust ADCC responses.

In summary, we show that passively-acquired infant ADCC activity correlates with protection and associates with better clinical outcomes in infected infants. Additionally, our work suggests that the combined impact of nAbs and ADCC correlates with decreased transmission. Furthermore, the correlations were not merely due to differences in IgG magnitude but most likely due to specific, as yet undefined, antibody properties. Future efforts should be aimed at investigating both the mechanisms for these associations and characterizing the antibodies that impart ADCC and potentially other effector activity. Our data highlights the benefits of ADCC and supports designing vaccines that can enhance ADCC in highly exposed infants.

CHAPTER V: IDENTIFYING PATIENT SAMPLES WITH HIGH ADCC BREADTH AND POTENCY

Background

The rapid mutation rate of HIV-1 has resulted in an extremely diverse population of variants both within an individual, as well as globally. The use of subtype classification is used to group together variants that are more genetically similar (Taylor et al., 2008). Most of the genetic diversity occurs in the *env* region with about 20% amino acid differences within a subtype and 35% between subtypes (Korber et al., 2001; R. M. Lynch et al., 2009). These amino acid changes in the *env* gene impact the composition and structure of the envelope (Env) protein that is targeted by antibodies. HIV-1 subtypes often cluster in certain geographically regions, with Subtype C being predominant in Sub-Saharan Africa (Hemelaar et al., 2006). One of the major challenges of developing an effective HIV-1 vaccine, is identifying ways to protect individuals from becoming infected by this wide variety of variants.

Neutralizing antibodies have been a major focus for vaccine development, and a small subset of people naturally develop broadly neutralizing antibodies (bnAbs) that can neutralize variants spanning different clades (Wibmer et al., 2015). Although protection against infection is seen with passive transfusion of nAbs in animal models (Barouch et al., 2013; Gautam et al., 2016; Horwitz et al., 2013; Klein et al., 2012; Moldt et al., 2012; Schiffner et al., 2013), clinical trials in humans along with studies in MTCT cohorts have not confirmed these findings (Baan et al., 2013; Ghulam-Smith et al.,

2017; J. B. Lynch et al., 2011; Martinez et al., 2020; Milligan et al., 2016; Pancino et al., 1998).

To date, there has been only one vaccine trial, the RV144 Thai Vaccine Efficacy Trial, that has shown any efficacy, a modest 31.2% (Rerks-Ngarm et al., 2009). Interest in studying ADCC has grown after analysis of this study determined that nAbs were not responsible for the conferred protection, but rather nnAbs capable of inducing ADCC (Haynes et al., 2012). Data presented in Chapter 4, along with work from other labs, suggest that ADCC is a correlate of protection (Mabuka et al., 2012; Milligan et al., 2015). Similar to bnAbs, there are likely antibodies capable of inducing broad and potent ADCC responses, which would be of great use in vaccine development. A potential way forward in the development of an effective vaccine is to determine if pre-existing broad and potent ADCC responses correlate with protection. Yet currently, there is not much known about the breadth of ADCC responses. Some studies have suggested that non-neutralizing antibodies (nnAbs) have limited breadth in recognizing cells infected with primary HIV-1 isolates (Bruel et al., 2017), while others show breadth across multiple subtypes (Madhavi et al., 2014). One consistency across studies, is that ADCC responses against T/F variants are lower than responses measured against lab-adapted stains (Bruel et al., 2016). Most studies to date have assessed breadth of ADCC responses of a small number of monoclonal nnAbs and bnAbs. However, little progress has been made to assess ADCC breadth present in human cohorts.

In this chapter, we will discuss the identification of broad and potent ADCC-inducing antibodies present in samples from the BAN Study cohort. We used a panel of

Envs that was previously determined to represent over 200 Envs from all areas of the world (DeCamp et al., 2014). ADCC activity in maternal plasma, infant plasma, and IgG isolated from breast milk was assessed against ten viruses in this panel. A breadth and potency score ($ADCC_{BP}$) was calculated for each sample, where breadth refers to the number of viruses the samples has ADCC activity against, and potency considers the magnitude of the response. BP score allows us to look at both of these parameters in a single value of 0 to 1. A score of 0 indicates the samples could not induce any ADCC against any of the viruses in the panel, and a score of 1 indicates the sample could induce 100% ADCC against the entire panel. Therefore, samples with BP scores closest to 1 would be the best at inducing ADCC across a range of HIV variants, and likely useful for future vaccine development. In Chapter 4 our data showed non-transmitting mother-infant pairs had higher autologous ADCC responses compared to transmitting mother-infant pairs, suggesting ADCC was a correlate of protection in MTCT of HIV-1. Therefore, we hypothesized that non-transmitting mother-infant pairs would also have broader and more potent ADCC responses compared to transmitting mother-infant pairs. Additionally, we sought to determine samples with elite ADCC activity, which we defined as the top 5% of broad and potent responses tested, that would provide a subset of samples for future studies focused on determining antibody characteristics necessary for conferring protective ADCC responses.

Results

5.1 Breadth and potency of ADCC-inducing antibodies does not differ based on transmission status.

Replication competent viruses were successfully generated incorporating 10 of the 12 Envs proposed by deCamp, *et al.* (DeCamp et al., 2014). Briefly, these reference Envs were inserted into an NL4-3 backbone using yeast gap repair methodology, as previously described (Ghulam-Smith et al., 2017). Prior to use in any of our assays, viruses were passaged through $CD4^+$ T cells. ADCC responses of infant plasma, maternal plasma, and IgG isolated from breast milk (BM IgG) were assessed against each of the ten viruses (Appendix 2-4). Unlike the experiments in Chapter 4, we did not test a serial dilution for each sample, rather we tested the top dilution only (1:50), resulting in percent ADCC for each virus. We have previously shown that AUC is strongly correlated with percent ADCC observed at the top dilution. $ADCC_{BP}$ was estimated for each sample by averaging the percentage of ADCC across all 10 viruses. Any % ADCC estimate below zero was set to zero prior to calculations. The average was then $\log_2$ transformed, yielding a score ranging from 0 to 1. No differences were seen between HEI (mean 0.36, range 0.17-0.52) and HEU infants (mean 0.37, range 0.14-0.55, $p = 0.74$, unpaired t test with Welch's correction, Fig. 5.1A) However, transmitting mothers had slightly more broad and potent plasma (mean 0.49, range 0.33-0.63, Fig. 5.1B) and breast milk $ADCC_{BP}$ (mean 0.35, range 0.14-0.55, Fig. 5.1C) compared to non-transmitting mothers (plasma mean 0.47, range 0.33-0.79; BM mean 0.30, range 0.15-0.49), although not

significant (maternal plasma p = 0.41; BM p = 0.06, unpaired t test with Welch's correction).

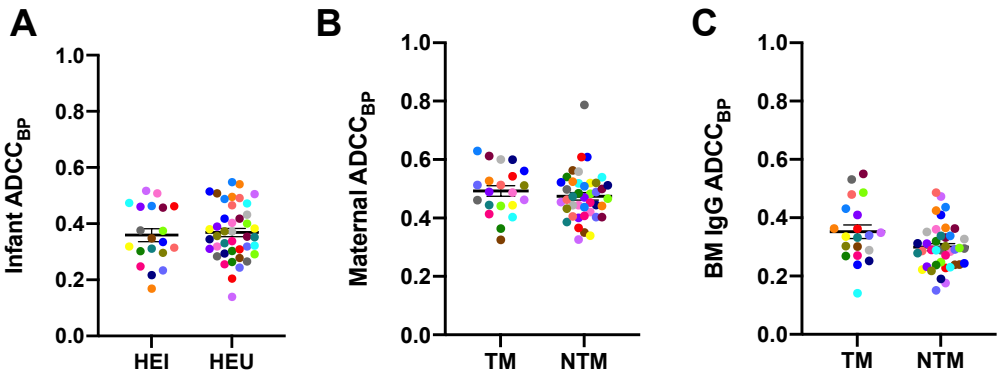

Figure 5.1. Broad and potent infant and maternal ADCC responses. HIV exposed infected (HEI) and HIV exposed uninfected (HEU) infants (A) and transmitting (TM) and non-transmitting (NTM) maternal plasma (B) and IgG isolated from breast milk (BM IgG) (C) broad and potent ADCC responses ($ADCC_{BP}$) shown in relation to transmission outcome. Colors signify matched pairs. Bars show mean and standard error.

5.2 Breadth and potency of neutralizing antibodies and ADCC-inducing antibodies does not impact transmission.

In Chapter 4, we showed a synergistic effect between autologous neutralization and ADCC that impacted transmission. We wanted to determine if the additive breadth and potency of these antibody responses would associate with reduced transmission, as $ADCC_{BP}$ alone did not. $Neut_{BP}$ and $ADCC_{BP}$ were summed before comparing HEI to HEU infants and TM to NTM mothers, respectively. Unlike with autologous responses, there were no differences in $Neut_{BP}$ + $ADCC_{BP}$ between HEI (mean 1.02, range 0.37-1.34) and HEU infants (mean 1.00, range 0.51-1.29, p = 0.63, unpaired t test with Welch's correction, Fig. 5.2A). TM mothers (mean 1.19, range 0.55-1.48) did have

higher $Neut_{BP}$ + $ADCC_{BP}$ compared to NTM mothers (mean 1.10, range 0.72-1.57), but this difference was not significant (p = 0.12, unpaired t test with Welch's correction, Fig. 5.2B).

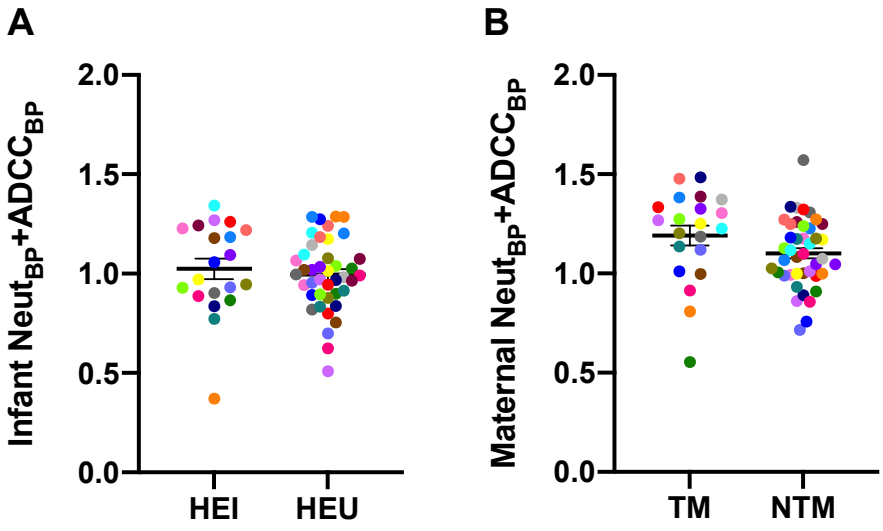

Figure 5.2. Infant and maternal broad and potent neutralization plus ADCC responses. Neutralization ($Neut_{BP}$) and ADCC breadth and potency ($ADCC_{BP}$) was summed for HIV exposed infected (HEI) and HIV exposed uninfected (HEU) infants (A) and transmitting (TM) and non-transmitting (NTM) mothers (B). $Neut_{BP}$ + $ADCC_{BP}$ responses shown in relation to transmission outcome. Colors signify matched pairs. Bars show mean and standard error.

5.3 Breadth and Potency of Infant ADCC-inducing antibodies does not impact infant outcomes.

In the cohort tested, 8 HEI and 6 HEU out of 63 infants acquired a serious adverse event (SAE) of a grade 4 or greater, or death. However, our analysis only included 61 infants, 7 HEI and 5 HEU, as there was insufficient infant plasma for PID 99 and PID 1666 to assess $ADCC_{BP}$ against all viruses in the panel. We have previously shown pre-

existing autologous ADCC responses were associated with better outcomes in infants that went on to acquire infection. We wanted to determine if the presence of broad and potent ADCC responses also modulated disease progression in those infants that acquired infection. To determine if $ADCC_{BP}$ impacted clinical outcomes, infants were separated into two groups, one where $ADCC_{BP}$ was less than median and the other where $ADCC_{BP}$

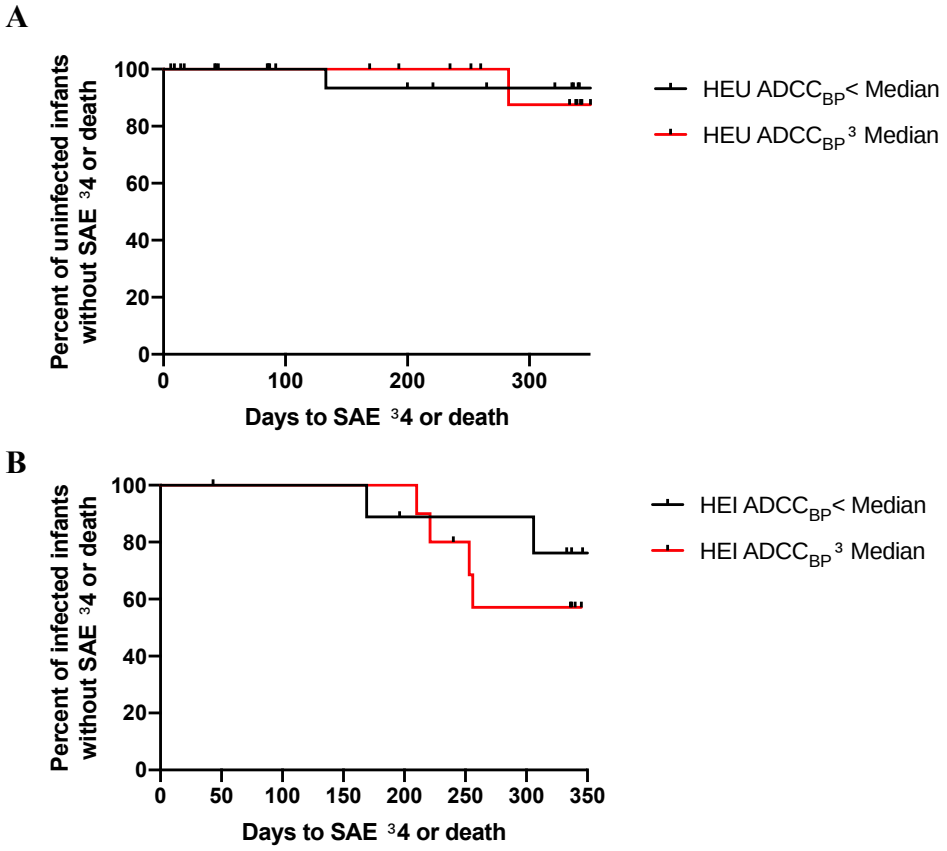

Figure 5.3. Morbidity is not associated with broad and potent ADCC response. Kaplan-Meir curves estimating times (days) to a grade 4 or greater serious adverse event (SAE) or death for HIV exposed uninfected (HEU) (A) and HIV exposed infected (HEI) infants (B) with $ADCC_{BP}$ greater than or equal to the cohort median (red) or less than the cohort median (black). Tick marks denote right censoring.

was greater than or equal to median. Similar to the analysis in Chapter 4, we assessed outcomes in infected and uninfected infants separately. Similar occurrences of SAE ≥ 4 or death were seen in both the high and low $ADCC_{BP}$ groups for uninfected infants (log-rank p = 0.87, HR: 0.85, 95%CI 0.08 to 9.04, Fig. 5.3A) and when assessing infected infants only (log-rank p = 0.44, HR: 0.58, 95%CI 0.13 to 2.59, Fig. 5.3B). Similar results were seen when assessing the relationship between infant outcomes and $ADCC_{BP}$ from maternal plasma and breast milk.

5.4 Heat maps reveal lack of unique ADCC fingerprint.

BP score was calculated to help us easily evaluate the breadth and potency of ADCC responses between transmitting and non-transmitting mother-infant pairs. We were also interested in comparing responses across the variants tested to see if patterns emerged. Heat map analysis provided a visual representation of the percent ADCC against each virus used to calculate $ADCC_{BP}$ for infant plasma (Fig. 5.4), maternal plasma (Fig. 5.5), and BM IgG (Fig. 5.6). Infant samples clustered into two groups with 100% bootstrap support, although those groups were comprised of similar amounts of infected and uninfected infants. Maternal samples had a small subset of 6 patients that were separated from the rest of the group with 100% bootstrap support. There was no distinction between transmitting and non-transmitting mothers (Fig. 5.5). Finally, BM IgG clustered into two groups with 100% bootstrap support. These groups were comprised of similar amounts of transmitting and non-transmitting mothers, suggesting that there is not a unique ADCC fingerprint that can distinguish between transmitting and non-transmitting mother-infant pairs.

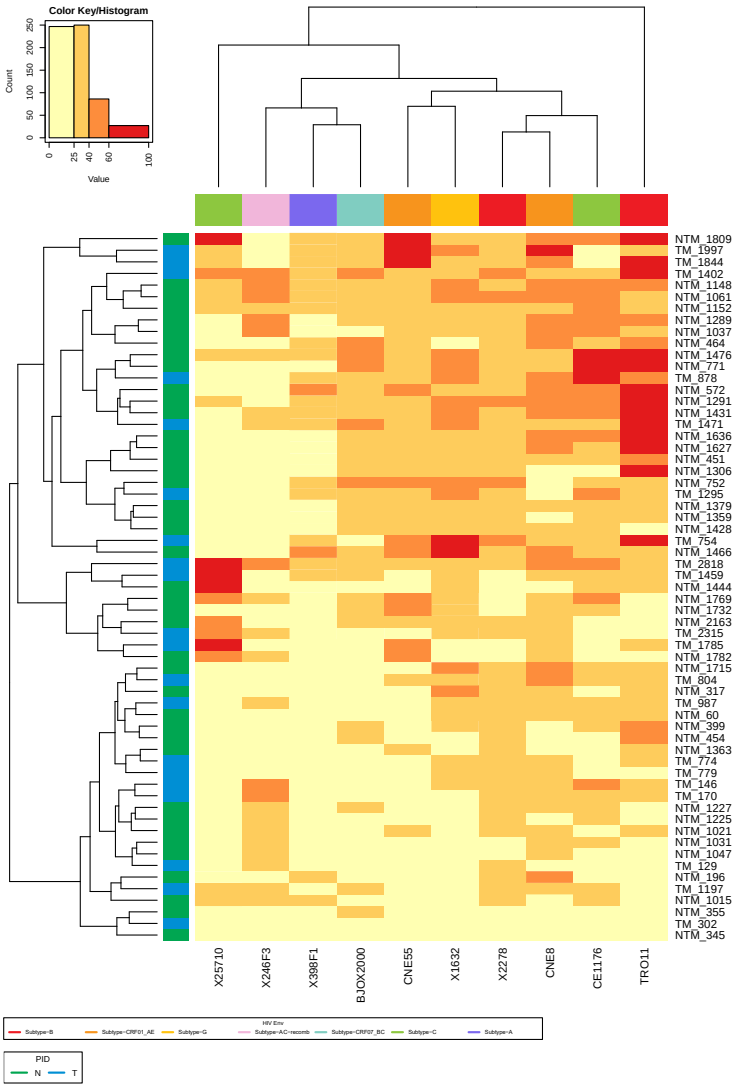

Figure 5.4. Heat map of infant ADCC responses against a heterologous global Env panel. Each square in the grid represents the average percent ADCC for each virus-plasma combination tested: <25% yellow; 25 to 40% light orange; 40 to 60% dark orange; >60% red. Virus subtypes are indicated by color: B, red; CRF01_AE, orange; G, yellow; AC_recomb, pink; CRF07_BC, teal; C, lime; A, purple. On the left green denotes non-transmitting and blue denotes transmitting mothers. The branches show the hierarchical clustering.

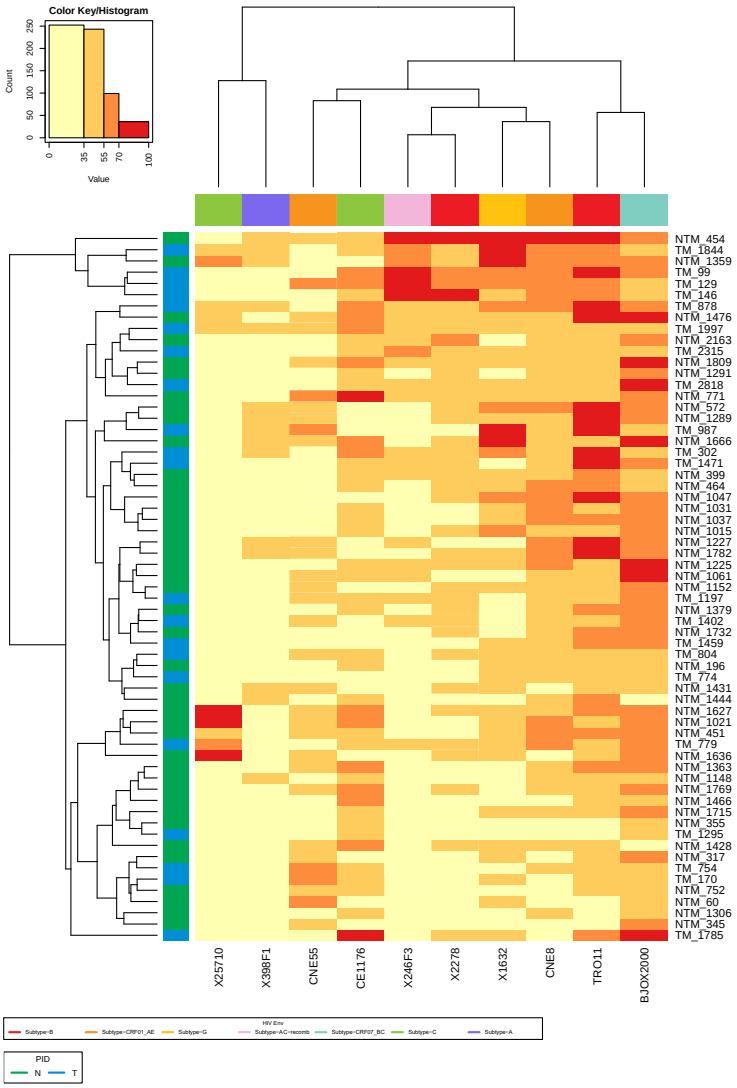

Figure 5.5. Heat map of maternal ADCC responses against a heterologous global Env panel. Each square in the grid represents the average percent ADCC for each virus-plasma combination tested: <35% yellow; 35 to 55% light orange; 55 to 70% dark orange; >70% red. Virus subtypes are indicated by color: B, red; CRF01_AE, orange; G, yellow; AC_recomb, pink; CRF07_BC, teal; C, lime; A, purple. On the left green denotes non-transmitting and blue denotes transmitting mothers. The branches show the hierarchical clustering.

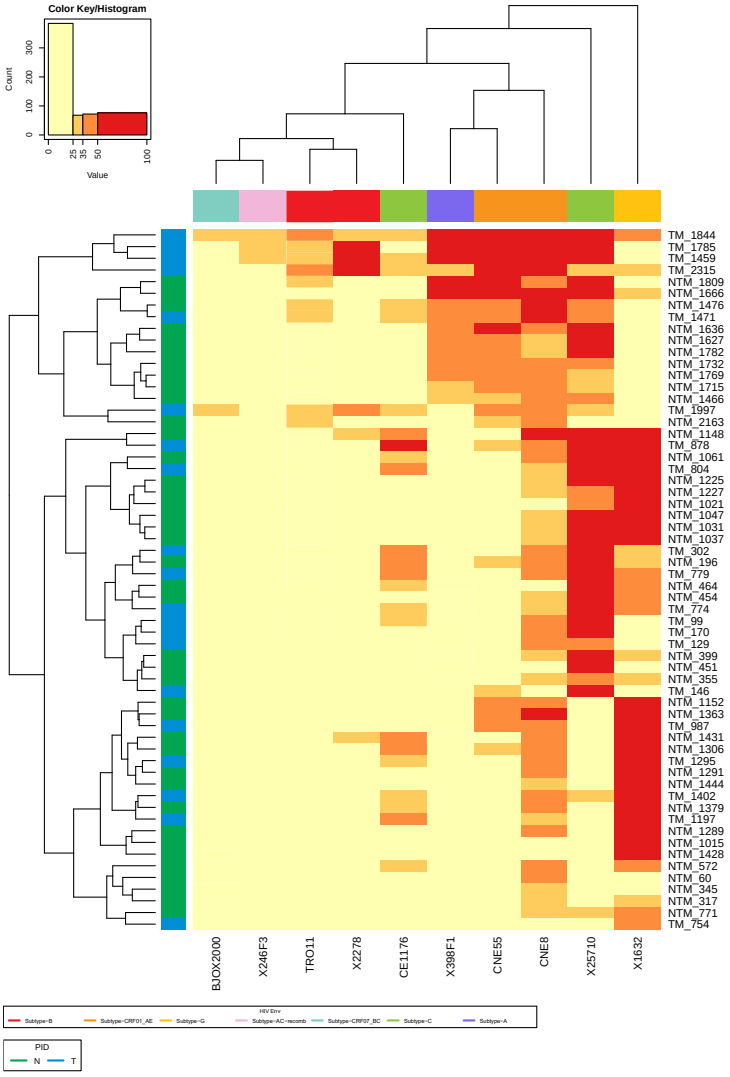

Figure 5.6. Heat map of BM IgG ADCC responses against a heterologous global Env panel. Each square in the grid represents the average percent ADCC for each virus-plasma combination tested: <25% yellow; 25 to 35% light orange; 35 to 50% dark orange; >50% red. Virus subtypes are indicated by color: B, red; CRF01_AE, orange; G, yellow; AC_recomb, pink; CRF07_BC, teal; C, lime; A, purple. On the left green denotes non-transmitting and blue denotes transmitting mothers. The branches show the hierarchical clustering.

5.5 Breadth and Potency of infant, but not maternal, ADCC and neutralizing antibodies are correlated.

We previously showed that autologous ADCC and neutralization responses were not correlated, indicating these antibody functions are independent activities. Furthermore, previous studies reported a lack of association between neutralization and ADCC, although those reports only considered responses against individual variants (Borggren et al., 2016). We wanted to see if the breadth and potency of these responses were correlated. As expected, maternal $Neut_{BP}$ and $ADCC_{BP}$ were not correlated ($p = 0.74$, $r = 0.04$, Spearman's correlation, Fig. 5.7B). However, infant $Neut_{BP}$ and $ADCC_{BP}$ were significantly correlated ($p = 0.007$, $r = 0.34$, Spearman's correlation, Fig. 5.7B), which has previously been shown for infant plasma ADCC when compared to neutralization breadth and neutralization potency individually (Milligan et al., 2015).

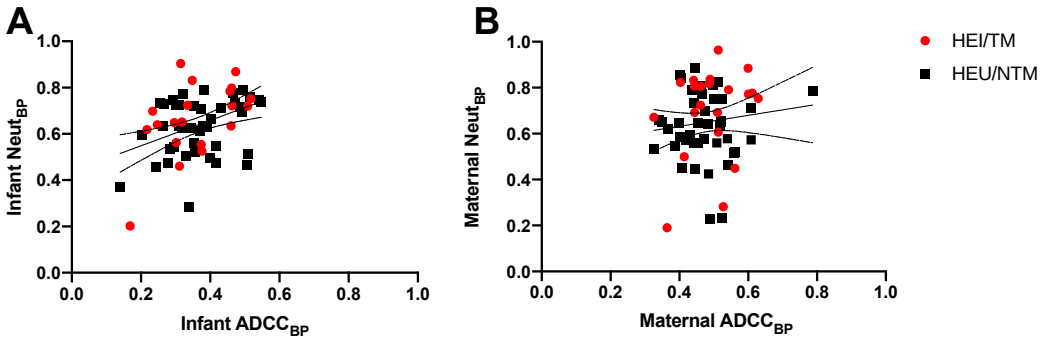

Figure 5.7. Infant and maternal broad and potent ADCC and neutralization responses. $ADCC_{BP}$ correlation with $Neut_{BP}$ among infants (A) and mothers (B). The red and black dots indicate TMs/HEI and NTMs/HEU infants respectively. Correlations were assessed using Spearman's statistic. Line indicates linear regression fit with 95% confidence interval.

5.6 Infant ADCC$_{BP}$ responses decline over time and get increasingly different from matched maternal ADCC$_{BP}$

Infants passively acquire antibodies from their mothers across the placenta and through ingesting breast milk (Fouda et al., 2011). Therefore, we expected to see infant responses mirror the waning of maternal antibodies over time. Similar to autologous responses, we saw a significant negative correlation between the breadth and potency of infant ADCC responses and the days postpartum that the sample was collected ($p < 0.0001$, $r = -0.52$, Spearman's correlation, Fig. 5.8A). Additionally, the difference between the maternal and infant ADCC$_{BP}$ response significantly increased over time, where the responses were most similar closest to birth ($p = 0.0003$, $r = 0.45$, Spearman's correlation, Fig. 5.8B).

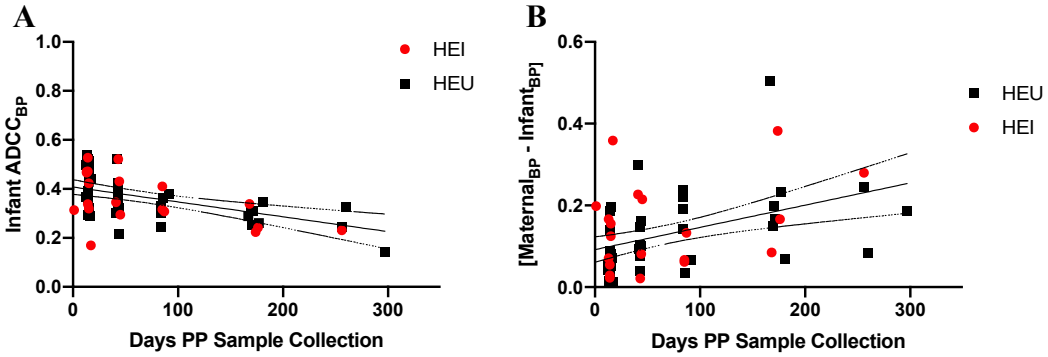

Figure 5.8. Infant ADCC$_{BP}$ and difference between maternal ADCC$_{BP}$ in relation to time after birth. Correlation between infant ADCC$_{BP}$ (A) relative to days after birth and the differences between infant and maternal ADCC$_{BP}$ over time after birth (B). The red and black dots indicate HEI and HEU infants, respectively. Correlations were assessed using Spearman's statistic. Line indicates linear regression fit with 95% confidence interval.

5.7 $ADCC_{AUC}$ and $ADCC_{BP}$ responses are only correlated in infant plasma.

Broad heterologous ADCC responses may not protect against acquisition because circulating variants in transmitting mothers may be resistant. It is possible there will be no association between heterologous responses and responses against the autologous maternal variants. Therefore, we wanted to determine if autologous ADCC responses against circulating maternal variants were correlated to the breadth and potency assessed in these samples. Infant autologous and heterologous ADCC responses were highly correlated ($p = 0.002$, $r = 0.47$, Spearman's correlation, Fig. 5.9A), however no associations were seen between autologous and heterologous ADCC responses in maternal plasma ($p = 0.15$, $r = 0.23$, Spearman's correlation, Fig. 5.9B) or IgG isolated from breast milk ($p = 0.51$, $r = 0.10$, Spearman's correlation, Fig. 5.9C).

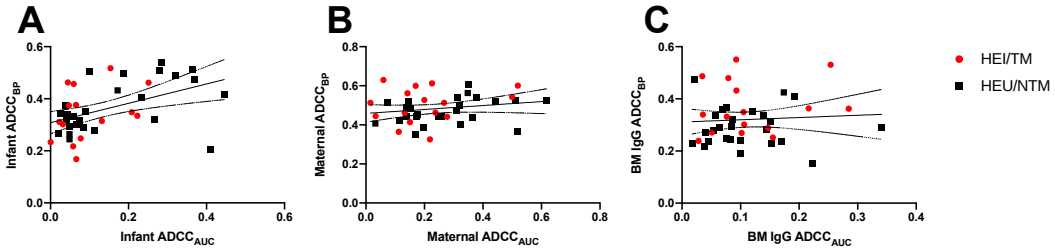

Figure 5.9. Heterologous and Autologous ADCC responses. Correlations between autologous ($ADCC_{AUC}$) and heterologous ($ADCC_{BP}$) ADCC responses in infants (A), mothers (B), and breast milk IgG (BM IgG) (C). The red and black dots indicate TM/HEI and NTM/HEU infants, respectively. Correlations were assessed using Spearman's statistic. Line indicates linear regression fit with 95% confidence interval.

5.8 Magnitude of Total IgG does not impact breadth and potency of ADCC-inducing antibodies.

Higher potency of ADCC responses could be a result of increased quantities of immunoglobulins present in the different plasma samples. To confirm that the differences among samples was due to the difference in quality and not quantity of the antibodies, we measured immunoglobulins in our BAN cohort samples. Total IgG present in infant plasma, maternal plasma, and breast milk was determined by summing IgG1, IgG2, IgG3, and IgG4 magnitudes that were acquired using a Human Isotyping Multiplex Assay. No associations were found between magnitude of total IgG and $ADCC_{BP}$ in infant plasma ($p = 0.25$, $r = 0.15$, Spearman's correlation, Fig. 5.10A), maternal plasma ($p = 0.22$, $r = -0.16$, Spearman's correlation, Fig. 5.10B), or breast milk ($p = 0.14$, $r = 0.20$, Spearman's correlation, Fig. 5.10C). This was the same observation seen when comparing total IgG to autologous ADCC responses against circulating maternal variants.

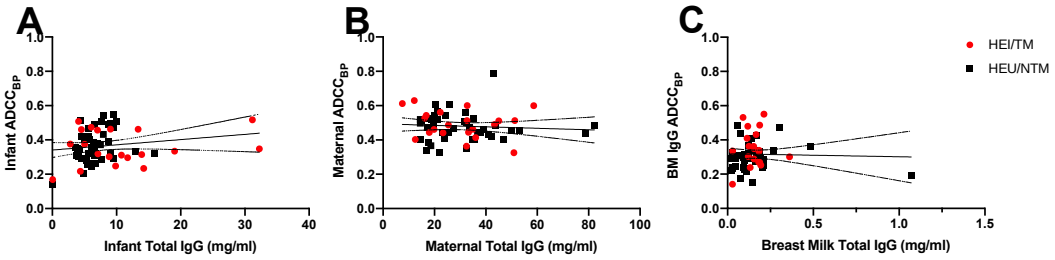

Figure 5.10. Immunoglobulin relation to breadth and potency of ADCC responses. Total IgG in relation to $ADCC_{BP}$ in infant plasma (A), maternal plasma (B), and breast milk IgG (C). The red and black dots indicate TM/HEI and NTM/HEU infants, respectively. Correlations were assessed using Spearman's statistic. Line indicates linear regression fit with 95% confidence interval.

Again, HIV-specific IgG was not measured and therefore it is possible that a correlation could be seen in that instance.

In the subset of infant samples tested for autologous ADCC, presented in the previous chapter, we saw a significantly higher IgG to IgA ratio present in the plasma of HEU infants compared to HEI infants. Previous studies have also suggested IgA may dampen the ADCC response (Haynes et al., 2012; Ruiz et al., 2016). Therefore, we wanted to determine if this association was seen when considering the entire cohort tested for heterologous responses. Although the trend remained the same, there was no longer a significant difference between HEI and HEU infant IgG/IgA ratios in infant plasma ($p = 0.07$, unpaired t test with Welch's correction). No differences in IgG/IgA ratios were seen between transmitting and non-transmitting maternal plasma ($p = 0.14$) or breast milk ($p = 0.56$, unpaired t test with Welch's correction). However, there was a strong correlation between IgG/IgA ratios and the breadth and potency of infant ADCC responses ($p = 0.0006$, $r = 0.43$, Spearman's correlation, Fig. 5.11A). Again, this association was only

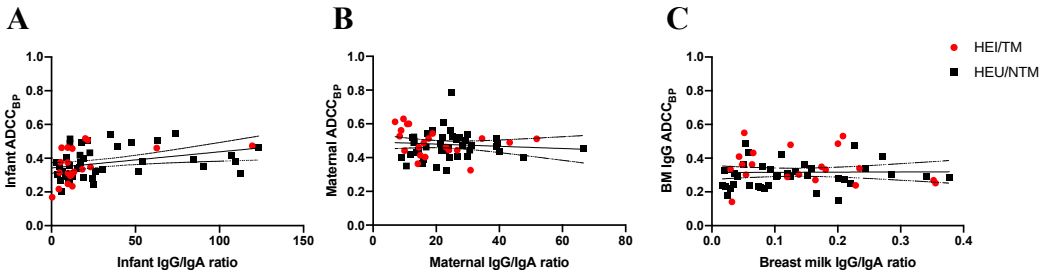

Figure 5.11. Immunoglobulin IgG/IgA ratios in relation to ADCC$_{BP}$. IgG to IgA ratios were correlated with ADCC$_{BP}$ in infant plasma (A), maternal plasma (B), and breast milk IgG (C). The red and black dots indicate TM/HEI and NTM/HEU infants, respectively. Correlations were assessed using Spearman's statistic. Line indicates linear regression fit with 95% confidence interval.

seen in infant plasma, and not maternal plasma (p = 0.42, r = -0.10, Spearman's correlation, Fig. 5.11B) or breast milk (p = 0.62, r = 0.07, Spearman's correlation, Fig. 5.11C), which was also the case for autologous responses.

5.9 $ADCC_{BP}$ is not associated with indicators of maternal disease progression.

High viral loads have been associated with the development of bnAbs (Moore et al., 2015). We were interested to identify factors that may be associated with the development of broad and potent ADCC responses. Therefore, we looked for associations between $ADCC_{BP}$ and maternal plasma viral load and maternal CD4+ T cell counts. No associations were seen between maternal $\log_{10}$ plasma viral load and $ADCC_{BP}$ in infant plasma (p = 0.13, r = 0.19, Fig. 5.12A), maternal plasma (p = 0.50, r = -0.09, Fig. 5.12B),

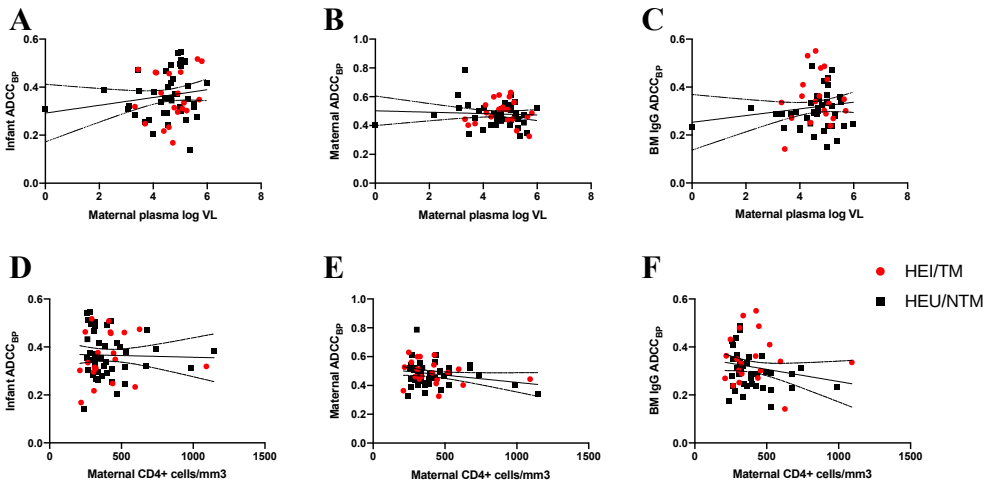

Figure 5.12. Maternal viral load and $CD4^+$ T cell counts do not impact the breadth and potency of ADCC responses. Infant $ADCC_{BP}$ (A&D), maternal $ADCC_{BP}$ (B&E), and BM IgG $ADCC_{BP}$ (C&F) were compared to maternal $\log_{10}$ plasma viral load (A-C) and $CD4^+$ cells/mm^3 (D-F). The red and black dots indicate TM/HEI and NTM/HEU infants, respectively. Correlations were assessed using Spearman's statistic. Line indicates linear regression fit with 95% confidence interval.

or BM IgG ($p = 0.42$, $r = 0.11$, Fig. 5.12C). This lack of association was also seen between maternal $CD4^+$ cells/mm^3 and $ADCC_{BP}$ of infant plasma ($p = 0.79$, $r = -0.03$, Fig. 5.12D), maternal plasma ($p = 0.16$, $r = -0.18$, Fig. 5.12E), and BM IgG ($p = 0.11$, $r = -0.21$, Spearman's correlation, Fig. 5.12F).

Discussion

In order to prevent transmission, antibodies need to block exposure strains. However, other than MTCT, it is difficult to predict exposure strains due to the immense diversity of HIV-1. Thus, it would be ideal to have an antibody with reactivity against a diverse range of variants for use in a vaccine. Here, we employed a panel of 12 Envs, of which we generated ten replication competent viruses, in order to determine $ADCC_{BP}$ for maternal plasma, infant plasma, and BM IgG that came from the BAN study. We had hypothesized that broad and potent ADCC responses would be more common in non-transmitting mothers as compared to transmitting mothers. This hypothesis was generated based on our findings that non-transmitting mother-infant pairs had higher autologous ADCC responses compared to transmitting mother-infant pairs. However, we did not see any differences in $ADCC_{BP}$ between transmitting and non-transmitting mother-infant pairs. This was further supported when assessing heat map analysis with bootstrapping where we did not identify a unique ADCC signature that could distinguish the two groups. This was an unexpected finding because the HIV exposed uninfected infants had higher responses against the autologous strains present in the corresponding mother compared to infants that acquired infection. Our data suggests that these antibodies are specifically targeted against the mother's strains; however, protected infants do not

necessarily have a breadth of responses against all variants. Furthermore, unlike autologous responses, broad and potent responses did not associate with better outcomes in infants regardless of their infection status. Our study supports that having ADCC responses that specifically target exposure strains are most important for preventing transmission and modulating disease outcomes. However, that does not rule out the protective nature of broad and potent ADCC-inducing antibodies, which would likely be useful in a vaccine. This highlights the arms race between the viral evolution and the development of antibodies, similar to how individuals can develop bnAbs that are ineffective against their own virus, but can potentially confer protection against newly exposed individuals.

Previous studies have suggested that monoclonal nnAbs have narrow breadth (Bruel et al., 2017), and bnAbs display higher levels of ADCC. Our data from patient plasma, however, did support breadth of ADCC responses against a number of clades, highlighting differences between monoclonal and polyclonal antibodies. Similarly, previous studies have observed higher ADCC mediated by sera compared to mAbs (Smalls-Mantey et al., 2012). Furthermore, we have shown that neutralization and ADCC capacity are unrelated. Because our study used patient plasma, the ADCC responses tested account for a heterogenous mixture of antibodies, including both nAbs and nnAbs, both of which could contribute to the breadth and potency of ADCC that we observed. Conversely, it is possible assessed responses are due to a single antibody with previously undefined characteristics. Furthermore, it is possible that the plasma contains a subset of antibodies capable of both broad and potent neutralization and ADCC responses.

Although ADCC and neutralization were not correlated, some of the samples with broad and potent ADCC responses displayed fairly high neutralization breadth and potency as well. Currently, we are unable to attribute ADCC activity to specific antibodies present in the plasma and more intensive studies would be necessary to address these questions.

It is well established that infants passively acquire antibodies from their mothers (Niewiesk, 2014), and as such we would expect maternal and infant ADCC responses to be well correlated. Surprisingly, maternal and infant $ADCC_{BP}$ were not correlated (data not shown), however, we did see a decrease in infant $ADCC_{BP}$ over time as well as an increasing difference between infant and maternal responses. The decrease in infant $ADCC_{BP}$ mirrors the waning of maternal antibodies, which is further supported by the increasing difference seen between maternal and infant responses. These data support that infants are acquiring these broad and potent antibodies from their mothers. The lack of correlation between maternal and infant $ADCC_{BP}$ could potentially be explained by differential placental transfer. Recent studies have suggested that the placenta has evolved to passively transfer antibodies that can confer the most protection in neonates, and determinants such as IgG subclass and glycan profiles play a role in this selective process (Jennewein et al., 2019; Martinez et al., 2019). We can speculate that this selective transfer can vary between mother-infant pairs which could account for the lack of association seen between maternal and infant broad and potent ADCC responses. Regardless, our data clearly supports that infant responses come from antibodies which they passively acquired from their mothers.

I had hypothesized that non-transmitting mother-infant pairs would have broader and more potent ADCC responses compared to transmitting mother-infant pairs. This hypothesis originated from non-transmitting mother-infant pairs having higher autologous ADCC responses. However, this hypothesis was not supported by our data as we did not see significant differences between $ADCC_{BP}$ of transmitting and non-transmitting pairs. Interestingly, $ADCC_{AUC}$ against autologous contemporaneous maternal strains and $ADCC_{BP}$ were highly correlated in infant plasma. This was unexpected considering we saw differences in autologous infant responses between HEI and HEU infant, but no differences when comparing $ADCC_{BP}$. With autologous and heterologous responses being correlated we would have expected to also see differences in $ADCC_{BP}$ between groups. Upon closer analysis, we noticed that this association between autologous infant $ADCC_{AUC}$ and heterologous $ADCC_{BP}$ was entirely driven by the HEU infants ($p = 0.013$, $r = 0.48$, Spearman's correlation). HEI infant autologous and heterologous ADCC responses were not correlated ($p = 0.24$, $r = 0.32$, Spearman's correlation). In this way, infants that lacked protective autologous ADCC responses may still have ADCC-inducing antibodies present in their plasma that can recognize a broad range of variants.

The development of bnAbs has been associated with higher viral loads, likely due to increased antigenic exposure (Moore et al., 2015). However, broad and potent ADCC responses were not associated with maternal plasma viral load or maternal CD4 count in our cohort, mirroring what we previously reported when examining autologous ADCC responses. Our results also align with previous findings reporting a lack of correlation

between ADCC, $CD4^+$ T cell count, viral load, and neutralization (Smalls-Mantey et al., 2012). Breadth and potency of ADCC responses were not associated with the magnitude of total IgG present in the samples, suggesting plasma with broader and more potent responses do not simply have more antibody available to induce a more robust response. We had determined that HEU infants had a higher IgG to IgA ratio compared to HEI infants, supporting others' suggestions that IgA may negatively impact ADCC (Haynes et al., 2012; Ruiz et al., 2017). Interestingly, we saw a strong correlation between infant IgG/IgA ratios and the breadth and potency of infant ADCC responses. This association was not seen in maternal plasma or breast milk. These data are likely driven by differences in potency as compared to breadth. If IgA does dampen the ADCC response as suggested, then the higher amounts of IgG in the infant plasma could overcome the effect of IgA and drive more robust responses. However, it is unlikely that the IgG/IgA ratio makes in impact on the breadth of ADCC responses. Instead, the quality of the antibodies, such as isotypes and glycan profile, likely contributes to the variation of breadth between patient samples.

In this chapter we tested 185 samples, including infant plasma, maternal plasma, and BM IgG. From these, we defined the top 5% broad and potent responses as having elite ADCC activity. These 9 elite ADCC responses were all maternal plasma from a mix of both transmitting and non-transmitting women: PID 99, 129, 146, 454, 572, 878, 1359, 1476, 1844. The highest $ADCC_{BP}$ score was from the maternal plasma of PID 454. This plasma was able to induce 100% ADCC against five of the ten viruses tested, with a range of 26 to 100%, resulting in a BP score of 0.77. Interestingly, the lowest responses

were against subtype C variant, Env 25710, which should be genetically closer to the circulating variants in the mothers compared to cross-clade variants. Furthermore, the ADCC responses from all maternal plasmas were, on average, lowest against this particular variant compared to all of the variants tested, suggesting that perhaps this particular Env isn't susceptible to ADCC. More in depth analysis must be done to determine the antibody characteristics that confer broad and potent ADCC responses.

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CHAPTER VI: CONCLUDING REMARKS

Summary of Major Findings

The work presented in this dissertation discussed the role of ADCC in the prevention of MTCT of HIV-1 and the development of new methodology necessary to address these questions. In Chapter 3, I discussed the development of a new cell line, MT4-CCR5-Luc. I transduced MT4 cells with CCR5, to increase infectability, and a Tat-inducible Luciferase reporter, to use luminescence as a read out of infection. I validated and optimized the MT4-CCR5-Luc cells for use as targets in an ADCC assay. I showed robust luciferase expression after productive infection with maternal HIV-1 variants. Our assay yielded similar ADCC estimates as those obtained from flow cytometric methods, which are commonly used in the field. In contrast to the flow cytometric assays, our assay is relatively high throughput, uses fewer cells and patient plasma, and is less expensive. The development of MT4-CCR5-Luc system provided us a novel tool to address the role of ADCC in the prevention of MTCT.

In Chapter 4, I assessed ADCC responses against maternal autologous viruses in samples from the control arm of the BAN study, where HIV-infected mothers either did or did not transmit the virus to their infants during breastfeeding. I determined that infant $ADCC_{AUC}$ responses against the exposure strains was a correlate of protection in MTCT of HIV-1. The data also showed that infected infants with high as compared to low ADCC had lower morbidity and mortality up to 1 year after birth, suggesting ADCC is also associated with better infant outcomes. Multiplex analysis determined similar levels of IgG were present across plasma samples, confirming the differences seen in ADCC

responses resulted from the presence of specific rather than the total antibody quantity. Interestingly, our data showed non-transmitting mother-infant pairs have higher IgG to IgA ratios compared to transmitting mother-infant pairs, suggesting IgA may negatively impact ADCC responses. Higher ADCC responses from IgG isolated from breast milk (BM IgG) compared to breast milk supernatant also supports this notion. This had previously been hypothesized after ADCC associations in the RV144 vaccine trial were shown only in individuals with low plasma IgA (Haynes et al., 2012).

In Chapter 5, I assessed the breadth and potency of ADCC responses ($ADCC_{BP}$) in maternal plasma, infant plasma, and BM IgG. Each sample was tested against a panel of 10 different Envs representing the global diversity of HIV-1. I hypothesized that non-transmitting mother-infant pairs would have more broad and potent responses compared to transmitting mother-infant pairs. In contrast to expectations, I observed similar $ADCC_{BP}$ between transmitting and non-transmitting mother-infant pairs. Additionally, broad and potent responses did not associate with lower infant morbidity and mortality up to 1 year after birth. Previous work assessing neutralization breadth and potency ($Neut_{BP}$) in this cohort showed higher $Neut_{BP}$ in transmitting as compared to non-transmitting mothers, along with a unique neutralization signature identified by bootstrap analysis (Ghulam-Smith et al., 2017). When heat maps were analyzed for maternal plasma, infant plasma, and BM IgG responses against heterologous virus, I did not observe a unique ADCC signature and there was no distinct separation between transmitting and non-transmitting mother-infant pairs. Furthermore, it was previously shown that infants born to mothers with high $Neut_{BP}$ had worse outcomes 1 year after birth compared to infants

born to mothers with low $Neut_{BP}$ (Ghulam-Smith et al., 2017). These data, along with data discussed in Chapter 4, suggest that the presence of antibodies capable of inducing ADCC, more so than neutralization, against the challenge variant are most important for preventing transmission and influencing infant outcomes. Furthermore, it is possible that transmitting mothers have more ADCC-resistant variants compared to non-transmitting mothers. This could potentially explain why we see differences based on transmission outcomes in autologous infant responses, but not in responses against heterologous variants.

Discussion

Based on the data detailed in this dissertation, along with conflicting results between human cohorts and animal studies, I propose that antibodies capable of mediating ADCC against a diverse range of variants will not be effective to prevent infection in a prophylactic vaccine. The antibodies will have to specifically target the exposure strains, and it remains unclear whether a vaccine can elicit ADCC responses against the diverse number of strains present in all of the potential transmitting individuals. ADCC is still an important immune mechanism as it has previously been shown that protection conferred by bnAbs in animal challenge models drastically decreased when FcR-binding was engineered out of the antibody (Hessell et al., 2007). Previous data from our lab did not see significant differences in nAb responses between HIV-exposed infected (HEI) compared to HIV-exposed uninfected (HEU) infants (Ghulam-Smith et al., 2017). However, I observed that HEI infants have significantly lower levels of combined neutralization and ADCC responses compared to HEU infants,

further supporting ADCC may augment neutralization to prevent transmission. NAbs likely act as a first line of defense, but alone are not enough to prevent transmission. High immunological pressure from nAbs drives viral escape exposing epitopes targeted by ADCC-inducing antibodies. In this way the ADCC-susceptibility of HIV variants may increase as the virus becomes more neutralization resistant. ADCC can then act as a second line of defense to clear cells that may have become infected by neutralization resistant variants, preventing the spread of infection and protecting the infants against transmission.

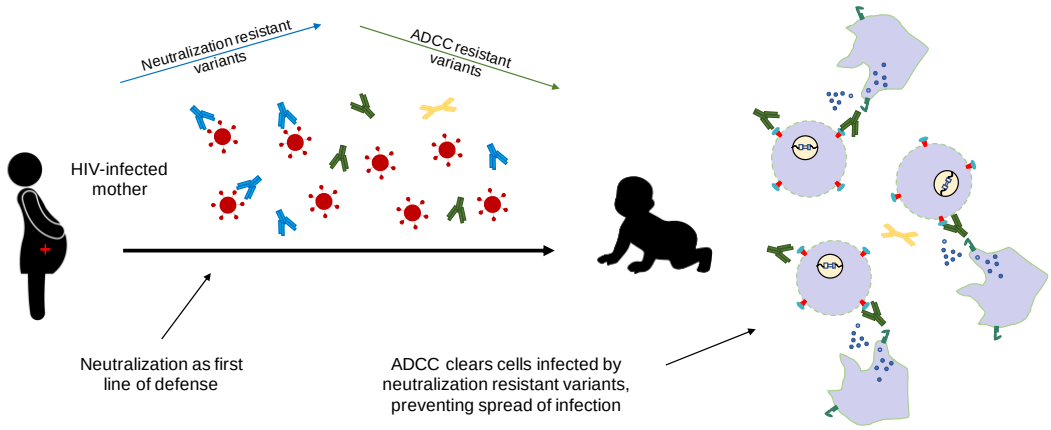


Illustration 4. Proposed Mechanism for how nAbs and ADCC-inducing Abs prevent MTCT of HIV-1. Infants passively acquire antibodies and virus from their infected mothers. Nabs act as a first line of defense to prevent infection. However, ADCC-inducing antibodies are necessary to clear cells that become infected by neutralization resistant variants. The immunological pressure resulting in neutralization resistant variants exposes epitopes targeted by ADCC-inducing antibodies, effectively increasing ADCC-susceptibility in the process.

Although the data in Chapter 4 suggests ADCC is a correlate of protection, there are concerns about how applicable these results will be to a protective vaccine. The major concern lies in the lack of association seen when assessing heterologous ADCC

responses. ADCC was protective only when the antibodies were raised against autologous virus. In Chapter 5, where the variants were changed, we lost the differences seen between HEI and HEU infants. This suggests that the viral variant is extremely important in determining the efficacy of the ADCC response. A vaccine developed to induce ADCC-mediating antibodies would likely fail without knowing the exposure variants. This may help explain why the RV144 vaccine trial had modest efficacy associated with ADCC responses, whereas the HVTN702 trial failed to provide any protection against acquisition (Rerks-Ngarm et al., 2009; UNAIDS, 2020b). The vaccine regiment in the HVTN702 trial was based on the RV144 regiment, but adapted for Subtype C virus, the most common subtype in South Africa where the HVTN702 trial took place (UNAIDS, 2020b). South Africa has the most HIV infections in the world, and therefore the largest genetic diversity in the circulating HIV variants. It is possible that the vaccine generated broad ADCC responses, but it was not adequate at targeting the vast amount of circulating variants in the African region. The work presented in this dissertation highlights the importance of continuing to study ADCC. If a vaccine does in fact need to elicit variant-specific ADCC responses, that poses a giant hurdle in the development of a feasible vaccine.

Significance

To our knowledge, no group has assessed autologous ADCC responses against maternal variants in infant plasma, maternal plasma, and breast milk samples. Our study is the most analogous to responses required against the challenge strains, helping to clarify the role of ADCC in the prevention of HIV acquisition. By testing ADCC

responses against breast milk supernatant in addition to IgG isolated from that sample, I was able to show data supporting IgA negatively impacts ADCC activity. In addition to autologous responses, we also examined heterologous responses against a panel of Envs that represent the global diversity of HIV-1. To date, no group has assessed patient ADCC breadth in plasma and breast milk. Our results are an initial step in determining the range of human ADCC responses, both in magnitude and breadth, which are currently undefined.

By assessing both autologous and heterologous responses, I observed that variations in the virus greatly impacts ADCC responses. The viruses in the global panel display a wide range of ADCC susceptibility. For example, global panel virus 25710 appears to be relatively ADCC-resistant when compared to the other viruses tested. This suggests that infected individuals may harbor viruses with a range of ADCC susceptibilities. It is possible that transmitting mothers harbor a greater proportion of ADCC-resistant strains, which could explain the lower ADCC responses present among the HEI infants against the contemporaneous maternal strains.

Finally, we have identified plasma samples that have elite ADCC activity. This subset of samples will help us concentrate our efforts to determine what characteristics are important for inducing a strong ADCC response. For instance, by isolating and characterizing the antibodies present in the samples with elite ADCC, we may identify antibody epitopes targeted by antibodies that mediate broad and potent ADCC. Furthermore, the highest breadth and potency was induced by maternal plasma from PID 454. This individual is extremely interesting as their plasma sample was able to induce

100% ADCC against five of the ten variants tested in the global panel. Additionally, we confirmed that this particular sample did not have dramatically more plasma IgG compared to other "non-elite" ADCC plasma. We further confirmed that the ADCC was mediated by IgG and not another plasma constituent. This strongly supports that the responses assessed in PID 454 were due to currently unidentified specific characteristics of the antibodies, and not simply explained by differences in IgG levels or another plasma constituent. The antibodies present in this plasma sample warrant further investigation.

Overall, the work presented in this dissertation provides insights into the role ADCC plays in the prevention of MTCT of HIV-1 by using methodology that allows us to assess responses against maternal variants. However, it is still unclear if this protection could be harnessed for use in a vaccine. The development of our new cell line has already contributed to advancing the field, as multiple groups have now incorporated using MT4-CCR5-Luc cells in their ADCC studies. Furthermore, identification of elite ADCC activity provides a starting point for in-depth characterization of ADCC-inducing antibodies. Comparisons between autologous and heterologous ADCC responses has opened new avenues for investigation, including considering the ADCC-susceptibility of circulating variants, which may have broader implications in the design and development of future vaccines.

Study Limitations

One limitation in our study is the small sample size, particularly for the analysis of the autologous ADCC responses. Virus was not successfully made for every mother in

our cohort, limiting the sample size for the analysis as discussed in Chapter 4. Furthermore, only 14 infants in our cohort had a SAE ≥4 or death, limiting the power of our survival analysis. This limited number of events also made us unable to perform a Cox proportional hazard model. Another limitation is that we only quantified total levels of immunoglobulins in our samples and we did not quantify HIV-specific antibodies. We assumed that by picking a consensus sequence for HIV-1 specific quantifications, we would not have results applicable to the circulating maternal strains. It is possible that the levels of HIV-specific antibodies are different between groups which could contribute to differences that were seen. Furthermore, information about infant viral load and $CD4^+$ T cell counts was not collected in the clinical trial. This limits our ability for further analysis to determine why infected infants with high ADCC have better clinical outcomes, which we had hypothesized may be due to ADCC reducing the number of infected cells therefore lowering the infants viral load and allowing better immune control. Our studies are correlative and they cannot prove causation. Finally, our study is based on a single cohort, and therefore needs to be repeated in other cohorts to confirm generalizability.

Remaining Questions

My work addresses one component of the ADCC response, the role of antibody. The project does not examine the presence and functional capacity of the effector cells. Healthy infant NK cells express high levels of perforin and granzyme B, which are necessary for inducing cell killing (Y.-C. C. Lee & Lin, 2013). However, it remains unclear if HIV infection changes the capacity for NK cell cytotoxic responses. ADCC-

inducing antibodies can only be effective if there are cells present capable of inducing killing. It would be interesting to assess differences in NK cell responses between HEI and HEU infants. One hypothesis is that if ADCC is protective then HEU infants would have better NK cells, in addition to better antibodies, compared to HEI infants. To test this hypothesis infant PBMCs would be required.

Our study focused on IgG-mediated ADCC responses. However, it remains unclear how IgA impacts ADCC activity. Data presented in Chapter 4 supports IgA having a negative effect on ADCC, as we saw higher responses from IgG isolated from breast milk compared to matched breast milk supernatant. High levels of IgA in breast milk suggests that IgA may be responsible for these differences. Additionally, we saw IgG/IgA ratios positively correlating with ADCC activity, further supporting that IgA negatively impacts the ADCC response. The mechanism behind how IgA may negatively impact IgG-mediated ADCC remains unknown. One possibility is IgA outcompetes IgG for binding Env expressed on the surface of infected cells. Our data, along with ADCC being implicated as a protective correlate in individuals with low plasma IgA in the RV144 vaccine trial, highlights the need to further investigate what appears to be an important component in inducing a strong ADCC response.

Future Directions

In order to develop a vaccine that induces protective ADCC responses, it is necessary to understand the characteristics, such as glycosylation patterns and binding sites, of the antibodies that prevent infection. In Chapter 5, breadth and potency was assessed in our cohort. The samples ranking in the top 5% for $ADCC_{BP}$ may contain

antibodies that effectively block diverse exposure variants. Epitope mapping will be performed to determine where on Env these antibodies are binding. Additionally, a number of factors can impact ADCC efficacy, one example being glycosylation in the antibody Fc domain. Furthermore, it is necessary to determine if a single antibody, or a pool of antibodies present in the sample is what's driving the ADCC response. Single cell sorting can be used to isolate B cells from frozen PBMC samples to generate monoclonal antibodies to further parse out the ADCC responses from the plasma.

We are currently working to test our new hypothesis that transmitting as compared to non-transmitting mothers harbor a greater proportion of ADCC-resistant strains. To address this question, we will use a pool of maternal plasmas, of mothers who were not included in the autologous studies, as a standard to test ADCC susceptibility of the maternal viruses isolated from transmitting as compared to non-transmitting mothers. We expect that ADCC responses will be higher against cells infected with non-transmitting maternal variants compared to cells infected with transmitting maternal variants. At the same time, we will assess a pool of bnAbs to determine their ADCC capacity against clinical variants. This bnAb pool will contain 3BNC117 (CD4bs), PGT121 (V3 glycan), PG9 (V1V2 loop), and 10E8 (MPER). Finally, we will individually test the maternal plasma from PID 454, which was the sample with the highest $ADCC_{BP}$ tested. These studies could explain why we saw significantly higher infant autologous responses, but no difference in heterologous responses. Additionally, if the maternal plasma from PID 454 induces ADCC against a majority of clinical variants, this sample contains antibodies that may block transmission. This would warrant further

investigation. Preliminary data against a limited number of variants suggests maternal plasma from PID 454 induces just as strong ADCC as the pool of maternal plasmas, suggesting it is the plasma sample driving the response seen in the pool.

The ADCC responses presented in this dissertation were assessed in patient plasma where the antibody composition is unknown. Future studies would need to address if these ADCC responses are mediated by a single antibody or a panoply of antibodies. In addition, the Env epitopes targeted by the samples observed to have elite ADCC have not been determined. Epitope mapping can be performed by measuring ADCC of bnAbs, with known binding sites, against the previously tested global panel viruses. In this way, the bnAb ADCC responses can act as a map to compare against the elite ADCC samples and maternal plasma from PID 454. Any matches to the bnAb response profile can help determine the epitopes targeted by the elite ADCC samples. This information can help inform antigen choice for vaccinations that would induce the development of ADCC-inducing antibodies. Careful planning for future experiments is necessary as these are limited historical samples. Additionally, more work should be done to look at ADCC responses in cohorts other than MTCT where we can still identify the exposure strains, such as in discordant couples to evaluate if ADCC is protective.

Our work demonstrates that ADCC is a correlate of protection in MTCT of HIV-1 and impacts the morbidity and mortality of infected infants up to one year after birth. Differences between ADCC responses induced against autologous maternal variants and unrelated heterologous variants has raised future questions surrounding the ADCC-susceptibly of HIV variants and the impact on transmission. ADCC and neutralization are

independent functions of antibodies and an effective vaccine will likely need to induce both responses to prevent transmission.

APPENDIX 1: AUTOLOGOUS ADCC AND NEUTRALIZATION DATA WITH PCR METHOD FOR MATERNAL ENV AMPLIFICATION.

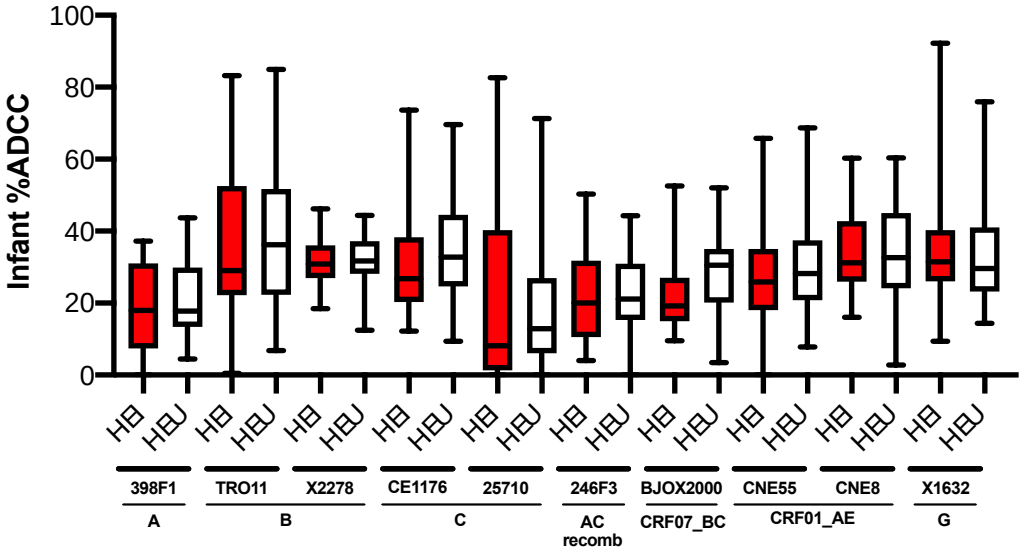
Appendix Table 1. Infant, maternal, BMS, and BM IgG $ADCC_{AUC}$ and $Neut_{AUC}$ with PCR method for maternal Env amplification.

ID	Outcome	PCR method: If SGA, # of amplicons combined		Infant ADCC AUC	Maternal $ADCC_{AUC}$	BMS ADCC AUC	BM IgG ADCC AUC	Infant Neut AUC	Maternal $Neut_{AUC}$
1152	NTM	SGA	5	0.17	0.25	0.06	0.12	0.28	0.24
1015	NTM	SGA	30	0.05	0.17	0.02	0.10	0.49	0.07
1782	NTM	SGA	29	0.02	0.07	0.02	0.15	0.32	0.18
1476	NTM	SGA	12	0.36	0.35	0.08	0.19	0.37	0.14
1021	NTM	SGA	1	0.07	0.31	0.00	0.05	ND	ND
1363	NTM	SGA	16	0.05	0.14	0.00	0.09	0.47	0.11
1666	NTM	SGA	2	0.28	0.51	0.12	0.17	0.43	0.04
1061	NTM	Bulk	--	0.19	0.14	0.03	0.08	0.13	0.00
1227	NTM	SGA	2	0.27	0.44	0.19	0.34	0.34	0.01
771	NTM	SGA	10	0.37	0.38	0.15	0.15	0.37	0.20
1715	NTM	SGA + Bulk	17	0.04	0.03	0.00	0.04	0.00	0.00
317	NTM	SGA	9	0.08	0.32	0.16	0.22	0.28	0.16
1444	NTM	SGA + Bulk	16	0.09	0.20	0.04	0.05	0.28	0.15
1379	NTM	SGA	30	0.06	0.31	0.00	0.06	0.29	0.14
1431	NTM	Bulk	--	0.32	0.36	0.16	0.14	0.27	0.27
2163	NTM	Bulk	--	0.04	0.15	0.00	0.04	0.25	0.19
60	NTM	SGA	10	0.11	0.17	0.00	0.17	0.14	0.24
1148	NTM	Bulk	--	0.10	0.18	0.00	0.02	0.25	0.15
399	NTM	SGA	16	0.08	0.15	0.02	0.08	0.00	0.15
1225	NTM	Bulk	--	0.02	0.32	0.03	0.08	ND	ND
355	NTM	Bulk	--	0.41	0.52	0.18	0.02	0.02	0.00
1037	NTM	Bulk	--	0.23	0.26	0.10	0.14	0.12	0.11
196	NTM	Bulk	--	0.04	0.11	0.01	0.07	0.20	0.15
1047	NTM	SGA	30	0.05	0.14	0.07	0.06	0.15	0.00
572	NTM	Bulk	--	0.28	0.35	0.07	0.10	0.27	0.13
1289	NTM	SGA	24	0.45	0.62	0.10	0.08	0.08	0.13
99	TM	SGA	12	ND	0.52	0.09	0.15	0.17	0.21
129	TM	SGA	18	0.06	0.17	0.00	0.16	0.31	0.23
146	TM	Bulk	--	0.22	0.14	0.04	0.03	0.33	0.27
170	TM	SGA	12	0.03	0.11	0.07	0.10	0.20	0.30

302	TM	SGA	3	0.07	0.20	0.06	0.22	0.15	0.10
774	TM	SGA	8	0.08	0.15	0.00	0.05	0.11	0.12
779	TM	SGA	21	0.00	0.02	0.00	0.04	0.00	0.20
804	TM	SGA + Bulk	14	0.02	0.03	0.00	0.08	0.04	0.07
878	TM	SGA	8	0.04	0.06	0.02	0.09	0.37	0.12
1295	TM	Bulk	--	0.21	0.22	0.01	0.11	0.21	0.33
1402	TM	Bulk	--	0.15	0.24	0.07	0.10	0.08	0.24
1459	TM	SGA	3	0.05	0.28	0.06	0.03	0.47	0.03
1785	TM	Bulk	--	0.07	0.13	0.07	0.25	0.09	0.02
1844	TM	SGA	2	0.06	0.23	0.03	0.09	0.00	0.00
1997	TM	SGA	3	0.25	0.50	0.13	0.28	0.34	0.28
2315	TM	Bulk	--	0.13	0.27	0.03	0.08	0.00	0.3

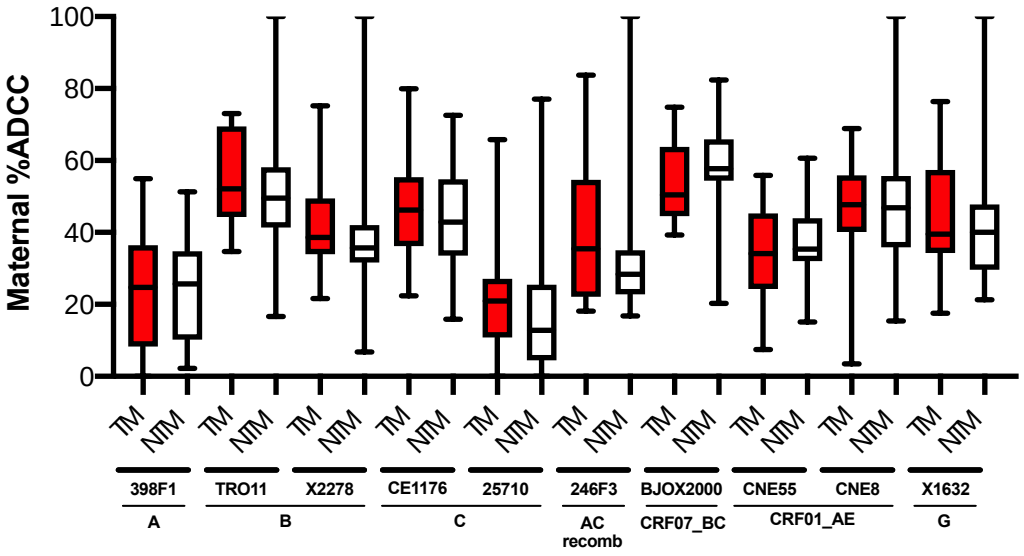
TM, transmission; NTM, no transmission; SGA, single genome amplification; BMS, breast milk supernatant; BM IgG, IgG isolated from breast milk; Neut, neutralization
ND: not done due to insufficient sample.

APPENDIX 2: INFANT HETEROLOGOUS ADCC RESPONSES AGAINST GLOBAL PANEL VARIANTS.



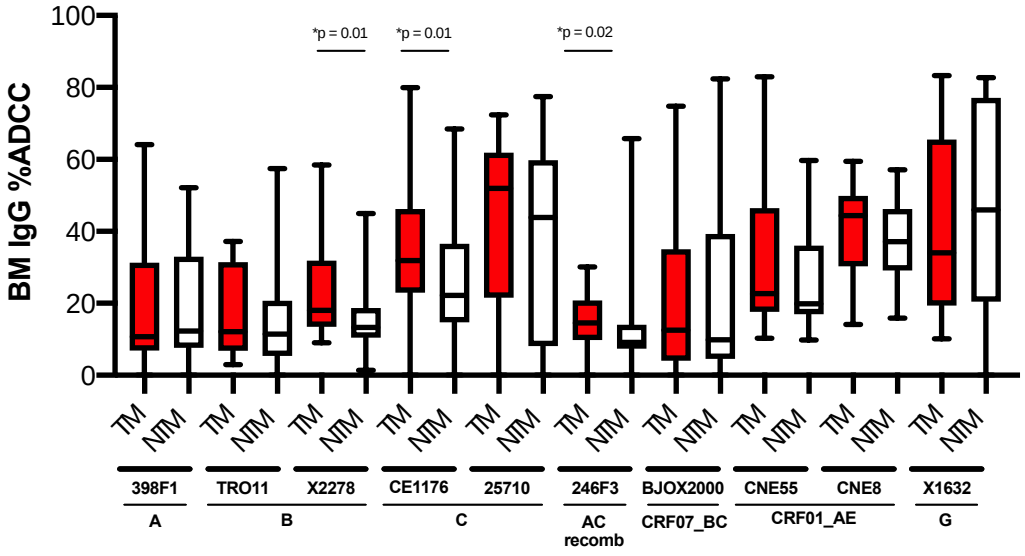
Appendix Figure 1. Infant heterologous ADCC responses against global panel variants. Percent ADCC against individual Envs in the reference panel among highly exposed infected and highly exposed uninfected infants. Virus and subtype are shown on the x axis. Red bars represent highly exposed infected infants (HEI); white bars represent highly exposed uninfected infant (HEU).

APPENDIX 3: MATERNAL HETEROLOGOUS ADCC RESPONSES AGAINST GLOBAL PANEL VARIANTS.



Appendix Figure 2. Maternal heterologous ADCC responses against global panel variants. Percent ADCC against individual Envs in the reference panel among transmitting and non-transmitting mothers. Virus and subtype are shown on the x axis. Red bars represent transmitting mothers (TM); white bars represent non-transmitting mothers (NTM).

APPENDIX 4: BM IGG ADCC RESPONSES AGAINST GLOBAL PANEL VARIANTS.



Appendix Figure 3. **BM IgG heterologous ADCC responses against global panel variants.** Percent ADCC from IgG isolated from breast milk (BM IgG) against individual Envs in the reference panel among transmitting and non-transmitting mothers. Virus and subtype are shown on the x axis. Red bars represent transmitting mothers (TM); white bars represent non-transmitting mothers (NTM). Statistically significant differences are labeled above individual Envs.

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