Evaluating the Impact of Antibody Epitope and Function on HIV-1 Vertical Transmission

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Abstract

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Despite significant advances in scientific research and the advent of treatment and prevention strategies over the past several decades, the HIV/AIDS pandemic continues to pose a significant threat to public health. Nearly 40 million people are estimated to be living with HIV, with 1.5 million new infections in 2021 alone. An effective HIV vaccine would help to reduce the global burden of HIV/AIDS. The humoral or antibody response is one aspect of the immune system that contributes to vaccine-mediated protection from pathogen infection. Studies in animal models have provided proof of concept that HIV antibodies can protect from infection and pathogenesis. However, these models are limited by the use of single challenge viruses and thus do not reflect the diversity of circulating HIV strains. Furthermore, species-specific differences between humans and animal models hinder an accurate assessment of antibody interactions with other arms of the immune system. Studies to identify immune correlates of

protection from HIV infection in humans are important for informing the design of an effective HIV vaccine.

Vertical transmission of HIV is a natural setting where the role of pre-existing antibodies on HIV acquisition can be directly studied. During gestation, infants receive maternal HIV-specific antibodies via placental antibody transfer and these antibodies remain in circulation after birth. Because HIV is found in breastmilk, breastfeeding infants are exposed to HIV in the presence of pre-existing HIV-specific antibodies. These passive antibodies can be measured directly in infant blood samples. We hypothesize that passively-transferred antibodies contribute to reduced vertical transmission risk.

To protect from infection by mediating a myriad of functions, antibodies must bind to specific targets. For HIV, antibodies targeting the Envelope (Env) surface glycoprotein are thought to be critical for protection because Env is required for efficient viral entry. Studies in animal models and humans have consistently demonstrated that antibodies capable of mediating antibody-dependent cellular cytotoxicity (ADCC), or killing of HIV-infected cells, can reduce HIV pathogenesis. Within the setting of breastfeeding transmission, our group previously demonstrated that passively-acquired ADCC correlates with improved survival of infants who acquire HIV, though this study was limited to a single cohort (the NBT cohort). This finding motivates additional studies to better define whether pre-existing ADCC antibodies can protect from infection in addition to reducing pathogenesis. The specific epitopes targeted by protective antibodies are also poorly defined. In this thesis, we sought to bridge these gaps by comprehensively examining whether antibody epitope or ADCC activity protect from HIV acquisition.

In Chapter II, we sought to reproduce prior findings of ADCC protection in NBT in a second cohort, CTL. We also used an ELISA that measures antibody potential to engage dimerized FcγRs IIa and IIIa to interrogate the activity of the RFADCC assay used previously. We found that ADCC activity measured via the RFADCC assay correlated with improved survival among infants living with HIV in the CTL and combined NBT/CTL cohorts. Dimeric FcγRIIa or FcγRIIIa binding also correlated with infant survival, indicating that both receptors may mediate the observed survival benefit of pre-existing HIV-specific antibodies. Finally, there was a trend between RFADCC activity, but not dimeric FcγRIIa or FcγRIIIa binding, and reduced infection risk, suggesting that the RFADCC assay may be measuring a distinct activity than the dimeric FcγR ELISA.

In Chapter III, we leveraged a high-throughput approach to determine whether specific epitopes targeted by plasma antibodies in breastfeeding mother-infant pairs correlate with reduced vertical transmission risk. We used phage display of HIV peptides from Env that focused on linear epitopes that are less well studied. Although we did not identify any direct correlates of reduced vertical transmission risk, antibodies targeting variable loops 1/2 and constant region 5 (C5) were associated with improved survival of infants who acquired HIV in the NBT cohort. We further validated the C5 results using a peptide ELISA for infants from both the NBT and CTL cohorts, where C5 peptide ELISA activity again correlated with improved survival. C5 peptide ELISA activity also correlated with lower setpoint viral load and delayed HIV acquisition, both predictors of infant survival. Altogether, these results provide new insights into the specificity of pre-existing antibodies that protect from HIV pathogenesis.

In Chapter IV, we investigated the specific properties of antibodies comprising a potent plasma ADCC response. We focused on a mother who had high plasma ADCC activity and who

did not transmit HIV to her infant despite several high-risk factors. We reconstructed 17 unique antibodies that all mediated ADCC and recognized epitopes across HIV Env. In competition experiments, several antibodies were required to recapitulate the plasma ADCC activity of the mother and her infant, providing evidence for a potent plasma ADCC response that is highly polyclonal.

Together, the studies presented in this thesis promote a deeper understanding of the role of antibodies in vertical transmission of HIV. By comprehensively examining both antibody epitope and effector function, this thesis provides a useful framework for studying the impact of multiple properties of antibodies on HIV transmission and pathogenesis. It is clear from the combined studies that antibody epitope and function both contribute to pre-existing antibody-mediated protection from viral pathogenesis.

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Chapter I

Introduction

HIV/AIDS Epidemic

The emergence of the HIV-1 (HIV)/AIDS epidemic has resulted in more than 80 million cases and over 40 million deaths[1]. In 2021 alone, there were nearly 40 million people living with HIV, with approximately 1.5 million new HIV infections and 650,000 AIDS-related deaths[1]. Infection with HIV invariably results in lifelong infection, owing to the integration of the proviral genome into the genome of infected cells[2]. The advent of highly effective combination antiretroviral therapy (ART) facilitated the ability to achieve long-term viral suppression. Access to ART thus greatly improved the health of people living with HIV (PLWH) and prevented progression to AIDS[3]. Furthermore, ART dramatically reduces infectiousness and thus also reduces the risk of HIV transmission to sexual partners and from mothers to infants[4,5]. The recent declaration that PLWH with undetectable viral load do not pose transmission risk to their sexual partner(s) (undetectable = untransmittable) provided hope that new HIV infections could be prevented entirely if viral suppression is achieved in all PLWH[6]. In combination with ART, other strategies as such education interventions have led to a 54% decrease in the incidence of new HIV infections since a peak in 1996[1].

Despite the effectiveness of ART, providing treatment to all PLWH remains challenging, as does long-term adherence to ART. While large-scale funding efforts such as the US President's Emergency Plan for AIDS Relief (PEPFAR) have had a significant impact on the access of ART and other services, HIV treatment has been insufficient to end the HIV/AIDS pandemic[1]. Furthermore, the incidence of HIV infections may be increasing in some parts of the world where rates had previously decreased, in part due to supply chain and other challenges due

to the SARS-CoV-2 pandemic, as well as waning funding over the past decade[1]. Thus, the inequalities in health services that drive the HIV epidemic in developing countries may be growing rather than shrinking. While the delivery of effective vaccine might be impacted by similar equity concerns, an effective vaccine with limited doses would not have the adherence issues and long-term financial needs inherent to lifelong ART. To guide the development of a preventative vaccine that could reduce the public health burden of the HIV/AIDS pandemic, there is a need to understand immune correlates of protection from HIV infection and pathogenesis.

Antibody structure and function

For many existing vaccines, antibodies are a correlation of protection[7]. Antibodies (Abs) or immunoglobulins (Igs) are Y- or T-shaped glycoproteins that comprise the secreted compartment of the humoral immune system[8]. Each Ig molecule is comprised of two identical heavy and two identical light chains. The heavy and light chains are linked post-translationally via multiple disulfide bridges. Heavy and light chains both contain variable and constant domains. Whereas the variable domains comprise the antigen binding fragment (Fab), the constant domains make up the crystallizable fragment (Fc), which is colloquially called constant region. There are five classes (isotypes) of Ig: IgM, IgD, IgG, IgA, and IgE, as determined by the antibody heavy chain. The IgD, IgE, and IgE classes form monomers, and the IgA and IgM classes can form dimers and pentamers, respectively. Naïve B cells initially express monomeric IgM and IgD[9]. Exposure to antigen results in B cell activation, leading to the expression of secreted Abs and class-switching to distinct isotypes of secreted Abs, which produces Abs of different classes with the same Fab domain. There are two types of light chains expressed in humans, lambda (λ) or kappa (κ), with only one light chain type used in each Ig molecule.

Within the Fab domain, the specific antigen-binding site for most Abs is typically comprised by the three aptly-named complementarity-determining regions (CDRs) of the heavy and light chains, although mutations in the framework regions (FRs) interspersed between the CDRs can influence antigen binding[9]. In the three-dimensional structure of an antigen-bound Fab, the CDRs are typically in close association within the main contact site. The CDR3 typically makes up the central Ab contact site with antigen.

In response to antigenic stimulation, B cells migrate to secondary lymphoid tissues and undergo a process of iterative and competitive selection for higher affinity binding antibodies, called affinity maturation[10]. Affinity maturation is largely driven by the accumulation of mutations in the Fab domain via somatic hypermutation (SHM), which involves a complex pathway initiated by the enzyme AID[8,10]. The selection for higher affinity Abs inherently results in missense mutations predominantly in the CDRs because mutations in these regions may most directly impact antigen binding. As such, mutations in the CDRs are often the focus of studies of antibody ontogeny.

In the context of pathogen infection, IgG is the most widely studied class because it is the most prevalent in the blood and extracellular fluid[8]. IgG heavy chains have three constant domains (CH1, CH2, CH3). Within CH1, the two heavy chains are also linked together through an additional disulfide bond. CH1 and CH2 are separated by a hinge region, imparting conformational flexibility to IgG molecules[9]. CH2 and CH3 represent the classical Fc domain, with the association between the two heavy chains further stabilized by non-covalent interactions. The CH2 domain contains a highly conserved glycosylation site at residue N297. The specific composition of the N297 glycan has been shown to modulate the interaction between Fc and Fc receptors (FcRs) expressed on immune cells[11,12].

Within the IgG class, there are four subclasses: IgG1, IgG2, IgG3, IgG4 (also the order of decreasing abundance)[13]. The different IgG subclasses are produced as a result of usage of separate IgG C-region (Cγ) genes after class-switching (also mediated by AID). Although the relative abundance of the IgG subclasses is partially due to the order of the Cγ locus (γ3 -> γ1 -> γ2 -> γ4), the class-switching process itself is influenced by the cytokine milieu produced in response to different immune stimuli[8,13]. In the case of viral infections (with protein antigens), B cells receive T cell help as a result of cross-presentation of antigen, and the B cell response is typically skewed towards class-switching to IgG1 or IgG3[13]. By contrast, the IgG response to polysaccharide antigens or glycans is frequently of the IgG2 subclass.

In addition to overall abundance, the IgG subclasses differ in several other properties. Notably, in most individuals, IgG3 has a shorter serum half-life (~7 days) compared to the other classes (~21 days). The IgG subclasses also differ in hinge length and therefore Fab arm flexibility: the IgG2/4 hinges (12 AA) are shorter that of IgG1 (15 AA), and IgG3 has a significantly longer hinge (~62 AA)[13]. This variable hinge length also impacts the ability of the different IgG subclasses to interact with FcRs, with improved interaction between FcRs and IgG subclasses with longer hinges (further discussed below). Finally, with respect to placental antibody transfer, an important consideration for this thesis, IgG1 and IgG3 have improved transport to the developing fetus[8,14]. This process is thought to be mediated by neonatal FcR (FcRn)-expressing cells within the placenta, although the full mechanism of placental Ab transfer is not completely understood[15]. FcRn is also involved in recycling of IgG within the blood, and IgG molecules with higher affinity Fc-FcRn interactions have improved half-life[9].

Fc Receptors and Antibody Functions

Abs can mediate several functions that may be critical to the control of viral infections. Perhaps most well-known, neutralization occurs when Ab molecules directly bind to pathogens and thereby prevent infection of target cells and/or entry via mucosal barriers[8]. In addition to acting independently to neutralize, Abs can bridge the humoral response to other arms of the immune system, mediating effector functions, via Fc interactions.

The numerous Fc-mediated effector functions of Abs include antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent monocyte/neutrophil phagocytosis (ADNP), and antibody-dependent complement deposition (ADCD)[16]. These functions are predominantly mediated by IgG class antibodies. ADCC, ADNP, and ADCD all require the interaction of the Ab Fc domain with subtype specific FcRs, for example FcγRs for IgG. The specific residues recognized by FcRs are in the CH2 and CH3 domains[12]. ADCC is primarily mediated by cells expressing FcγRIIIa (CD16a), predominantly natural killer (NK) cells and activated monocyte populations[12,17]. ADNP is mediated by cells expressing FcγRIIa (CD32a), including neutrophils, monocytes, and other cell types. Some cell types can also mediate ADCC via FcγRIIa. Initiation of Fc-mediated effector functions requires cross-linking of FcRs by antigen-bound antibodies (discussed in[18]). In this regard, the engagement of both IgG Fab domains greatly increases antibody functional affinity and promotes FcR crosslinking. Antibody complex crosslinking increases with higher levels of surface antigen, until saturating levels of antigen prevent binding of both Fab domains. Thus, optimal concentrations of antigen and antibodies are critical to driving high levels of Fc-mediated effector functions.

There are a number of allotypic variants of the FcγRs which affect the affinity of these receptors for IgG[13]. For FcγRIIa, there is amino acid polymorphism at residue 131, with a higher

receptor affinity for IgG in individuals with H131 than R131. Similarly, the V158 variant of FcγRIIIa has a slightly higher affinity for IgG than the F158 variant. The presence of these higher affinity variants has been shown to directly impact antibody effector functions mediated by these receptors, yet the role of FcγR polymorphism in HIV infection is unclear[12,19–22].

HIV-Specific Antibodies

HIV Envelope Structure and Entry

The focus of this thesis will be on antibodies recognizing the Envelope (Env) glycoprotein, as this is the only protein expressed on the surface of virions and it is required for efficient viral entry into target cells[2,23]. Furthermore, many of the reported epitopes for isolated HIV-specific monoclonal antibodies (mAbs) are within Env[24]. On the viral surface, the complete form of HIV Env is a trimer of heterodimers of gp120 and gp41 subunit proteins, with 7-14 Env trimers per viron[25]. The *env* gene encodes a 160 kDa polypeptide (gp160) which is post-translationally cleaved into gp120 and gp41 by furin endoprotease downstream of an RXK/RR motif[26]. After cleavage, the gp120 and gp41 subunits associate together non-covalently to form the Env trimer. Whereas the gp120 subunit is involved in recognition and binding to the viral receptor, CD4, and co-receptor, CCR5 and/or CXCR4, the gp41 subunit is involved in fusion between the viral and cell membranes[23].

Within the gp120 subunit, Env has five more-conserved constant regions (C1-C5) and five variable loops (V1-V5), which tend to be more heterogeneous in sequence and length among circulating HIV strains. The structure of Env partially depends on ten highly-conserved disulfide bonds, nine in gp120 and one in gp41, with several of these bonds forming the loop structures of C1-C4 and V1-V4[27]. Env also has approximately 30 N-linked glycosylation sites (N-X-S/T motif), although there is considerable variation across strains, the vast majority of which are

within the gp120 subunit and can promote viral escape from antibodies[27–29]. The gp41 subunit has an ectodomain, transmembrane domain, and cytoplasmic tail. The gp41 ectodomain is comprised of a fusion peptide (FP) and two helical domains (HR1 or the N-heptad repeat [NHR] and HR2 or the C-heptad repeat [CHR]), which are connected by a flexible loop containing the only disulfide bonds within gp41, named the disulfide loop or immunodominant epitope (IDE)[30].

Engagement of Env with CD4 induces structural changes in V1/V2 and V3, as well as promotes formation of a bridging sheet which facilitates engagement of the viral coreceptor, which is most often CCR5 for transmitted viruses[23]. The coreceptor binding site (CoRBS) of CCR5-topic viruses is in the V3 loop, centered on a GPGR/Q motif[28]. Engagement of the CoRBS results in another structural rearrangement involving the formation of a six-helix bundle by the gp41 NHR and CHR domains, exposing the fusion peptide and bringing the viral and host membranes close together[31]. This second phase of rearrangement occasionally results in shedding of gp120 monomers from the virion, which can bind to other CD4-expressing cells.

Epitopes of HIV-specific antibodies

Studies have described HIV-specific antibodies which target epitopes across gp120 and gp41. Several binding studies have examined whether antibodies to specific Env epitopes correlate with protection from HIV infection (discussed below). In the initial "closed" conformation, only the V1/V2 loop and CD4 binding site (CD4bs) are accessible[32]. Engagement of CD4 results in exposure of the V3 loop and formation of the bridging sheet, which contains multiple CD4-inducible (CD4i) or "open" conformation epitopes. The CD4i epitopes are divided into clusters A-C and are targeted by antibodies which can be particularly potent mediators of ADCC (discussed further below)[33,34]. There is also an additional open conformation epitope within V2 (V2i)[35]. Some Env epitopes appear to be immunodominant in the setting of HIV

infection and vaccination, including V3 and the gp41 IDE. Although a complete Env trimer is required for infection, gp120 or gp41 monomers can be incorporated into virions and gp41 stumps can remain on the cell surface after viral entry, which may partially explain the prevalence of antibodies targeting inaccessible epitopes[16]. There is also evidence that native Env trimers are unstable and can decay over time[36].

Within gp41, early studies described antibodies targeting six distinct clusters of epitopes, with clusters I (the IDE) and II (HR2) being most immunogenic[37–39]. Gp41 cluster III was later defined as the membrane-proximal external region (MPER) and is a target of neutralizing antibodies. Additional epitopes targeted by antibodies include glycosylation sites within gp120 (part of the glycan shield termed the "high mannose patch"), particularly within V3, and the trimer "apex" surrounding the N160 glycan in V1/V2[40,41]. Antibodies targeting these epitopes have garnered significant attention because many show broad neutralization capacity. They are also peculiar because glycans are typically immunosuppressive to antibody responses. Most recently, several additional potent neutralizing antibodies targeting the interface region between gp120 and gp41 have been described[42].

Functions mediated by HIV-specific Antibodies

HIV-specific antibodies are capable of mediating a number of functions, and in some cases multiple functions (e.g. neutralizing antibodies can sometimes mediate ADCC)[43]. Antibodies can be neutralizing or non-neutralizing. Even among neutralizing antibodies, not all Abs neutralize every variant due to viral escape (i.e., structural dissimilarity) at the core epitope or inaccessibility of the epitope on the closed Env trimer. In some cases, steric hindrance between the antibody Fc region and HIV Env prevent binding to an otherwise accessible epitope, as evidenced by the ability of Fab fragments but not intact IgG to neutralize HIV[44]. Many groups

have focused on characterizing neutralizing antibodies (nAbs) which can prevent infection by diverse and heterologous circulating variants, known as broadly neutralizing antibodies (bnAbs), eliciting bnAbs via vaccination remains challenging (discussed below)[36].

In addition to neutralization, HIV-specific antibodies can mediate several effector functions, including ADCC, ADCP, and ADNP. Some studies have also measured the antibody-dependent cell-mediated virus inhibition (ADCVI) mediated by HIV-specific antibodies, which is the conglomerate of several effector functions mediated by multiple cell types. Whereas neutralization must occur prior to viral entry, most antibody-mediated effector functions occur after entry or late in the viral replication cycle via antibody binding to antigen on the cell surface. Regardless of function, binding is the initial pre-requisite for all antibody-mediated activity. Binding affinity has been shown to varying degrees to correlate with neutralization and/or Fc-mediated effector functions[45–51]. In addition to this requirement, to initiate Fc-mediated effector functions, the Fc region must be accessible to FcRs on the surface of immune cells[18,33,52]. Thus, the mode of antibody attachment to its epitope is critical factor in the ability to mediate effector functions. Consequently, both neutralizing and non-neutralizing antibodies (nnAbs) targeting the same epitope can mediate drastically different of effector functions[53]. A recent study provided evidence for an additional influence of antigen-induced allosteric cooperativity on Fc accessability[54].

Viral proteins can also impact antibody mediated effector functions by limiting antigen exposure on the cell surface. HIV Nef and Vpu downregulate CD4 expression on the surface on HIV-infected cells, limiting Env engagement of the CD4 during viral replication and thus decreasing the exposure of CD4i epitopes[55]. Nef also limits $CD4^+$ T cell surface exposure of NK cell activating receptors. Consequently, both of these functions limit ADCC activity directed

against HIV-infected cells[56,57]. Although antibodies targeting CD4i epitopes are appealing because they mediate high ADCC levels against antigen coated cells, they show reduced effectiveness against HIV-infected cells. Thus, the role of ADCC directed against CD4i epitopes in viral clearance may be limited, unless strategies are developed to overcome viral downregulation of these epitopes.

Antibodies Arising in HIV Infection

The antibody response to HIV arises shortly after the establishment of infection and continues to provide immune pressure on the virus throughout the course of infection (reviewed in [16,58,59]). The initial antibody response arises 1-2 weeks after infection in the form of IgM and IgG antibodies targeting gp41. This response is centered on the IDE which connects the two HR domains of gp41 and contains the single, critical disulfide linkage within gp41[60]. It is hypothesized that the early presence of gp41 antibodies is due to stimulation of memory B cells specific for microbial antigens[61]. Antibodies recognizing the gp120 subunit arise ~3 weeks post-infection, initially targeting the V3 loop. Potent neutralizing and ADCC antibodies to both immunodominant epitopes and more conserved cites, the CD4bs, CD4i epitopes, and the gp41 MPER were not detected within 40 days of infection in one study, but are detected within the first few months of acute infection[16,58]. In fact, antibodies capable of autologous neutralization, which refers to neutralization of the infecting strain of an individual, are infrequently seen within the first three months of infection. Consistent with an inability to neutralize, these early Abs typically do not bind functional Env trimer on virions[62]. As such, the majority of immune pressure and reduction of plasma viremia in early infection is thought to be driven by the early T cell response[59]. In addition to IgG, Env-specific IgA antibodies are expressed at mucosal barriers earlier infection, but these antibodies tend to wane over time.

In individuals that mount a nAb response to HIV, there is a continuous inter-host evolutionary arms race between the neutralizing Ab response and viral escape from this response[63]. In a subset of adults (20-40%), bnAbs develop after 2-4 years of infection. There is evidence bnAbs may develop more frequently and faster in infants[64,65]. The ability of bnAbs to neutralize diverse HIV variants and provide protection from sensitive variants in humans (discussed below) makes them an appealing target for elicitation via vaccination[66]. However, viral escape from nAbs invariably occurs in infecting individuals as replicating viruses gain mutations which promote resistance to neutralization over time, despite the tendency of bnAbs to target more conserved or functional constrained sites of Env[67]. The extensive diversity of circulating HIV strains, particularly within *env*, presents a significant challenge for vaccine development (reviewed in [68]). The between-clade amino acid difference in Env is 22-44%, and thus the neutralization sensitivity of circulating viruses varies widely across clades. Even within infected individuals, HIV diversifies at a rate of 1% per year in response to immune response. The resulting quasispecies of transmittable viruses circulating during chronic infection can likewise have varying levels of neutralization sensitivity. As such, immunization strategies must overcome the increasing diversity of global HIV variants.

Many characterized HIV bnAbs also possess features which are immunologically unfavorable and thus difficult to elicit, such as high levels of SHM, atypically long CDRs, and poly- or auto-reactivity[42,63]. Furthermore, despite the ability of bnAbs to potently neutralize diverse HIV variants, the presence of a broadly neutralizing response does not correlate with reduced viremia. In fact, bnAbs responses are observed more frequently in individuals with higher viral loads and lower $CD4^+$ T cell counts, which may suggest a requirement of high antigenic stimulation for the development of these responses[16].

Whereas most early nAbs are capable of autologous neutralization but do not neutralize diverse variants, early ADCC-capable antibodies can mediate killing against diverse HIV subtypes[16]. This may be due to the tendency of early ADCC Abs to target well conserved regions of Env, including CD4i epitopes and gp41 cluster II[38]. The robustness of the ADCC response compared to early nAbs suggest that early ADCC may also contribute to the control of viremia. In this regard, there is some evidence that early ADCC responses may predict slower disease progression[69–71]. Although the focus of studies on viral escape during chronic infection has been on nAbs, there is also evidence that ADCC-mediating Abs can exert immune pressure[43,72,73]. Previous studies are mixed on the evolution of the ADCC response over time. In some studies, ADCC activity was found to remain relatively constant through the course of infection[16]. By contrast, one study reported Fc-mediated effector functions, including ADCC, waned over the course of infection, in parallel with a decrease in IgG3 subclass antibodies and the emergence of nAbs[74]. A complicating factor in this context is the effects of HIV Nef and Vpu, which will dampen the effects of some ADCC antibodies (as discussed earlier).

HIV Control in Humans, Animal Models, and HIV Vaccine Trials

Natural History Studies in Humans

Early studies of individuals with HIV compared antibody responses between individuals who progressed quickly to AIDS ("rapid progressors") and those who did not ("slow progressors"). Although the present utility of these studies is limited by distinct methodology and the inability to control for predictors of HIV pathogenesis, such as setpoint viral load and infection duration, they provided key initial insights into aspects of humoral immunity that influence HIV disease progression. Several studies recognized that bnAbs appeared to arise earlier in rapid progressors, providing a potential link between antigenic burden and the

development of neutralization breadth[75]. Other studies focused on distinguishing between epitopes targeted by antibodies in rapid vs. slow progressors, identifying potential a correlation between antibodies targeting epitopes such as V3, C5, or the gp41 disulfide loop and HIV progression[76–78]. In terms of antibody effector function, many early studies suggested a potential correlation between early ADCC activity and slowed disease progression[71,79,80]. In addition to ADCC, other studies support a potential role of ADCVI and ADNP in reduced HIV pathogenesis, but these studies are limited[80].

More recently, the focus of natural history studies has shifted to individuals who maintain low levels of HIV viremia in the absence of ART, termed long-term non-progressors (LTNPs), and a rare subset of these individuals who also maintain high $CD4^+$ T cell counts, termed elite controllers[81]. Like older natural history studies, several studies of LTNPs support a role for antibody effector functions, particularly ADCC, and not neutralization in protection from HIV pathogenesis. As discussed earlier, many groups have suggested that LTNPs do not produce bnAbs more frequently, yet some bnAbs have been isolated from these individuals[67]. Nonetheless, elite controllers often produce antibodies capable of autologous neutralization and exhibit a more intact humoral response compared to progressing individuals. Several studies have identified an association between certain HLA alleles and elite control, but this is thought to influence CD8+ T cell control and not the antibody response[81]. By contrast, there does not appear to an association between FcyR alleles and elite controller status[82]. Compared to progressors, elite controllers express a higher proportion of IgG3 subclass antibodies, which may correspond to improved antibody effector functions and polyfunctionality[83,84]. Indeed, some studies have identified higher levels of ADCC activity in elite controllers[49,55,85–87]. In one study, LTNP individuals had a broader ADCC response than progressing individuals[87]. There is also

some evidence that elite controllers have a skewed antibody glycosylation profile, which leads to higher levels of ADCC activity[88,89]. In combination, these studies support a role for antibody-mediated effector functions, particularly ADCC, in LTNP status.

Studies in Animal Models

Studies in animal models have been critical for evaluating vaccine and therapeutic candidates, as well as allowing for a mechanistic understanding of potential correlates of protection. Of available models of HIV infection and pathogenesis, non-human primates (NHPs, particularly rhesus macaques) and humanized mice are highly utilized. Rhesus macaques (RMs) share a high degree of genetic similarity with humans and infection with some clones of SIV recapitulate many aspects of HIV infection in humans[90]. More recently, recombinant SIVs expressing HIV Env (SHIVs) have allowed for more reliable assessment of vaccine candidates and therapeutics. It is important to note, however, that differences between human and macaque FcRs prevent full assessment of antibody effector functions in this model[91]. While mice are more genetically divergent from humans, the amenability of mice to genetic manipulation enabled the generation of humanized mouse models which recapitulate varying degrees of HIV infection[90]. In addition to species differences, important caveats of many animal studies include: 1) the use of a high dose of challenge virus, which does not reflect natural HIV transmission, and 2) the use of a single challenge virus which does not reflect the genetic diversity of HIV strains[92].

A number of studies in animal models support a protective role of antibodies in HIV infection[17,33,92,93]. *In vitro* and passive immunization studies in animal models have shown convincingly that nAbs can protect SIV/SHIV acquisition if present at sufficient concentration within 24 hours of viral challenge[80]. Several vaccine studies in NHPs also show that vaccine-elicited nAbs can protect from infection. However, in many studies sterilizing immunity is only

achieved if there is a high degree of similarity between the vaccine strain and challenge virus[92]. A seminal study using Fc-defective variants of bnAb b12 also showed that Fc-mediated effector functions contribute to b12-mediated protection in RMs[94]. A recent follow-up study suggested this protection may be mediated by complement or effector cells[95]. Another recent study showed that VRC01 class-switched to IgG2 provided less protection than its IgG1 counterpart[96]. Although some bnAbs do not depend on Fc-mediated effector functions, it is estimated that such functions can account for nearly half of bnAb-mediated control of viremia[97]. Several additional studies using Fc-defective mAb variants support a role of Fc-mediated effector functions in protection from infection in animal models, including reduced viremia and an increased number of challenges to establish infection[45,98,99].

Apart from evaluating the contribution of effector functions to nAb efficacy, many passive immunization and vaccine studies support a protective role of nnAbs in animal models[17]. In one study, high levels of ADCC elicited by attenuated SIV infection led to complete protection from heterologous and neutralization-resistant viral challenge[100]. Other studies have correlated ADCC levels with slowed disease progression, reduced viremia, a reduced number of transmitted/founder variants, and a greater number of challenges to establish infection[101–107]. Importantly, several of these studies involved the use of nnAbs, which suggests that ADCC alone may confer partial protection from HIV/SIV acquisition and pathogenesis[18]. The direct role of NK cells in protection from HIV/SIV pathogenesis is supported by a recent study showing that NK cell depletion prior to SIV challenge led to higher levels of viremia during acute infection[108]. Another recent study evaluated the protective potential of passive immunization with vaccine-elicited antibodies from heterologous challenge in recipient animals. Animals that received plasma with signatures of polyfunctionality, ADCC/ADCP potential, and potent glycosylation

profiles were transiently protected from viral challenge[109]. Although more sparse, there is also evidence that effector functions other than ADCC confer partial protection against infection in animal models[16,110]. Indeed, two recent studies, among others, have highlighted a potential role for antibody-mediated phagocytosis in reduced infection risk in animal models[111,112].

Vaccine Studies in Humans

The focus of many HIV vaccine trials has been the induction of neutralizing antibodies and/or protective cytotoxic T cell responses[42]. To date, 8 vaccine efficacy trials have been completed and one trial is ongoing. Importantly, many of these trials failed to elicit bnAbs and nAb levels did not correlate with reduced infection risk[33,113]. Only the RV144 trial based in Thailand showed statistically significant protection from HIV infection, with estimated 60% protection at 12-months post-vaccination and 31.2% efficacy at 42 months[114]. This trial used a canarypox vector based ALVAC vaccine expressing Gag, protease, and Env with a gp120 boost. In an initial multivariate correlates of protection analysis, increased binding antibodies to V1V2 and decreased Env-specific IgA antibodies correlated with protection from infection[115]. In interaction analyses, a combination of low IgA binding and ADCC activity, nAbs levels, IgG avidity, or Env-specific $CD4^+$ T cells also correlated with protection[115,116]. Although other vaccine trials did not show any efficacy overall, ADCVI levels correlated with reduced infection risk in the Vax004 trial[113].

The RV144 trial provided hope that an improved version of the vaccine platform would show greater efficacy in additional studies. However, the HVTN 702 trial, which used a similar regimen to RV144, did not show any efficacy in a high-risk population in Sub-Saharan Africa[117]. Although immune correlates analyses for the HVTN 702 trial is not complete, differences in immune profiles elicited by the vaccines, the sensitivity of circulating strains to ADCC, and

overall HIV acquisition risk are hypothesized to contribute to the reduced efficacy of HVTN 702 compared to RV144[117–120]. As such, the role of non-neutralizing antibodies and ADCC in protection from HIV acquisition is still unclear.

In addition to active vaccination trials, several studies have examined whether passive vaccination can protect from HIV acquisition. The HVTN 703 and 704 trials examined whether infusions of CD4bs bnAb VRC01 protected from HIV acquisition in two at-risk populations[66]. HVTN 703 and 704 only had 8.8% and 26.6% overall efficacy. However, protective efficacy against VRC01-sensitive HIV isolates was ~75%, suggesting that bnAb passive vaccination has the potential to be effective if sufficient bnAb levels and breadth can be achieved[42]. Safety studies of some additional passive vaccination regimens have recently been completed or are currently underway (e.g. see ongoing IMPAACT network studies)[121].

Vertical Transmission of HIV-1

Vertical transmission or mother-to-child transmission (MTCT) of HIV is a unique setting in which to study the role of antibodies in HIV transmission and pathogenesis because infants passively receive maternal HIV-specific antibodies via placental antibody transfer during pregnancy. Vertical transmission an occur *in utero*, peripartum, or during breastfeeding. Importantly, the setting of vertical transmission merits additional attention because there are nearly two million children currently living with HIV, most of whom acquire HIV vertically, and children make up a disproportionately high number of AIDS-related deaths[1,122]. Furthermore, children living with HIV are particularly affected by unequal access to ART, with only half of children currently on ART compared to 76% of adults[1].

In an ART naïve setting, where breastfeeding is common, 30-45% of infants of HIV+ mothers acquire HIV. Breastfeeding transmission is particularly high during early life because

HIV viral loads are higher in colostrum than mature breast milk[123]. In addition to breastfeeding, other risk factors for vertical transmission include maternal plasma viral load and CD4 count, genitourinary tract viral levels, mastitis (for breastfeeding), and maternal genitourinary lesions or infections[5,124]. Several studies have shown that initiation of ART in mothers with HIV during late pregnancy and/or post-natal infant prophylactic treatment dramatically reduces the risk of vertical transmission[5,122]. Nevertheless, ART remains unavailable for many children and adolescents, who comprise a disproportionate number of new HIV infections[125]. Mothers who acquire HIV during pregnancy and/or begin ART late in pregnancy also remain at risk of transmitting HIV until viral suppression is achieved[14]. Thus, the development of an efficacious HIV vaccine could further reduce the burden of HIV on the vulnerable pediatric population[14].

A Role for Binding Antibodies in Vertical Transmission of HIV

Because many infants born to mothers with HIV do not acquire HIV, even in an ART-naïve setting, many groups have hypothesized that additional maternal factors, including antibodies, may protect infants from HIV acquisition. This could include antibodies present in the mother and/or antibodies that the mother transfers to her infant during gestation. The latter may be particularly relevant because they are present in the exposed infant. Placental antibody transfer predominantly occurs during the third trimester, and passively-transferred antibodies remain functional and in infant circulation for several months after birth[126–129]. Studying breastfeeding transmission in infants who test negative at birth thus allows for assessment of the role of pre-existing HIV-specific antibodies circulating at the time of HIV exposure in HIV acquisition. This setting therefore provides a rare opportunity to study an aspect of humoral immunity to HIV which may inform active vaccine and therapeutic design.

Several studies have identified correlations between maternally-derived, Env-specific antibody responses and altered MTCT risk. In addition to total levels of gp120- or gp41-specific antibodies, epitopes previously implicated in MTCT risk include: V3, CD4bs, V1/V2, HR1, IDE, MPER, and the gp41 cytoplasmic domain[130–142]. However, other studies identified opposing associations or no association with MTCT risk[126,131,134–136]. The inconsistency between these studies may be due to sample and study design differences (discussed below). Another consideration is the use of antigens from lab-adapted HIV strains, which do not typically reflect the diversity of circulating HIV strains, which will inherently bias the relevance of a given assay readout to a study cohort. Finally, many studies do not characterize the fine epitope of plasma antibodies and thus limit the ability to define specific correlates of protection. A comprehensive study to map the epitopes targeted by passively-acquired antibodies at high resolution may more decisively determine the influence of specific epitopes on vertical transmission outcomes.

A Role for Antibody Function in Vertical Transmission of HIV

Regarding function, many studies have examined whether antibody activities correlate with protection from MTCT, with a particular focus on neutralization and ADCC due to results from animal and vaccine studies (summarized in Table 1.1). Data on the role of neutralizing antibodies on vertical transmission are also conflicting: some studies suggest levels of neutralizing antibodies protect from vertical transmission[131,133,139,143–146], whereas many have observed an association with increased MTCT risk or no effect[128,129,136,147–152]. Although fewer studies examined the role of ADCC in MTCT prior to the RV144 trial, several studies support a role for ADCC activity in reduced MTCT risk[129,140,148,153] and/or reduced pathogenesis in HIV+ infants[129,144,148,154]. In one study, ADCC activity in breastmilk of mothers also correlated with reduced MTCT risk[155]. However, it is important to note that not all studies support a role for

ADCC in altered MTCT risk[131,151,156,157]. The role of ADCC in MTCT thus remains unclear. Studies on the influence of Fc effector functions other than ADCC on vertical transmission are limited, although a recent study supports a role of strong FcγR engagement in protection from MTCT[158].

Table 1.1. Summary of studies on the role of antibody function in vertical transmission.
Table summarizing results previous studies examining whether specific antibody activities correlate with protection from, risk, or no association with HIV vertical transmission or HIV pathogenesis. Table lists references for indicated associations.

Antibody Activity	Protection	Risk	No association
Neutralization	131, 133, 139, 143-146	150	128, 129, 136, 147-152, 227
ADCC	129, 140, 144, 148, 153-155		131, 151, 156, 157

Caveats of Prior Studies

The inconsistency between vertical transmission studies may be due to many factors. Some studies used infant cord blood instead of plasma from early life, which may not accurately reflect plasma responses[159]. Other studies may include infant samples from several months after delivery, when passively-transferred antibodies have largely waned and thus may not significantly impact MTCT[126,128,129]. Many early studies used maternal plasma as a surrogate for infant samples. Late pregnancy plasma may represent passively-transferred antibodies in infants because placental antibody transfer occurs during this time, yet placental antibody transfer may be impaired in mothers with HIV and HIV-specific antibodies may be less efficiently transported across the placenta[126,160,161]. There is also evidence that dependence of placental antibody transfer on FcR interactions may skew the Fc effector functions of antibodies in infants compared to mothers[162]. Whereas antibodies in maternal circulation may provide selective pressure on viruses, antibodies in early infant life may have a more direct role in HIV acquisition or pathogenesis in infants[122,163]. In studies using both maternal and infant samples, there is a

typically a high degree of concordance between paired mother-infant responses, but the impact of antibodies on MTCT and outcome may differ based on sample origin[129,133,134,144,150,164]. The use of maternal and early infant samples may therefore provide additional insights into the effect of both immune compartments on MTCT.

Other factors may also explain differences between studies. Importantly, vertical transmission studies are often limited in size, which may limit the ability to detect associations between antibody activities and outcomes. Differences in location and timing of cohort studies inherently means the HIV strains circulating are not the same in each cohort, which can impact epitope-specific viral sensitivity to neutralization and ADCC[68,119]. Finally, many early studies do not account for confounders, such as maternal viral load, which strongly correlates with transmission risk, and the inclusion of *in utero* or late transmission cases, where the impact of passively-transferred antibodies may be less strong[124,129,165,166].

MTCT Cohorts Studied in this Thesis

This thesis utilized samples from two cohorts based in Nairobi, Kenya: the Nairobi Breastfeeding Trial (NBT)[167] and the Cytotoxic T Lymphocyte (CTL)[168] cohort (summarized in Table 1.2). Both cohorts enrolled mothers with HIV in late pregnancy, and mother-infant pairs were followed for two years in NBT or one year CTL, with CTL infants who acquired HIV followed for an additional year. As the original studies took place before the widespread availability of ART, NBT participants did not receive ART. Mothers in the CTL cohort received a short course of zidovudine in late pregnancy, consistent with the standard of care at the time. However, the relatively short half-life of zidovudine limits the impact of this treatment on breastfeeding transmission[169–171]. This enables examination of the role of antibodies in breastfeeding transmission without a confounding role for ART in the CTL cohort.

In order to focus on the role of passively-transferred antibodies specifically in breastfeeding transmission of HIV, a sub-cohort of NBT mother-infant pairs was selected from based on the following criteria (described in [129]): 1) HIV negative infant testing at birth, 2) documented breastfeeding for at least three months or until HIV transmission, and 3) availability of infant sample from the first two weeks of life, when passively-transferred antibody levels are high[126]. 72 mother-infants met these criteria, of which 21 infants acquired HIV (listed as HIV+ in figures) and 51 remained HIV-exposed, uninfected (HEU). When the same selection criteria were applied to CTL cohort participants, 161 mother-infant pairs initially met these criteria. To reduce the number of samples and thereby increase experiment feasibility, the CTL sub-cohort design was further modified to a case-control design and 78 pairs were randomly selected. This resulted in 87 mother-infant pairs after adding all infection cases back in, with 15 infants who acquired HIV and 72 who remained HEU. The similar design of both studies allows for the use of combined data from both cohorts to increase analytical power. In both cohorts, maternal and infant samples were collected, which allows us to determine whether antibodies in either mothers or infants influence vertical transmission. This thesis will use maternal plasma from late pregnancy and infant samples from early life to focus on passively-acquired antibody responses directly in infants or antibodies which were passively transferred during gestation.

Table 1.2. Overview of NBT and CTL cohorts.
Table summarizing differences between the NBT and CTL cohorts. Both cohorts were based in Nairobi, Kenya. Studies recruited women living with HIV during late pregnancy.

Cohort	Study Period	Follow-Up Period	ART?	N For Thesis Chapters
NBT	1992-1998	2 years for all participants	No	51 HEU infants, 21 infants who acquired HIV
CTL	1999-2002	1 year for all participants; 2 years for infants who acquired HIV	Zidovudine during late pregnancy in mothers	72 HEU infants, 15 infants who acquired HIV

Goals for this thesis

The goal of the chapters described in this thesis is to determine whether HIV-specific antibodies influence vertical transmission or pathogenesis of HIV. Studying breastfeeding transmission, where infants are exposed to HIV in the presence of passively-acquired, HIV-specific antibodies that be measured by studying samples near the time of birth, may aid in the identification of immune correlates of protection which have remained elusive. To address the limitations of previous studies (discussed above), I will use next-generation techniques and more relevant antigens to take a comprehensive approach, examining the role of both antibody epitope and effector function in transmission. Where possible, I will also account for known transmission risk factors.

A previous study from our group was among the first to show a protective role of passively-acquired antibodies in humans[129]. In this study, passively-acquired ADCC activity, but not neutralization, correlated with improved survival of HIV+ infants. HIV-exposed infants also had higher passively-acquired ADCC activity than HIV+ infants, though this was not statistically significant. Because this study was limited to a single cohort, additional work is needed to determine whether these results are reproducible. As the specific properties of protective ADCC antibodies, including their epitope, were also not identified, defining these properties may provide additional insights in a protective antibody response in humans.

In Chapter II, I will build upon prior work from our group by expanding this study to a second cohort and using distinct measures of ADCC potential. In Chapter III, I will determine whether binding targets of HIV-specific antibodies are associated with HIV transmission risk or pathogenesis by using high-throughput phage display of Env peptides. In Chapter IV, I will focus on the case of a non-transmitting mother with high transmission risk factors to identify and

characterize ADCC mediating plasma antibodies which may have contributed to protection from HIV transmission. Collectively, the chapters described in this thesis will promote a deepened understanding of the role of antibodies in vertical transmission.

Chapter II

Improved HIV-positive infant survival is correlated with high levels of HIV-specific ADCC activity in multiple cohorts

Sections of text in this chapter have been modified from the following manuscript:

Yaffe ZA, Naiman NE, Slyker J, Wines BD, Richardson BA, Hogarth PM, Bosire R, Farquhar C, Ngacha DM, Nduati R, John-Stewart G, Overbaugh J. Improved HIV-positive infant survival is correlated with high levels of HIV-specific ADCC activity in multiple cohorts. *Cell Rep Med*. 2021; 2(4):100254. doi: 10.1016/j.xcrm.2021.100254.

Introduction

Non-neutralizing antibodies (nnAbs) can mediate several cytotoxic effector functions, such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent phagocytosis (ADP), which play an important role in the control of infectious diseases[172]. NnAbs, particularly ADCC-mediating Abs, have been associated with protection from SIV acquisition or progression to AIDS in several studies of macaques, a commonly used model for human HIV-1 (HIV) infection[55]. While early studies in humans also suggested that ADCC activity correlates with protection from HIV infection and/or slowed disease progression, human data on the role of HIV-specific ADCC-mediating Abs in infection and disease is still somewhat limited[80]. Understanding the protective capacity of pre-existing nnAbs in HIV-exposed and infected individuals may help guide the design of an effective HIV vaccine[125].

Our team has leveraged the unique setting of mother-to-child transmission (MTCT) to specifically investigate the role of ADCC-mediating Abs on outcomes in infants of mothers living with HIV[122]. Maternal HIV-specific Abs are particularly relevant in this setting because they enter the infant circulation via transfer across the placenta and remain in circulation for months after birth[126,127,129,147]. We previously showed that passively-acquired infant plasma ADCC activity positively correlated with infant survival, although maternal plasma ADCC

activity did not; nor did neutralization breadth and potency of infant or maternal plasma[129]. In addition, maternal breast milk HIV-specific ADCC activity inversely correlated with breastfeeding MTCT risk[155]. These findings were observed using samples from the Nairobi Breastfeeding Trial (NBT), which was conducted prior to the use of antiviral therapy for prevention of MTCT, and using HIV gp120 envelope protein in the rapid and fluorometric ADCC (RFADCC) assay, which may measure other non-neutralizing activities as well as ADCC[173–176].

Given the very limited data on immune correlates of protection in humans, we asked whether the findings from NBT were generalizable to other cohorts and reproducible using a different ADCC assay. In a second cohort: the Cytotoxic T Lymphocyte (CTL) cohort[168], we again identify a significant association between passively-acquired gp120-specific ADCC activity and infant survival. We also expanded our detection methods to include a dimeric recombinant soluble FcγR ELISA (dimeric FcγR), in addition to the RFADCC assay, to measure the ADCC potential of passively-acquired Abs[177,178]. The dimeric FcγR ELISA results agreed with those of the RFADCC assay for both cohorts, and combined analyses using data from both cohorts identified a strong association between passively-acquired ADCC activity and reduced infant mortality using both assays. These results provide further evidence for the therapeutic potential of pre-existing ADCC-mediating Abs in individuals that become HIV infected.

Materials and Methods

Human Subjects

Plasma samples used in this study were from the Nairobi Breastfeeding Trial (NBT) and Cytotoxic T Lymphocyte (CTL) cohort. These studies enrolled HIV+ mothers late in pregnancy from 1992-1998 for NBT and 1999-2002 for CTL. Individuals were enrolled in late pregnancy,

and mother-infant pairs were followed for two years of life in the NBT and for one year for CTL, with the exception that CTL infants who became infected in the first year were followed for an additional year[167,168]. Both studies were carried out before the widespread availability of antiretroviral therapy (ART). Whereas all NBT participants did not receive ART, in CTL nearly all mothers received a short course of zidovudine prior to delivery for prevention of infant infection, which was consistent with contemporaneous standard of care. Approval to conduct this study was provided by Kenyatta National Hospital - University of Nairobi Ethics and Research Committee, the Fred Hutchinson Cancer Research Center Institutional Review Board, and/or the University of Washington Institutional Review Board. Study participants provided written informed consent prior to enrollment and for use of their data and samples for future studies.

Cell Lines

CEM.NKR cells (RRID:CVCL_X622; originally derived from female human T-lymphoblastoid cells) were obtained from NIH AIDS Reagent Program (catalog #458) and grown at 37°C in RPMI 1640 media with added penicillin (100 U/mL), streptomycin (100 µg/mL), amphotericin B (250 ng/mL), L-glutamine (2mM), and fetal bovine serum (10%). These cells were not further authenticated in our hands.

Study Design and Plasma Samples

In both groups, a sub-cohort was selected for inclusion in this study based on infant criteria described in Milligan et al, which included HIV negative DNA/RNA testing at birth, breastfeeding history ≥ 3 months or until time of transmission, and availability of an infant sample from the first week of life[129]. All eligible 72 cases from the NBT cohort were included in the study. In the CTL cohort, 161 mother-infant pairs initially met the same criteria, only 15 of which included infants who acquired HIV. Given that this large number of primarily non-

transmitting pairs would make running all samples in parallel with the RFADCC assay difficult, the design was amended to a case-control design through selection of a random sample of 78 from the 161 mother-infant pairs. Six of 15 infected and 72 of 146 uninfected cases were randomly selected. The nine remaining infected cases were added back in after selection. In order to account for the non-random addition of infected cases, all samples were weighted according to Borgan II weighting[179]. Infected cases were assigned a weight of 1, CTL HEUs were assigned a weight of 2.028, and all NBT samples were assigned a weight of 1. All infant samples from the CTL cohort were plasma, whereas 61 and 11 infant samples from NBT were cord blood and plasma, respectively. All samples were heat inactivated at 56°C for 1 hour. Insufficient volume remained for plasma of one infant in the NBT study to run the dimeric FcγR ELISAs on this sample. These cohorts will also be utilized in Chapters III and IV.

Rapid and Fluorometric ADCC Assay

The rapid and fluorometric ADCC (RFADCC) assay was carried out as described previously[173,180]. Briefly, CEM.NKR cells were stained with PKH26 cell linker dye (Sigma-Aldrich) and CFSE cytosolic dye (Vybrant CFDA-SE Cell Tracer Kit, Invitrogen). Double-stained cells were then coated with Clade A/D BL035.W6M.ENV.C1 gp120 (Immune Tech; GenBank Accession #DQ208480) at a ratio of 1.5ug gp120 per 100,000 cells for 1 hour at room temperature. Coated cells were washed and a total of 5,000 target cells were added to wells containing 100uL of plasma diluted at 1:5000 in RPMI media or 100-500ng/mL of control mAbs. After a 15-minute incubation, PBMCs from HIV negative donors were added at a ratio of 50:1 effector to target cells. RFADCC activity was allowed to occur for four hours at 37ºC before cells were washed and fixed in 1% paraformaldehyde (Santa Cruz Biotechnology). Data were acquired via flow cytometry (BD Symphony). PKH and CFSE were detected in the PE and

FITC channels, respectively. Data were analyzed using FlowJo (v.9.9, Treestar). ADCC was defined as the percentage of PKH+, CFSE- cells out of total PKH+ cells after subtracting the activity mediated against uncoated target cells (background), which was set to 3-5%. ADCC activity was normalized to that mediated by Anti-HIV Immune Globulin (HIVIG, NIH ARP, Catalog #3957).

Dimeric FcγR ELISA

The ELISA using dimeric recombinant soluble FcγRIIIa and FcγRIIa ectodomains (dimeric FcγR) was adapted from Wines et al[177]. Maxisorp 384-well plates were coated at 4°C overnight with BL035.W6M.ENV.C1 gp120 at 1ug/mL in PBS. Plates were washed five times between all steps in PBS-0.02% Tween-20-1mM EDTA (PBSE). Plates were blocked with 1% human serum albumin (Sigma Aldrich) in PBSE at 37°C for 1 hour. All samples/reagents used in subsequent steps were diluted in 1% bovine serum albumin (Sigma Aldrich) in PBSE. Plasma samples at 1:50, 1:100, or 1:200 dilutions and mAb controls were then incubated at 37°C for 1 hour. Biotinylated dimeric rsFcγRIIa variant H131 or rsFcγRIIIa variant V158 were prepared as previously described and added at 0.2μg or 0.1μg/mL, respectively, for 1 hour at 37°C[177]. Next, plates were incubated with high sensitivity streptavidin-HRP (Pierce) at 1:10,000 dilution for 1 hour at 37°C. After a final wash, 1-Step TMB-Ultra (Thermo Fisher) was added to the wells. The reaction was stopped 5-10 minutes later using 1M H_2SO_4 (Sigma Aldrich). Absorbance was measured at 450nm optical density. The activity measured for wells coated with HIVIG was used to normalize measurements across experiments, after subtracting the background activity of wells in which no plasma/mAb was added. The area under the curve (AUC) for the activities at each dilution normalized to that of HIVIG was calculated and divided by 1000 for use in subsequent analyses.

Statistical Analysis

Raw data was processed in Microsoft Excel. Statistical analyses were performed using RStudio (RStudio Team 2018) or GraphPad Prism v8. Graphs were generated using GraphPad Prism. In place of a single viral load, an area under the curve (AUC) for maternal viral loads was calculated to adjust for the potential impact of ART on maternal viral load and therefore HIV transmission risk. Linear regression on adjacent viral load timepoints was used to impute missing viral loads where necessary. For the CTL cohort, all AUC calculations used viral loads from pregnancy week 32 (P32), delivery, and one month postpartum. For the NBT cohort, AUC calculations used viral loads from P32 and delivery, week eight postpartum, or week 14 postpartum, where available. AUCs were divided by the total measurement time interval to account for the difference in measurement interval between samples. The AUC/week of maternal viral load was used in all subsequent transmission and survival analyses.

A binomial logistic regression of assay activity and viral load on infant infection status was performed to determine whether these factors were associated with HIV transmission. To determine whether assay activity was associated with survival of infants with HIV, assay activity and viral load were used as predictors in Cox proportional-hazards models of infant survival time. For models controlling for maternal CD4 count with or instead of viral load, the CD4 count closest to delivery was selected if data from multiple timepoints were available. Kaplan-Meier survival curves were also used to compare HIV+ infants with assay activity at/above or below the median via the log-rank test. Statistical significance was defined as a p value less than 0.05 and, where multiple comparisons were made, a Benjamini-Hochberg correction with a false discovery rate of 0.05 was applied. In all figures, N corresponds to the number of individuals.

Except where noted, all data represent the mean of two biological replicates, with two technical replicates in each experiment. Additional details can be found in the figure legends.

Results

RFADCC activity of HIV+ infants using distinct gp120 antigens

Because the correlation in ADCC activity and infant outcome was previously detected in the NBT cohort using the RFADCC assay, we used this same assay in the current study, with the goal of determining the generalizability of the association between RFADCC activity and infant outcomes. At the time of enrollment, clade A was dominant in Nairobi, Kenya, and clade D and recombinants were also common[181]. We used gp120 derived from a clade A/D variant (BL035) isolated from an infant with HIV in the NBT cohort as the antigen in the studies of this cohort and showed that it gave results representative among gp120s from diverse strains in the RFADCC assay[129]. To further validate this, we tested gp120 from a clade A strain, BG505, and observed a statistically significant association between passive ADCC antibodies and infant survival, as seen previously with BL035 and clade B SF162 gp120 antigens (Figure 2.1)[129,164]. In addition, we showed that prior results with SF162 gp120 antigen were reproducible in this study (Figure 2.1). Together these findings demonstrate a consistent association between infant ADCC activity and clinical outcome independent of the gp120 antigen tested.

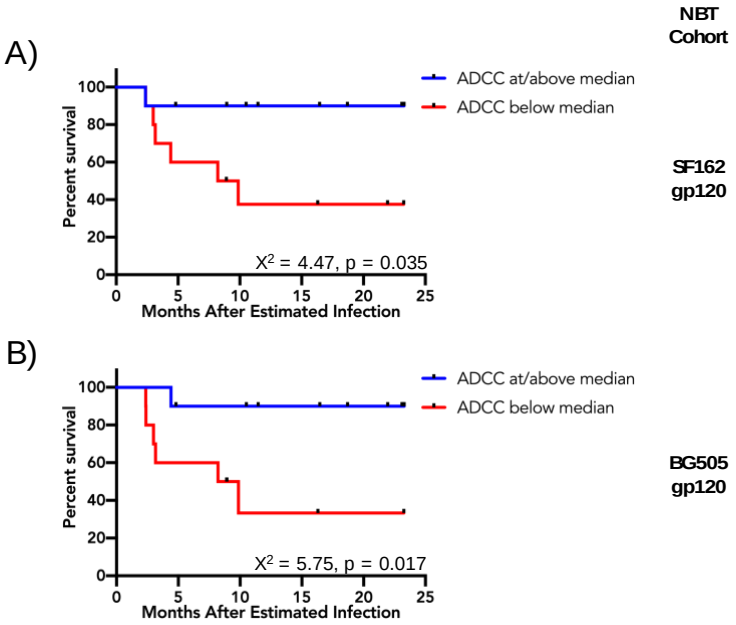

Figure 2.1. Association between gp120-specific RFADCC activity and HIV+ infant outcome for two other antigens in the NBT cohort.
Kaplan-Meier survival curves for HIV+ infants with passively-acquired ADCC activity at/above (blue) or below (red) the median from the NBT cohort (N = 20), using either clade B SF162 (panel A) or clade A BG505 (panel B) gp120 as the antigens. Data are results from one (BG505) or two (SF162) biological replicates, with two technical replicates each. Black dots indicate time of censoring. All p-values are statistically significant after Benjamini-Hochberg correction using a false discovery rate of 0.05.

RFADCC activity of infants in the CTL cohort

Using the RFADCC assay and BL035 gp120 as the antigen, we measured the ADCC activity of 87 infants from the CTL cohort who were defined as HIV-negative at birth, 15 of whom subsequently tested positive and the other 72 remained HIV exposed, uninfected (HEU). As with the NBT cohort, we selected infants who tested HIV-negative at birth so that we could assess the passively-transferred levels of HIV-specific antibodies without interference from de novo responses. We included only infants with samples available from the first week of life, during which time both HIV breastfeeding transmission risk and passive-acquired ADCC activity are high[129,167]. We examined the relationship between passively-acquired ADCC levels in the first weeks of life with both infection outcome and clinical outcome (in those infants who subsequently acquired HIV (HIV+) in the CTL cohort). There was not a statistically significant difference in ADCC activity between HEU and HIV+ infants (p = 0.73, Figure 2.2A), nor was

passively-acquired ADCC activity associated with HIV acquisition risk in logistic regression analysis (Odds Ratio (OR) 1.00, p = 0.99, Table 2.1). By contrast, among HIV+ infants in the CTL cohort, those with ADCC activity above the median generally had longer survival times (Figure 2.2B) and there was a statistically significant association between pre-existing ADCC activity and improved survival after adjusting for maternal viral load (Hazard Ratio (HR) 0.97**,** p = 0.017; Cox proportional-hazards model, Table 2.2).

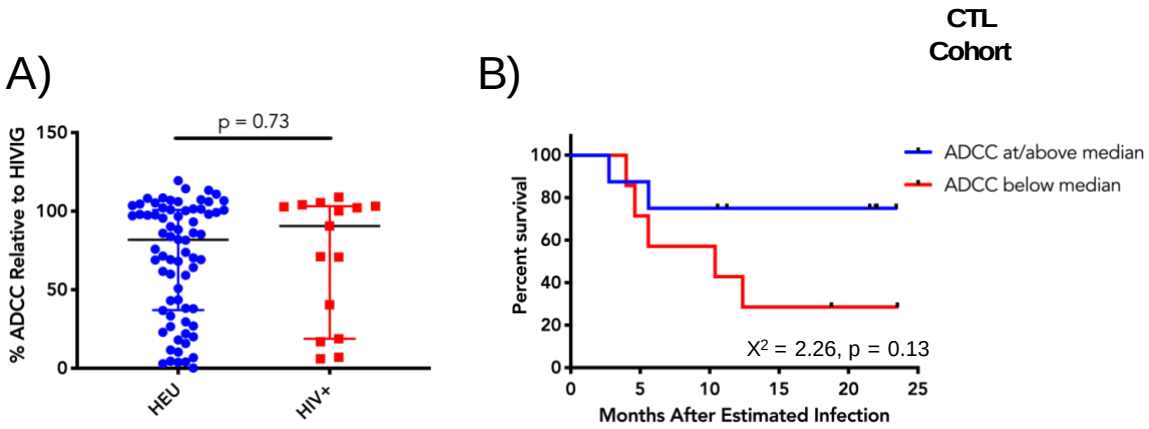

Figure 2.2. Evaluation of gp120-specific RFADCC activity with infant infection status and HIV+ infant outcome in the CTL cohort.
(A) Normalized RFADCC activity of HIV-exposed, uninfected (HEU, blue, N = 72) and HIV+ (red, N = 15) infants in the CTL cohort. Median assay activity compared by the Mann-Whitney U test. B) Kaplan-Meier survival curve for HIV+ infants with passively-acquired ADCC activity at/above (blue) or below (red) the median from the CTL cohort (N = 15). Clade A/D BL035 gp120 used as antigen. Black dots indicate time of censoring. Data from two technical and biological replicates.

Table 2.3. Summary of logistic regression analyses of odds of infant infection by assay activity.
Table describing the results of binomial logistic regression analysis of infant infection status by assay activity, adjusting for maternal viral load, for the NBT, CTL, or combined cohorts. OR: odds ratio; CI: confidence interval. All statistics rounded to the nearest two significant figures. The H131 and V158 variants were used for FcyRIIa and FcyRIIIa, respectively. Data from two technical and biological replicates, apart from only one biological replicate performed for HEU infants in the CTL cohort.

Assay	Cohort	OR (95% CI)	p value
RFADCC	NBT	0.99 (0.97, 1.01)	0.31
	CTL	1.00 (0.99, 1.01)	0.99
	Combined	0.99 (0.98, 1.00)	0.062
Dimeric FcyRIIa ELISA	NBT	1.03 (0.92, 1.16)	0.58
	CTL	0.98 (0.88, 1.09)	0.68
	Combined	1.05 (0.97, 1.12)	0.21
Dimeric FcyRIIIa ELISA	NBT	1.03 (0.90, 1.18)	0.63
	CTL	0.95 (0.86, 1.05)	0.32
	Combined	1.00 (0.93, 1.08)	0.96

Table 2.4. Summary of Cox proportional-hazards models of infant survival.
Results of Cox proportional-hazards modeling using assay activity + AUC maternal viral load as inputs and survival time + death as outcomes. HR: hazard ratio; CI: confidence interval. All statistics rounded to the nearest two significant figures. The H131 and V158 variants were used for the dimeric FcyRIIa and FcyRIIIa ELISAs, respectively. *Statistically significant after Benjamini-Hochberg correction using a false discovery rate of 0.05. Data from two technical and biological replicates.

Assay	Cohort	HR (95% CI)	p value
RFADCC	NBT	0.94 (0.89, 0.99)	0.024*
	CTL	0.97 (0.94, 0.99)	0.017*
	Combined	0.97 (0.95, 0.99)	0.029*
Dimeric FcyRIIa ELISA	NBT	0.81 (0.67, 0.97)	0.020*
	CTL	0.74 (0.51, 1.07)	0.11
	Combined	0.83 (0.74, 0.93)	0.0017*
Dimeric FcyRIIIa ELISA	NBT	0.80 (0.66, 0.97)	0.024*
	CTL	0.77 (0.62, 0.95)	0.017*
	Combined	0.82 (0.73, 0.92)	0.00061*

RFADCC activity of infants from combined cohorts

Because we used similar selection criteria for infants from both cohorts, we combined the RFADCC results obtained for HIV+ infants in the NBT and CTL cohorts (total N = 36) to determine whether there was an association between passively-acquired ADCC activity and infant survival in the combined cohorts. Indeed, in the combined cohorts, we observed a statistically significant association between passively-acquired ADCC activity and HIV+ infant survival when we compared infants with high (≥ median) versus low (< median) ADCC activity ($X^2 = 7.81$, p = 0.0052, Figure 2.3) and when we used continuous data in Cox proportional-hazards modeling adjusted for maternal viral load (HR 0.97**,** p = 0.029, Table 2.2).

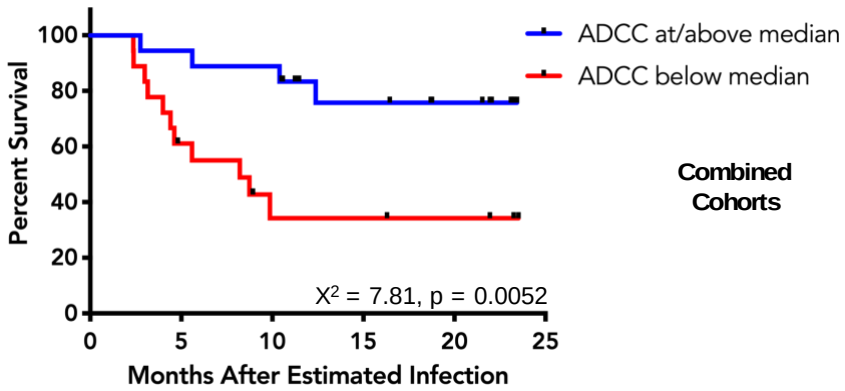

Figure 2.3. Association between gp120-specific RFADCC activity and HIV+ infant outcome. Kaplan-Meier survival curve for HIV+ infants with passively-acquired ADCC activity at/above (blue) or below (red) the median from both the NBT and CTL cohorts (N = 36). Clade A/D BL035 gp120 was used as antigen. Black dots indicate time of censoring. Data from two technical and biological replicates.

Dimeric FcγR ELISA activity as an additional measure of ADCC

We next examined whether the effector potential of gp120-specific plasma antibodies measured by a different assay were correlated with infant outcome in the NBT cohort. For this, we employed the dimeric FcγR ELISA, which was developed to mimic the cross-linking of Fc receptors that occurs during initiation of effector functions[177]. We used FcγRIIa, which is found on neutrophils, monocytes, and many other effector cell types, and FcγRIIIa, which is expressed by natural killer (NK) cells, $CD16^+$ monocytes, and other leukocytes[17,182,183]. There was no

difference in median dimeric FcγR activity between HEU and HIV+ infants in the NBT cohort for either FcγRIIa (p = 0.63) or FcγRIIIa (p = 0.90) via Mann-Whitney U test. Activity measured by this assay was also not associated with MTCT risk in logistic regression analysis for either FcγRIIa (OR 1.03, p = 0.58) or FcγRIIIa (OR 1.03, p = 0.63; Table 2.1) after adjustment for maternal viral load. There was improved survival in HIV+ infants with higher activity measured in the dimeric FcyR ELISA that was statistically significant for both FcγRIIa (HR 0.81**,** p = 0.020) and FcγRIIIa (HR 0.80**,** p = 0.024) in the NBT cohort (Table 2.2; Fig 2.4). All of these results are consistent with data from the RFADCC assay[129].

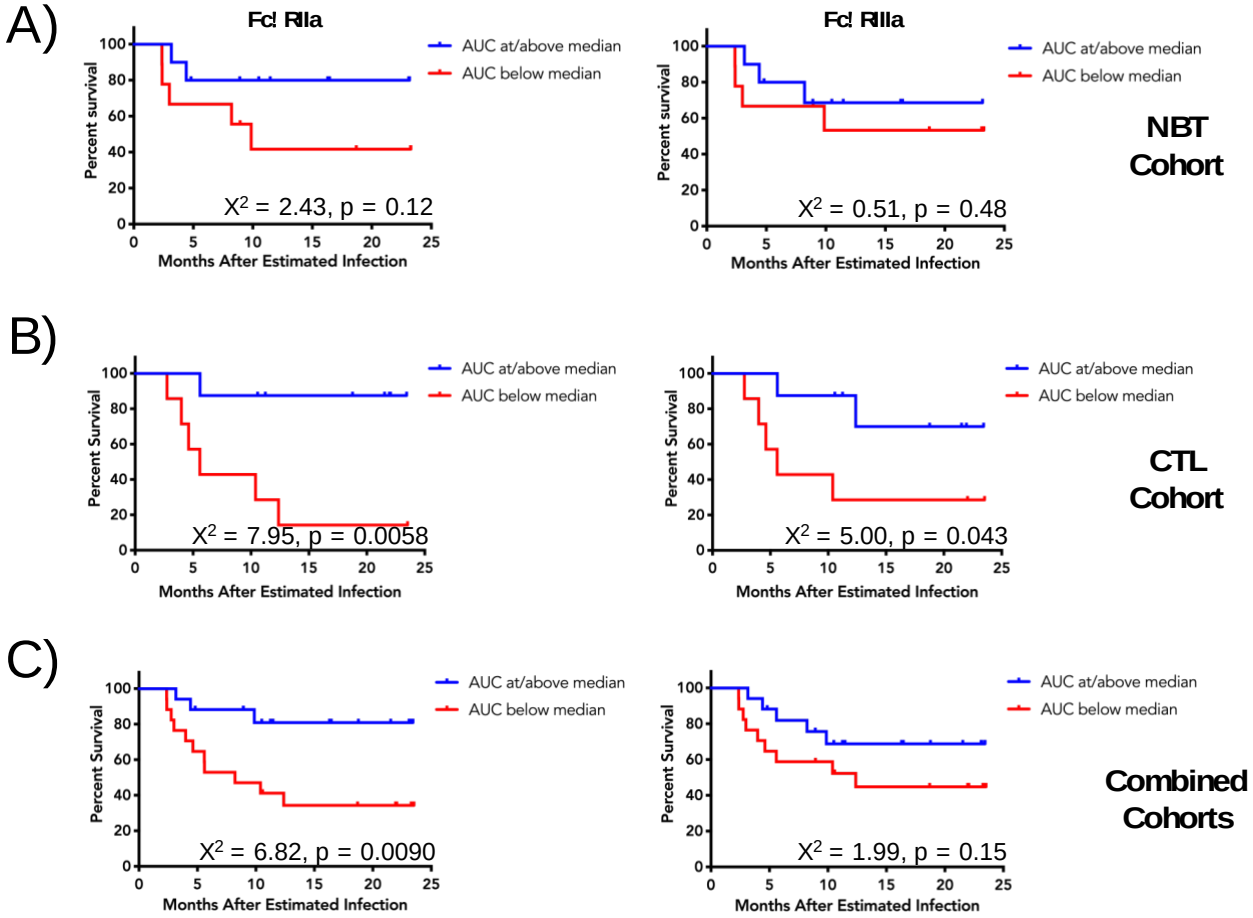

Figure 2.4. Association between dimeric FcγR activity and HIV+ infant outcome for the NBT and CTL cohorts.

Kaplan-Meier survival curves for HIV+ infants with dimeric FcγRIIa (variant H131, left panels) or FcγRIIIa (variant V158, right panels) ELISA activity at/above (blue) or below (red) the median from the NBT cohort (top panels, N = 20), CTL cohort (middle panels, N = 15), or the combined cohorts (lower panels, N = 35). Clade A/D BL035 gp120 used as antigen. Data from two technical and biological

replicates, apart from only one biological replicate performed for HEU infants in the CTL cohort. Black dots indicate time of censoring. P-values for FcγRIIa in the CTL and combined cohorts are statistically significant after Benjamini-Hochberg correction using a false discovery rate of 0.05.

We also examined ADCC activity using the dimeric FcγR ELISA assay in the CTL cohort, focusing on only the HIV+ infants and survival as an outcome, given the lack of association between ADCC activity and infection risk as previously described for the NBT cohort[129] and reported here for the CTL cohort (Figure 2.2A and Table 2.1). In Cox proportional-hazards modeling for the CTL cohort, there was a trend between FcγRIIa activity and infant survival (HR 0.74**,** p = 0.11) and a statistically significant association of FcγRIIIa activity with infant survival (HR 0.77**,** p = 0.017). Importantly, there was a statistically significant association between the activity of both receptors with survival when the two cohorts were combined: FcγRIIa (HR 0.83**,** p = 0.0017) and FcγRIIIa (HR 0.82**,** p = 0.00061). In combined survival analyses excluding the eight NBT infants infected after 6 months, when passive antibodies may be less relevant, there was still a statistically significant association between RFADCC (p = 0.021), dimeric FcγRIIa (p = 0.017), or dimeric FcγRIIIa (p = 0.0067) activity and infant survival. Kaplan Meier curves are shown in Figure 2.4 and Cox proportional-hazards survival analyses are summarized in Table 2.2. The results of models controlling for maternal CD4 count or CD4 count and viral load instead of viral load only were highly similar (Table 2.3).

Table 2.5. Summary of Cox proportional-hazards models of infant survival, adjusting for multiple confounders.
Results of Cox proportional-hazards modeling using assay activity + AUC maternal viral load, maternal CD4 count, or AUC maternal viral load + CD4 count as inputs and survival time + death as outcomes. HR: hazard ratio; CI: confidence interval. All statistics rounded to the nearest two significant figures. The H131 and V158 variants were used for FcγRIIa and FcγRIIIa, respectively. Data from two technical and biological replicates. Statistical significance defined as a p value less than 0.05.

		Adjusted for viral load		Adjusted for CD4 count		Adjust for viral load + CD4 count	
Cohort	Assay	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value
MB	RFADCC	0.937 (0.886, 0.991)	0.023	0.934 (0.877, 0.995)	0.034	0.936 (0.888, 0.987)	0.014
CTL		0.967 (0.940, 0.994)	0.017	0.984 (0.964, 1.00)	0.12	0.968 (0.941, 0.997)	0.034
Combined		0.980 (0.962, 0.998)	0.029	0.980 (0.963, 0.998)	0.030	0.979 (0.960, 0.998)	0.027
MB	Dimeric FcγRIIa	0.807 (0.674, 0.966)	0.020	0.838 (0.707, 0.993)	0.042	0.792 (0.657, 0.955)	0.015
CTL		0.737 (0.507, 1.07)	0.11	0.796 (0.601, 1.05)	0.11	0.768 (0.550, 1.07)	0.12
Combined		0.834 (0.745, 0.934)	0.0017	0.869 (0.781, 0.966)	0.0094	0.834 (0.744, 0.933)	0.0016
MB	Dimeric FcγRIIIa	0.801 (0.661, 0.972)	0.024	0.827 (0.691, 0.988)	0.037	0.787 (0.646, 0.960)	0.018
CTL		0.772 (0.624, 0.954)	0.017	0.784 (0.644, 0.955)	0.016	0.785 (0.636, 0.970)	0.025
Combined		0.816 (0.726, 0.916)	0.00061	0.839 (0.752, 0.936)	0.0017	0.818 (0.728, 0.918)	0.00068

ADCC activity of surviving and non-surviving HIV+ infants

As a final comparison of outcomes, we examined whether passively-acquired RFADCC or dimeric FcγR ELISA activity differed between surviving and non-surviving HIV+ infants.

Indeed, surviving HIV+ infants had significantly higher median RFADCC activity than infants that did not survive during the follow-up period (Figure 2.5A), as well as significantly higher ELISA activity for dimeric FcγRIIa (Figure 2.5B) and FcγRIIIa (Figure 2.5C). All comparisons between surviving and non-surviving infants were also significant in the separate cohorts (data not shown), with the exception of NBT infants for dimeric FcγRIIIa (p = 0.06).

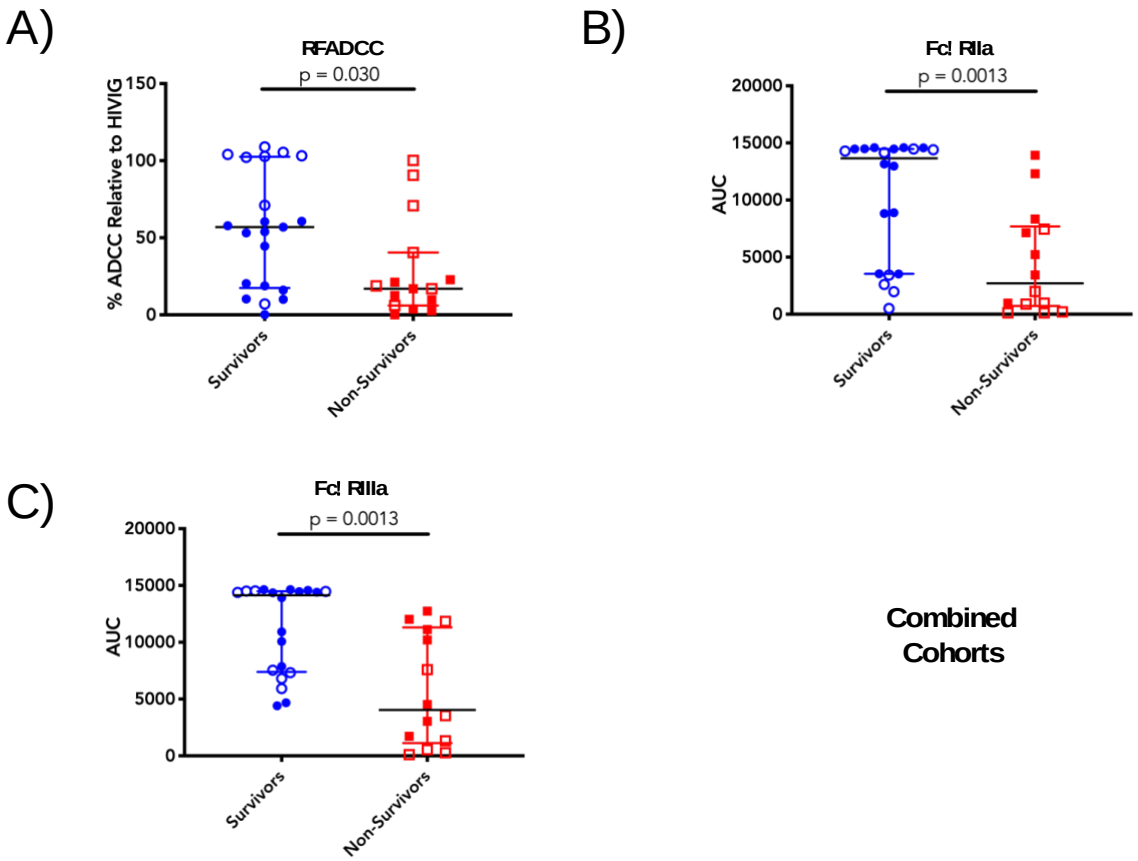

Figure 2.5. Comparison of gp120-specific RFADCC or dimeric FcγR activity in surviving and non-surviving HIV+ infants.
(A) Normalized ADCC activity of surviving (blue, N = 20) and non-surviving (red, N = 15) HIV+ infants in the NBT and CTL cohorts. (B) AUC normalized dimeric FcγRIIa (H131 variant) ELISA activity for surviving (blue, N = 20) and non-surviving (red, N = 14) HIV+ infants in the NBT and CTL cohorts. (C) AUC dimeric FcγRIIIa (variant V158) ELISA activity for surviving (blue, N = 20) and non-surviving (red, N = 14) HIV+ infants in the NBT and CTL cohorts. Closed symbols are infants from the NBT cohort and open symbols are infants from the CTL cohort. Sample for one HIV+ infant from the NBT cohort ran out before ELISAs were run. Median assay activity of groups in all panels was compared by the Mann-Whitney U test. Clade A/D BL035 gp120 used as antigen. All p-values are statistically significant after Benjamini-Hochberg correction using a false discovery rate of 0.05. Data from two technical and biological replicates.

Discussion

It has been challenging to define correlates of protection from HIV infection and disease progression in humans, where it will be necessary to protect against viruses that exhibit extensive diversity, which is difficult to capture in animal models[184]. Despite this, growing evidence suggests that Fc-mediated effector functions may play an important role in protection and disease[17,80]. For example, it was shown in the RV144 trial that V1V2-specific nnAbs correlated with protection from infection and that vaccinees exhibited engagement of FcγRIIa and FcγRIIIa by Abs recognizing a broad range of cross-clade antigens[115,178]. With regard to disease outcome, long-term non-progressor status has also been associated with the development of *de novo* ADCC-mediating Abs[85–87]. It has been difficult to study both protection and disease progression simultaneously in the context of naturally circulating HIV. MTCT is a unique context in which the effect of pre-existing nnAbs on infection risk and disease outcome can be studied in the presence of endemic circulating HIV strains. Here, we demonstrate that passively-acquired ADCC antibody activity measured by two different assays, and in two different cohorts (individually or combined), is correlated with improved clinical outcome in infants who acquire HIV.

Passively-acquired ADCC activity, measured with the RFADCC assay, was associated with improved HIV+ infant survival in the CTL cohort, similar to what was observed previously in the NBT cohort with this assay[129]. Importantly, this association was highly significant when the data using this assay from the two cohorts were combined. Collectively, these findings suggest that the RFADCC assay is measuring a biologically relevant activity in this setting, although the specific nature of this activity in this assay remains poorly defined. This method uses gp120-coated target cells, which may not completely resemble the epitopes presented by

HIV-infected cells, and it measures both NK cell and monocyte-mediated effector functions[174–176]. The findings presented here highlight the importance of better understanding the biological mechanisms underlying the activity that correlates with improved clinical outcomes.

The relationship between ADCC and infant outcome was also observed within both cohorts and in a combined analysis with a second assay that measures the ability of close-proximity antigen-antibody complexes to engage dimeric FcγRs, mimicking the clustering of FcγR required for activation of innate cells that mediate effector functions[177,185]. The dimeric FcγR ELISA has previously been shown to correlate with multiple methods of measuring ADCC[178,186]. While ADCC activity is classically thought to be mediated by NK cells via FcγRIIIa, a growing amount of literature supports a potential role of FcγRIIa and monocytes in promoting ADCC and protective effects against HIV[12,172,175,178,187]. Our finding that FcγRIIa binding was also associated with infant survival is consistent with the importance of multiple cellular triggers for Ab effector functions in the context of HIV infection. Taken together, the highly concordant results among both cohorts and both assays may suggest that Fc-mediated effector functions, such as ADCC, play a role in limiting disease progression in infants with HIV.

The positive impact of passively-acquired antibodies on infant infection is not unique to HIV. For example, ADCC-mediating maternal antibodies are associated with decreased HSV dissemination[188]. Similarly, the degree of NK cell activation by RSV-specific maternal antibodies was inversely associated with infant RSV disease severity[189]. ADCC has also been associated with improved outcomes in previous HIV MTCT studies; however, some of these studies were limited by imprecise determination of infant infectious status or by the study of maternal antibodies, rather than the direct passive antibody levels in the infant[140,144,154,155]. Here,

we provide strong support for a role of effector functions mediated by pre-existing antibodies in improving HIV clinical outcome. Given there is cross-species variability in immunoglobulin-FcR interactions that could impact the relevance of study of HIV-specific ADCC antibodies in experimental systems, these human studies provide critical information for understanding which antibody activities are important for vaccine and therapeutic design[91,185].

This study has several limitations. Of the 35 infants that acquired HIV in our study, 7 had an estimated infection time of six months of age or greater, when passively-acquired antibodies will have largely waned[122]. As described earlier, this did not appear to impact overall results because when the survival analyses were restricted to infants that acquired HIV before six months of age, the results were similar to those of the entire cohort(s). However, a larger study focused on infants at risk and infected within the first 2 months of life would more directly examine the impact of passive antibodies. A final caveat is the use of the RFADCC assay, which relies upon coated target cells, and an ELISA-based assay to measure ADCC activity mediated by passively-transferred antibodies. Although it has been argued that these approaches are less biologically relevant than infection-based assays, both assays have been shown to be highly correlated with other measures of ADCC[177,178]. Importantly, RFADCC activity was also associated with outcome measures in a number of human studies[129,155,190,191].

Addendum

In this addendum, I will describe additional experiments examining the role of passively-acquired ADCC in infant outcomes. Although the RFADCC used in some of the experiments described above correlates with improved HIV+ infant survival, it is limited by the use of antigen coated cells, which are not representative of the context of antigen exposure on HIV-infected cells (as discussed above and in the Conclusions section of this thesis). In collaboration with Dr. Andrés Finzi, we examined whether passively-acquired ADCC directed against HIV-infected primary $CD4^+$ T cells also correlates with HIV+ infant survival in the NBT cohort. In these experiments, primary cells were infected with transmitted/founder virus CH58 with the L139A Env mutation, which promotes sampling of open Env conformations[192]. Consistent with earlier results, infants with higher ADCC activity survived longer than infants with low ADCC activity (Figure 2.6A). Infants with high cell-surface binding antibody levels also survived longer (Figure 2.6B).

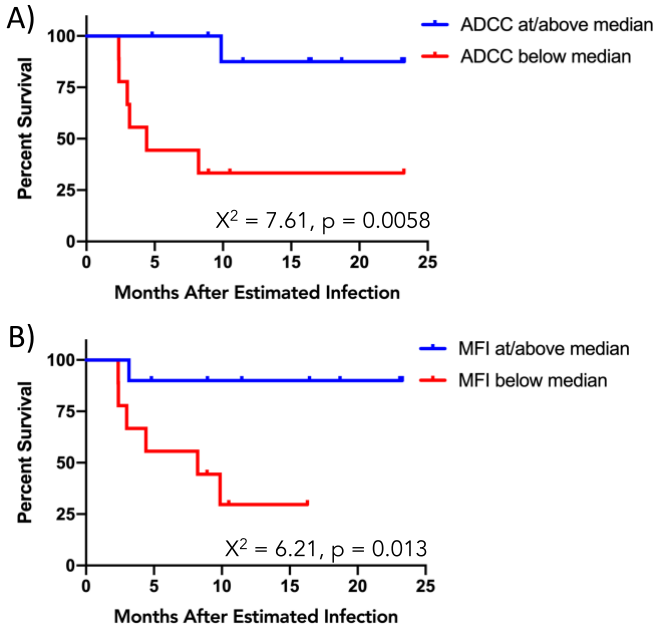

Figure 2.6. Comparison of HIV-infected cell ADCC activity or surface binding antibody levels in surviving and non-surviving HIV+ infants.
Kaplan-Meier survival curves for HIV+ infants with CH58 L193A-infected primary $CD4^+$ T cell ADCC activity (A) or cell surface binding antibody levels (B) at/above (blue) or below (red) the median from the

NBT cohort (top panels, N = 19). Black dots indicate time of censoring. Data from three replicate experiments.

In Cox proportional-hazards models, cell surface binding antibody levels were associated with improved HIV+ infant survival (HR = 0.98, p = 0.0044), whereas ADCC was not (HR = 0.93, p = 0.13). Although these results were limited to a single cohort and a smaller subset of HIV+ infants (19 out of 21), they provide modest support for a role of ADCC activity in improved survival. There is also strong evidence that surface antibody levels, which typically correlate with ADCC activity, may contribute to the survival benefit of passively-acquired antibodies in the NBT cohort, consistent with results from an earlier study from our group[129].

The experiments described earlier in this chapter focused on ADCC activity specific to the gp120 subunit of Env. Evidence from animal studies suggests that ADCC directed against gp41 may also correlated with improved outcomes[106,107]. We therefore examined whether ADCC activity directed against the gp41 subunit of Env also correlated with HIV+ infant survival in the NBT and CTL cohorts. We used the RFADCC assay for these experiments because gp120-specific activity in this assay correlated with infant survival in both cohorts. Whereas higher gp41-specific ADCC activity was not associated with improved survival in Kaplan-Meier curves for the individual cohorts, infants with higher ADCC activity survived significantly longer than infants with low ADCC activity in a combined cohort analysis (Figure 2.7).

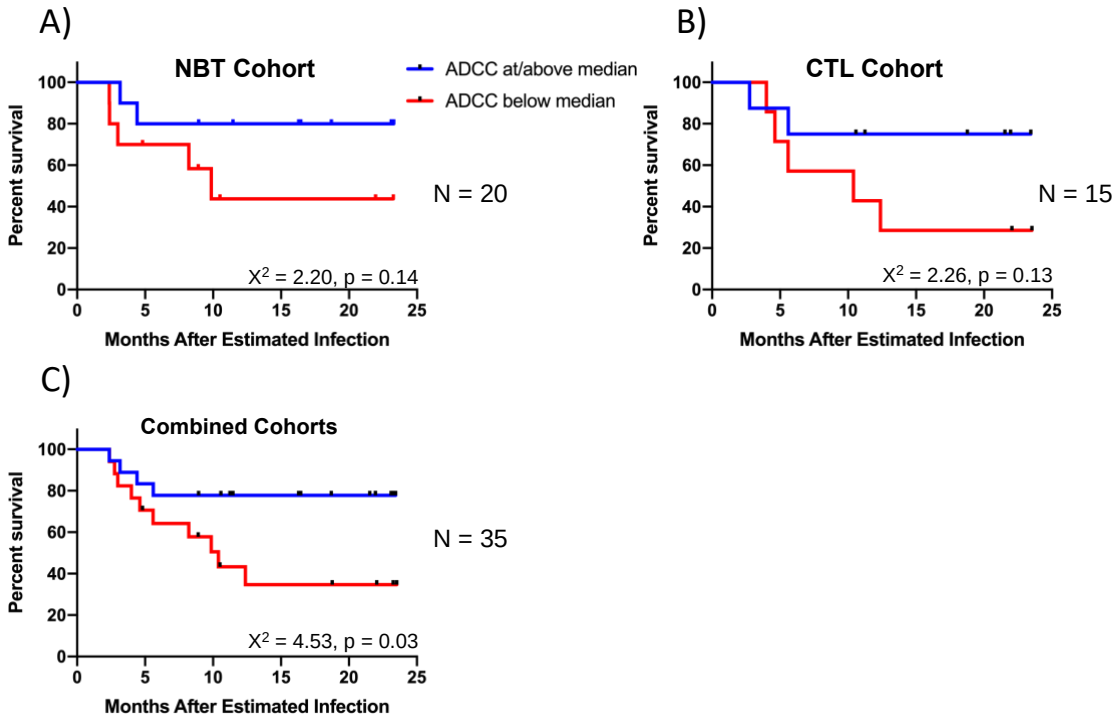

Figure 2.7. Comparison of gp41-specific ADCC activity in surviving and non-surviving HIV+ infants from the NBT and CTL cohorts.
Kaplan-Meier survival curve for HIV+ infants with passively-acquired ADCC activity at/above (blue) or below (red) the median from the NBT (A), CTL (B), or combined cohorts (C). Clade C ZA1197 gp41 ectodomain was used as antigen. Black dots indicate time of censoring. Data from two technical and biological replicates.

There was also a statistically significant association between passively-acquired gp41-specific ADCC activity and improved HIV+ infant survival in Cox proportional-hazards models of survival for the NBT (HR = 0.96, p = 0.017), CTL (HR = 0.75, p = 0.036), and combined cohorts (HR = 0.95, p = 0.013). Altogether, these results provide evidence for a potential role of passively-acquired ADCC directed against the gp41 subunit in improved HIV+ infant survival. Identifying the epitope targets of gp41-specific antibodies in plasma may provide novel correlates of protection.

Chapter III

Passively-acquired C5-specific antibodies are associated with improved survival of infants that acquire HIV

The text in this chapter has been modified slightly from a manuscript entitled "Passively-acquired C5-specific antibodies are associated with improved survival of infants that acquire HIV," which is in preparation.

Introduction

As the search for an effective HIV vaccine continues, there remains a need to identify immune correlates of protection from HIV acquisition and/or pathogenesis. While animal models have been fruitful for proof-of-concept studies, accurate measurement of protection from HIV infection requires human studies. The setting of HIV vertical transmission, or mother to child transmission (MTCT), is unique in this regard, because HIV-specific antibodies are passively-transferred to infants during gestation and remain in circulation after birth [126]. For this reason, numerous studies of vertical transmission have examined whether the quantity and/or qualities of maternal and passive antibodies correlate with infant outcomes. Some studies have suggested binding antibodies targeting variable loop 3 (V3), the CD4 binding site, and/or the gp41 ectodomain correlate with vertical transmission risk, although these studies are based primarily on antibodies present in the mother and results have been variable [131,134,138,141].

The role of passively-transferred HIV-specific antibodies in infant outcomes can best be evaluated in cases where infants are exposed through breastfeeding and test HIV negative at birth, so that passive antibodies can be measured around the time of HIV exposure. Here, we examined epitopes beyond the well-studied and immunodominant antigenic sites identified in previous studies to characterize antibody responses to linear epitopes outside of these domains in two Kenyan cohorts of breastfeeding mother-infant pairs [44]. Using a high-throughput screen of

plasma from one cohort, we identified passively-acquired responses to variable loop 1 and 2 (V1V2) and constant region 5 (C5) as predictors of improved survival in infants that acquired HIV during the study (HIV+). In an analysis combining binding results from two cohorts, C5 peptide ELISA activity correlated with improved HIV+ infant survival, delayed HIV acquisition, and lower setpoint viral load.

Materials and Methods

Human Subjects

Plasma samples used in this study were from the Nairobi Breastfeeding Trial (NBT) and Cytotoxic T Lymphocyte (CTL) cohort. These studies enrolled HIV+ mothers late in pregnancy from 1992-1998 for NBT and 1999-2002 for CTL. Individuals were enrolled in late pregnancy, and mother-infant pairs were followed for two years of life in the NBT and for one year for CTL, with the exception that CTL infants who became infected in the first year were followed for an additional year [167,168]. Both studies were carried out before the widespread availability of antiretroviral therapy (ART). Whereas all NBT participants did not receive any ART, in CTL nearly all mothers received a short course of zidovudine prior to delivery for prevention of infant infection, which was consistent with contemporaneous standard of care. Study participants provided written informed consent prior to enrollment and for use of their data and samples for future studies.

Study Design

In both the Nairobi Breastfeeding Trial (NBT) and Cytotoxic T Lymphocyte (CTL) cohorts, a sub-cohort was selected for inclusion in this study based on infant criteria, which included an HIV negative DNA/RNA test at birth, breastfeeding history ≥ 3 months or until time of transmission, and availability of an infant sample from the first week of life to measure passive antibody[129,193]. 71 mother-infant pairs met these selection criteria. For the infants who

acquired HIV during the follow-up period, the estimated time of infection was defined as the midpoint between the last negative and first positive HIV-1 DNA/RNA PCR test. Approval to conduct this study was provided by Kenyatta National Hospital - University of Nairobi Ethics and Research Committee, the Fred Hutchinson Cancer Center Institutional Review Board, and/or the University of Washington Institutional Review Board.

Phage Display of HIV Env Peptides

Phage display immunoprecipitation sequencing (PhIP-Seq) was performed as previously described[194]. An oligonucleotide pool encoding 1,369 peptides was generated for the *env* ectodomain and transmembrane domain (HXB2 AA numbering 30-704) from six HIV-1 strains: 9032.08_A1 (Genbank Accession # EU576114; B41), BF520.W14M.C2 (KX168094; BF520), BG505.W6.C2 (DQ208458; BG505), BL035.W6M.C1 (DQ208480; BL035), QA013.70I.H1 (FJ866134; QA013), and ZA1197.MB (AY463234; ZA1197). The BF520, BG505, and BL035 strains were isolated early in infection from infants enrolled in the NBT cohort. Oligonucleotides encoded 38 amino acid tiles, with 36 amino acid overlap between adjacent tiles. The oligonucleotide pool was synthesized by Twist Bioscience (San Francisco, CA). Amplified phage at 2 x 10^5-fold representation were incubated with each heat inactivated plasma sample (10µL^{-1} of a 1:10 dilution) for 20 hours at 4°C on an orbital shaker. After overnight incubation, antibody-bound phage were immunoprecipitated using protein A- and -G-coated beads (Thermo Fisher Scientific, Waltham, MA). Samples were then prepared for multiplexed sequencing on an Illumina MiSeq or HiSeq 2500 with 125bp single end reads using the rapid run setting, as described [194]. Demultiplexing, read alignment, and enrichment calculations were performed as described [194]. Alignment was performed with Bowtie 1.3, with up to 2 mismatches allowed. See 'Data and Code Availability' for more information. To directly compare the enrichment of

peptides with the same starting residue, the amino acid sequences of the library strains were aligned using the Los Alamos National Laboratory HIVAlign tool and the relative position of the first amino acid in each peptide compared to the largest library strain (B41) was used for plotting purposes.

Peptide ELISA

Plasma samples were added to plates coated overnight with neutrAvidin and then a biotinylated C5 peptide (Biotin-SELYKYKVVKIEPLGIAPTAAKRRVVQREKR; HXB2 AA 481-511; CPC Scientific). An ELISA to detect HIV peptide specificity in plasma was adapted from a previously described protocol, as follows [195]. 384-well MaxiSorp plates (Millipore Sigma, Burlington, MA) were coated with neutrAvidin (Thermo Fisher Scientific) at 5 μg mL^{-1} in sterile water and allowed to dry overnight at 37°C. Plates were washed four times between all steps with PBS-0.05% Tween 20 (Thermo Fisher Scientific; PBST) wash buffer. The following morning, plates were blocked with 3% bovine serum albumin (BSA) in PBST for 1 hour at 37°C. Plates were then coated with biotinylated B41 C5 peptide at 2 μg mL^{-1} in PBS for 1 hour at 37°C. All samples/reagents used in subsequent steps were diluted in 3% BSA in PBST. Plates were blocked again for 1 hour, then 6 five-fold serial dilutions of plasma starting at 1:100 dilution were applied and incubated at 37°C for 1 hour. Next, Goat anti-human IgG-HRP (Sigma-Aldrich, St. Louis, MO) was applied at 1:2500 dilution for 1 hour at 37°C. After a final wash, 1-Step TMB-Ultra (Thermo Fisher Scientific) pre-warmed to room temperature was added to the wells. The reaction was stopped after 2-5 min using 1M H_2SO_4 (Sigma-Aldrich). Absorbance was measured at 450nm optical density. Serial dilutions of C5-specific mAb 1331A and flu-specific mAb FI6v3 were included as positive and negative controls [196,197].Statistical analysis was performed using GraphPad Prism or RStudio. All data represent the mean of two

biological replicates. Principal components analysis (PCA) was performed using enrichment data from all infants in the NBT cohort to identify regions explaining high variance with the entire dataset, defined as the regions with the greatest loading vectors in the first two principal components. For ELISAs, the area under the curve (AUC) was divided by 1000 for use in subsequent analyses.

Quantification and Statistical Analysis

PhiP-Seq heatmaps were generated with the NumPy, pandas, Matplotlib, and seaborn Python packages. [198]. Raw ELISA data was processed in Microsoft Excel (RRID:SCR_016137). ELISA graphs were generated using GraphPad Prism v9 (RRID:SCR_002798). The enrichment for peptides in each identified region was summed for each strain ("summed enrichment") and then averaged over the number of strains (6) to compare differences in aggregate responses between individuals for each region. For ELISA data, the area under the curve (AUC) for each sample serial dilution was calculated in GraphPad Prism using the trapezoid rule and assuming a baseline of zero after background activity was subtracted. A binomial logistic regression of region summed enrichment and viral load on infant infection status was performed to determine whether these factors were associated with HIV transmission. To determine whether region summed enrichment or ELISA AUC were associated with HIV-infected infant survival, they were used as predictors in univariate Cox proportional-hazards models of infant survival and survival time.

Data and Code Availability

The PhIP-Seq sequencing data set generated during this study is publicly available (Short Read Archive submission in progress). Enrichment data for PhIP-Seq experiments is available upon request. The python API "phippery" was used to process the demultiplexed sequencing

data, align reads to the reference oligonucleotide sequences, and calculate enrichment. "phippery" is available at (https://github.com/matsengrp/phippery). The custom RStudio code generated and used in this study for survival and transmission analysis is available upon request.

Ethics

Human subjects: Approval to conduct this study was provided by Kenyatta National Hospital - University of Nairobi Ethics and Research Committee, the Fred Hutchinson Cancer Research Center Institutional Review Board, and/or the University of Washington Institutional Review Board. Study participants provided written informed consent prior to enrollment and for use of their data and samples for future studies.

Results

We first tested infant samples and matched maternal plasma from the Nairobi Breastfeeding Trial (NBT) to measure responses to HIV Env peptides using a high throughput phage display approach. All 71 infants were defined as HIV negative at birth, of whom 20 infants acquired HIV during the study (HIV+) and 51 remained HIV-exposed, uninfected (HEU). Infant samples were from the first two weeks of life to focus on passively-acquired Ab responses circulating near the time of infection for HIV+ infants. Using an Env library lacking V3 and IDE peptides, epitopes targeted by passively-transferred antibodies in infant plasma included C1, the C terminus of V1 and beginning of V2 (V1V2), and C5 within the gp120 subunit, as well as peptides within the C-heptad repeat (CHR; Figure 3.1). Nearly all individuals showed responses to C5, whereas responses to other epitopes were less common. Maternal plasma samples from the third trimester of pregnancy showed similar responses to infants (Figure 3.2).

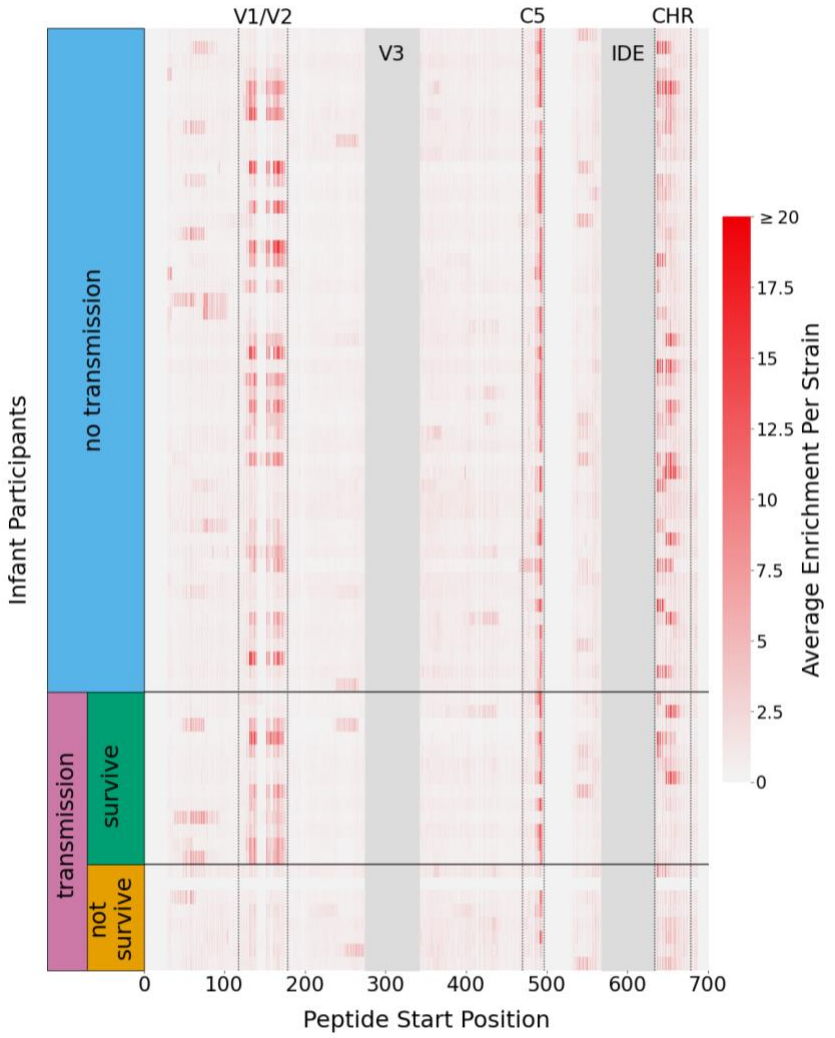

Figure 3.1. Env phage display responses in NBT cohort infants.
Heatmap showing the average enrichment for peptides at each position across the Envelope Protein. Enriched peptides are shown red, with darker shades indicating higher enrichment (binding), as indicated by the scale on the right, for infants in the NBT cohort (N = 71). Peptides are ordered by start position of the N-terminal amino acid, beginning at HXB2 amino acid 31 and spanning amino acid 704. Regions excluded from the library, V3 and the gp41 disulfide loop, are indicated in light gray. Rows indicate plasma samples and are group by infant HIV acquisition status and survival for HIV+ infants (N = 20). Insufficient sample remained for one HIV+ infant – this sample is shown in white (no data) to maintain consistency between figures for infant and maternal data (Figure 3.2). Within each group, individuals are ordered by ascending maternal viral load. Dotted vertical lines indicate regions contributing high variance in PCA, with epitopes indicated above: V1V2 (HXB2 AA 125-210), C5 (HXB2 AA 462-511), and the gp41 CHR (HXB2 AA 617-696). Data are from two replicate PhIP-Seq experiments.

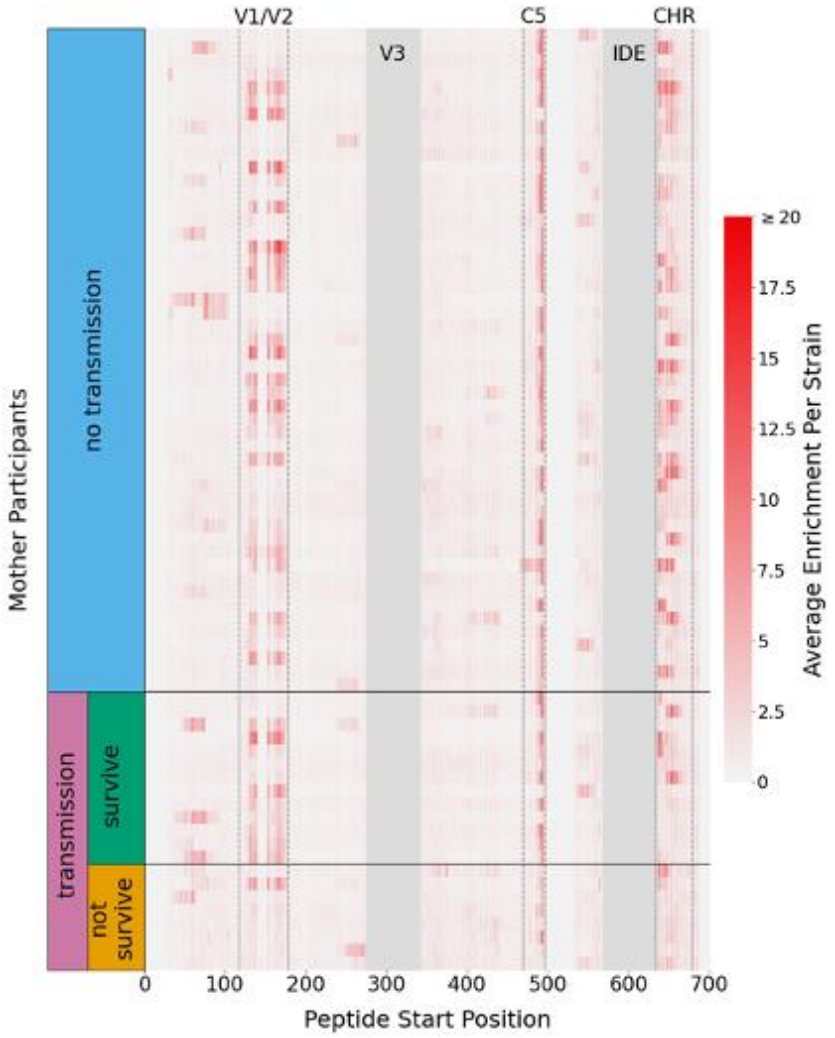

Figure 3.2. Env phage display responses in NBT cohort mothers.
Heatmap showing the average enrichment for peptides at each position across the Envelope Protein. Enriched peptides are shown red, with darker shades indicating higher enrichment (binding), as indicated by the scale on the right, for infants in the NBT cohort (N = 71). Peptides are ordered by start position of the N-terminal amino acid, beginning at HXB2 amino acid 31 and spanning amino acid 704. Regions excluded from the library, V3 and the gp41 disulfide loop, are indicated in gray. Rows indicate plasma samples and are group by infant HIV acquisition status and survival for HIV+ infants (N = 20). Within each group, individuals are ordered by ascending maternal viral load. Dotted vertical lines indicate regions contributing high variance in PCA, with epitopes indicated above: V1V2 (HXB2 AA 125-210), C5 (HXB2 AA 462-511), and the gp41 CHR (HXB2 AA 617-696). Data are from two replicate PhIP-Seq experiments.

PCA was then used to identify differences in passively-acquired Ab responses between HEU and HIV+ infants. Three regions contributed high variance in responses among infant samples: V1/V2, C5, and the gp41 CHR (Figure 3.3). In order to compare differences in the

aggregate responses to these regions between individuals while also limiting multiple hypothesis testing, we summed the enrichment values for strain-specific peptides spanning each region and averaged the enrichment values from each library strain. We next examined whether summed enrichment for any of these regions was associated with infant infection status or clinical outcome. Maternal and passively-acquired infant responses to any region were not associated with HIV acquisition risk in binomial logistic regression analysis, adjusted for maternal viral load. By contrast, there was an association between improved HIV+ infant survival and aggregate infant responses to V1/V2 (Hazard Ratio [HR] = 0.84, p = 0.046) and C5 (HR = 0.95, p = 0.048), but not the CHR, in Cox proportional-hazards models of infant survival, adjusted for maternal viral load. There was a trend for maternal plasma responses to C5 and infant survival, but V1/V2 was not a significant predictor (C5 HR = 0.95, p = 0.060; V1V2 HR = 0.97, p = 0.18).

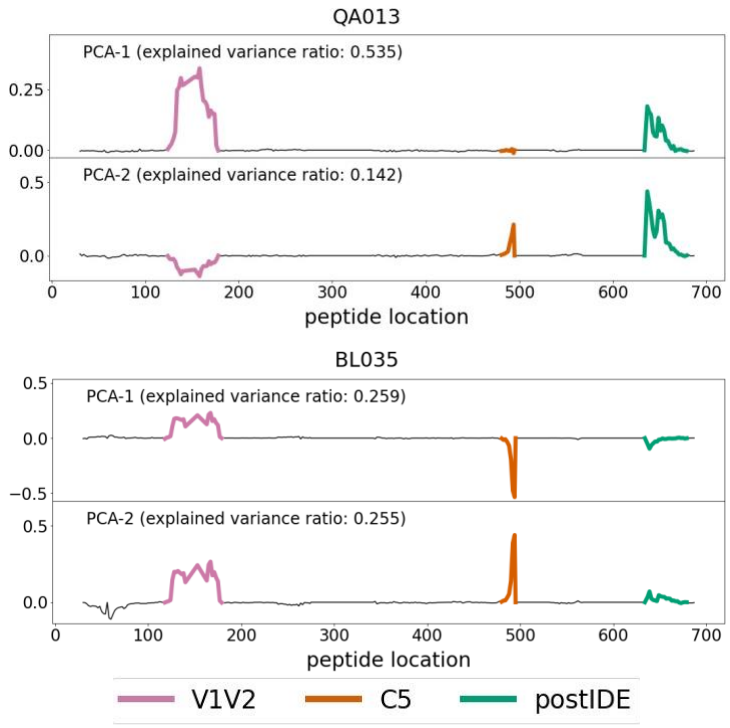

Figure 3.3. Principal components analysis (PCA) for representative library strains.
Loadings for peptides for the first two principal components are shown, with the 1st amino acid of each peptide indicated on the x-axis. Regions selected as explaining high variance for each strain are colored according to the lower legend: V1V2 (lilac), C5 (orange), and the gp41 C-heptad repeat (green). These PCA regions were used to define intervals for summed enrichment calculation in Methods. Representative

results for two strains are shown, with the strains indicated at the top of each plot. The explained variance ratio for each principal component is shown in each plot.

To further address whether C5-specific antibodies correlated with infant survival, we synthesized a peptide spanning the C-terminal end of C5 and tested plasma antibody binding to this peptide via ELISA. Surviving HIV+ infants showed a trend for higher C5-specific binding activity measured by ELISA than non-surviving infants, although this did not reach statistical significance ($p = 0.067$; Figure 3.4). In a Cox proportional-hazards model of infant survival adjusted for maternal viral load, C5-specific ELISA activity was associated with improved infant survival (HR = 0.90, $p = 0.031$). C5-specific ELISA activity also correlated with C5 enrichment (Spearman $r = 0.59$, $p = 0.0061$; Figure 3.5), suggesting that the peptide ELISA measured similar responses to those detected via phage display.

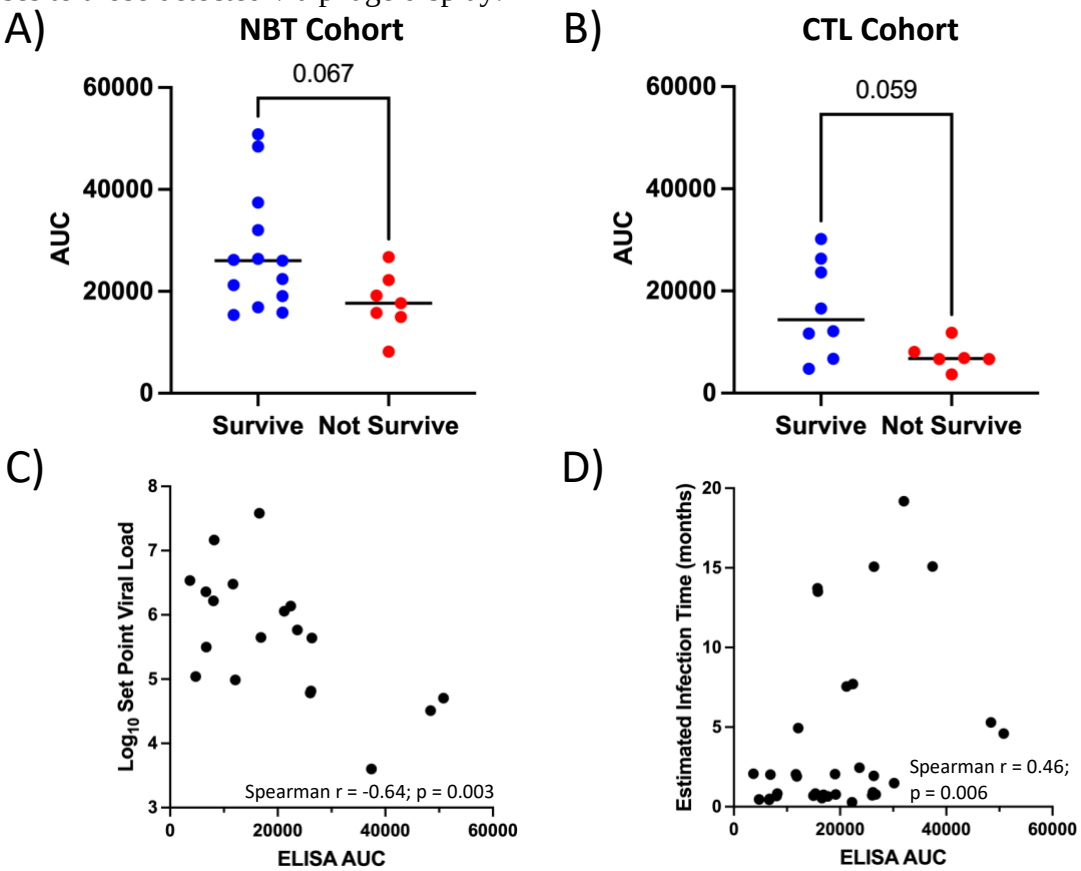

Figure 3.4. Correlation of C5 peptide ELISA activity with HIV+ infant clinical measures.

A) C5 peptide ELISA activity of surviving (blue, N = 13) and non-surviving (red, N = 7) HIV+ infants in the NBT cohort. B) C5 peptide ELISA activity of surviving (blue, N = 8) and non-surviving (red, N = 6) HIV+ infants in the CTL cohort. Median activity of groups in A-B were compared by Mann-Whitney U test. Data are from two technical and biological replicates. C) Plot of combined cohort C5 peptide ELISA activity (x axis) vs. Log_{10} set point viral load (y axis) for infants with viral load data available (N = 20). D) Plot of combined cohort C5 peptide ELISA activity (x axis) and estimated time of HIV infection for HIV+ infants (N = 34), as defined in Methods.

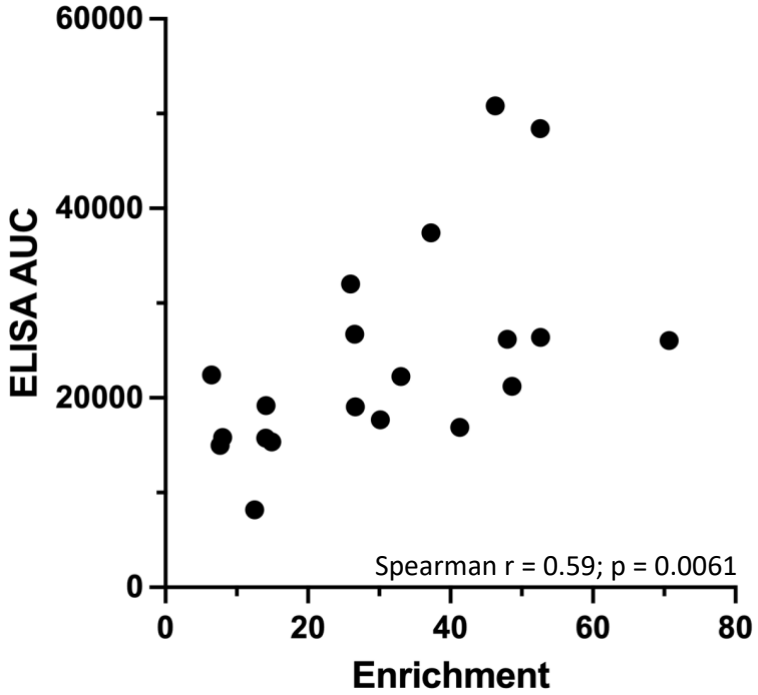

Figure 3.5. Correlation between C5 enrichment and C5 peptide ELISA activity in NBT HIV+ infants.
The Spearman correlation between aggregate C5 responses and C5 peptide ELISA activity in HIV+ infants (N = 20) from the NBT cohort.

To further validate the correlation between C5-specific antibody responses and HIV+ infant survival, we repeated the C5 peptide ELISA using early plasma from a second cohort (CTL) of infants who acquired HIV during breastfeeding (n = 14). There was also a trend for higher C5-specific ELISA activity in surviving compared to non-surviving infants (p = 0.059; Figure 3.4B). In a Cox proportional-hazards model, C5-specific ELISA activity trended with HIV+ infant survival (HR = 0.63, p = 0.068). Because the selection criteria for infants included from each study were similar (as discussed in Methods), we combined the ELISA results from

both cohorts to determine whether C5-specific ELISA activity correlated with infant survival in the combined cohort (n = 34). In this combined analysis of a larger sample size, C5 ELISA activity was also associated with improved HIV+ infant survival in a Cox proportional-hazards model (HR = 0.91, p = 0.0097).

We next examined whether C5-specific responses correlated with other measures of protection, including estimated infection time, setpoint viral load, or peak viral load, all of which have previously been associated with decreased survival and/or increased HIV pathogenesis in infants [199,200]. Setpoint and peak viral load estimates were available for 9 of 20 infants in the NBT cohort and 11 (setpoint) or 14 (peak) of 14 infants in the CTL cohort. Among this subset of 20 or 23 infants, C5 ELISA activity was inversely correlated with setpoint viral load (Spearman r = -0.64, p = 0.003; Figure 3.4C) and trended inversely with peak viral load (Spearman r = -0.38, p = 0.079; Figure 3.6). Furthermore, C5 ELISA activity correlated with the estimated time of HIV acquisition for the HIV+ infants (Spearman r = 0.46, p = 0.004; Figure 3.4D).

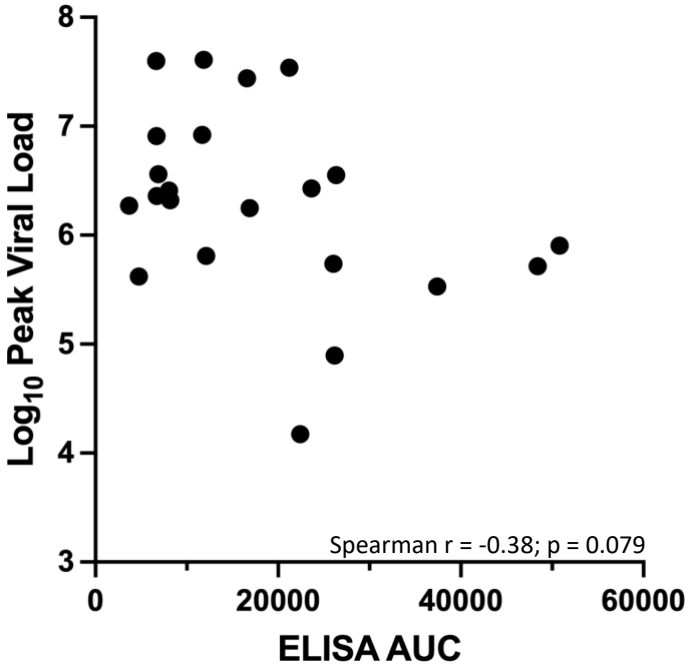

Figure 3.6. Correlation between C5 peptide ELISA activity and peak viral load in HIV+ infants. The Spearman correlation between C5 peptide ELISA activity and Log_{10} peak viral load in HIV+ infants the combined NBT and CTL cohorts (N = 23).

Discussion

In this study, we used phage display of Env ectodomain peptides to assess whether maternal or passively-acquired infant antibody responses to specific peptides were associated with HIV vertical transmission or HIV+ infant survival in two Kenyan cohorts. Plasma samples showed consistent responses to peptides spanning the V1V2, C5, and CHR regions of Env. Passively-acquired responses to both V1/V2 and C5 were associated with improved HIV+ infant survival. Further interrogation of the C5 responses showed that levels of C5-specific antibodies are also associated with various measures of protection.

Our conclusion that passively-acquired C5-specific Ab responses are associated with improved survival in HIV+ infants is supported by results from two cohorts and the use of orthogonal methods. In addition to the association with survival in combined analyses, C5-specific ELISA activity correlated inversely with setpoint viral load and estimated infection time. As both of these measures are known predictors of infant survival, the correlation of C5-specific Ab activity with these measures may explain how such passively-transferred Abs might directly impact infant survival. It is noteworthy that several early studies of human cohorts support a role of C5-specific Abs in slowed disease progression [76,77]. In one study, rapid progressors exhibited higher responses to a C5 peptide than non-progressors at early timepoints, but this response waned in rapid progressors and was maintained or gained in non-progressors [77]. In another study, slow progressing individuals showed higher early antibody responses to C5 than rapid progressors [76]. The consistency of the results presented here provides support for a role of C5-specific antibodies in improved clinical outcome.

There are several limitations of this study. The limited length of peptides in phage display does not allow for capture of responses to conformational epitopes. The phage display library

used in this study also did not capture responses to V3 or the gp41 disulfide loop, but this was by design to improve detection of other less dominant responses. V1/V2 responses correlated with improved survival in the NBT cohort, although these results are limited to only one cohort and phage display of V1/V2 peptides. Given previous findings that V1/V2-specific antibody levels correlated with vaccine efficacy in the RV144 trial, and vaccine efficacy was dependent on specific residues in V1/V2, the results of the present study could be pursued further [201,202]. Finally, viral load data were not available for all HIV+ infants and the statistical power of these analyses was therefore limited within each single cohort, though C5 ELISA activity correlated with setpoint viral load in the combined cohort, even with this small sample size.

Overall, these findings raise the question of how C5-specific Abs directly impact HIV pathogenesis. Several described C5-specific mAbs are capable of mediating antibody-dependent cellular cytotoxicity (ADCC)[203], which is also a correlate of infant survival [193], but the functional properties of C5-specific plasma Abs have not been thoroughly evaluated in human cohorts. An alternative hypothesis that has been proposed is that C5-specific Abs reduce immune activation stemming from the homology between the C5 and human HLA proteins, though this hypothesis has not been widely tested [204]. In either case, further cohort and molecular studies of C5-specific antibodies are merited to determine whether such antibodies can be leveraged to inform immune responses that could contribute to vaccine protection.

Addendum

The results described above in Chapter III are based on experiments using an HIV Env phage display library excluding peptides from the immunodominant V3 and IDE regions. I will describe additional experiments and data relevant to this chapter in this addendum. In addition to the library lacking V3/IDE, we also tested paired mother-infant samples from the NBT cohort for responses to peptides spanning the full Env ectodomain (Env Full). In addition to responses to V1/V2, C5, and the gp41 CHR, we also detected strong responses to the V3 and IDE regions in both infants (Figure 3.7) and mothers (Figure 3.8) for the BG505 strain, among others, consistent with their high immunogenicity and linear conformation.

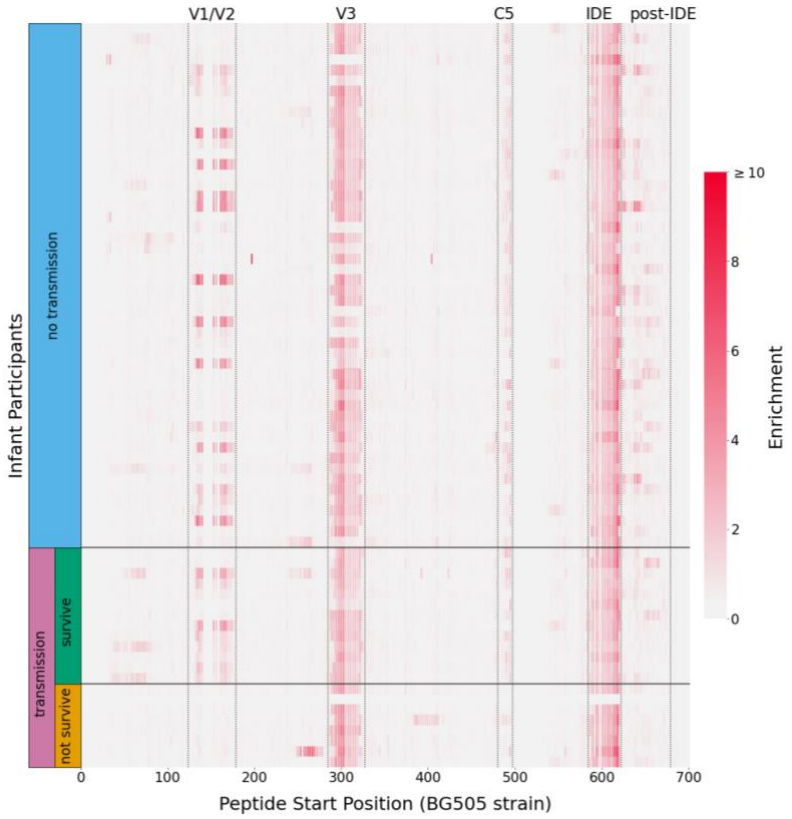

Figure 3.7. Full Env ectodomain phage display responses in NBT cohort infants.
Heatmap showing the average enrichment for peptides at each position across the Envelope Protein. Enriched peptides are shown red, with darker shades indicating higher enrichment (binding), as indicated by the scale on the right, for infants in the NBT cohort (N = 71). Peptides are ordered by start position of the N-terminal amino acid, beginning at HXB2 amino acid 31 and spanning amino acid 704. Rows indicate plasma samples and are group by infant HIV acquisition status and survival for HIV+ infants (N = 20). Within each group, individuals are ordered by ascending maternal viral load. Dotted vertical lines

indicate regions contributing high variance in PCA, with epitopes indicated above: V1V2 (HXB2 AA 125-210), V3 (HXB2 AA 271-351), C5 (HXB2 AA 462-511), the gp41 IDE (HXB2 AA 564-639), and CHR (HXB2 AA 617-696). Data are from two replicate PhIP-Seq experiments.

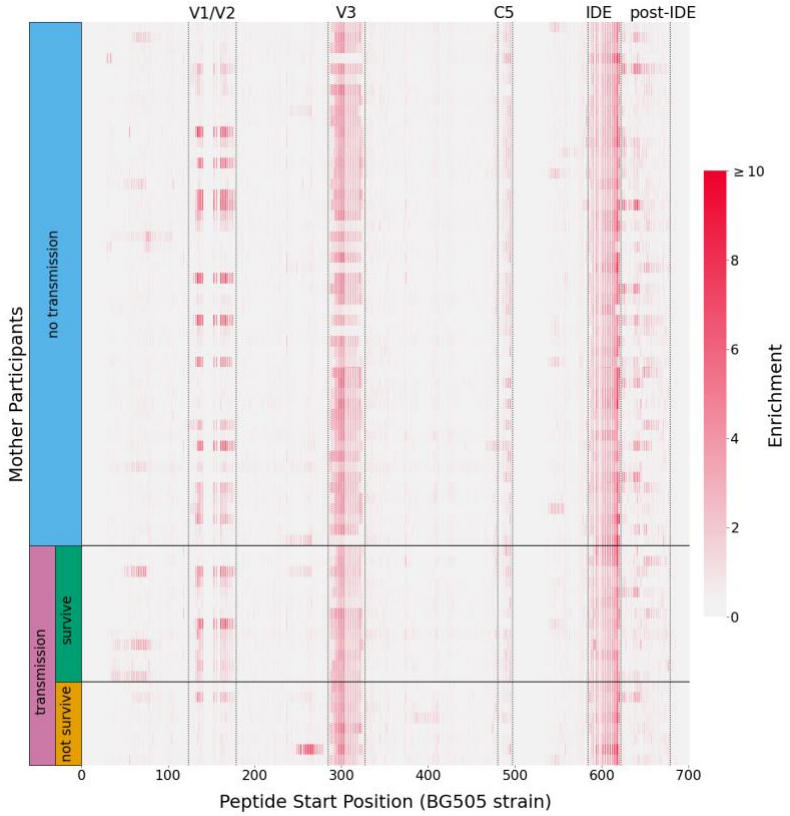

Figure 3.8. Full Env ectodomain phage display responses in NBT cohort mothers.
Heatmap showing the average enrichment for peptides at each position across the Envelope Protein. Enriched peptides are shown red, with darker shades indicating higher enrichment (binding), as indicated by the scale on the right, for mothers in the NBT cohort (N = 72). Peptides are ordered by start position of the N-terminal amino acid, beginning at HXB2 amino acid 31 and spanning amino acid 704. Rows indicate plasma samples and are group by infant HIV acquisition status and survival for HIV+ infants (N = 20). Within each group, individuals are ordered by ascending maternal viral load. Dotted vertical lines indicate regions contributing high variance in PCA, with epitopes indicated above: V1V2 (HXB2 AA 125-210), V3 (HXB2 AA 271-351), C5 (HXB2 AA 462-511), the gp41 IDE (HXB2 AA 564-639), and CHR (HXB2 AA 617-696). Data are from two replicate PhIP-Seq experiments.

As with the data from the no V3/IDE library, we used PCA to identify regions contributing high variance across samples, which included V1V2, V3, C5, IDE, and CHR (indicated in Figures 3.7 and 3.8). We again calculated aggregate responses across strains to determine whether responses to any region were associated with vertical transmission risk or HIV+ infant survival, while also limiting multiple hypothesis testing. Responses to the CHR trended with reduced HIV acquisition risk in infant (Odds Ratio (OR) = 0.99, p = 0.064), but not

maternal samples (OR = 0.99, p = 0.11) in binomial logistic regression adjusted for maternal viral load, though the effect size was small. Responses to other epitope regions were not associated with vertical transmission risk. By contrast, responses to several regions were associated with HIV+ infant survival (Table 3.1). Similar to the No V3/IDE results, responses to C5 and V1V2 correlated with improved HIV+ infant survival for both infant and maternal samples (C5) or infant samples only (V1V2). Surprisingly, there was also a correlation between infant responses to the IDE and decreased HIV+ infant survival, with a trend for maternal samples. Finally, there was a trend for a correlation between V3 responses and decreased HIV+ infant survival for infant samples only.

Table 3.1. Summary of Cox-proportional hazards models of infant survival, adjusted for maternal viral load.

Results of Cox proportional-hazards models using regional responses + AUC maternal viral load as inputs and HIV+ infant survival time + death as outcomes. HR: hazard ratio. All statistics rounded to the nearest two significant figures. Data from two biological replicates. Rows are ordered by descending P value.

sample	region	HR	P value
Infant	C5	0.75	0.018
Mother	C5	0.79	0.020
Infant	V1V2	0.64	0.034
Infant	IDE	1.09	0.037
Mother	IDE	1.06	0.089
Infant	V3	1.09	0.098
Mother	V1V2	0.94	0.18
Infant	CHR	0.81	0.20
Mother	V3	1.05	0.22
Mother	CHR	0.94	0.32

In preliminary analyses, we also averaged enrichment across strains with high sequence similarity and enrichment that was strongly correlated to limit multiple hypothesis testing while also preserving responses that were strain-specific. In this revised analysis, the most significant predictors of infant survival were IDE_combo (BG505, BF520; HR = 1.07, p = 0.0078), V3_combo (BG505, BL035, ZA1197; HR = 1.10, p = 0.016), and C5_B41 (HR = 0.70, p =

0.035). We observed a high degree of co-linearity between the V3 and C5 responses, but not the IDE and C5. Similarly, in a multivariable Cox proportional-hazards models of survival, V3 (HR = 1.08, p = 0.16) and C5 (HR = 0.78, p = 0.20) were not significant predictors. By contrast, in a multivariable Cox proportional-hazards models of survival, IDE (HR = 1.08, p = 0.017) and C5 (HR = 0.65, p = 0.028) were both significant predictors of survival, albeit with opposite effects.

Based on the multivariate survival analysis results, we opted to determine whether responses to C5_B41 and IDE_combo were also significant predictors of survival in a multivariate model for infants from the CTL cohort. We tested plasma from all CTL infants for responses to these regions using the Env Full library in PhIP-Seq. Surprisingly, in a multivariate Cox proportional-hazards model of HIV+ infant survival, IDE_combo was a significant predictor of survival (HR = 1.10, p = 0.024) but C5_B41 was not (HR = 1.06, p = 0.55).

We sought to further validate the IDE results in both cohorts via peptide ELISA. To this end, we coated plates with a biotinylated IDE peptide from the BG505 strain (Biotin-SGG-LRDQQLLGIWGCSGKLICTTNVPWNSSWSNRN), as described above for the C5 peptide ELISA. Surprisingly, in both cohorts, surviving HIV+ infants had significantly higher IDE peptide ELISA activity, the opposite of what was seen in PhIP-Seq experiments (Figure 3.9).

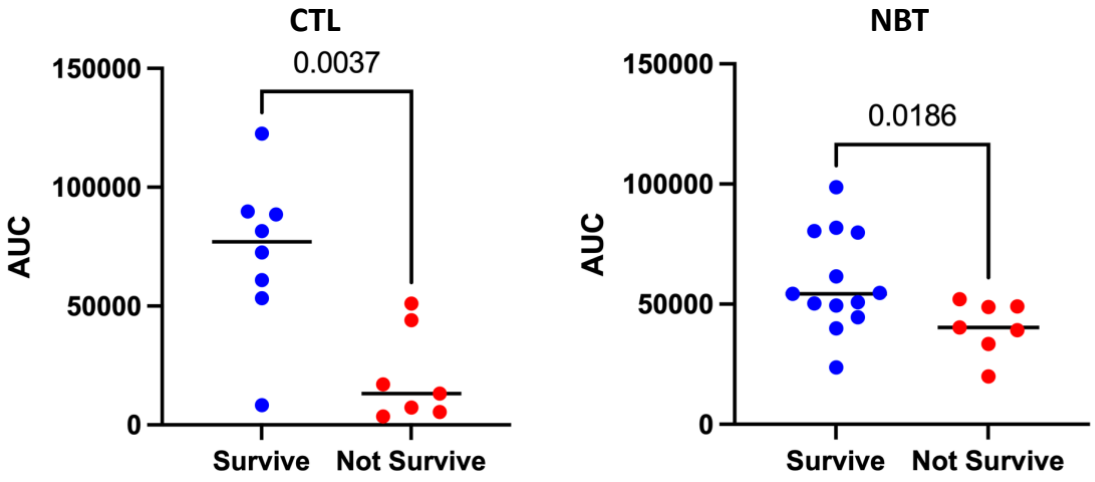

Figure 3.9. Comparison of IDE peptide ELISA activity among surviving and non-surviving HIV+ infants from the NBT and CTL cohorts.
A) IDE peptide ELISA activity of surviving (blue, N = 13) and non-surviving (red, N = 7) HIV+ infants in the NBT cohort. B) IDE peptide ELISA activity of surviving (blue, N = 8) and non-surviving (red, N = 7) HIV+ infants in the CTL cohort. Median activity of groups in A-B were compared by Mann-Whitney U test. Data are from two technical and biological replicates.

In a Cox proportional-hazards model, IDE peptide ELISA activity was also associated with HIV+ infant survival in the NBT (HR = 0.95, p = 0.044) and CTL (HR = 0.96, p = 0.015) cohorts. We hypothesized that the opposing results may be due to differences in total levels of gp41-specific antibodies, which could skew the results of the more sensitive ELISA. We therefore tested infant plasma samples for gp41-specific ELISA activity, using ZA1197 (clade C) gp41 monomer as the antigen. Whereas surviving HIV+ infants had significantly higher gp41-specific ELISA activity (p = 0.0080 by Mann-Whitney U test), there was no difference in ELISA activity among NBT cohort infants (p = 0.24).

The discordance of the PhIP-Seq and peptide ELISA results for the IDE region are puzzling. On the other hand, the consistency of the assay-specific results across the NBT and CTL cohorts suggest these assays may be measuring distinct antibody responses to the IDE. Several factors may explain these conflicting results. The disulfide linkage within the IDE, which is highly conserved across HIV strains, is known to impact binding of some IDE-specific antibodies[38]. There is mixed evidence on whether phage displayed peptides properly form disulfide bonds during replication in host bacteria. As the IDE peptide used in the ELISAs was synthesized with the disulfide bond intact, examining the effect of reducing this bond on the ELISA results would reveal whether it is contributing to the observed difference across assays. It is also unclear whether the specific conformation of the IDE, which is known to be highly heterogeneous, differs between presentation via N-terminal fusion to the T7 phage minor capsid protein and via biotinylated peptide binding to streptavidin-coated plates in ELISA[205].

Chapter IV

Reconstruction of a Polyclonal ADCC Antibody Repertoire from an HIV-1 Non-Transmitting Mother

The text in this chapter has been modified slightly from a manuscript entitled "Reconstruction of a Polyclonal ADCC Antibody Repertoire from an HIV-1 Non-Transmitting Mother" which has been submitted to Cell Reports and is currently under review.

Introduction

Antibodies (Abs) prevent infection and modulate the immune response to pathogens via several mechanisms, preventing infection by neutralizing the virus and clearing infected cells through Fc-mediated effector functions such as antibody dependent cellular cytotoxicity (ADCC). Accumulating evidence, including numerous natural history studies as well as a vaccine clinical trial, implicates ADCC in both reduced HIV-1 transmission and pathogenesis[55]. Passive antibody studies in animal models using Abs where ADCC function was ablated have also suggested a role for Fc-mediated activity in protection[45,96]. However, results in animal models are mixed, potentially because they are confounded by less efficient signaling of human antibodies in these models[91]. Thus, human studies remain central to understanding the role of ADCC responses in HIV infection.

In early studies, ADCC responses have been associated with reduced viremia and slowed disease progression in individuals living with HIV[80]. Similarly, elite controllers have been reported to have broader and higher magnitude ADCC than HIV progressors[190]. Numerous correlative studies of the relationship between ADCC activity and disease outcome in chronic HIV infection have also suggested a beneficial role of ADCC Abs in reduced HIV pathogenesis[71,85,87,129,140,144,154,155,190]. Recent studies from varied of transmission settings continue to support these early reports[72,148,191,193,206,207]. A role for ADCC in an HIV vaccine

setting remains controversial because there is mixed evidence from vaccine studies to date. The RV144 vaccine trial found an association between a combination of low plasma IgA and high plasma ADCC and protection from HIV infection[114,115]. However, a follow-up vaccine trial, HVTN 702, using a similar regimen in a distinct population, did not demonstrate any efficacy[117].

The one setting where there are consistent findings of a role for ADCC responses is in protection from mother to child transmission (MTCT) of HIV. MTCT is a unique setting through which the impact of circulating HIV-specific Abs on HIV acquisition and pathogenesis can be directly studied[122]. Infants receive maternal HIV-specific Abs from their mothers during the third trimester of pregnancy via placental antibody transfer, and these Abs remain present in plasma for several months after birth[126,127,129]. Within the setting of HIV MTCT, our group has shown in two different cohorts that ADCC mediated by passively-transferred antibodies in infant plasma is correlated with improved survival of infants that acquire HIV[129,193]. Moreover, passively-acquired ADCC activity correlated with decreased HIV acquisition risk in a combined cohort analysis[193]. More recently, Thomas et al showed in a distinct cohort that HIV-exposed, uninfected infants had higher passively-acquired autologous ADCC-mediating Abs than infants who acquired HIV during follow-up[148,208]. There was also a correlation between passively-acquired ADCC-mediating Abs and improved survival of infants that acquired HIV. Collectively, these studies demonstrated a correlation between passively acquired, Ab-mediated ADCC and improved clinical outcome in three different cohorts using three distinct assays. In each of these studies, there was no evidence for a role of neutralizing antibodies (nAbs) in protection from MTCT or infant disease progression[129,147,148]. In combination, these studies support a clinical benefit of pre-existing ADCC activity within the context of HIV MTCT.

Despite the lack of a comprehensive approach to study ADCC antibodies, several major targets of ADCC mAbs have been identified in the various studies noted above. Although ADCC mAbs targeting nearly all major Env epitopes have been described, the most common targets of gp120-specific ADCC mAbs include variable loop 3 (V3) and CD4-inducible (CD4i) epitopes, which are only exposed on the Env trimer after CD4 engagement[16,43]. Guan et al further divided the CD4i epitopes into three major clusters (A, B, and C) based on competition experiments using prototypic mAbs A32 or C11, E51-M9, and 17b or 19e, respectively[34]. Since then, additional CD4i mAbs have been described with epitopes that do not completely overlap with the prototypic cluster mAbs[34]. Within gp41, early studies identified two distinct regions that are targeted by ADCC-capable, non-neutralizing Abs[38,209]. Prior studies suggest that the majority of gp41-specific antibodies in HIV-infected individuals bind to two regions, the C-C loop and C-heptad repeat, also referred to as clusters I and II, respectively[37–39,210]. Cluster I, a predominantly linear epitope, is targeted by prototypic mAbs 246-D and 50-69, whereas cluster II is discontinuous and is recognized by prototypic mAbs 98-6 and 167-7.

Even with increasing evidence that ADCC may play a protective role within the setting of MTCT, the specific characteristics of the individual Ab lineages that contribute to plasma ADCC are not well studied. In several instances, mAbs capable of mediating ADCC were identified incidentally using methods optimized to identify nAbs[211,212]. Other reports have focused on the cumulative plasma ADCC response and/or on the Abs targeting a specific epitope[72,186,195,213]. These efforts have been fruitful in identifying potent ADCC mAbs targeting distinct epitopes, but they fall short in failing to comprehensively evaluate plasma ADCC responses to both HIV Envelope (Env) subunits[43]. Although gp120-specific ADCC has most consistently been associated with protection, ADCC directed against the gp41 subunit correlated with protection or

reduced viremia in multiple animal studies[106,107]. As such, few studies have taken a holistic approach to identify the lineages of mAbs that comprise plasma ADCC responses and how these Abs interact to promote potent cell killing.

In this study we sought to identify the mAbs that contributed to the passively-acquired plasma ADCC repertoire by sorting memory B cells circulating during late pregnancy, as the majority of placental Ab transfer occurs during the third trimester, thereby serving as a proxy identifying for passively-acquired mAbs in an infant[126]. We selected a mother, MG540, who was HIV seropositive at enrollment in the Nairobi Breastfeeding Trial (NBT), had a high viral load and did not transmit HIV to her infant, BG540[167]. We identified 20 ADCC-capable mAbs representing 14 clonal families and targeting both gp120 and gp41 epitopes of Env. In competition ADCC experiments, combinations of mAbs recapitulated the majority of the plasma ADCC responses of MG540 and the passively-acquired plasma ADCC of her infant, BG540. Single epitope-specific Abs that mediate ADCC did not individually contribute significantly to the overall plasma ADCC response, whereas combinations of isolated Abs targeting distinct epitopes largely recapitulated the plasma activity, which suggests the MG540 plasma ADCC response is functionally polyclonal in nature.

Materials and Methods

Data and Code Availability

The PhIP-Seq sequencing data set generated during this study is publicly available under BioProject ID PRJNA870920. GenBank accession numbers for MG540 antibody sequences are OQ054484 to OQ054517. The python API "phippery" was used to process the demultiplexed sequencing data, align reads to the reference oligonucleotide sequences, and calculate enrichment. "phippery" is available at (https://github.com/matsengrp/phippery). Clonal family

inference was performed with the partis software package (available at https://github.com/psathyrella/partis/) paired clustering method, treating wells as droplets.

Human Plasma and PBMC Samples

Plasma and peripheral blood mononuclear cell (PBMC) samples were collected from mother MG540, an individual living with HIV who enrolled in the Nairobi Breastfeeding Trial (NBT), or her infant, BG540[167]. As the NBT was carried out before the availability of antiretroviral therapy (ART), NBT participants did not receive ART. The infecting virus of MG540 was clade A based on available envelope sequences[181]. Approval to conduct this study was provided by Kenyatta National Hospital - University of Nairobi Ethics and Research Committee, the Fred Hutchinson Cancer Research Center Institutional Review Board, and/or the University of Washington Institutional Review Board. Study participants provided written informed consent prior to enrollment for use of their data and samples for future studies.

Cell Lines

CEM.NKR cells (RRID:CVCL_X622; originally derived from female human T-lymphoblastoid cells) were obtained from NIH AIDS Reagent Program (ARP; Germantown, MD, catalog #458) and grown at 37°C in RPMI 1640 media with added penicillin (100 U/mL), streptomycin (100 µg/mL), amphotericin B (250 ng/mL), L-glutamine (2mM), and fetal bovine serum (10%; all from Thermo Fisher Scientific). FreeStyle 293F cells (Thermo Fisher Scientific, Waltham, MA, catalog #R790-07; RRID:CVCL_D603) were obtained from Invitrogen and grown at 37°C in Freestyle 293 Expression Media (Thermo Fisher Scientific) according to the manufacturer's instructions. These cells were not further authenticated in our hands.

B Cell Sorting

An MG540 PBMC sample from pregnancy week 34 (P34) was thawed as previously described after storage in liquid nitrogen[214]. Cells were stained on ice for 30mins using a cocktail of anti-CD19-BV510, anti-IgD-FITC, anti-IgM-FITC, anti-CD3-BV711, anti-CD14-BV711, and anti-CD16-BV711. Cells were then washed once and resuspended in fluorescence-activated cell sorting (FACS) wash (1x PBS, 2% FBS) prior to loading onto a BD FACS Aria II cell sorter. Memory B cells were identified and sorted as $CD3^-$ $CD14^-$ $CD16^-$ $CD19^+$ IgD^- IgM^-. B cells were sorted into B cell medium (Iscove's modified Dulbecco's medium [GIBCO, subsidiary of Thermo Fisher Scientific], 10% heat-inactivated low-IgG FBS [Life Technologies, subsidiary of Thermo Fisher Scientific], 5 ml GlutaMAX [Life Technologies], 1 ml MycoZap plus PR [Lonza, Basel, Switzerland]). Immediately following sorting, memory B cells were plated at 8 B cells in 55 µL per well into 11 x 384-well plates in B cell medium supplemented with 100 U mL^{-1} interleukin-2 (IL-2; Roche, Basel Switzerland), 50 ng mL^{-1} IL-21 (Invitrogen), and 1 x 10^5 cells mL^{-1} irradiated 3T3-CD40L feeder cells (ARP Cat #12535; RRID:CVCL_1H10) using a Tecan automated liquid handling system. Cultured B cells were incubated for 11 days at 37°C in a 5% CO2 incubator based on the protocol by Huang et al[215]. At day 10 of incubation, IgG was detected by ELISA in 53% of a random sample of wells at >10ng mL^{-1} and 51% > 100ng mL^{-1}.

B Cell Culture Harvest and Supernatant ELISA

On day 11, B cells culture supernatants were aspirated and divided among two additional 384-well plates (20 µL per duplicate) by a Tecan automated liquid handling system. The B cells remained in their original plates, were resuspended in 20 µL RNA storage buffer (15 mM Tris pH 8 and 10 U murine RNAse inhibitor, New England Biolabs [NEB], Ipswich, MA), and were frozen at -80°C until needed. An ELISA to detect HIV specificity in cell supernatants was adapted from a previously described protocol, as follows[195]. Maxisorp 384-well plates (Nunc,

subsidiary of Thermo Fisher Scientific) were coated with 1 µg mL^{-1} BG505.W6M.ENV.B1 (BG505) gp120 (Cambridge Bio, Brookline, MA, Genbank #ABA61515.1), 0.5 µg mL^{-1} C.ZA.1197MB gp41 ectodomain (Immune Tech, New York, NY, Genbank #AYA63234.1), and 1 µg mL^{-1} BG505.SOSIP.664 T332N (from BG505.W6.C2 Env, Genbank #DQ208458.1; kindly provided by Kelly Lee) in 1x PBS overnight at 4°C. Plates were washed four times between steps with PBS-0.05% Tween 20 (Thermo Fisher Scientific; PBST) wash buffer. All samples/reagents used in subsequent steps were diluted in B cell media (as above). Plates were blocked with B cell media for 1 hour at 37°C. B cell supernatants were then applied and incubated at 37°C for two hours. Next, Goat anti-human IgG-HRP (Sigma-Aldrich, St. Louis, MO) was applied at 1:2500 dilution for 1 hour at 37°C. After a final wash, 1-Step TMB-Ultra (Thermo Fisher Scientific) was added to the wells. The reaction was stopped after 15 min using 1M H_2SO_4 (Sigma-Aldrich). Absorbance was measured at 450nm optical density. Positive ELISA activity was defined was an absorbance greater than twice the background activity of wells incubated with B cell media alone.

Rapid and Fluorometric ADCC Assay

The rapid and fluorometric ADCC (RFADCC) assay was carried out as described previously[173,180]. Briefly, CEM.NKR cells were stained with PKH26 cell linker dye (Sigma-Aldrich) and CFSE cytosolic dye (Vybrant CFDA-SE Cell Tracer Kit, Invitrogen). Double-stained cells were then coated with gp120 or gp41 at a ratio of 1.5µg antigen per 100,000 cells for 1 hour at room temperature. Coated cells were washed and a total of 5,000 target cells were added to wells containing 100µL of plasma diluted at 1:5000 in RPMI media or control mAbs at 100 – 500 ng mL^{-1}. After 15 min, PBMCs from HIV negative donors were added at a ratio of 50:1 effector to target cells. RFADCC activity was allowed to occur for four hours at 37ºC

before cells were washed and fixed in 2% paraformaldehyde in PBS (Santa Cruz Biotechnology, Dallas, TX). Data were acquired via flow cytometry (BD Symphony or Fortessa X50; BD Biosciences, Franklin Lakes, NJ). PKH and CFSE were detected in the PE and FITC channels, respectively. Data were analyzed using FlowJo (v.10.1, Treestar, RRID:SCR_008520). ADCC was defined as the percentage of PKH^+, $CFSE^-$ cells out of total PKH^+ cells after subtracting detected "background" activity mediated against uncoated target cells, which was consistently set to 3-5%. ADCC activity was normalized to that mediated by Anti-HIV Immune Globulin (HIVIG, NIH ARP, Catalog #3957). For serial dilution experiments, gp120-specific and gp41-specific mAbs were tested at 0.05 – 750 ng $mL^{-1}$and 0.15 – 2500 ng mL^{-1}, respectively. The concentration at which 50% of maximal ADCC activity was detected (the 50% effective concentration; EC_{50}) was determined for each mAb by fitting a sigmoidal dose-response curve to the data and interpolating.

Multiplex Supernatant RFADCC Assay

The RFADCC assay described above was adapted for use with B cell culture supernatants and multiple antigens. To simultaneously detect ADCC mediated against gp120 and gp41 coated cells, CEM.NKR cells were first labelled with CFSE to reduce batch effects. CFSE-labelled cells were then labelled with PKH26 or CellVue Claret cell linker dye (Sigma-Aldrich), which has similar properties to PKH26 and is spectrally compatible with both PKH26 and CFSE[216,217]. PKH-26- and CFSE-labelled cells were coated with ZA1197 gp41, whereas Cellvue Claret- and CFSE-labelled cells were coated with BG505 gp120. The remaining protocol steps of the protocol were followed as described above. Cellvue Claret was detected in the APC channel. Gp41-specific ADCC was defined as the percentage of PKH^+ $CFSE^-$ Cellvue $Claret^-$ cells after subtracting background activity. Gp120-specific ADCC was defined as the percentage of Cellvue

Claret^{+} CFSE^{-} PKH^{-} cells after subtracting background activity. Positive ADCC was defined as activity greater than twice the background.

Reconstruction of Antibodies

Wells demonstrating HIV-specific ELISA activity and gp120- or gp41-specific RFADCC activity were selected for antibody gene amplification and cloning. Plates containing frozen B cells for wells of interest were thawed on ice. Reverse transcription-PCR amplification of IgG heavy and light chain variable regions was performed as previously described, followed by nested chain-specific amplification PCRs[215]. Two distinct primer sets were used to minimize PCR bias and maximize recovery of HIV-specific antibodies[218,219]. Functional sequences were identified from second round amplified sequences using IMGT V-QUEST[220]. Where sufficient sequencing for the 5' constant region was available, we determined IgG subclass; all heavy chain sequences were identified as IgG1 subclass. Amplified variable region sequences were cloned into corresponding IgG1, IgK, or IgL expression vectors[215]. The Freestyle MAX system (Thermo Fisher Scientific) was used to co-transfect paired heavy and light chain expression plasmids for sequences isolated from the same well, and IgG was purified as described[195]. Where multiple possible heavy and light chains pairs were present, all possible combinations were generated. To produce G236R/L328R (GRLR) FcγR null binding variants of select mAbs, heavy chain variable regions were cloned into an IgG1 expression vector containing the G236R/L328R substitutions.

Sequence Analysis and Clonal Inference

Second round nested PCR sequences for heavy and light chains were grouped into clonal families (stemming from a single rearrangement event) and annotated with the B cell immunoglobulin analysis program, partis, using the paired clustering option (https://github.com/psathyrella/partis and https://arxiv.org/abs/2203.11367). This uses a Hidden

Markov Model, together with a variety of more heuristic, but faster, steps, to first group together sequences from among each single (heavy or light) chain. It then incorporates pairing information by splitting apart such single-chain clusters whose partner clusters are incompatible.

Pseudovirus Production and Neutralization Assays

TZM-bl-based neutralization assays and production of pseudoviruses carrying the Q23Δ*env* backbone were performed as previously described[64]. For MG540 plasma, the NT_{50} represents the reciprocal dilution of plasma that results in 50% reduction of virus infectivity. For mAbs, the IC_{50} represents the final concentration in μg mL^{-1} which led to 50% neutralization of the indicated virus.

Phage Display Immunoprecipitation Sequencing

To determine the targets of mAbs with linear epitopes, we used phage display immunoprecipitation sequencing (PhIP-Seq), as previously described[180,221,222]. An oligonucleotide pool was generated for the *env* ectodomain and transmembrane domain (HXB2 AA 30-704) from six HIV-1 strains on GenBank: 9032.08_A1 (EU576114; B41), BF520.W14M.C2 (KX168094; BF520), BG505.W6.C2 (DQ208458; BG505), BL035.W6M.C1 (DQ208480; BL035), QA013.70I.H1 (FJ866134; QA013), and ZA1197.MB (AY463234; ZA1197). The BF520, BG505, and BL035 strains were isolated early in infection from infants enrolled in the NBT cohort. Oligonucleotides encoded 38 amino acid tiles, with 36 amino acid overlaps between adjacent tiles. Amplified phage at 2 x 10^5-fold representation were incubated with each sample mAb (10ng total at 1μg mL^{-1}) in technical duplicate. After overnight incubation, phage were immunoprecipitated using protein A- and -G-coated beads (Pierce, a subsidiary of Thermo Fisher Scientific). Samples were then prepared for multiplexed sequencing on an Illumina MiSeq or HiSeq 2500 with 125bp single end reads using the rapid run setting, as

described[194]. Demultiplexing, read alignment, and enrichment calculations were performed as described[194]. Alignment was performed with Bowtie 1.3, with up to 2 mismatches allowed. See 'Data and Code Availability' for more information.

Gp120, gp41, and SOSIP ELISAs

ELISAs using plates coated with BG505 gp120, ZA1197 gp41, BG505 T332N SOSIP, and CNE8 SOSIP were performed similarly to the supernatant ELISAs described above. 384-well MaxiSorp plates (Nunc) were coated with single gp120 or SOSIP antigens at 1 µg mL^{-1} or gp41 protein at 0.5 µg mL^{-1}. All subsequent steps were performed using 10% non-fat milk (RPI, Mt. Prospect, IL) in PBST. mAbs were tested in serial dilutions at 31.25 – 1000ng mL^{-1}.

Competition ELISA

To determine the epitope of mAbs that did not map to a linear epitope via PhIP-Seq, we used antibodies with known epitopes in a competition binding assay, as previously described[195,223]. Briefly, 384-well MaxiSorp plates were coated with either BG505 gp120 or ZA1197 gp41, as detailed above. Plates were then blocked with 3% BSA in PBST. Serial dilutions of antibodies of interested were added at 9.76 ng mL^{-1} to 10 µg mL^{-1} and incubated at 37ºC for 15 mins. Biotinylated (BT) antibodies were then added without washing and samples were incubated at 37ºC for 45 mins. Next, plates were incubated with Pierce High Sensitivity Streptavidin-HRP (Thermo Fisher Scientific) at 1:10000 dilution for 1 hour at 37°C. After a final wash, 1-Step TMB-Ultra (Thermo Fisher Scientific) was added to the wells. The reaction was stopped 1-10 minutes later using 1M H_2SO_4 (Sigma-Aldrich). Absorbance was measured at 450nm optical density. Percent inhibition was calculated as: 100 * [(AUC in presence of mAb of interest) / (scaled average absorbance of no mAb control and flu-specific mAb FI6v3)]. Antibodies were biotinylated using the EZ-Link Sulfo-NHS Biotinylation Kit (Thermo Fisher

Scientific), according to the manufacturer's instructions. Empirically determined optimal concentrations of the biotinylated mAbs were: 100ng mL^{-1} (MG540.90, 167-7, QA255.067), 250ng mL^{-1} (MG540.37, QA255.006), 500ng mL^{-1} (VRC01), and 750ng mL^{-1} (A32, C11, 17b).

Flow Cytometry-Based Analysis of Cell Surface Staining and ADCC

Cell surface mAb binding and ADCC responses mediated by mAbs (5 µg mL^{-1}) were measured at 48h post-infection as previously described[224,225]. Cells infected with HIV-1 primary isolates (CH58 WT, CH58 *nef*$^{-}$ *vpu*$^{-}$, or BG505 T332N) were stained intracellularly for HIV-1 p24, using the Cytofix/Cytoperm fixation/permeabilization kit (BD Biosciences, Franklin Lakes, NJ) and a fluorescent anti-p24 mAb (phycoerythrin [PE]-conjugated anti-p24, clone KC57; Beckman Coulter/Immunotech) at a 1:100 dilution. BG505 T332N infected cells were also stained with anti-CD4-FITC mAb (FITC anti-human CD4 Antibody, Biolegend, San Diego, CA) at a 1:100 dilution, and p24+CD4-low cells were chosen for the analysis. Infected cells were identified by gating the living cell population on the basis of the viability dye staining. The percentage of cytotoxicity was calculated using the following formula: [(% of $p24^{+}$ cells in targets plus effectors) – (%$p24^{+}$ cells in targets plus effectors plus Abs)] / (% of $p24^{+}$ cells in targets). For cell surface antibody staining, primary $CD4^{+}$ T cells were incubated with antibody samples at 5 µg mL^{-1} for 30 mins at 37°C. Cells were then washed with PBS and stained with goat anti-human (AlexaFluor 647; Jackson ImmunoResearch, West Grove, PA) secondary antibody for 20 min in PBS. A32, 17b, and 3BNC117 were used as positive controls. A GRLR variant of 3BNC117 was used as a negative control.

Competition ADCC Assay

The competition ADCC assay was performed using a modified version of the RFADCC assay, as previously described[164]. Target cells were coated with either BG505 gp120 or ZA1197

gp41, as detailed above. Coated target cells were added to wells containing 50 µL total of a GRLR variant of a competitor antibody or an equivalent volume of media[226]. The solution was mixed by pipetting up and down repeatedly and the cells were incubated for 15 mins at RT in the dark, allowing for competitor antibodies to bind. Next, 50 µL of plasma at a previously optimized dilution (1:5000 for gp120 and 1:2000 for gp41) or an indicated mAb at the indicated effective concentration (EC) was added to the wells. Wells were mixed again and incubated for 15 mins at RT in the dark before adding effector cells. The concentration of GRLR mAb added was empirically determined via serial dilution by testing for complete abrogation of wildtype antibody ADCC when run at the EC_{95} concentration. GRLR variants were added at the following concentrations: 12.5 µg mL^{-1} (MG540.7, -17, -50, -63, -82, and -90), 25 µg mL^{-1} (MG540.37, -56, and -86). For wells containing multiple GRLR variants, each GRLR mAb was added at a higher working concentration so that the final concentration in 50µL remained the same optimal final concentration above. Percent ADCC inhibition was calculated as: 100 * [(plasma ADCC in the presence of the indicated GRLR mAbs) / (plasma ADCC in the absence of GRLR mAbs)]

Quantification and Statistical Analysis

Raw data was processed in Microsoft Excel (RRID:SCR_016137) or GraphPad Prism v9 (RRID:SCR_002798). Graphs were generated using GraphPad Prism. PhiP-Seq heatmaps were generated with the NumPy, pandas, Matplotlib, and seaborn Python packages. Except where noted, all data represent the mean of two biological replicates, with two technical replicates in each experiment. Data were normalized and/or averaged across replicates using Microsoft Excel or GraphPad Prism. Enrichment was calculated as previously described for PhIP-Seq data[198]. Additional details can be found in the figure legends.

Results

Supernatant screening and mAb reconstruction

HIV-1 non-transmitting mother MG540 was selected from the Nairobi Breastfeeding Trial (NBT), which was conducted prior to the availability of antiretroviral therapy for prevention of MTCT[167]. We selected individual MG540 as a case where transmission did not occur despite the presence of known factors that correlate with transmission, including high plasma viral load (>10^5 copies mL^{-1} in late pregnancy) and high breastmilk viral levels (>10^4 copies mL^{-1} at delivery) during at least 10 months of breastfeeding[122,123,227]. In addition, MG540 had gp120-specific plasma ADCC in the top quartile for the NBT cohort at pregnancy week 34 (P34)[129]. Her infant, BG540, born at gestational week 41, also had passively acquired plasma ADCC in the top quartile at week of life 0 (W0) compared to other infants in the cohort, a feature that is associated with reduced HIV acquisition risk and pathogenesis[148,193]. Maternal monoclonal antibody (mAb) sequences present during late pregnancy, when the majority of placental antibody transfer occurs, were reconstructed by sorting memory B cells from an MG540 PBMC sample collected at P34, ~7 weeks before the birth of BG540[126]. This sample, stored since 1994, contained 10.8 x 10^6 live cells with 81% viability. Based on fluorescent antibody staining, 3.9% of single cells were B cells ($CD19^+$ $CD3^-$ $CD14^-$ $CD16^-$), and of these, 41.6% of cells were memory B cells (IgM^- IgD^-). For this study, we ultimately sorted 23,856 memory B cells into 11 x 384-well plates at a density of eight cells per well, which typically results in 1-2 viable cells per well, and cultured for 11 days to promote IgG expression.

To select for memory B cells that expressed HIV-binding antibodies that mediate ADCC, we developed a two-step protocol to screen first for antigen binding and then, within positive wells, for ADCC activity. For binding assays, we included gp120 (clade A BG505), gp41 (clade

C ZA1197), and Env trimer (BG505 T332N SOSIP) antigens in order to maximize detection of Env-specific antibodies. Screening well supernatants for HIV-specific IgG via ELISA identified 257 (8.23% of total) wells with activity >2x above the background. The highest activity subset (n = 192 wells) of these wells was subsequently screened for gp120- and gp41-specific ADCC activity via a multiplexed version of the RFADCC assay, which correlates well with clinical outcomes, and using the same antigens (Figure 4.1)[129,174,190,193]. Ninety-five of 192 wells (49.5%) demonstrated RFADCC activity >2x above background. Of these, 70% mediated gp120-specific ADCC, whereas 30% were active against gp41. We attempted mAb reconstruction for all 95 wells, yielding 20 pairs of heavy and light chain sequences (21% reconstruction success) and 18 unique mAbs representing 14 clonal families (Table 4.1). Identical heavy chain sequences were obtained for two distinct pairs of wells, which hereafter will only be referred to by a single identifier. Heavy and light chain nucleotide somatic hypermutation (SHM) ranged from 6.9-19.3% and 3.0-14.6% and complementarity determining region three (CDR3) length ranged from 10-31 and 9-15 amino acids, respectively. All mAbs were initially tested to confirm HIV specificity via ELISA: eight families were gp120-specific and six were gp41-specific. All of the gp120-specific families bound to both stabilized BG505 T332N (clade A) and CNE8 (CRF01) SOSIP trimers, whereas only one of the six gp41-specific families weakly bound to both trimers (Figure 4.2).

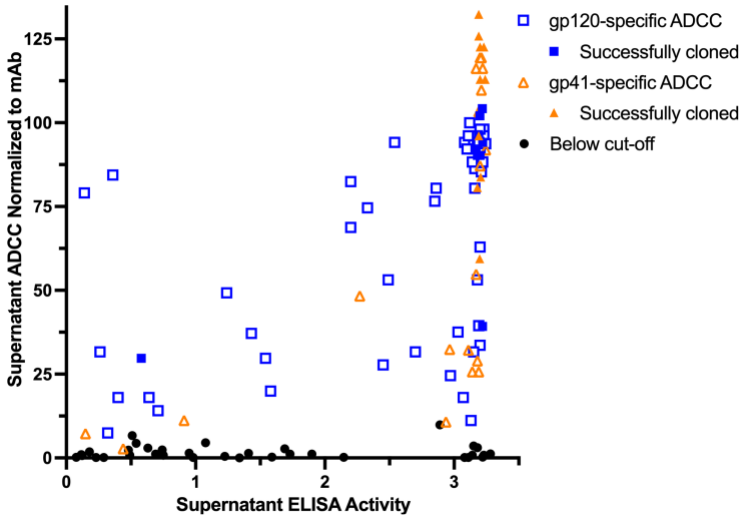

Figure 4.1. HIV-binding and ADCC activity of primary B cell culture supernatants.
Comparison of ELISA and RFADCC activity for B cell culture supernatants with HIV-specific ELISA activity (>2x background), where background-subtracted ELISA activity (x-axis) is plotted against RFADCC activity normalized to positive control mAbs C11 (gp120) or 167-7 (gp41; y-axis). Symbols indicate whether each well had gp120- or gp41-specific RFADCC activity and whether a mAb was successfully cloned from the indicated well (closed symbols). MAb reconstruction was only attempted for wells with RFADCC activity two-fold above the respective gp120- or gp41-specific background activities, which are distinct.

Table 4.1. Characteristics of reconstructed MG540 mAbs.
Distinct, isolated MG540 P34 mAbs (rows) and their characteristics (columns) are reported. Clonally related mAbs are grouped into families. Antibodies are grouped by target, gp120 or gp41, as confirmed by ELISA (BG505 gp120 and ZA1197 gp41). Results of qualitative studies are also reported: ELISA activity against BG505 T332N and CNE8 SOSIP trimers, epitope mapping by PhIP-seq and ELISA, and RFADCC activity. NT: not tested, V3: variable loop 3, IDE: immunodominant epitope. RFADCC peak activity (% normalized to HIVIG) and EC_{50} (ng/mL) are averaged between two replicate experiments. Asterisks (*) by mAb IDs indicate mAbs selected for GRLR variant generation. +++: Saturating ELISA activity at all/most concentrations, ++: activity above background for all concentrations, +: activity above background for some concentrations, -: no activity above background. See Figure 4.2 for representative SOSIP ELISA data and Table 4.2 for neutralization data.

MG540 P34 Ab Rearrangement Characteristics									SOSIP ELISA		Epitope Mapping		RFADCC	
									Clade A	CRF01				
Family	ELISA Target	mAb ID	Heavy Chain	CDRH3 Length	VH SHM (%nt)	Light Chain	CDRL3 Length	VL SHM (%nt)	BG505.C2 T332N	CNE8	PhIP-Seq	ELISA	Peak Activity	EC_{50}
1	gp120	MG540.7*	V1-69 D3-9 J3	26	12.7	KV1-9 J5	9	5	++	++	negative	C11	140	2.4
2		MG540.16	V5-51 D2-15 J4	18	13.8	LV3-1 J2	12	14	+++	++	V3	NT	135	9.9
		MG540.67		18	12.7		12	13.4	NT	NT	V3	V3	133	8.0
		MG540.90*		18	14.3		12	14.6	+++	+	V3	V3	141	6.2
3		MG540.17*	V3-21 D3-9 J4	23	7.8	KV1-5 J2	12	6.5	++	-	negative	negative	130	6.7
4		MG540.48	V1-2 D3-10 J5	14	8.7	LV8-61 J3	12	7.6	++	++	negative	A32	146	3.7
5		MG540.50*	V1-69 D3-10 J5	18	10.3	KV3-20 J4	11	5.5	++	+++	negative	A32	145	2.8
6		MG540.51	V1-69 D3-16 J4	28	14.9	KV3-20 J3	11	4.6	++	++	negative	C11	133	3.8
7		MG540.82*	V1-69 D6-13 J4	10	13.6	KV3-11 J5	12	6.2	+++	+++	negative	17b	38.7	59
8		MG540.84	V5-51 D3-16 J4	19	9.9	LV1-51 J2	15	8.6	+++	++	V3	V3	130	11
9	gp41	MG540.32	V1-3 D2-15 J3	21	18.3	KV3-15 J2	11	10.9	+	-	IDE	NT	141	33
		MG540.86*		21	19.3		11	5.9	+	+	IDE	IDE	138	23
10		MG540.37*	V1-2 D2-21 J6	20	8.8	KV4-1 K2	11	10	-	-	negative	Cluster II	134	47
11		MG540.56*	V4-39 D1-26 J4	15	14.3	LV2-14 J3	11	8.3	-	-	negative	Cluster II	136	37
12		MG540.61	V1-69 D4-17 J6	25	6.9	KV2-28 J3	11	3	-	-	negative	Cluster II	158	56
13		MG540.63*	V3-64 D5-12 J6	22	13.9	KV2-29 J4	11	7.7	-	-	IDE	IDE	122	19
14		MG540.88	V3-30 D3-3 J6	31	14.8	LV1-40 J3	13	11.7	-	-	negative	Cluster II	141	42

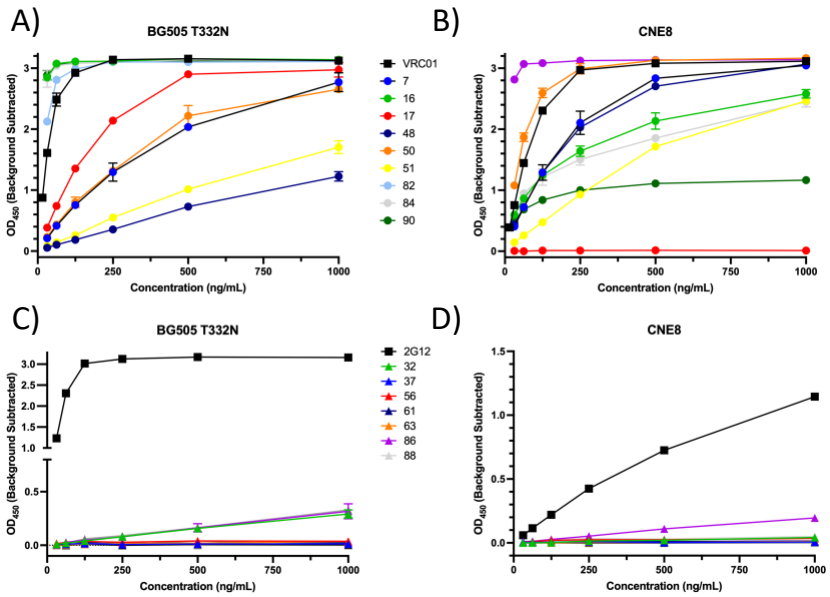

Figure 4.2. Representative SOSIP ELISA data.
Raw background-subtracted ELISA activity (OD450) of the mAbs plotted against antibody concentration (ng/mL). Gp120- (A, B) and gp41-specific (C-D) mAbs were tested for ELISA activity against BG505 T332N (A,C) or CNE8 (B,D) SOSIP trimer-coated wells. Data are representative of two independent experiments. Gp120-specific positive control ADCC mAbs were C11 and A32 and gp41-specific positive controls were QA255.067 and 167-D. 2G12 was also included as an Env trimer-specific positive control. 2G12 activity was much higher than the other mAbs in (C) so the axis was split to more clearly display activity for all mAbs.

ADCC activity of mAbs

Reconstructed mAbs were next tested for ADCC activity using the RFADCC assay. We selected this assay because RFADCC activity has been shown to correlate with infant outcomes[129,193]. We used the same antigens as in the supernatant screening: BG505 gp120 and ZA1197 gp41. BG505 Env is a particularly relevant antigen because it is an early infection isolate from an infant in the NBT cohort and it is closely related to MG540 *env* sequences from P34 (~88% pairwise nucleotide identity). To determine the potency of each mAb, we tested serial dilutions of each antibody and quantified the effective concentration for 50% maximal killing (EC_{50}) as well as peak (maximum) killing. Most of the gp120-specific mAbs potently mediated ADCC against BG505 gp120 coated target cells, with activity comparable to cluster A mAbs C11 and A32, which are prototype cluster A mAbs that targeted CD4i sites (Figure 4.3A)[228,229]. With the exception of less potent mAb MG540.82 (EC_{50} = 59 ng mL^{-1}), the EC_{50} of

the gp120-specific mAbs ranged from 2.4 – 11 ng mL^{-1}, with several mAbs more potent than C11 (6.1 ng mL^{-1}) but not A32 (1.7 ng mL^{-1}). All gp120 mAbs exhibited similar maximum ADCC activity, again with the exception of mAb MG540.82, which mediated markedly lower ADCC (>3-fold) compared to the other mAbs. Similarly, the gp41-specific mAbs strongly mediated ADCC against ZA1197 gp41-coated cells (Figure 4.3B), with similar potency to the cluster I mAb QA255.067 and the cluster II mAb 167-7[38,46,180]. The gp41-specific mAbs had EC_{50} values ranging from 19 – 98 ng mL^{-1}, in line with those of QA255.067 (18 ng mL^{-1}) and 167-7 (68 ng mL^{-1}). The gp41-specific mAbs also mediated similar maximum ADCC to each other, apart from MG540.66, which is clonally related to more potent MG540.32 and .86 (family 9). The ADCC EC_{50} and peak activities of all mAbs are summarized in Table 4.1.

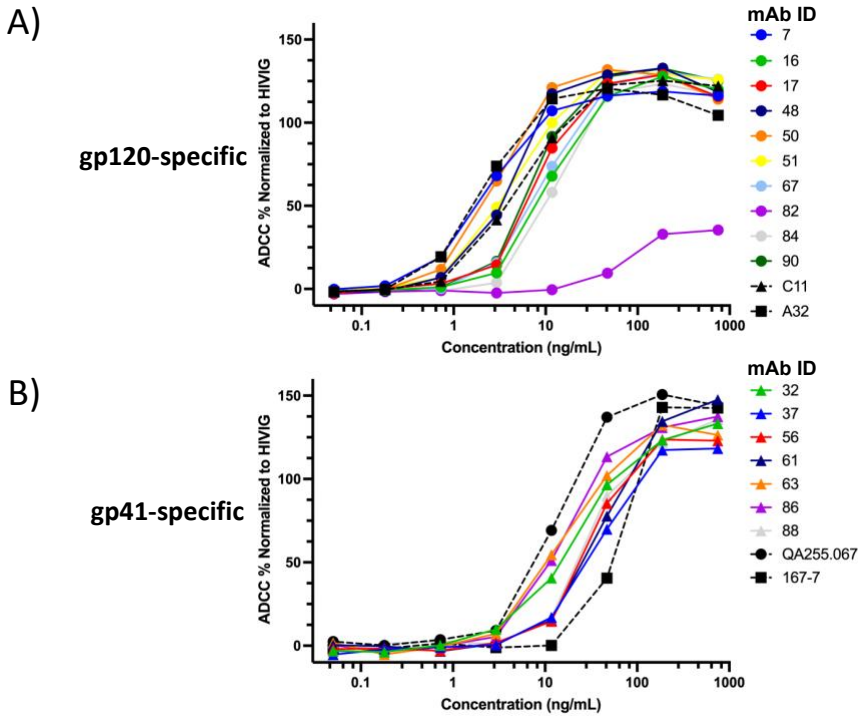

Figure 4.3. RFADCC activity and potency of reconstructed MG540 mAbs.
Raw background-subtracted ELISA activity (OD450) of the mAbs plotted against antibody concentration (ng/mL). Gp120- (A, B) and gp41-specific (C-D) mAbs were tested for ELISA activity against BG505 T332N (A,C) or CNE8 (B,D) SOSIP trimer-coated wells. Data are representative of two independent experiments. Gp120-specific positive control ADCC mAbs were C11 and A32 and gp41-specific positive controls were QA255.067 and 167-D. 2G12 was also included as an Env trimer-specific positive control. 2G12 activity was much higher than the other mAbs in (C) so the axis was split to more clearly display activity for all mAbs.

Although the RFADCC assay is a valuable measure of antibody effector function due to its correlation with clinical outcomes, there is not agreement in the field on whether it specifically detects ADCC or a combination of effector functions[174,175]. More importantly, the use of gp120- or gp41-coated cells as targets in the RFADCC assays presents epitopes that are less accessible within the setting of HIV infection, largely due to CD4 downregulation by Nef and Vpu, as well as decreased surface Env expression[55]. To examine the potential of MG540 mAbs to mediate ADCC of infected cells, we tested whether the mAbs could mediate ADCC against HIV-infected primary $CD4^+$ T cells (Figure 4.4). We compared the binding (Figure 4.4A) and ADCC (Figure 4.4B) mediated against cells infected with a wildtype or *nef*$^-$ *vpu*$^-$ CH58 (clade B) infectious molecular clone commonly used in this assay[57,230]. Nearly all mAbs showed high levels of binding and ADCC against *nef*$^-$ *vpu*$^-$ CH58-infected cells, comparable to positive controls A32 and/or 17b, except for .82. A few showed (MG540.50, .51) even higher levels, comparable to a positive control that recognizes the CD4 binding site within the closed state of Env, 3BNC117 (Figure 3A and B, orange bars)[231]. By contrast, most mAbs showed markedly reduced binding to WT CH58-infected cells, in agreement with expected reduced epitope accessibility. Because several MG540 mAbs, .16, .50, .67, .84, .86, and .90, showed ADCC levels above the negative control 3BNC117 GRLR mAb, despite binding levels near background (Figure 4.4A-B, green bars), we tested this subset of mAbs for binding and ADCC against cells infected with BG505 T332N, the strain used in the RFADCC assay and more closely related to MG540 *env* sequences. These mAbs displayed binding levels (MFI: 416-466) ~3-fold above the no Ab control (MFI: 148), albeit at ~5-fold lower levels than 3BNC117 (MFI: 2208; Figure 4.4C). Similarly, there was measurable ADCC against BG505-infected cells, with activity ranging from 6-13%, compared to 3BNC117, which showed ~15% ADCC (Figure 4.4D). This

was consistently above the levels of the negative control (~1%), suggesting that this subset of antibodies mediates low levels of ADCC against BG505-infected cells. Some mAbs mediated negative levels of ADCC in this assay, likely due to killing of cells binding shed gp120[55].

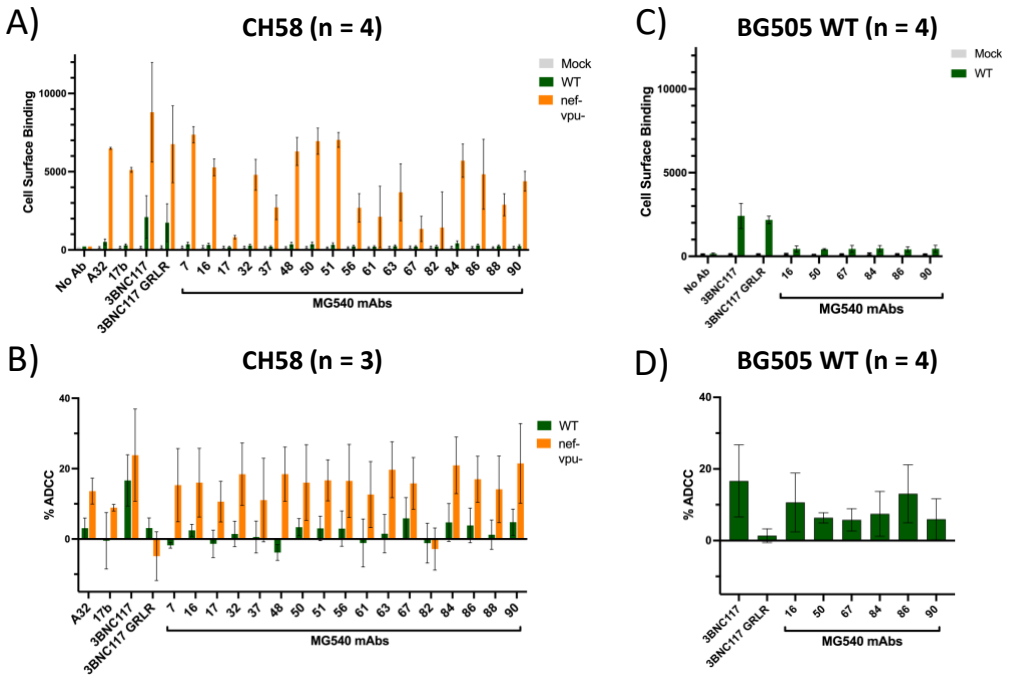

Figure 4.4. Several MG540 mAbs bind and mediate ADCC against primary isolate infected CD4$^+$ T cells.
Surface antibody binding (A,C) and ADCC (B,D) of indicated mAbs was evaluated in CH58- (A,B) or BG505 T332N-infected (C,D) CD4$^+$ T cells. Binding and ADCC were assessed in mock, WT, and *nef vpu*$^-$ virus-infected cells depending on the assay and infecting variant, as indicated. Positive control mAbs included were A32, 17b, and 3BNC117. Negative controls include no antibody and a GRLR variant of 3BNC117. Mean and standard deviation are shown. Number of replicate experiments performed are indicated, except for MG540.50 which was tested in two replicate experiments in (C-D).

Defining MG540 mAb epitopes

We mapped the epitope of each reconstructed mAb using a combination of phage immunoprecipitation sequencing (PhIP-Seq) and competition ELISAs to identify both linear and conformational mAb epitopes. For PhIP-Seq, we used an HIV-specific phage display library expressing 38-mer Env peptides from six HIV strains. In some cases, Abs recognized well known linear epitopes in V3 region of gp120, centered on the GPxQ/R motif (mAbs MG540.16, .67, .84, .90), or the C-C loop (immunodominant epitope or IDE) of gp41 (mAbs MG540.32, .63, .66, .86; Figure 4.5A). Importantly, MG540 P34 and BG540 W0 plasma samples did not strongly

enrich for responses for linear epitopes outside V3 or the IDE, suggesting that V3 and IDE-specific mAbs make up the majority of the linear epitope-directed plasma repertoire.

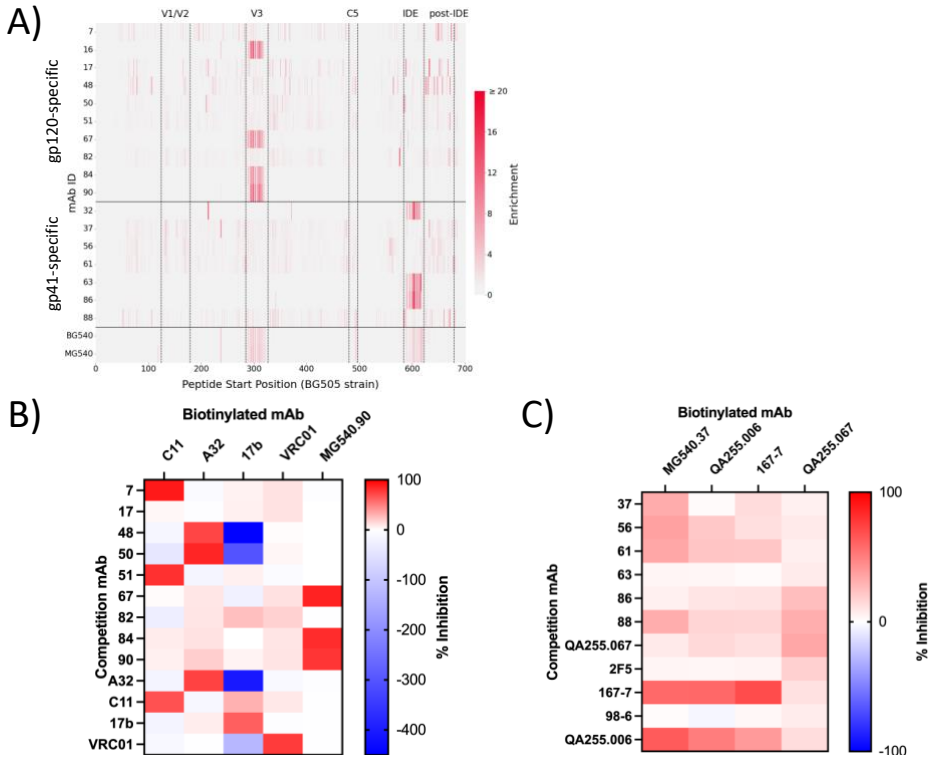

Figure 4.5. Epitope mapping of MG540 mAbs.
Heatmap (A) showing the degree of enrichment of each peptide in red, with darker shades indicating higher enrichment (binding), as indicated by the scale on the right. Peptides are ordered by start position of the N-terminal amino acid, beginning at HXB2 amino acid 31 and spanning amino acid 704. MG540 mAbs are stratified by gp120 or gp41 specificity. Plasma samples are shown at the bottom. Dotted vertical lines indicate known linear Env epitopes, with the epitope indicated above. The Heatmaps (B,C) displaying the degree of competitive inhibition of biotinylated mAb ELISA activity using BG505 gp120- (B) or ZA1197 gp41-coated plates (C) are shown for a subset of mAbs. Competitor antibodies indicated in rows and biotinylated antibodies are listed as columns. Data are representative of two replicate experiments. Inhibition calculation is described in Methods. See Figures 4.6 and 4.7 for additional epitope information and binding enhancement data.

Many of the mAbs directed to linear epitopes bound to peptides from multiple HIV strains (Figure 4.6). Among the V3-specific mAbs, MG540.84 bound to all strains tested except clade D QA013. Clonal family 2 (mAbs MG540.16, .67, .90) did not bind peptides from strains BF520 (clade A) and QA013. The strains differ in the residues upstream of the core binding motif: SIR/HI for B41, BG505, BL035, and ZA1197, GEHM for QA013, and SVHL for BF520, which may explain the observed differences in binding. These strain-specific results have

similarly been reported for other V3 mAbs[198]. MG540 and BG540 plasma also did not show detectable enrichment for V3 peptides from the QA013 strain, supporting strain-specificity of mAbs directed against this site.

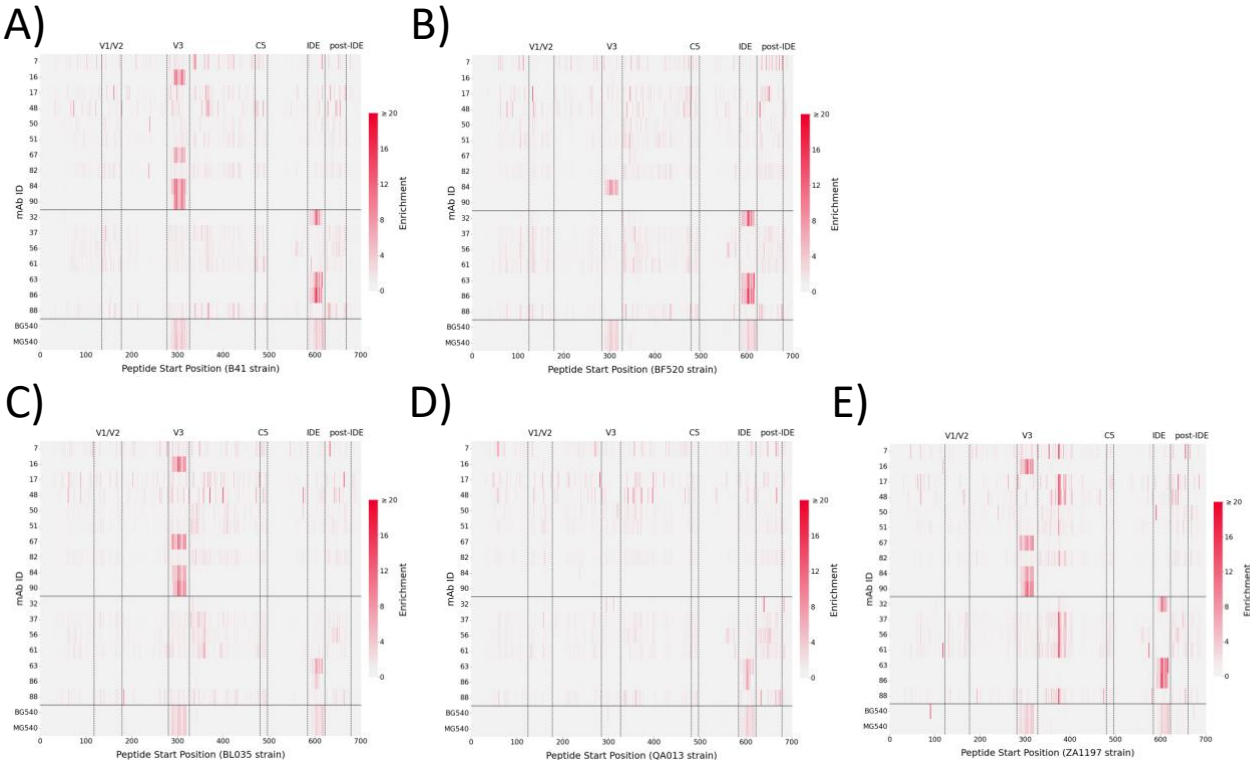

Figure 4.6. PhIP-Seq data for additional library strains.
Peptide enrichment (red intensity) data for Env strains B41 (A), BF520 (B), BL035 (C), QA013 (D), and ZA1197 (E) are shown for representative MG540 mAbs. Full HIV strain information provided in Methods. Peptides ordered based on start position of the N-terminal amino acid.

Among the IDE-specific mAbs, MG540.63 and .86 displayed responses to peptides from all strains. By contrast, mAbs MG540.32 and .66, which are clonal to MG540.86, exhibited reduced responses to the clade A/D BL035 and QA013 IDE, with no binding by mAb MG540.32. Similarly, mAb MG540.32 bound to fewer epitopes overall within the IDE, which might suggest that it has a narrower epitope than the other IDE mAbs. At the center of the C-C loop, the BL035 and QA013 strains contain a negatively charged histidine at site 602 (HXB2 numbering), whereas all other strains utilize polar leucine or isoleucine residue. These data are in

agreement with a study reporting that mutations at this site reduce mAb binding responses in PhIP-Seq[198].

Since the remaining mAbs bound to gp120 or gp41 monomers but did not target linear epitopes represented by 38-mer peptides, we tested them for recognition of conformational epitopes targeted by previously described HIV-specific ADCC antibodies. As many potent ADCC mAbs target CD4-inducible (CD4i) regions of gp120, we focused on biotinylated variants of CD4i mAbs in competition ELISAs (Figure 4.5B). Competition was observed between five MG540 mAbs and CD4i mAbs: mAbs MG540.7 and .51 competed with C11, mAbs MG540.48 and .50 competed with A32, and MG540.82 competed with 17b. As expected based on the PhIP-Seq data, the V3-specific mAbs (MG540.67, .84, .90) competed with each other. Only one of the gp120-specific mAbs, MG540.17, did not exhibit clear competition with the biotinylated mAbs.

In the course of competition experiments, we noted that A32 and A32-like mAbs MG540.48 and .50 enhanced 17b ELISA activity, indicating that these mAbs may improve 17b epitope accessibility, similar to how stabilizing mutations or CD4-mimetics have been shown to enhance 17b affinity for Env expressed on HIV-infected cells[56,213,232]. Although previous studies suggest enhancement of A32 binding to gp120 monomer by 17b, we did not observe enhanced binding mediated by 17b in initial competition experiments[233]. By contrast, there was a modest increase in A32 binding to gp120 monomer coated wells by C11 and C11-like mAb MG540.51. When a lower concentration of biotinylated-A32 was used for competition, there was improved binding of A32 by these mAbs (Figure 4.7).

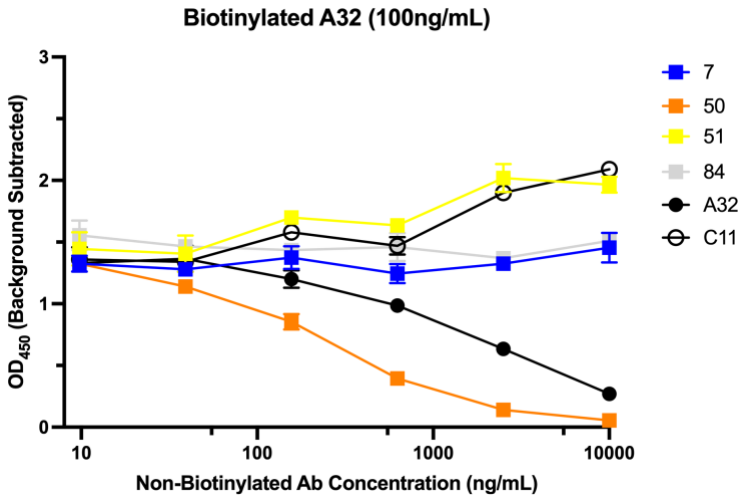

Figure 4.7. Enhancement. Of A32 binding in competition ELISA.
Background-subtracted ELISA activity of 100 ng/mL biotinylated A32 mAb is shown for BG505 gp120-coated wells pre-incubated with the indicated MG540 and control mAbs at the indicated concentrations (x-axis). Data is from a single experiment, with two technical replicates per condition.

As PhIP-Seq identified the cluster I-specific mAbs (MG540.32, .63, .66, .86), we used mAbs specific for discontinuous cluster II, QA255.006 and 167-7, as competitors in a gp41 competition ELISA to determine if the remaining mAbs were directed against this region[38,180]. Indeed, all four of the remaining mAbs, MG540.37, .56, .61, and .88, exhibited moderate competition of QA255.006 and/or 167-7 binding, indicating that they likely target cluster II (Figure 4.5C). As these results would suggest that the mAbs compete with each other, we also performed competition experiments using biotinylated mAb MG540.37. There was clear competition by mAbs MG540.37, .56, .61, .88, 167-7, and QA255.006 with MG540.37, which supported the cluster II specificity of these mAbs. Of note, mAb MG540.88 interestingly also competed with QA255.067, an IDE-specific mAb. MG540.37 was the only non-IDE mAb that did not compete with QA255.006, which suggests it may have a lower affinity or non-overlapping epitope within cluster II.

Altogether, mapping experiments identified the epitopes targeted by all of the isolated MG540 mAbs except for MG540.17. In total, the 14 clonal families recognized six non-overlapping Env regions, across both gp120 and gp41.

Neutralization activity of mAbs

Because ADCC-capable Abs that bind HIV Env can be neutralizing, we tested the ability of all mAbs to neutralize a small panel of cross-clade, heterologous viruses, many of which were only weakly neutralized by MG540 P34 plasma (Table 4.2). Most reconstructed mAbs were non-neutralizing. The two V3-specific families, 2 and 8, strongly neutralized the two tier 1 viruses tested, SF162 (clade B) and Q461.D1 (clade A), with IC_{50} values ranging from < 0.625 to 5.4 μg mL^{-1} but did not neutralize the tier 2 viruses tested.

Table 4.2. Heterologous virus neutralization by MG540 mAbs and plasma.
Neutralization of heterologous HIV viruses by MG540 P34 plasma (top row) and MG540 mAbs. Reported IC_{50} values are the reciprocal dilution of plasma or mAb concentration in μg/mL. Antibodies that did not neutralize the Tier 1 viruses SF162 and Q461.D1 were not tested against additional viruses. Data are the average of two replicate experiments. NT: not tested.

MG540 P34 Ab characteristics		Neutralization					
		n/a	Tier 1		Tier 2		
			Clade B	Clade A	Clade A	Clade C	Clade D
Family		SIV	SF162	Q461.D1	BG505.C2 T332N	QC406.F3	QD435.A4
plasma	MG540 P34 plasma	<1:100	>3200	528.0	<1:100	<1:100	<1:100
1	MG540.7	>20	>20	>20	>20	NT	NT
2	MG540.16	>20	<0.625	3.1	>20	>20	>20
	MG540.67	>20	>20	5.4	>20	>20	>20
	MG540.90	>20	<0.625	2.7	>20	>20	>20
3	MG540.17	>20	>20	>20	>20	NT	NT
4	MG540.48	>20	>20	>20	>20	NT	NT
5	MG540.50	>20	>20	>20	>20	NT	NT
6	MG540.51	>20	>20	>20	>20	NT	NT
7	MG540.82	>20	>20	>20	>20	NT	NT
8	MG540.84	>20	<0.625	0.8	>20	>20	>20
9	MG540.32	>20	>20	>20	>20	NT	NT
	MG540.86	>20	>20	>20	>20	NT	NT
10	MG540.37	>20	>20	>20	>20	NT	NT
11	MG540.56	>20	>20	>20	>20	NT	NT
12	MG540.61	>20	>20	>20	>20	NT	NT
13	MG540.63	>20	>20	>20	>20	NT	NT
14	MG540.88	>20	>20	>20	>20	NT	NT

Contribution of mAbs to overall plasma ADCC

We performed competition experiments with the isolated MG540 mAbs to determine how they contribute to overall plasma ADCC. For this, we selected one mAb representing each epitope to distinguish between the epitopes targeted (*indicated in Table 1) and generated GRLR

variants, which introduces mutations that prevent binding to FcγRs and thus disrupt ADCC activity[226]. For clonal families or mAbs targeting the same epitope, we selected the most potent mAb to reduce redundancy in evaluating the impact of each epitope on plasma ADCC, resulting in five distinct mAbs for gp120 and four for gp41.

We first validated the ability of serial dilutions of the GRLR variants to inhibit ADCC by its wildtype counterpart and used these results to define a concentration that resulted in at least 85% reduction in wildtype mAb ADCC for downstream studies (Figure 4.8). We next evaluated "cross-competition" between the GRLR variants and wildtype antibodies targeting distinct epitopes (Figure 4.9). As expected, the GRLR variants again potently reduced wildtype mAb ADCC (4-25% of mock activity), with little competition between most gp120 mAbs targeting distinct epitopes (which we define here as >75% of mock activity; maximum mock standard deviation = 4.5%). The exception is the GRLR variant of mAb MG540.50, which also strongly competed with mAb .17, although .17 GRLR did not compete with mAb .50 (Figure 4.9A). These results suggest there may be partial overlap between the epitope of mAbs MG540.17 and .50, which has been reported for other CD4i mAbs[34]. However, we also cannot rule out steric hindrance of a binding site by the bound mAb that is not due to an overlapping epitope. As a result, we opted to include both MG540.17 and .50 in subsequent competition experiments.

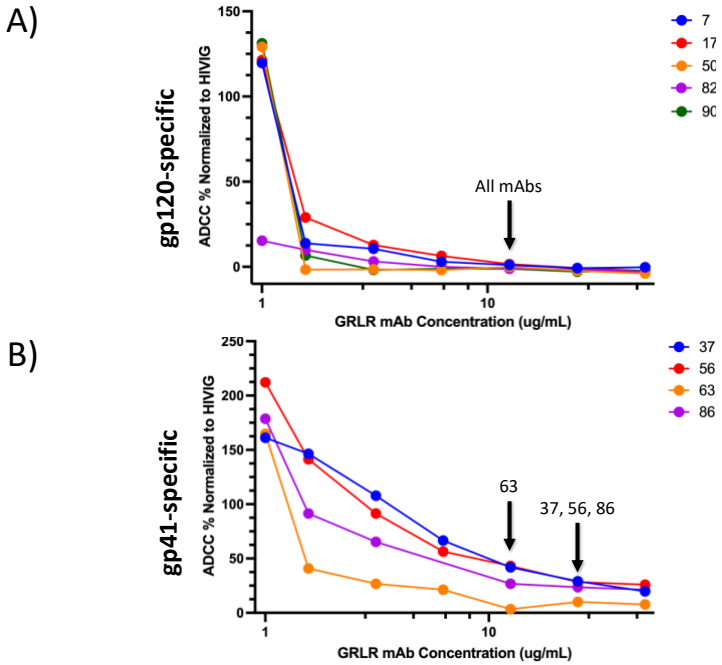

Figure 4.8. Validation of GRLR variant competition of wildtype mAb ADCC.
Relative ADCC of the indicated gp120- (A) or gp41-specific (B) wildtype mAbs in the presence or absence of the corresponding GRLR mAb variant at the indicated concentration (µg/mL). WT mAbs were added to target cells at their EC_{95} concentrations. A placeholder value of 1 was used for the no competition condition to allow for plotting on a logarithmic scale. The GRLR mAb concentrations selected for subsequent competition experiments are indicated by labeled arrows. Data are representative of two replicate experiments.

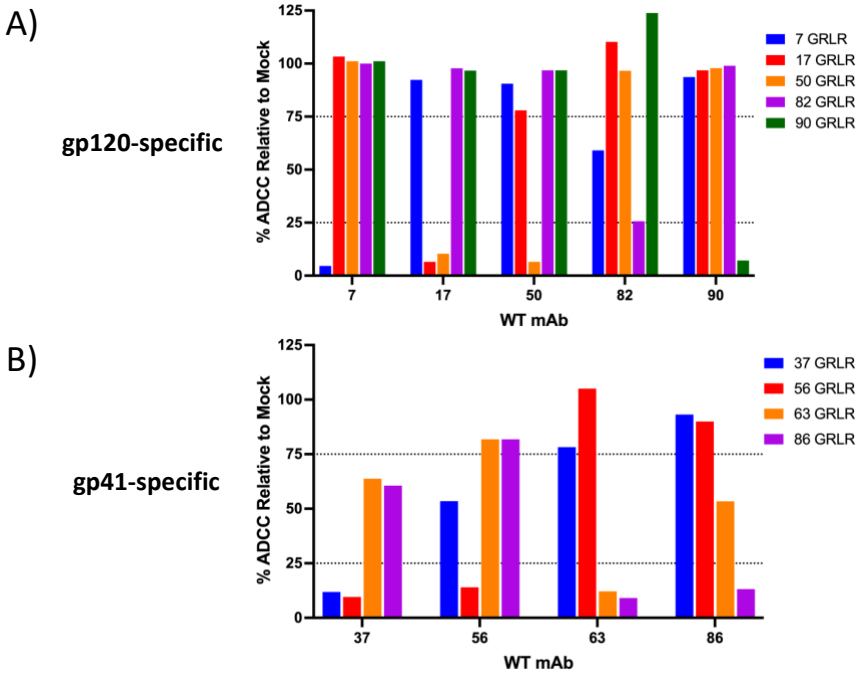

Figure 4.9. mAb GRLR variants exhibit cross-competition of ADCC activity.
Relative ADCC of the indicated gp120- (A) or gp41-specific (B) wildtype mAbs in the presence or absence of the corresponding GRLR mAb variant at the indicated concentration (µg/mL). WT mAbs were added to target cells at their EC_{95} concentrations. A placeholder value of 1 was used for the no

competition condition to allow for plotting on a logarithmic scale. The GRLR mAb concentrations selected for subsequent competition experiments are indicated by labeled arrows. Data are representative of two replicate experiments.

For gp41, the GRLR mutants showed similar inhibition of wildtype mAbs as seen with the gp120 mAbs (86-88%). The Cluster I-specific gp41 mAbs, MG540.63 and .86, competed with each other, as did the cluster II mAbs (MG540.37 and -56), consistent with epitope mapping experiments (Figure 4.9B). The degree of competition was not equally bidirectional, with mAbs MG540.56 and .86 more potently inhibiting (<25% of mock) ADCC mediated by their respective gp41 cluster counterparts (<75% of mock), MG540.37 and .63. Due to the high degree of nonreciprocal cross-competition between the gp41-specific mAbs, we opted to include all four mAbs in plasma competition experiments to maximize our chance of recapitulating the ADCC response.

After validating the effectiveness of the GRL R variants to inhibit epitope-specific ADCC, we tested the ability of combinations of GRLR mAbs to inhibit either gp120- or gp41-specific plasma ADCC (Figure 4.10). The maximum inhibition by a single antibody (bars with only one shaded box in lower chart) was only 10%, suggesting that none of the mAbs dominate the MG540 plasma ADCC response. Among the gp120-specific mAbs, pre-incubation with combination of 3-5 mAbs (indicated by shaded boxes in lower chart) accounted for upwards of 55% of plasma ADCC, demonstrating that these mAbs collectively make up the majority of the gp120-specific plasma ADCC repertoire (Figure 4.10A). Overall, competitions involving more GRLR mAbs resulted in higher ADCC inhibition, although this trend was influenced by the specific mAbs present in each competition. In terms of epitope-specific contribution, combinations containing C11-like mAb MG540.7 tended to show the greatest ADCC inhibition. Conversely, combinations containing mAbs MG540.17 or .82 were somewhat lower ranking overall in terms of hierarchy for inhibition.

Compared to the gp120-specific antibodies, the gp41-targeting mAbs accounted for a greater proportion of total MG540 gp41-specific plasma ADCC (Figure 4.10D). Pre-incubation with all 4 mAbs led to a 78% reduction of gp41 plasma ADCC. Single mAb competitions resulted in 14-26% ADCC inhibition, with cluster II mAb MG540.56 having the greatest impact. Similarly, competitions containing mAb MG540.56 yielded more ADCC inhibition overall. Taken together, the plasma competition experiments indicate that the isolated mAbs largely account for MG540 plasma ADCC activity, with the gp41 mAbs resulting in near-complete abrogation of plasma ADCC.

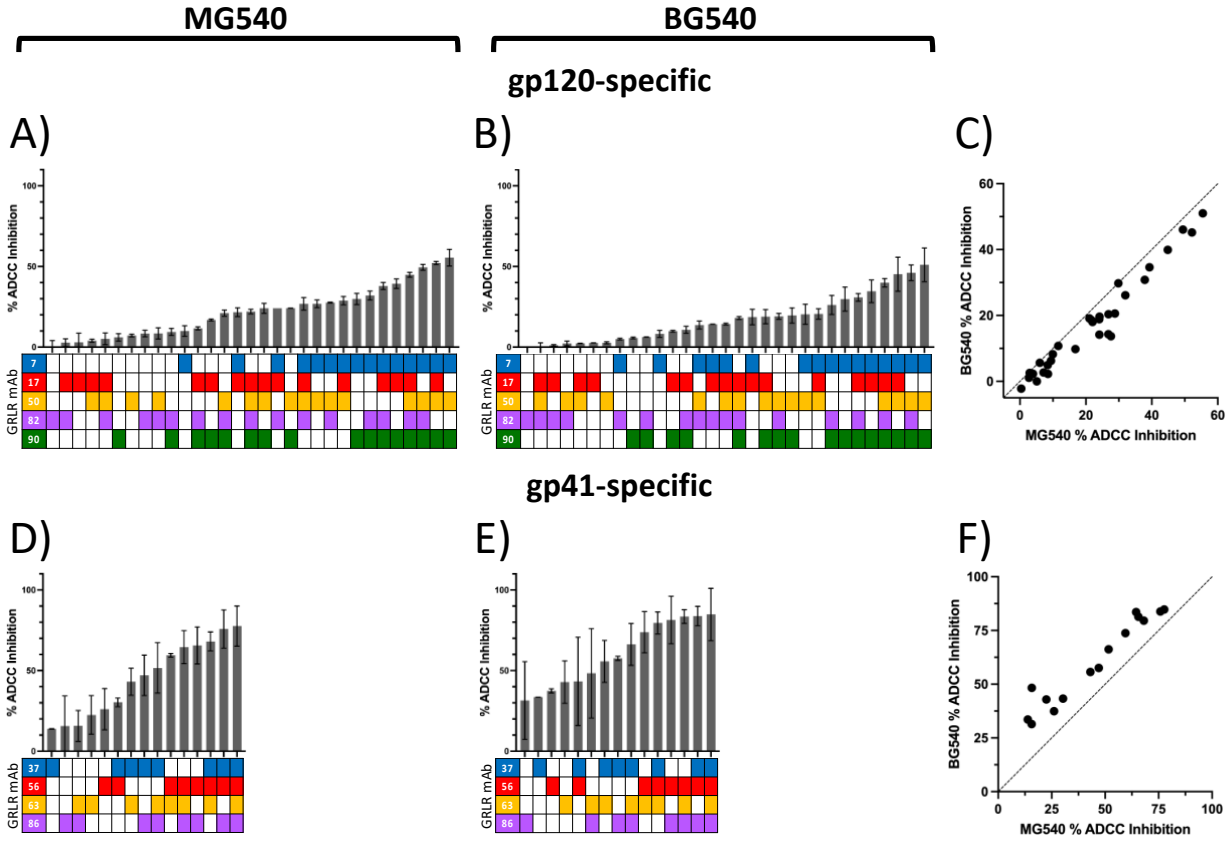

Figure 4.10. Results of competition ADCC experiments with GRLR mAbs and MG540 P34 or BG540 W0 plasma.
Inhibition of MG540 P34 (A-B) or BG540 W0 (D-E) plasma gp120- (A-C) or gp41-specific (D-F) RFADCC activity by target cell pre-incubation with the indicated combinations of GRLR mAb variants. Below each bar, the shaded charts indicate which mAbs were present in the competition. Conditions are ordered by ascending degree of average plasma ADCC inhibition. Inhibition calculation is described in Methods. Panels C and F show correlations between GRLR mAb plasma gp120- (C) or gp41-specific (F) ADCC competitions for MG540 and BG540. Mean ADCC inhibition values from maternal (A-B; MG540) and infant (D-E; BG540) plasma ADCC competition assays for gp120 (A,D) and gp41(B,E)

were compared for all conditions, which includes single and combined mAbs. Dashed lines indicate line of identity. Mean and standard deviation from two replicate experiments are shown.

We also examined whether the selected MG540 GRLR variants similarly recapitulated the passively-acquired plasma ADCC of her infant, BG540. We tested the same combinations of GRLR MG540 mAbs for the ability to inhibit BG540 gp120- or gp41-specific ADCC using plasma from the first week of life. The GRLR mAbs recapitulated BG540 gp120-specific plasma ADCC to a strikingly similar extent (Figure 4.10B). Single mAb competitions reduced plasma ADCC by only up to 8%. A combination of three to five mAbs inhibited gp120 plasma ADCC by 30 to 51%, suggesting that the five GRLR mAbs recapitulate the BG540 plasma ADCC response to a similar degree as for MG540. As with MG540, C11-like mAb MG540.7 appeared to contribute the most to plasma ADCC, followed by V3-specific mAb MG540.90. Antibodies MG540.17 and .82 again did not appreciably reduce gp120-specific ADCC compared to competitions lacking these antibodies.

Similar to MG540, the gp41-specific GRLR variants recapitulated BG540 W0 plasma ADCC to a higher degree than for gp120-specific ADCC (Figure 4.10E). Combinations of two to four GRLR mAbs inhibited BG540 plasma ADCC by 81-85%. Single mAb competitions resulted in 31-43% ADCC inhibition, a higher proportion than observed for MG540 plasma. Competitions containing mAb MG540.56 again yielded greater ADCC inhibition, suggesting that contributes highly to passively-acquired plasma ADCC activity in BG540.

In addition to recapitulating the level of ADCC activity mediated by plasma to a similar degree for MG540 and BG540, the contribution of each combination of GRLR mAbs was statistically significantly correlated across these individuals (Spearman r = 0.96 for gp120 and 0.96 for gp41). The gp120 competitions were slightly skewed towards having a higher impact on MG540 plasma ADCC (Figure 4.10C). Conversely, the gp41-specific competitions consistently

had a greater impact on BG540 passively-acquired plasma ADCC activity when compared to MG540 (Figure 4.10F). Altogether, these data suggest a high degree of concordance between the ADCC activity recapitulated by the GRLR mAbs for MG540 and BG540.

Enhancement between antibodies

There was some evidence to suggest enhancing interactions between antibodies in competition ADCC experiments. For example, there was an increase of 110 - 124% of mock ADCC activity for mAb MG540.82 when target cells were pre-incubated with the GRLR variants of mAb MG540.17 and .90 (Figure 4.9A). To explore this further, we tested wildtype mAbs at a lower EC_{50} concentration rather than saturating at EC_{95} concentrations. Indeed, there was stronger evidence for enhancement between multiple MG540 mAbs. This increased activity was most evident between MG540.82 and .90, which resulted in >260% ADCC relative to mock competition. The ADCC mediated by MG540.82 was also higher when cells were pre-incubated with MG540.17 (161% of mock) and .50 (131% of mock), although to a lesser extent (Figure 4.11). In order to probe these results further, we also tested for ADCC enhancement by the GRLR variants against BG505 T332N-infected $CD4^+$ T cells. Compared to MG540.82 alone (2% ADCC), co-incubation with GRLR variants of MG540.17, .50, and .90 resulted in a > 4-fold increase in ADCC (9-19%) against BG505 T332N-infected cells (Figure 4.12).

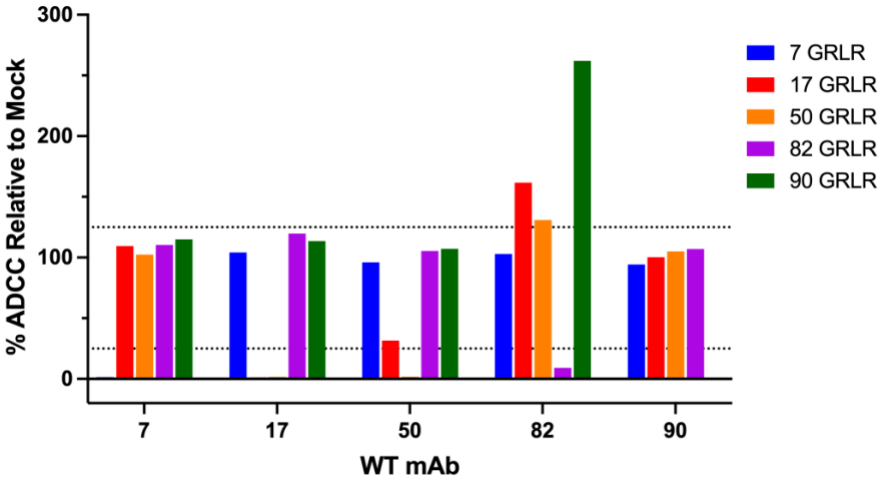

Figure 4.11. Cross-clonotype competition or enhancement by GRLR mAbs at EC_{50} wildtype mAb concentrations.
Additional competition experiments using wildtype antibodies at the corresponding EC_{50} concentration. Data points indicate the relative ADCC activity of the indicated wildtype antibody in the presence of GRLR variants normalized to the activity of the mock (no GRLR antibody) condition. Dashed lines indicate 25% and 125% of mock competition ADCC. Data are from two replicate experiments.

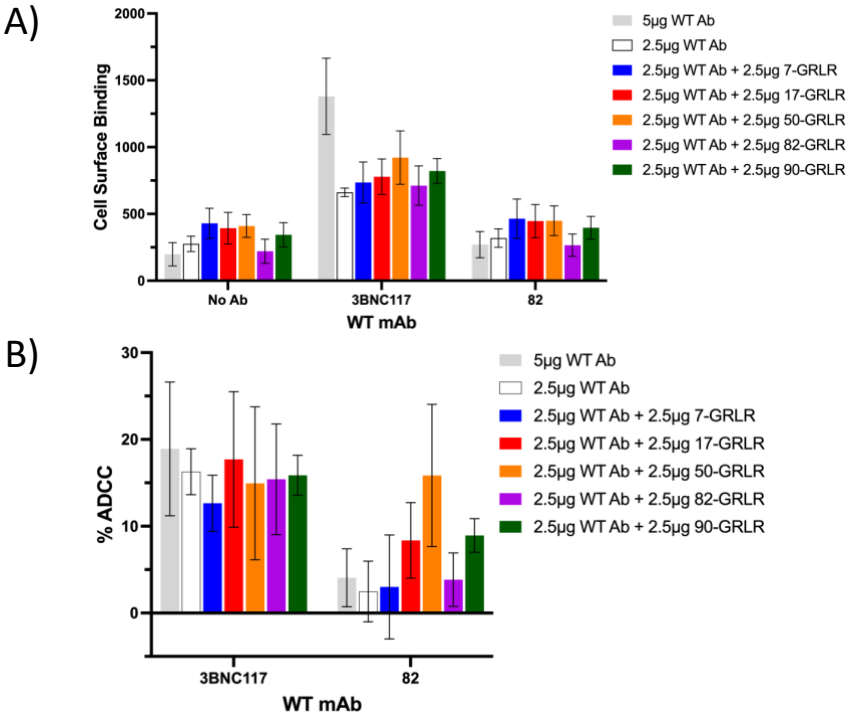

Figure 4.12. GRLR mAb ADCC enhancement experiments using BG505-infected $CD4^+$ T cells.
Surface antibody binding (A) and ADCC (B) of indicated mAbs against BG505 T332N-infected primary $CD4^+$ T cells in the presence or absence of the indicated GRLR mAbs. The antibody listed at the bottom indicates the wildtype antibody tested. The right legend indicates the combination of mAbs tested and their concentrations. 3BNC117 was included as a positive ADCC control. Data are the average and standard deviation of five replicate experiments, except for MG540.7-GRLR and .17-GRLR, which were only tested three times.

Discussion

In this study, we designed a two-step screening approach to identify and reconstruct ADCC-mediating HIV-specific mAbs from sorted memory B cells isolated from a non-transmitting mother living with HIV, MG540. Individual MG540 was selected because she did not transmit HIV to her infant despite a high viral load and nearly a year of breastfeeding in the absence of anti-retroviral therapy, which was not available at the time. In total, we recovered 14 unique lineages, represented by 18 mAbs, which targeted a diversity of epitopes and together provide a thorough assessment of the ADCC-capable mAbs produced by circulating memory B cells during chronic HIV in this individual. These mAbs account for a large fraction of the late pregnancy plasma ADCC within MG540 as well as the passively-acquired ADCC activity of her infant, BG540, with no gp120-specific mAb contributing more than 10%. These data suggest the polyclonal collection of MG540 mAbs were the dominant passively-transferred ADCC antibodies circulating at the time of HIV exposure in BG540.

Whereas numerous studies have identified neutralizing Abs by culturing sorted B cells and testing the cell supernatants in neutralization assays, we opted to screen the supernatants via ELISA and the RFADCC assay to identify wells with B cells expressing ADCC-capable antibodies[215,234]. Of the wells with HIV-specific ELISA activity, nearly half mediated ADCC against target cells coated with gp120 or gp41 monomer, likely reflecting the common elicitation of ADCC-capable antibodies in HIV infection[235,236].

The mAbs reconstructed in this study recognized six non-overlapping epitopes amongst the gp120 and gp41 subunits of Env, including both CD4i (A32-, C11-, and 17b-like) and non-CD4i epitopes (V3, clusters I and II). One mAb, MG540.17, could not be mapped but based on non-reciprocal competition with a CD4i epitope targeting mAb, it may target a CD4i epitope that

may partially overlap with that of A32. This finding is consistent with earlier studies describing ADCC mAbs that recognized CD4i epitopes that do not completely overlap with prototypic cluster A and C mAbs, thereby supporting the classification of more nuanced CD4i epitopes[34,237]. The numerous epitopes targeted by the MG540 mAbs expand upon previous attempts to characterize the ADCC repertoire of HIV-infected individuals by recovering a highly polyclonal collection of mAbs targeting diverse epitopes. Many such studies recovered mAbs targeting only one or two epitopes or only CD4i epitopes, which might indicate that ADCC is largely driven by a monoclonal Ab response[34,180,195,212,237]. However, in several instances the ADCC-capable mAbs were identified by happenstance using approaches to screen for nAbs, and as such may have suffered from an inability to comprehensively detect broad lineages of nnAbs.

We selected the RFADCC assay for initial ADCC screening because activity in this assay has repeatedly been associated with positive clinical outcomes, including improved infant survival in MTCT studies[129,155,190,193,238]. This assay also correlates well with other measures of ADCC and is tractable for high throughput screening[178]. The ADCC-capable mAbs isolated in this study potently mediated ADCC against monomer coated cells in this assay. Many of the gp120 mAbs had an ADCC EC_{50} and peak activity comparable to prototype CD4i ADCC mAbs A32 and C11. The 17b-like mAb MG540.82 appears to be an outlier in that its lack of potency differs from previous studies suggesting most mAbs targeting CD4i epitopes are particularly potent mediators of ADCC[43]. Regarding gp41 specificity, the mAbs we identified were noticeably less potent than the gp120-specific mAbs per the RFADCC assay.

While RFADCC activity correlates with positive clinical outcomes, it may measure multiple effector functions and may not recapitulate the epitopes presented by HIV-infected $CD4^+$ T cells[176,239]. In an assay that measures killing of infected cells, many of the MG540 mAbs

also reproducibly mediated ADCC against *nef*$^{-}$ *vpu*$^{-}$ CH58-infected CD4^{+} T cells. Surface antibody binding and ADCC activity was relatively diminished against WT CH58 infected cells, which suggests some of the mAb epitope targets are less accessible on the surface of cells in which CD4 is downregulated, which is expected for CD4i antibodies. Nonetheless, ADCC activity was detected for six of the MG540 mAbs against both WT CH58- and BG505 T332N-infected cells. These findings provide evidence that the isolated mAbs likely mediate ADCC *in vivo*. This subset of six MG540 mAbs join those already described in the literature as capable of mediating ADCC against distinct HIV variants, including tier 2 viruses, even in the absence of neutralization capacity[129,180].

An important goal of this study was to define the repertoire of antibodies that contributed to the ADCC activity of Abs passively transferred to BG540. Competition ADCC experiments indicated that combinations of the reconstructed mAbs recapitulated the majority of contemporaneous MG540 plasma ADCC. The same was true for BG540 using plasma from the first week of life, and the relative contribution of each antibody was highly correlated between MG540 and BG540. Although single MG540 mAbs did not contribute significantly to overall ADCC activity, mAbs targeting CD4i epitopes appeared to make the largest impact on overall gp120-specific plasma ADCC, in line with earlier studies[43]. However, the limited contribution of single mAbs in this individual differs significantly from previous studies suggesting that the majority of plasma ADCC can be attributed to a single epitope, particularly those targeted by A32, C11, and 17b[237,240]. By contrast, in this study, combinations of up to five MG540 mAbs targeting distinct epitopes blocked MG540 gp120-specific plasma ADCC by 55%, suggesting that the MG540 plasma ADCC repertoire is polyclonal. Given this study, which is the first to fully interrogate the ADCC repertoire in a non-transmitting mother and define a polyclonal

response in this individual, future studies are needed to determine if this is more typical of non-transmitting mothers. The GRLR variants of the mAbs isolated here may be useful for this purpose.

Although representatives of the MG540 mAbs captured the majority of the plasma ADCC activities of MG540 and BG540, the tested mAbs did not achieve complete inhibition of plasma ADCC activity, particularly for gp120. For this there are two likely explanations: 1) it is possible that the full set of MG540 mAbs comprising the plasma ADCC repertoire were not recovered, and/or 2) the GRLR mAbs used in plasma competition ADCC experiments blocked epitope-specific ADCC, but, at the same time also mediated enhancement of ADCC targeting distinct regions of Env, thereby confounding the perceived degree of ADCC inhibition. The latter possibility is supported in part by mAb competition experiments presented here that show evidence for enhancing effects between some combinations of MG540 mAbs, but further work is needed to fully understand how mAbs synergize to potently mediate ADCC.

This detailed study focused on characterizing ADCC antibodies from a single individual to provide a deeper understanding of the qualities and synergy of ADCC-capable mAbs comprising a potent plasma ADCC response. We expanded upon prior studies evaluating the contribution of mAbs targeting single epitopes to overall plasma ADCC by testing the ability of combinations of up to five mAbs isolated from this individual to recapitulate plasma ADCC. Notably, we present a case where ADCC responses appear to be highly polyclonal, which differs from prior studies indicating that plasma ADCC is driven by mAbs targeting a single epitope, particularly CD4i epitopes[237,240]. Given recent studies suggesting a protective role of effector functions other than ADCC, future studies to determine the broad range of effector functions mediated by the MG540 mAbs described here may be informative of their overall functional

activity[98,111]. Finally, the tools developed here, including the supernatant screening approach and the GRLR mutants of the MG540 mAbs, could be used to further probe human plasma for ADCC responses directed against a variety of epitopes.

Chapter V

Conclusions and Future Directions

As there is currently no protective HIV vaccine, human studies are invaluable for providing relevant correlates of protection from HIV infection and pathogenesis. Infants who are exposed to HIV via breastfeeding also passively receive HIV-specific antibodies from their mothers during gestation. The setting of breastfeeding exposure thus provides a unique opportunity to determine whether these pre-existing antibodies influence vertical transmission. The goal of this thesis was to investigate whether the epitope or effector function of pre-existing antibodies in breastfeeding infants contribute to reduced HIV acquisition risk and pathogenesis.

In Chapter II, two antibody measures, passively-acquired RFADCC and dimeric FcγR ELISA activity, correlated with improved survival of HIV+ infants from both cohorts, further supporting a role for ADCC in improved HIV outcomes. Importantly, similar findings were also observed in a recent study from another cohort[148]. In Chapter III, passively-acquired antibodies targeting C5 correlated with improved survival, suggesting that epitope contributes to improved outcomes in both cohorts. Excitingly, C5 peptide ELISA activity also correlated with lower setpoint viral load and delayed HIV acquisition, providing hints on a potential mechanism by which pre-existing antibodies can influence the course of HIV infection. In Chapter IV, we sought to link the role of antibody epitope and effector function by characterizing ADCC-mediating antibodies isolated from HIV non-transmitting mother MG540. Only a combination of the reconstructed antibodies recapitulated MG540 plasma ADCC and that of her infant, suggesting a polyclonal ADCC repertoire. The remaining sections will describe future directions for the work described in this thesis and provide additional context based on recent studies.

The role of antibody effector function in HIV vertical transmission

The data presented in chapter II provide support for a role of pre-existing ADCC in reduced HIV pathogenesis in the setting of vertical transmission. These results are consistent with earlier studies reporting that ADCC correlates with improved outcomes in a variety of settings. However, it is important to recognize that the results described here are still limited to a relatively small population. Although there is strong evidence for a role of ADCC in reduced HIV pathogenesis, it also remains unclear whether ADCC antibodies alone can promote sterilizing immunity to HIV. As ADCC is typically directed against cells which have already been infected, the window of opportunity to prevent systemic HIV infection is quite small[80]. Animal models have shown convincingly that non-neutralizing antibodies are capable of non-sterilizing immunity, but evidence for complete protection is limited[108,206]. A recent vertical transmission study implicates a role for ADCC sensitivity of strains in the degree of protection mediated by ADCC antibodies, suggesting the bar for ADCC-mediated protection from infection by diverse HIV strains may be quite high[149].

Another complicating factor is Nef/Vpu downregulation of CD4-inducible epitopes, which are common targets of potent ADCC antibodies[55]. Assays using antigen-coated cells (e.g. RFADCC) or Nef/Vpu-defective viruses allow for routine measurement of ADCC activity, but they may overestimate the *in vivo* ADCC activity of plasma antibodies or mAbs. To address this, we have collaborated with Andrés Finzi to measure ADCC activity mediated by mAbs and plasma samples against HIV-infected primary $CD4^+$ cells. In Chapter IV, most MG540 mAbs mediated ADCC against primary cells infected with a Nef/Vpu-defective virus, but ADCC activity against WT virus-infected cells was reduced. These results suggest that many of these antibodies preferentially recognize an open Env conformation and would have reduced activity

in vivo. We have also worked with the Finzi lab to determine whether ADCC activity against primary cells infected with CH58 containing the L193A mutation, which promotes sampling of open conformations, also correlates with HIV+ infant survival[192,241]. Indeed, infants with higher passively-acquired ADCC survived longer than infants with low ADCC activity, though this was limited to the NBT cohort. It will be important to further validate these results in the CTL cohort and to determine whether ADCC against WT virus-infected cells also correlates with survival.

Although RFADCC activity trended with reduced infection risk in this thesis, this was not the case for dimeric FcyR ELISA activity. Investigating differences in the activities measured by these assays may help to more clearly define the mechanisms involved in antibody-mediated protection from HIV pathogenesis. There is evidence that the RFADCC assay measures both NK cell and monocyte-mediated effector functions, which might suggest a role for multiple antibody effector functions in improved outcomes[174–176]. Indeed, recent studies have suggested that effector functions other than ADCC or polyfunctional activity may contribute to antibody-mediated protective effects[83,95,98,104,109,158,242–244]. Finally, there is increasing recognition for the role of antibody glycosylation in modulating both Fc effector functions and, in the setting of vertical transmission, placental antibody transfer[110,158]. Further work is therefore needed to dissect the role of specific effector function vs. polyfunctional potential in protection mediated by non-neutralizing antibodies. Investigating multiple effector functions and the glycosylation profiles of HIV-specific plasma antibodies would define whether there are specific facets of the humoral immune response which most strongly contribute to antibody-mediated protection.

The role of antibody epitope in HIV vertical transmission

Whereas several studies have examined whether antibodies targeting immunodominant epitopes (i.e. V3 and the gp41 IDE) correlate with vertical transmission[131–136,138–142], other

epitopes are less well studied. In Chapter III, we used an Env phage display library excluding V3 and IDE to map responses to other linear epitopes, including V1/V2, C5, and the CHR.

To our knowledge, only one study has examined the C5 antibody responses in HIV-exposed infants[130]. There, no difference was observed in C5 peptide reactivity between HEU and HIV+ infants, yet this response was only compared qualitatively and used infant samples from variable timepoints, which may confound results given that these antibodies decay over time. Here, passively-acquired antibody responses to C5 correlated with improved HIV+ infant survival, which is consistent with natural history studies suggesting that C5-specific antibodies protect from disease progression in adults[76–78]. The correlation of C5 peptide ELISA activity with delayed HIV acquisition supports an additional protective role of these antibodies in the setting of HIV transmission. Further work is warranted to identify the properties of C5-specific passively-acquired antibodies in infants and to determine whether these findings are generalizable to other populations.

As several studies have described C5-specific antibodies capable of mediating ADCC, a natural question is whether C5-specific plasma ADCC also correlates with HIV+ infant survival or delayed HIV acquisition in the NBT and CTL cohorts[203,245]. In addition, C5-specific antibodies could be isolated from the mother of an NBT cohort infant who had high C5 passive antibody levels to determine the characteristics of mAbs targeting the linear epitope in C5 and whether these antibodies mediate ADCC.

Consistent with previous studies, passively-acquired antibodies targeting V1/V2 were not associated with vertical transmission risk[131,134]. However, responses to V1V2 were associated with improved survival of NBT cohort HIV+ infants. Intriguingly, IgG responses to scaffolded V1V2 were inversely correlated with HIV acquisition risk in the RV144 trial, with increased

vaccine efficacy against strains containing K169 and V172[201,202,246,247]. Importantly, the follow up HVTN 702 trial in South Africa did not show any efficacy[117]. This may have been influenced by a lower match frequency for residue 169 between the vaccine strain and circulating strains compared to RV144[117,248]. Among the strains included in the phage display library used in Chapter III, most utilize K169 and V172 and most sequences from Kenya available on the LANL database contain cationic R/K169 and V172. Although these similarities might suggest that the V1V2 Ab response in this study shares epitope requirements to RV144 vaccinees, further work is warranted to validate these results and to determine whether these findings are observed in distinct cohorts. Experiments using V1V2 peptides with mutations at residues 169 and 172 would provide insights into the role of V1V2 sequence in the response measured in this thesis.

In addition to defining sequence requirements, it is important to more clearly characterize the V1V2 antibody described in Chapter III. Several classes of V2 epitopes have been described: V2i, V2p, and V2q[249]. Of these, only V2p is linear, and it is recognized by mAbs isolated from RV144 vaccinees. The V2 antigens used in prior vertical transmission studies did not preferentially present V2p epitopes and therefore the impact of V2p-like antibodies may have been missed[131,134,249,250]. As V2i and V2q are conformational, we hypothesize that the antibody response detected in Chapter III is likely specific for V2p. Competition experiments using different classes of V2-specific antibodies or V2 constructs presenting distinct epitopes would shed light on the specific plasma response measured in phage display experiments. As the specific activities of V2-specific antibodies differs by epitope type, these experiments would also provide clues on the function of V2-specific passively-acquired antibodies in HIV+ infants[250].

There was also no correlation between CHR responses with vertical transmission risk. A previous study from our group suggested that antibodies targeting antigens spanning the gp41

ectodomain were associated with increased vertical transmission risk[134]. As plasma Ab responses to the gp41 CHR were not associated with vertical transmission in Chapter III, these data suggest that Ab binding a distinct portion of the gp41 ectodomain, a non-linear epitope within the CHR, or total levels of gp41-specific Abs may explain the correlation with vertical transmission.

Polyclonality of the plasma ADCC response

An understanding of the epitope targets of protective antibodies is critical to guiding vaccine design. Whereas passive immunization with single nAbs protected from infection in some studies in animal models, evidence supporting the protective efficacy of single mAbs in humans, where diverse variants circulate, in limited[92]. The recent HVTN703/704 clinical trials observed incomplete protection by passive immunization with a single mAb with potent neutralization activity, VRC01[66]. VRC01 also mediates ADCC[251]. The limited efficacy was attributed to viral resistance to VRC01 and insufficient serum antibody levels[66]. In combination with bnAb treatment studies revealing rapid selection of escape mutations, these results suggest single mAbs are likely insufficient to confer complete protection[252].

In the case of non-neutralizing ADCC antibodies, there is also limited evidence that ADCC directed against a single epitope is sufficient for full protection from infection. Conceptually, the same issues are present as for bnAbs, namely that the mAb must recognize the epitope across strains. CD4i epitopes are of thus of high interest owing to their relatively high degree of conservation across strains and the ADCC potency of prototype CD4i mAbs, including A32 and C11[92]. Many studies have thus examined whether antibodies targeting a specific epitope (particularly CD4i epitopes) drive the plasma responses where ADCC correlates with protection. Although two early studies suggested that ADCC targeting an A32-like epitope comprises the majority of plasma ADCC, competition experiments in these studies used A32 Fab, which may

not perform in a similar manner to intact IgG[18,237,240]. Other studies are also mixed on whether ADCC directed against specific epitopes drives the overall plasma ADCC response. For example, our group showed that C11-like antibodies account for a varying level of plasma ADCC in infected individuals[195]. In another study, A32- or C11-like ADCC activity only accounted for the majority of plasma ADCC in individuals with low plasma ADCC activity[164]. Consistent with these two studies, the MG540 mAbs described in Chapter IV, while potent, did not individually recapitulate MG540 plasma ADCC. Rather, several mAbs were required to capture the majority of MG540 plasma ADCC activity, providing strong evidence for a polyclonal repertoire. A potential next step would be to determine whether combinations of mAbs tested in Chapter IV also recapitulate plasma ADCC in other NBT mothers and infants to a similar degree and if the level of competition corresponds with protection from vertical transmission or HIV pathogenesis.

Because the mAb combinations tested did not fully recapitulate MG540 plasma ADCC, further work is also needed to identify additional antibodies that may also contribute to MG540 plasma ADCC. As the mAbs isolated in Chapter IV were from 10 of 50 total B cell culture plates, there is an opportunity to screen additional B cell cultures and to reconstruct mAbs with distinct epitope targets or increased ADCC potency. These experiments may provide a more complete understanding of the polyclonal repertoire comprising a protective ADCC response.

In addition to contributing to potent plasma ADCC, there is also evidence for potential synergy, or enhancement of effector functions, by antibodies targeting distinct epitopes[253,254]. For example, A32-like antibodies have been shown to enhance binding and ADCC by CoRBS antibodies such as 17b[233]. In Chapter IV, A32-like antibody MG540.50 promoted enhanced ADCC mediated by 17b-like antibody MG540.82, in agreement with these prior studies. There

was also evidence for enhancement of MG540.82 ADCC by V3-specific and C11-like antibodies, supporting a novel role for antibodies recognizing additional epitopes in improved ADCC. As the mode of enhancement was not identified, further work to define the dynamics of improved ADCC may reveal mechanisms for increasing antibody-mediated protection. The observed enhancement of ADCC against HIV-infected primary cells also raises the question of antibody synergy could be leveraged to promote neutralization in addition to cell killing. There is strong evidence that CD4 mimetics, which induce open conformations of Env on the surface of infected cells, increase ADCC activity of CD4i mAbs even in the presence of Nef/Vpu, but the utility of this approach for preventative strategies is limited by the need for consistent CD4 mimetic levels[56]. The enhancement of ADCC mediated by CD4i mAb observed in Chapter IV provides hints that inducing open Env conformations may be possible with antibodies alone and may thus be elicited via vaccination. Additional work to identify the specific mechanism of the observed enhancement may inform novel approaches to provide broad protection from infection.

Conclusion

The results described in this thesis further support a role for both antibody epitope and effector function in protection from HIV pathogenesis. This thesis also provides powerful tools for understanding the importance of antibodies targeting distinct epitopes that comprise the plasma antibody repertoire. Additional work to investigate the findings described in this thesis, including the functional capacity of V1/V2 and C5 mAbs, the role of ADCC vs. distinct effector functions in reduced HIV pathogenesis, and the contribution of distinct antibody lineages to the plasma ADCC repertoire, could identify antibody properties than can be harnessed to improve HIV vaccine design.

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VITA

Zachary (Zak) A. Yaffe was born in Philadelphia, Pennsylvania, but grew up in Rockville, Maryland. He received his B.A. from Princeton University (2011-2015) and majored in Molecular Biology. For his undergraduate thesis research, Zak studied pseudorabies virus (PRV) replication and transport in Lynn Enquist's laboratory. After graduating from Princeton, Zak returned home and completed a two-year post-baccalaureate intramural research training award (IRTA) in the lab of Daniel Douek at the NIH Vaccine Research Center. In the Douek Lab, Zak worked on a project to understand the effects of type I interferon antagonism in chronic SIV infection of rhesus macaques. Since 2017, Zak has been enrolled in the Medical Scientist Training Program (MSTP; MD/PhD) at University of Washington. He completed research rotations in the labs of Julie Overbaugh and Hans-Peter Kiem. After finishing the first two years of medical school, Zak joined Julie Overbaugh's lab for his PhD research. Upon completing his PhD, Zak will return to medical school to do clinical rotations. After finishing medical school, Zak plans to pursue residency training in internal medicine. He hopes to continue studying the role of antibodies in protecting humans from infectious diseases.