Isolation and characterization of HIV-1 neutralizing antibodies from infants

Cassandra Simonich

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Reading Committee:
Julie Overbaugh, Chair
Denise Galloway
Shiu-Lok Hu

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Abstract

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

Developing a protective HIV vaccine remains a key goal of prevention efforts. Broadly neutralizing antibodies (bnAbs) that recognize diverse circulating strains of HIV are thought to be an important component of a protective vaccine but eliciting such responses remains elusive. bnAbs that have been isolated from HIV-infected adults exhibit several unusual features that present obstacles to their elicitation such as the requirement for high levels of somatic hypermutation (SHM) and long-term antibody maturation for broad activity. Efforts are ongoing to characterize the development of bnAbs following infection to guide immunogen design and vaccine strategies to elicit them despite these immunological barriers. HIV-infected infants develop bnAbs with more rapid kinetics than adults, suggesting that infant responses could inform a more straightforward path to achievable vaccine targets. To understand these unique pediatric responses, we isolated and characterized infant HIV-neutralizing antibodies (nAbs) and defined the development of an infant-derived bnAb.

First, we isolated nAbs contributing to a broad plasma response at one year post-infection from infant BF520. We observed that polyclonal nAbs with low SHM contribute to plasma neutralizing activity. Additionally, we isolated one infant-derived bnAb that targets a known conserved site of vulnerability on HIV envelope (Env) commonly targeted by adult bnAbs. This infant bnAb is distinct from adult bnAbs in that it utilizes different germline genes, has very low SHM, and importantly, lacks rare insertions and deletions that have been shown to be critical for the activity of most adult antibodies targeting this epitope. The identification of this infant bnAb illustrates that HIV-1-specific neutralization breadth can develop without prolonged affinity maturation and extensive SHM.

Next, we characterized the ontogeny of the infant-derived bnAb by reconstructing the naïve progenitor and developmental intermediates. We observed that the naïve antibody recognizes HIV Env, which contrasts with precursors of many adult bnAbs. The ability to neutralize viruses from multiple clades developed within six months of infection and the full breadth of the mature antibody was reached with as little as 3% SHM. Remarkably, substitutions in the kappa chain enabled both autologous and heterologous neutralization. This study provides a template for the design of vaccine strategies to elicit similar responses by identifying Env isolates that could be used as sequential immunogens.

Finally, we isolated and characterized nAbs contributing to plasma breadth at two years post-infection from infant BG505. This infant is of particular interest to the HIV field because the Env of the transmitted virus from BG505 has been extensively characterized in numerous structural and vaccine studies, and understanding the nAbs that developed following infection with this Env complements those studies. We isolated polyclonal nAbs from multiple expanded clonal families contributing to heterologous neutralizing activity.

These studies showed that infant antibodies have features distinct from adult antibodies, including several that may be amenable to better vaccine responses. This work represents the first isolation and characterization of a HIV-bnAb from an infant and highlights the potential of the infant immune system to develop polyclonal nAbs against HIV. Future studies should focus on characterizing nAbs contributing to broad plasma responses in additional infants to inform how to elicit bnAbs by vaccination.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADCC: antibody-dependent cell-mediated cytotoxicity

ADCP: antibody-dependent cell-mediated phagocytosis

ADCVI: antibody-dependent cell-mediated viral inhibition

AID: activation-induced cytidine deaminase

AIDS: acquired immunodeficiency syndrome

ART: antiretroviral therapy

BCR: B cell receptor

bnAb: broadly neutralizing antibody

CD4bs: CD4 binding site

CDR: complementarity determining region

CRF: circulating recombinant form

ELISA: enzyme-linked immunosorbent assay

Env: envelope

FR: framework region

HIV: human immunodeficiency virus

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

IgG: immunoglobulin G

mAb: monoclonal antibody

MPER: membrane proximal external region of HIV Env

MTCT: mother-to-child transmission

nAb: neutralizing antibody

PBMC: peripheral blood mononuclear cell

SHM: somatic hypermutation

UCA: unmutated common ancestor

V1/V2: variable regions 1 and 2 of HIV Env

V3: variable region 3 of HIV Env

VH: IgG heavy chain variable domain

VL: IgG light chain variable domain

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DEDICATION

This thesis is dedicated to Bonnie Mathieson (1945-2018),

who was an enthusiastic supporter of junior investigators, women in science,

and research on HIV mother-to-child transmission

Chapter I

Introduction

Global epidemiology of HIV

The human immunodeficiency virus (HIV) pandemic contributes significantly to the global burden of disease. Since the beginning of the pandemic, approximately 76 million people have become infected with HIV, and around 35 million people have died of the disease. Globally, an estimated 37 million adults and children were living with HIV in 2016 (1).

Since the discovery of HIV in 1983 (2), tremendous advancements have been made toward treatment and prevention of infection. Antiretroviral therapy (ART) has reduced HIV-associated morbidity and mortality in infected individuals. ART has also proven to be an effective prevention strategy, reducing the risk of transmission through viral suppression in infected individuals (3, 4). Treatment of pregnant women and infants born to HIV-positive mothers has decreased mother-to-child transmission (MTCT) (5). ART is also used as pre-exposure prophylaxis in high risk groups (6).

Global efforts to end the HIV/AIDS epidemic have focused on HIV diagnosis, treatment and viral suppression (1). The UNAIDS estimates that new infections in adults declined by 11% from 2010 to 2016 (1), and the number of new infections among children decreased by 48% between 2009 and 2014 (5). Despite these successes, significant gaps in the care continuum remain. While approximately 70% of people living with HIV were aware of their HIV status in 2016, only 53% of people living with HIV globally were accessing ART and 44% of all people living with HIV were virally suppressed (**Figure 1.1**) (1, 7). An estimated 1.8 million people were newly infected with HIV in 2016 including 160,000 new pediatric infections.

Sub-Saharan Africa has the highest global burden with 64% of the new infections in 2016 and over 25 million adults and children living with HIV (1). Moreover, HIV infection increases the morbidity and mortality of other diseases endemic to sub-Saharan Africa including malaria and tuberculosis (8, 9).

Despite the potential of ART to prevent new infection, barriers to HIV testing and treatment along with challenges with adherence to ART contribute to continued HIV transmission. Additional strategies such as development of a prophylactic vaccine are crucial to HIV prevention.

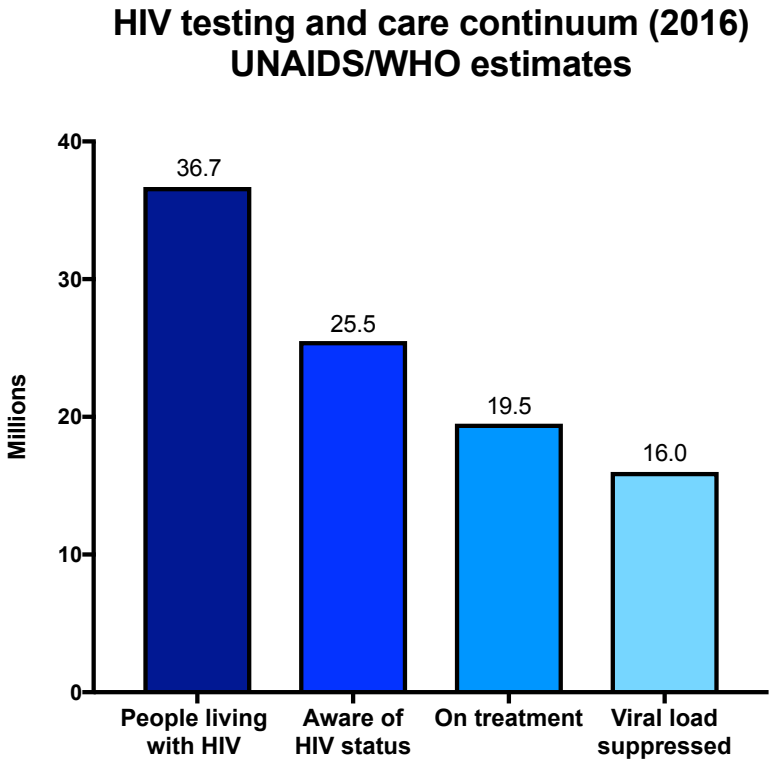

Figure 1.1 2016 Global HIV testing and care continuum

Figure adapted from (7). Number of individuals globally who are living with HIV, aware of their HIV status, on ART, and virally suppressed.

HIV genetic diversity

Multiple independent zoonotic transmissions of simian immunodeficiency virus (SIV) from non-human primates into humans have resulted in HIV type 1 (HIV-1) groups M, N, O and P and HIV type 2 (HIV-2) groups A-H (10). A member of the *Lentivirus* genus of the *Retroviridae* family, HIV is an enveloped virus with 2 copies of a single-stranded, positive sense RNA genome. Retroviruses synthesize double-stranded proviral DNA using the viral RNA as a template. This process results in high mutation and recombination rates because the enzyme reverse transcriptase is low-fidelity, lacks a proof-reading mechanism and can switch between the two template RNA genomes during reverse transcription (11, 12). In combination with high rates of viral replication (13), this process facilitates immune evasion through rapid evolution and high genetic diversity of HIV. Within an infected individual, viral sequences can differ by up to 10% (14).

Globally, HIV-1 group M is responsible for the pandemic and has diversified since the onset of the epidemic which is estimated as around the beginning of the twentieth century (15). Based on the extensive global sequence diversity of the envelope (Env) protein, HIV-1 group M is divided into subtypes or clades A-D, F-H, J and K (16) as well as an increasing number of circulating recombinant forms (CRFs) (17). Env sequences differ by 8-17% within subtypes and 17-35% between subtypes at the amino acid level (14). To be effective, a vaccine will need to account for the substantial diversity of circulating HIV-1 variants to protect against transmission. The challenge this presents is highlighted when considering another highly diverse virus, influenza. Influenza vaccines are administered annually and vary in their effectiveness from year to year (18). It is estimated that the global sequence diversity of influenza within a given year is similar to the diversity of HIV-1 within an infected individual at a single time point (14).

B cells and antibodies

The importance of antibodies in preventing infections by viral pathogens is exemplified by the ability of maternal antibodies to protect neonates against many viral infections (19). Antibodies (Ab) are the primary correlate of protection for most licensed vaccines against viral pathogens, many of them mediating protection through virus neutralization (20). Thus, HIV vaccine research has focused on eliciting Env-specific neutralizing antibody responses. This thesis focuses on the ontogeny and characteristics of HIV-neutralizing antibodies, therefore relevant antibody and B cell biology will be discussed in this section.

Antibodies can be membrane bound as the B cell receptor (BCR) or secreted and are composed of two heavy chains and two light chains that make up 3 functional domains: 2 identical antigen binding domains (Fab) and a "Fragment crystallizable" Fc domain that mediates effector function. Each heavy and light chain consists of variable and constant domains. The variable domain of one heavy and one light chain together make up one antigen binding site and each Ab has two antigen binding sites (**Figure 1.2**). The heavy chain constant region determines the antibody isotype (IgM, IgD, IgG, IgE and IgA) and effector function. IgG is the most abundant antibody isotype in the blood and mediates HIV-specific neutralizing activity in infected individuals (21-23), and is therefore the focus of the studies presented in this thesis.

During B cell development, the heavy and light chain variable domains are generated through a process of somatic recombination or V(D)J recombination. The variable domains of the heavy and light chain each contain three hypervariable loops, called complementarity-determining regions (CDRs), that together form the antigen contact site or paratope that is specific for a particular epitope. The CDR3s span the recombination junctions between gene segments and are the most variable in both sequence and length. The relatively invariant

framework regions (FR) provide the structural support of the antibody (24) (**Figure 1.2**).

Throughout the course of an immune response, antibody affinity increases through a process of affinity maturation in germinal centers where B cells undergo iterative rounds of clonal expansion, somatic hypermutation of variable domains and selection of high affinity mutants (25). Mutations are introduced by the enzyme AID (activation-induced cytidine deaminase) and through repair of AID-induced lesions. AID is preferentially targeted to mutational hotspots through specific sequence motifs (26). Mutations predominantly occur in the CDRs, which contact the antigen, and not in the FRs because they have fewer AID hotspots and mutations could disrupt the Ab structure and are less likely to affect antigen binding (27-29). High affinity B cells that exit the germinal center differentiate into memory B cells and long-lived plasma cells. Memory B cells remain in circulation and are primed to respond to re-exposure to antigen. Long-lived plasma cells home to the bone marrow and sustain high levels of Ab secretion into the blood (25). Memory B cells and plasma cells contribute to long-lived humoral immunity.

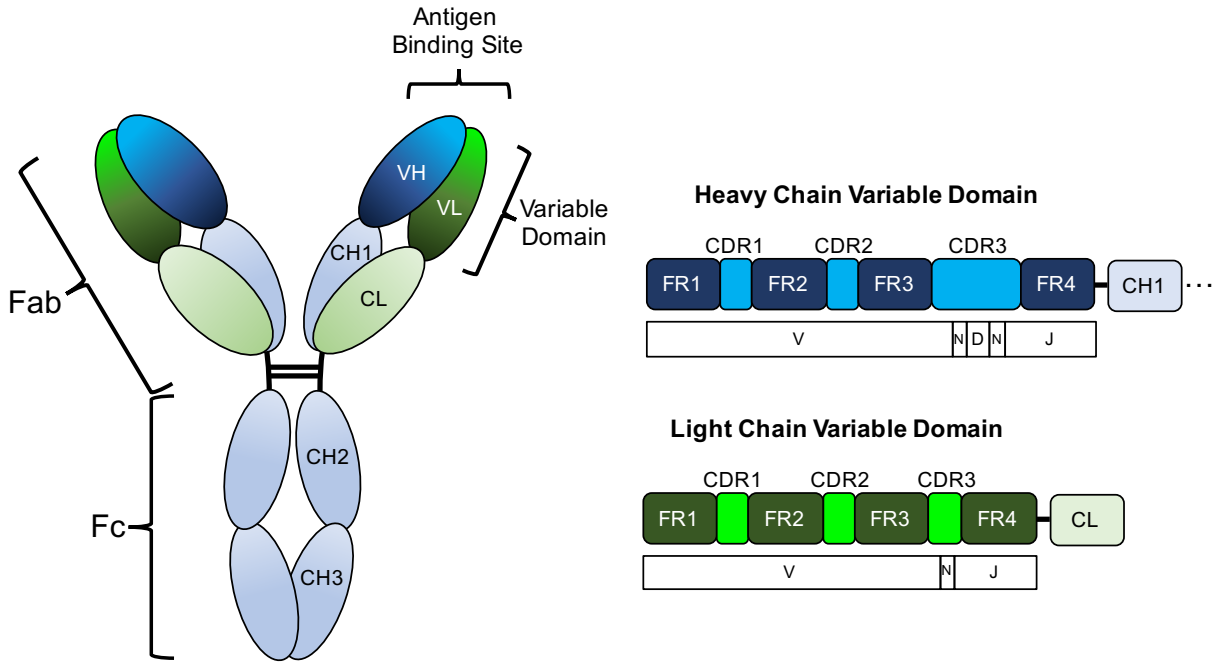

Figure 1.2 Antibody structure

Structure of IgG1. (A) An antibody is composed of two identical heavy (blue) and two identical light chains (green) connected by disulfide bonds. Antibodies have 3 functional domains, two antigen binding domains (Fab) and a "Fragment crystallizable" (Fc) domain. Each heavy chain consists of one variable domain (VH) and three constant domains (CH1, CH2, CH3). Each light chain has a variable (VL) and constant (CL) domain. (B) Heavy and light chain variable domains are generated by V(D)J recombination and contain four framework regions (FR) and three complementarity-determining regions (CDR). The CDRs are hypervariable and form the antigen binding site.

HIV-1 envelope glycoprotein

The envelope (Env) spike embedded in the viral membrane is encoded by *env,* which is one of three genes shared by all retroviruses: *gag, pol* and *env.* Structural proteins encoded by *gag* mediate assembly, packaging and release of viral particles, and *pol* encodes enzymes involved in viral replication. Additionally, HIV-1 has two genes *tat* and *rev* encoding proteins that regulate viral transcription, and four accessory genes *nef, vif, vpr* and *vpu* that promote immune evasion and virus spread (30). Env is the target of neutralizing antibodies, the focus of this thesis, and therefore warrants further discussion.

Envelope is synthesized as a gp160 polyprotein precursor that is cleaved by host cell proteases into gp120 and gp41 subunits. Heterodimers of the gp120 surface subunit and gp41 transmembrane subunit form Env trimers on the surface of the virion (**Figure 1.3A**). Gp120 contains five conserved domains (C1-C5) and five variable regions (V1-V5), and gp41 consists of 6 major domains (**Figure 1.3B**). Env trimers mediate viral entry into target CD4+ T cells, macrophages and monocytes through gp120 binding to the cellular receptor (CD4) and co-receptor (CCR5 or CXCR4) and gp41-promoted fusion of the viral and host cell membranes (30).

The Env trimer is essential for infection of host cells and is, therefore, the target of neutralizing antibodies that prevent virions from infecting host cells. In turn, Env has evolved a number of strategies to evade the human antibody response. The variable regions of gp120 form disulfide-bonded loops (with the exception of V5) and play a role in immune evasion by masking the more conserved regions (31, 32), which can be appreciated on a recently solved structure of the Env trimer (33-35) (**Figure 1.3C**).

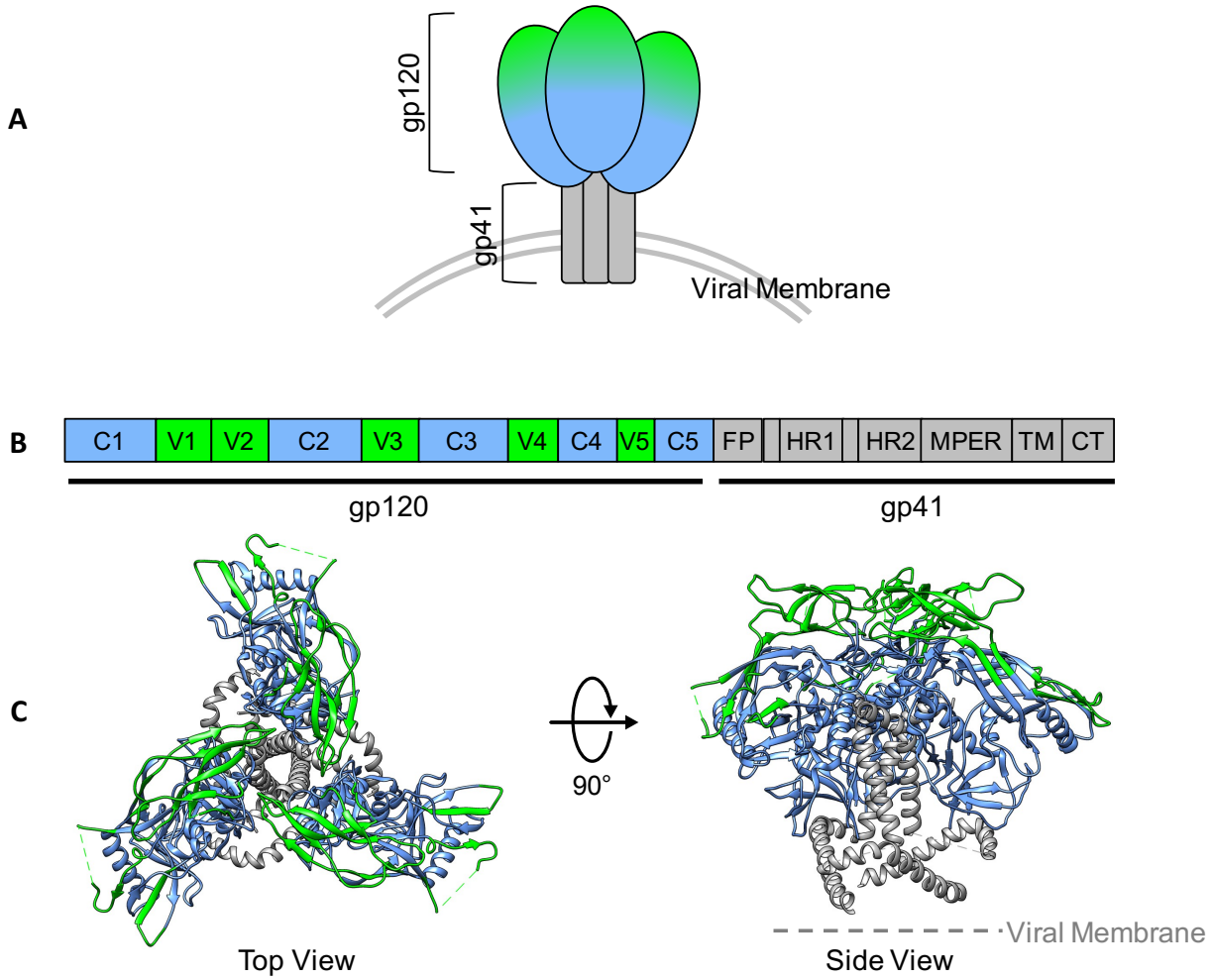

Figure 1.3 HIV-1 envelope

(A) Schematic of the HIV-1 Env spike on the virion surface. Env is a trimer of gp120 (green and blue) and gp41 (gray) heterodimers. (B) Gp120 consists of 5 constant domains (C1-C5) (blue) interspersed with 5 variable domains (V1-V5) (green) and gp41 (gray) contains the fusion peptide (F), heptad repeat 1 and 2 (HR1 and HR2), membrane proximal external region (MPER), transmembrane anchor (TM), and cytoplasmic tail (CT). (C) Structure of the ectodomain of the HIV-1 Env trimeric spike (PDB accession number 4NCO (33)), from a top view (left) and side view (right). Gp120 is shown in blue with variable loops in green and gp41 is in gray.

In addition to the genetic heterogeneity of the variable regions, Env is heavily glycosylated with host-derived glycans that are generally non-immunogenic and shield the underlying protein from immune surveillance (31, 32). Carbohydrates contribute approximately 50% of the molecular weight of the Env trimer with around 90 N-linked glycans per trimer, most of which are found on gp120, each with a median of 25 potential N-linked glycosylation sites (PNGS) (14, 31, 36, 37). PNGS are commonly added, removed or shifted to evade antibody responses (38-43).

Structural characterization of the pre-fusion closed conformation of the Env trimer (33, 34), the target of most neutralizing antibodies, has allowed a greater appreciation of the contribution of sequence variability and glycosylation to Env immune evasion. Conformational masking by variable regions and glycans restricts access to conserved structures such as the receptor binding site (33, 44-47). Modeling glycans onto the trimer structure shows that only ~3% of the protein surface is accessible to antibodies and only ~2% with low sequence variability is accessible (48) (**Figure 1.4A and B**). In contrast, influenza has ~14% of the HA glycoprotein surface accessible to antibodies (48).

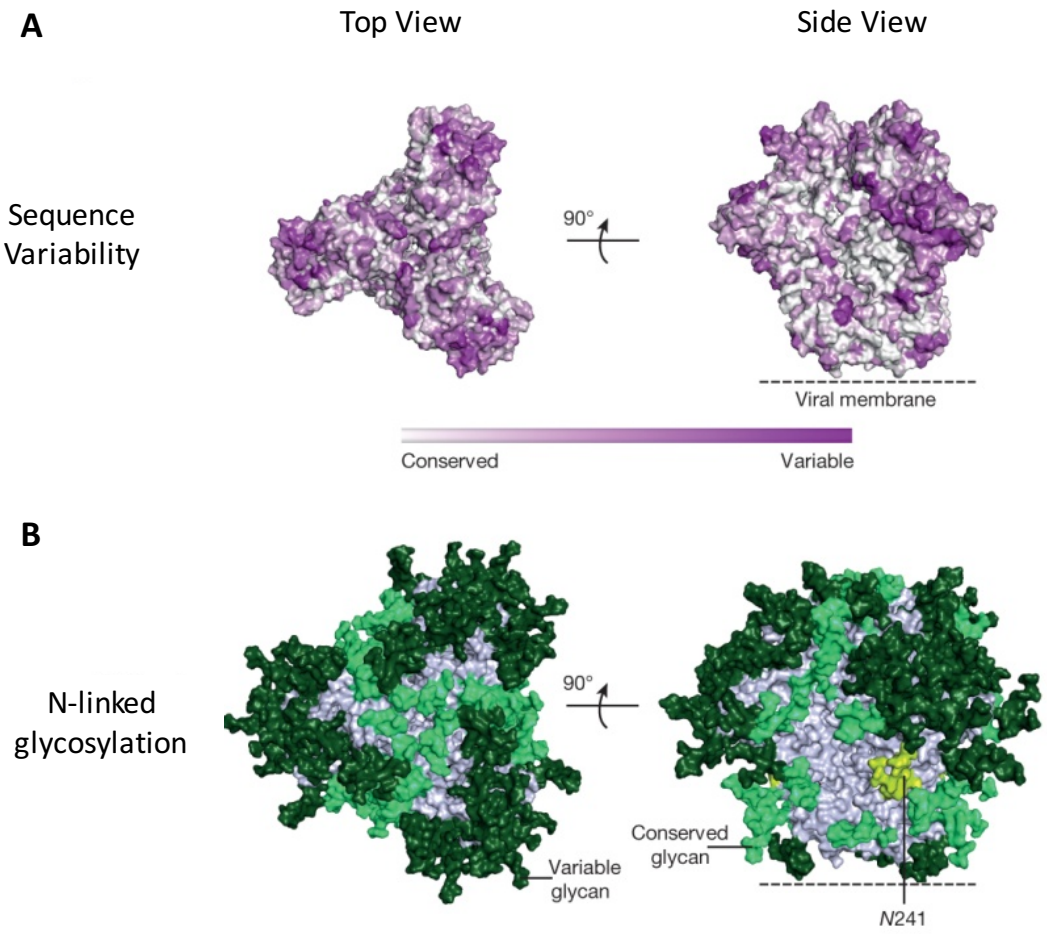

Figure 1.4 HIV-1 envelope sequence variability and glycan shield

Figure modified from (48). (A) Sequence variability of HIV-1 Env based on 3,943 HIV-1 strains. (B) Env N-linked glycans are depicted in light green (greater than 90% conservation across isolates), dark green (variable; less than 90% conservation across isolates) and yellow shows a conserved N-linked glycan not present on this isolate.

Recent studies suggest that most of the glycosylation sites on the Env trimer are occupied (49). The unusually dense glycan clustering on the trimer results in incomplete enzymatic processing in the Golgi creating patches of high mannose (oligomannose) glycan structures in addition to processed complex-type glycans and glycans in intermediate processing states (50). The ability of antibodies to target these glycans will be discussed in a later section; however, microheterogeneity in glycoforms can affect sensitivity to neutralizing antibodies (37, 50, 51).

Impressively, HIV Env has additional ways to thwart antibody recognition. HIV is unique in that each virion only has about 8-14 Env trimers on the surface(52-55), and only require 2-3 functional trimers for viral entry (56, 57). To make another comparison to flu, 450 glycoprotein spikes decorate the virion surface (58). The low density of Env spikes prevents bivalent antibody binding which limits avidity and potency (59). Additionally, the surface of the virion has non-functional forms of Env including as gp120/gp41 monomers and gp41 stumps. The majority of HIV-specific B cells make antibodies to these denatured proteins and only a small fraction produce neutralizing antibodies that bind the functional trimer (60, 61).

These extraordinary immune evasion tactics make the HIV-1 Env glycoprotein a challenging target for neutralizing antibodies.

Antibody development in response to HIV-1 infection

HIV-1 infection is established by a single or limited number of transmitted founder viruses (62, 63) that rapidly evolve into a diverse quasispecies (64). Acute infection is characterized by high viral replication and peak plasma viral load around 3 weeks after transmission with a subsequent decline to a set-point viral load (65). Initial B cell responses develop within weeks following HIV-1 transmission and produce binding antibodies (22). While these early binding antibodies have the potential to mediate Fc-dependent functions such as antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated viral inhibition (ADCVI) and antibody-dependent cell-mediated phagocytosis (ADCP), there is no evidence for selective pressure exerted on Env (62). Binding antibodies precede the development of neutralizing antibody responses, which are detected months after transmission (38, 66-68). In general, neutralizing antibodies prevent host cell infection by inhibiting Env receptor/co-receptor binding or viral fusion with target cells.

Most HIV-infected individuals develop high titers of autologous neutralizing antibodies that target regions of Env with relatively high sequence variation and drive rapid viral evolution and escape (38, 68-71). These nAbs are specific to the infecting, autologous strain and are not cross-reactive with strains isolated from other individuals, heterologous strains. HIV readily escapes strain-specific responses through amino acid substitutions, insertions and deletions and changes in glycosylation in the targeted epitope or in distal regions resulting in conformational masking (38, 69-73). The rapid diversification of Env, ~0.6-1% per year (74, 75), allows the virus to stay one step ahead of the humoral immune response. Most circulating viruses are resistant to contemporaneous nAbs (38, 68, 69, 71, 76). Subsequent nAbs develop that neutralize escape variants resulting in an ongoing race between viral escape and antibody development.

In some HIV-infected individuals, this process drives the development of cross-reactive neutralizing antibodies (77). Viral evolution in response to autologous nAb pressure has been shown to create or expose conserved epitopes through the addition (78, 79) or deletion of glycans (80). Autologous nAb pressure also promotes viral diversification, which drives strain-specific nAbs to recognize multiple immunotypes that emerge due to neutralization escape (80-89) (**Figure 1.5**). As a result, some nAbs are able to accommodate variability surrounding accessible conserved sites on the Env trimer leading to the ability to neutralize a broad range of viral strains. Broadly neutralizing antibodies (bnAbs) demonstrate neutralization of diverse circulating strains of HIV from multiple clades (cross-clade) that are difficult-to-neutralize (tier 2 and 3) (90, 91). While there isn't a defined set of criteria to determine if an antibody is "broadly neutralizing," for the purposes of this thesis breadth is defined as the ability of a mAb to neutralize >30% of tier 2 viruses in a cross-clade panel. Induction of broadly neutralizing antibodies is a major goal of HIV-1 vaccine development.

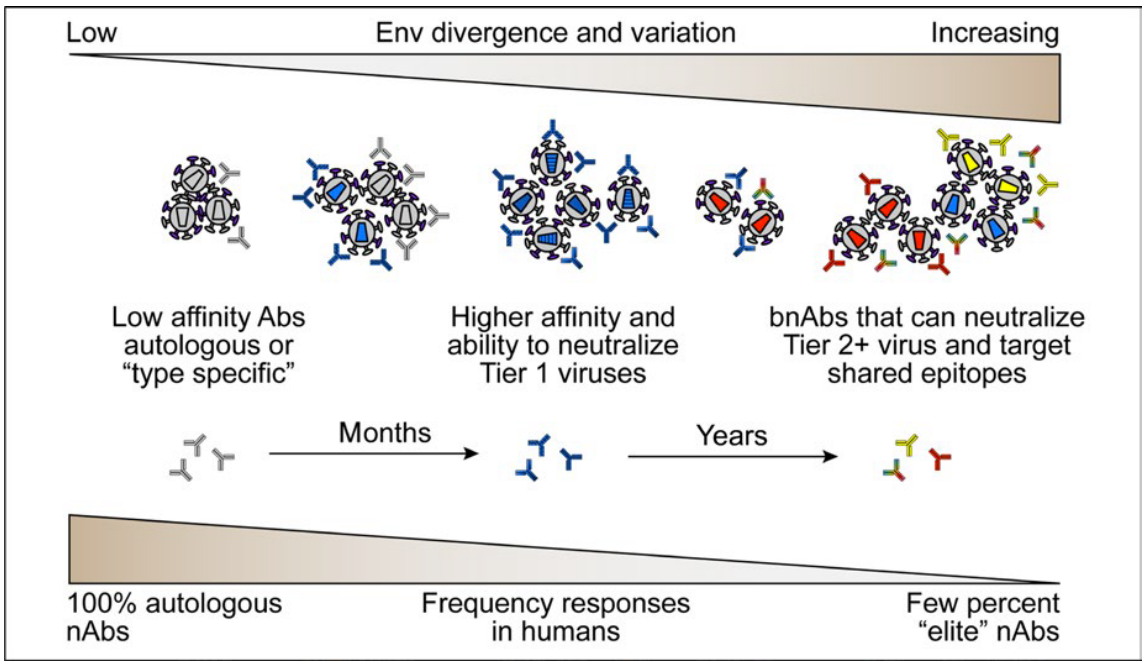

Figure 1.5 Neutralizing antibody development in response to HIV-1 infection

Figure from (92). Most HIV-infected individuals develop autologous or "type specific" neutralizing antibodies. As the virus escapes autologous nAbs, Env sequence diversity increases, and the escape variants are targeted by developing nAbs. This ongoing race between virus and antibody evolution leads to the development of broadly neutralizing antibodies in a subset of individuals.

HIV broadly neutralizing antibodies

Screening of large cohorts has revealed that many HIV-infected individuals develop some level of cross-neutralizing activity and that plasma neutralization breadth is a continuum (93-103). There are a range of estimates of breadth, though a recent study by Hraber et al. shows that approximately 50% of chronically HIV-1 infected individuals produce plasma antibody responses capable of neutralizing 50% of isolates from a panel of cross-clade global isolates after ~2-4 years of infection (93).

Factors associated with the development of bnAb responses

A number of viral and host factors are associated with the development of broadly neutralizing antibody responses to HIV infection. High levels of prolonged antigenic stimulation seem to be necessary as high viral load and longer duration of infection are associated with bnAb development (94-96, 98, 99, 101, 102, 104). Low CD4+ T cell counts, which indicate disease progression, have also been associated with bnAb development (94, 96, 104), and may reflect prolonged antigen exposure or a decrease in negative regulators of auto- or poly-reactive B cell development (95). As described in the previous section, the interplay between virus and antibody evolution shapes bnAb development (105), and viral diversity is associated with bnAb evolution (80-84, 95, 101) . Viral diversification as a driver of bnAbs is also supported by the setting of superinfection where individuals who are sequentially infected with distinct HIV strains develop broader responses (81, 106, 107). Antigen load, duration of virus exposure and viral diversity are closely linked, though a recent study demonstrated that they independently drive bnAb development (95). B cell responses require CD4+ T cell help in germinal centers, thus the quality of CD4+ T cell responses (108), highly functional CD4+ T-follicular helper cells (109-111), and enhanced germinal center activity (112, 113) are also associated with bnAb development. Other factors that influence bnAb responses include host genotype (94), HIV subtype (94, 95) and ethnicity (95). Many bnAbs have autoreactive (bind specific self-antigen) and/or polyreactive (bind a variety of dissimilar self- and non-self antigens) properties. A recent study suggests that immune perturbations associated with HIV-infection contribute to bnAb development as individuals who make bnAbs have a higher frequency of blood autoantibodies and a lower frequency of regulatory CD4+ T cells (111).

Isolation of bnAbs from HIV-infected adults

The development of new techniques for identifying HIV-1-specific single B cells and antibody cloning has allowed for the isolation of a number of bnAbs from adults (114-118). First-generation bnAbs were isolated before 2009 using B cell immortalization (119) and phage display (120, 121) technologies. Second-generation bnAbs with greater breadth and potency were first isolated by limiting-dilution B cell culture and subsequent high-throughput screening of culture supernatants for neutralizing activity (122). Many additional bnAbs have been isolated using this method (84, 85, 89, 123-128). B cell culture paired with functional screening doesn't require prior knowledge about the epitope specificity of the plasma response, thus it is a useful tool in cases where plasma mapping studies suggest the response is polyclonal. A disadvantage of this method is that it requires previously frozen B cells to survive in culture under conditions that stimulate proliferation and antibody secretion: alas, there is a high attrition rate (129).

Another method commonly employed to isolate bnAbs is the use of targeted portions of Env as bait to perform single-cell sorting of B cells using flow cytometry (51, 82, 85, 87, 130-134). This method doesn't require long-term B cell survival. However, the specificity of isolated antibodies is highly dependent on the chosen bait. For instance, the resurfaced Env gp120 subunit (RSC3), which presents the conserved receptor binding site, was used to isolate bnAbs targeting the CD4-binding site (82, 87, 130, 135-138).

Surface-expressed Env was successfully used to identify HIV-specific B cells expressing bnAbs (132). Soluble, native-like Env trimers are thought to expose most epitope targets of bnAbs and have been utilized as bait to identify bnAbs targeting a variety of epitopes (139-143), though these trimers do not contain a gp41 bnAb target, the membrane proximal external region (MPER).

In addition to the development of methods to identify HIV-specific B cells, another key advance was that the antibody variable regions from B cells of interest can be amplified and sequenced using primer sets that provide coverage of most known variable region genes and are robust to SHM (84, 131, 144).

Epitope targets of bnAbs

Characterization of isolated bNAbs has provided important insights for vaccine development such as identifying conserved sites of vulnerability on Env. bnAbs isolated to date target six regions on Env: the CD4-binding site (CD4bs) (82, 87, 121, 127, 130, 131), gp120 V1V2-glycan region (84, 89, 122, 124), V3-glycan region (51, 85, 123, 133, 134, 139, 143, 145), gp120-gp41 interface (125), gp41 fusion domain (140, 141), and gp41 membrane proximal external region (MPER) (126, 146, 147)(**Figure 1.6**).

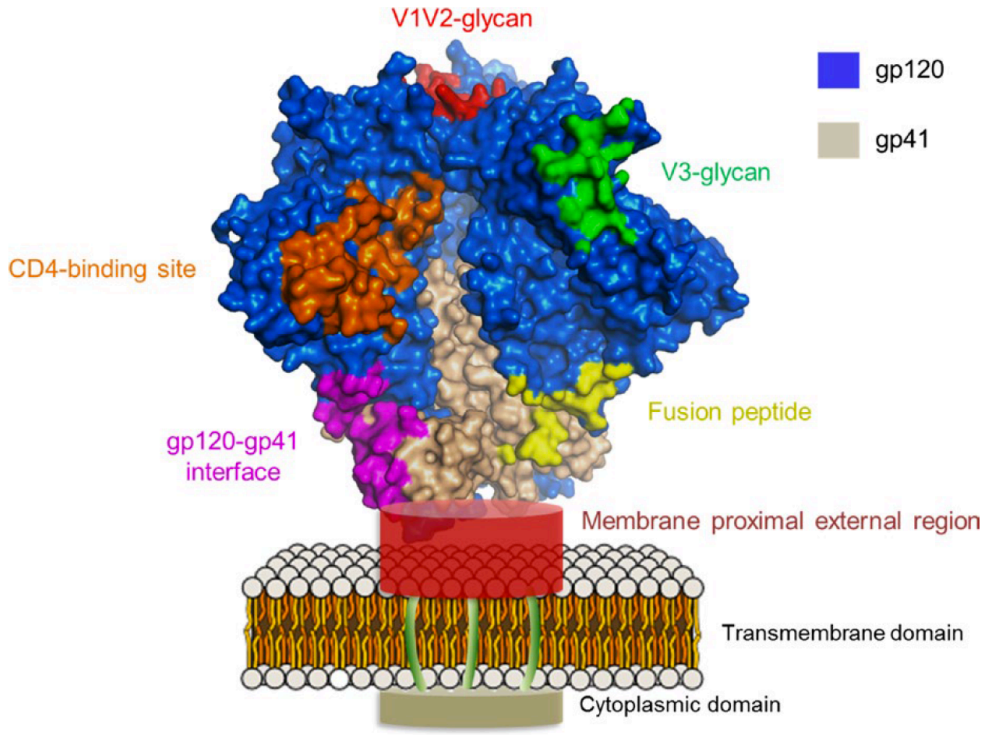

Figure 1.6 Epitope targets of bnAbs on the HIV Env trimer

Figure from (148). gp120 is shown in blue and gp41 in beige. bnAbs target 6 conserved sites of vulnerability: the CD4bs (orange), V1/V2 glycan region (red), V3 glycan region (green), gp120-gp41 interface (pink), gp41 fusion peptide (yellow), and gp41 membrane proximal external region (red).

Many bnAbs are able to counter the glycan shield evasion strategy of HIV Env. The Env glycoprotein can be distinguished as non-self by incorporation of glycans into glycopeptide targets; V1/V2-glycan, V3-glycan and gp12-gp41 interface bnAbs bind combinations of glycans and nearby protein (149-152). Additionally, the dense glycan clustering on Env results in incomplete cellular processing and patches of high mannose glycans that are distinct from host glycans and allow the immune system to recognize them as non-self (36, 49, 50).

Neutralization breadth can be mediated by predominant monoclonal responses targeting a single specificity, which has been described for plasma responses in a number of HIV-infected individuals (71, 94, 96, 99, 153, 154) as well as for isolated bnAbs that recapitulate plasma breadth and/or match the specificity of the dominant plasma response (82, 84, 85, 89, 122, 123, 130, 131, 133, 134). Though recently there is a greater appreciation for polyclonal responses, which have been suggested by plasma mapping studies in a subset of cases where breadth develops (80, 94, 96, 154-157), and through mAb isolation (21, 88, 125, 132, 158).

Protection afforded by bnAbs

There is relatively little evidence that nAbs and bnAbs restrict viral replication or impact disease progression. Neutralizing antibodies that develop in HIV-infected individuals exert selective pressure on the virus as discussed above, and studies have shown that bnAbs cause extinction of susceptible virus, but HIV rapidly escapes (82, 88, 159). One study demonstrated concomitant viral rebound and reduction in nAbs when B cells were depleted suggesting a role for nAbs in viral control (160). Additionally, a recent study implicated bnAbs in contributing to HIV control in an elite controller (143). Nevertheless, in most cases, nAbs and bnAbs do not appear to impact disease progression in infected individuals (101, 103, 104, 161). This is not surprising considering that many of the factors associated with the development of bnAbs are associated with disease progression.

When used as passive therapy in HIV-infected humans, bnAbs transiently reduce viremia with viral rebound associated with the emergence of escape variants (162-164). This highlights the remarkable ability of HIV to rapidly evolve and evade nAb responses. Interestingly, bnAbs have been shown to augment subsequent antibody development (165).

The promise of protection by bnAbs when present prior to virus exposure comes from animal models. Several studies in non-human primate models of infection have demonstrated that bnAbs protect against intravenous, intrarectal and intravaginal virus challenges at concentrations that are achievable through vaccination (92, 166). bnAbs can also clear infection shortly after challenge (167). Whether or not bnAbs can protect humans from HIV infection is currently being tested in an important proof-of-concept trial (168).

The ability of bnAbs to prevent transmission in animal models has added momentum to efforts to induce bnAbs by vaccination.

Unusual features of bnAbs and obstacles to bnAb vaccine elicitation

Determining the number of broadly neutralizing antibodies that have been isolated from HIV-infected adults is difficult due to a lack of a clear definition of what constitutes a broad antibody, differences in virus panels used to screen isolated nAbs, and the isolation of many clonally related bnAbs from some individuals. At least 50 bnAbs have been isolated (114-116, 148, 169, 170) and all characterized bnAbs have at least one unusual feature including extensive somatic hypermutation (SHM) (171), unusually long heavy chain CDR3 loops (170), and auto/polyreactivity (172, 173) that may present significant obstacles to bnAb induction by immunization (173). Table 1.1 summarizes unusual features of a representative set of adult-derived bnAbs.

Table 1.1 Unusual features of representative adult-derived bnAbs

Yellow highlighting indicates an unusual feature. NR = not reported
*poly/autoreactivity found by (172)

Epitope target	mAb ID	VH % SHM (nt)	CDRH3 AA length	Auto/poly reactivity	Reference
CD4bs	VRC01	32	12	Yes*	(130)
	3BNC117	26	10	Yes	(132)
	CH103	17	13	Yes	(82)
	8ANC131	26	16	Yes	(131)
V1V2-glycan	PG9	13	28	Yes*	(122)
	PG16	13	28	No	(122)
	PGT145	18	31	NR	(123)
	CAP256-VRC26	14	35	No	(84)
	PGDM1400	24	32	No	(139)
V3-glycan	PGT121	20	24	No	(123)
	PGT128	20	19	Yes	(123)
	PGT135	19	18	NR	(123)
	10-1074	17	24	NR	(51)
gp120-gp41 interface	PGT151	20	26	No	(128, 149)
	35O22	35	14	Yes	(125)
	8ANC195	28	20	NR	(131, 174)
Fusion Peptide	N123-VRC34	15	13	NR	(141)
MPER	10E8	21	20	Yes*	(126)

In the context of vaccination, SHM is restricted to about 8% (175). Of the dozens of bnAbs that have been described, all but three have extensive somatic hypermutation, which is thought to be a result of iterative rounds of SHM and affinity maturation in response to viral escape over years of infection (171). The resulting high degree of mutations as well as unusual mutations in the conserved framework regions (176) and rare insertions and deletions (177) are necessary for the neutralization breadth and potency of many of these antibodies (82, 84, 131, 150, 176-180). The three known bnAbs with heavy chain variable region SHM <10% each have other unusual features: the MPER-directed Abs M66.6 (181) and 4E10 (172, 182) are

autoreactive, and the V3-glycan directed DH270 lineage required a rare AID mutation that look several years to acquire (85). Most bnAbs are enriched with improbable AID mutations that are important for neutralization breadth (183). Recently characterized V3-glycan bnAbs have the lowest levels of SHM among adult bnAbs and still are greater than 10% (133). There are also developmental intermediates of a V3-glycan directed bnAb with lower SHM, but these require rare indels for activity (179). V1V2-glycan targeting CAP56-VRC26 lineage Abs with lower SHM require unusually long CDRH3 loops (84).

Another unusual feature is that many bnAbs share is long heavy chain CDR3 loops ≥24 amino acids (84, 89, 122-124, 128, 136, 149, 184), whereas the average CDRH3 length in human B cells is around 13-16 residues (185-187). Naïve B cells with very long CDRH3 loops of ≥28 amino acids are rare, only ~0.4% of circulating naïve B cells (188). Some bnAbs use such long CDRH3s to reach the CD4bs (82). V1V2-glycan and V3-glycan bnAbs require long CDRH3s to penetrate the glycan shield and interact with the underlying gp120 protein (150-152), and MPER-directed 10E8 has a long CDRH3 loop that reaches conserved hydrophobic residues on gp41 (126). Interestingly, the antibody repertoire of cows contains an abundance of antibodies with extremely long CDRH3s, and bnAbs were readily induced by vaccination with Env trimer (189). In humans, antibodies with extremely long CDR3s are more likely to be autoreactive (190). Many bnAbs exhibit autoreactivity (172, 173, 191), and tolerance mechanisms limit the development of poly/autoreactive B cells, which has led some to suggest that transient immune modulation may be necessary to induce such antibodies by vaccination (173, 192-194).

Ontogeny of bnAbs in HIV-infected adults

Eight studies have carefully examined the complex developmental pathways of CD4bs, V1V2- and V3-glycan, and MPER-directed bnAbs in HIV-infected adults (82, 84, 85, 87-89,

127, 133, 191). These studies revealed the importance of virus-antibody co-evolution in driving the development of breadth. A common theme uncovered is that B cell lineages co-operate where strain-specific lineages drive viral evolution leading to the generation of viral escape variants that are targeted by bnAb lineages (88, 133). This has led to the concept of B cell lineage immunogen design. The goal is to identify sequential immunogens that stimulate the naïve precursors and lineage intermediates of bnAbs to initiate appropriate B cell lineages and guide affinity maturation toward the development of neutralization breadth (**Figure 1.7**) (148). In some cases, this approach is complicated by the observation that the inferred naïve precursors (also referred to as UCA = unmutated common ancestor) of many bnAbs lack detectable binding to recombinant HIV envelope and thus require the design of germline-targeting immunogens for vaccines (85, 134, 178, 179, 195, 196).

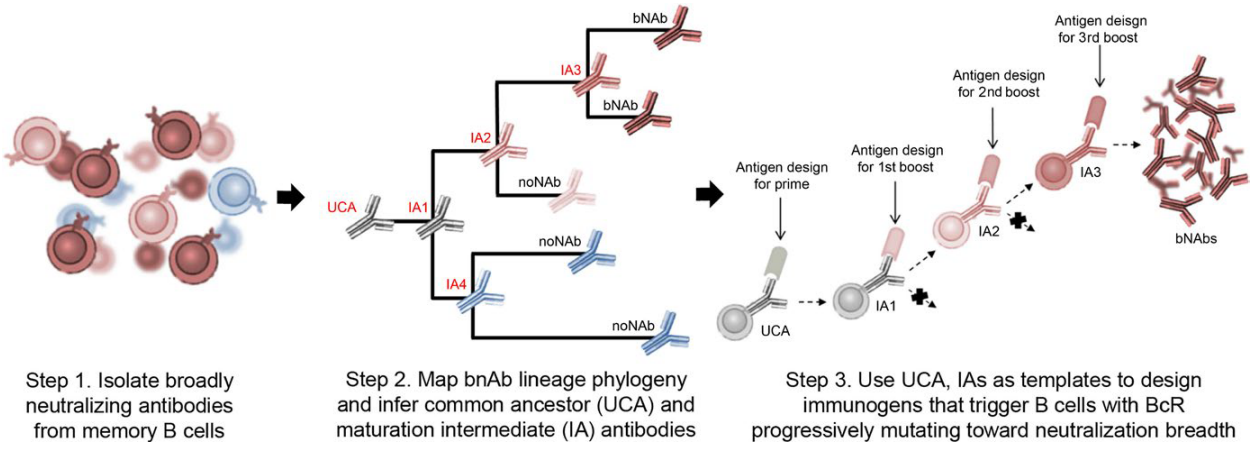

Figure 1.7 B cell lineage immunogen design

Figure from (148).

Immunization strategies that mimic lineage pathways defined for bnAbs have recently been attempted in animal models. Using this approach, cross-clade nAbs were elicited in knock-in mice carrying the inferred germline precursors of bnAbs (197) and sporadically in rabbits and non-human primates (198). The availability of soluble, native-like Env trimers has also led to progress in animal models towards eliciting autologous tier 2 neutralizing antibodies in response to immunization. The trimer used for many of the studies is based on an infant-derived transmitted virus, BG505 (199, 200). To date, there has not been success in eliciting bnAbs in humans using vaccine candidates.

V3-glycan targeting bnAbs

Chapters II and III focus on an antibody targeting the V3-glycan epitope, therefore bnAbs targeting this site will be discussed further.

The V3-glycan region of Env is commonly targeted in different individuals (94, 201), and bnAbs that recognize this epitope have been isolated from 12 donors (Table 1.2) (51, 85, 119, 123, 133, 134, 143, 202) suggesting this may be a good target for vaccines.

The V3-glycan bnAb epitope is centered around the N332 glycan, commonly called the N332-supersite (150), with variable bnAb dependence on surrounding glycans and amino acid residues in the conserved ^{324}GDIRQAH^{330} motif near the C-terminus of the V3 loop (202).

The N332-glycan is part of the "high-mannose patch" of the Env trimer (49), and these glycans, particularly the N332 and N301 glycans, act as a shield for the ^{324}GDIR^{327} peptide at base of gp120 V3 loop, which is part of the CCR5 co-receptor binding site (202). bnAbs that are able to target this conserved site use long CDRH3 loops to both bind the glycans and reach through the glycan shield and contact the peptide (123, 150, 152). All of the isolated bnAbs take

distinct structural approaches to attack this site with slightly different angles of approach and variable dependence on surrounding glycans and amino acid residues (116, 143, 196, 202, 203), which highlights the ability of the human immune system to recognize this conserved epitope as non-self. Impressively, some of these bnAbs exhibit promiscuous glycan recognition with the ability to bind both high-mannose and complex N-type glycans (51) while others are able to neutralize when the N332 glycan site is shifted to N334 (204). The mechanism of neutralization for V3-glycan bnAbs, though not precisely known, is thought to be allosteric inhibition of CD4 binding (205).

Overall, the V3-glycan class of bnAbs is a focus of vaccine development because these responses are commonly elicited, the bnAbs have diverse V(D)J gene usage, and they contain somewhat lower SHM compared to bnAbs directed to other epitopes. Progress has been made toward characterizing the development of V3-glycan bnAbs in adults (85, 133, 179). As mentioned above these studies revealed details about antibody-virus co-evolution that informs immunization strategies. For instance, Bonsignori et al. demonstrated a correlation between Env V1 loop length and neutralization breadth and suggest that immunization strategies should include Env variants with progressively longer V1 loops (85). In many cases, vaccine strategies are complicated by the observation that the naïve BCR or UCA of these antibodies doesn't bind recombinant Env monomer or trimer (85, 133, 134). Alam et al. synthesized a germline-targeting construct for some of these bnAbs (134). Despite this impressive progress, all of these adult-derived V3-glycan bnAbs have at least one if not several unusual features including high SHM, rare AID mutations, insertions/deletion, poly/autoreactivity and a lack of UCA binding to Env that may present significant obstacles to their elicitation (Table 1.2).

Table 1.2 V3-glycan bnAb characteristics

V3-glycan bnAbs have been isolated from 12 adult donors. Clonal families of antibodies are grouped and alternately shaded. Empty cells in the UCA-Env Binding and Poly-Autoreactive columns indicate that these were not determined. *sequence not available

bnAb	Donor	Time pi	Isolation Method	Heavy Chain VDJ	CDRH3 AA length	VH % SHM (nt)	Light Chain VJ	VL % SHM (nt)	Insertions/ Deletions (nt)	UCA-Env Binding	Poly-Auto-reactive	References
QA013.2	QA013	6.2 years	Culture	V3-7 D6-6 J5	21	21.2	LV1-40 LJ3	11.5				(206)
VRC41.02	CH765	unknown	Bait	V4-39 D3-3 J4-1	21	19.6	KV3-20 KJ4	13.1	none		No	(134)
VRC41.01	CH765	unknown	Bait	V4-39 D3-3 J4-1	21	21	KV3-20 KJ4	13.8	none		Yes	(134)
DH563	CH765	unknown	Bait	V4-39 D3-3 J4-1	21	22.3	KV3-20 KJ4	12.1	none		Weak	(134)
DH270.1	CH848	205 weeks	Culture	V1-2 D3-22 J4	20	5.6	LV2-23 LJ2	5.2	none; rare AID mutation	No		(85)
DH270.5	CH848	232 weeks	Bait	V1-2 D3-22 J4	20	11.1	LV2-23 LJ2	11.5	none; rare AID mutation	No		(85)
DH270.6	CH848	234 weeks	Bait	V1-2 D3-22 J4	20	12.9	LV2-23 LJ2	7.6	none; rare AID mutation	No		(85)
BG18	EB354	~28 years	Bait	V4-4*	21	21.2	LV3-25*	17.7	*			(143)
BG1	EB354	~28 years	Bait	V3-49*	22	27.2	KV1-49*	19.9	*			(143)
NC37	EB354	~24 years	Bait	V1-46*	19	28.8	KV3-20*	27.9	*			(143)
PCDN-27B	PC76	27 months	Bait	V4-34 D3-3 J5	22	10.5	KV3-20 KJ1	7.8	none		No	(133)
PCDN-27D	PC76	27 months	Bait	V4-34 D3-3 J5	22	11.2	KV3-20 KJ1	9.2	none		Weak	(133)
PCDN-33A	PC76	33 months	Bait	V4-34 D3-3 J5	22	13	KV3-20 KJ1	11.3	none	Minimal	No	(133)
PCDN-38A	PC76	38 months	Bait	V4-34 D3-3 J5	22	14.4	KV3-20 KJ1	11.3	none		No	(133)
PCDN-38B	PC76	38 months	Bait	V4-34 D3-3 J5	22	17.2	KV3-20 KJ1	12.1	none		No	(133)
PGDM11	IAVI14	not stated	Bait	V3-11 J3	21	20.6	KV2-24 KJ1	14.6	VH -6 CDRH1			(202)
PGDM14	IAVI14	not stated	Bait	V3-11 J3	21	11.4	KV2-24 KJ1	10.9	VH +3 CDRH1 and -15 FR1			(202)
PGDM21	IAVI82	not stated	Bait	V4-34 J6	20	22.5	KV3-20 KJ5	14.9	VH +12 CDRH2			(202)
PGDM31	IAVI26	not stated	Bait	V1-8 J6	24	15.6	KV3-20 KJ3	17.7	none			(202)

bnAb	Donor	Time pi	Isolation Method	Heavy Chain VDJ	CDRH3 AA length	VH % SHM (nt)	Light Chain VJ	VL % SHM (nt)	Insertions/ Deletions (nt)	UCA-Env Binding	Poly-Auto-reactive	References
10-1074	IAVI17	not stated	Bait	V4-59 D3-3 J6	24	17.2	LV3-21 LJ3	17.4	VL -12 FWR1 and +9 FWR3	No		(51)
PGT121	IAVI17	>3 years	Culture	V4-59 D3-3 J6	24	19.65	LV3-21 LJ3	19.0	VL -21 FR1 and +9 FR3		No	(123, 172, 179)
PGT122	IAVI17	>3 years	Culture	V4-59 D3-3 J6	24	19.65	LV3-21 LJ3	17.4	VL -21 FR1 and +9 FR3			(123)
PGT123	IAVI17	>3 years	Culture	V4-59 D3-3 J6	24	22.46	LV3-21 JLJ3	23.3	VL -21 FR1 and +9 FR3			(123)
PGT125	IAVI36	>3 years	Culture	V4-39 D3-16 J5	19	19.79	LV2-8 LJ2or3	15.8	VH +18 CDRH2 and VL -15 CDRL1	No	Yes	(123, 172, 207)
PGT126	IAVI36	>3 years	Culture	V4-39 D3-16 J5	19	17.71	LV2-8 LJ2or3	9.2	VH +18 CDRH2 and VL -15 CDRL1			(123)
PGT127	IAVI36	>3 years	Culture	V4-39 D3-16 J5	19	15.81	LJ2-8 LJ2or3	9.2	VH +18 CDRH2 and VL -15 CDRL1			(123)
PGT128	IAVI36	>3 years	Culture	V4-39 D3-16 J5	19	20.27	LV2-8 LJ2or3	9.2	VH +18 CDRH2 and VL -15 CDRL1		Yes	(123, 172, 207)
PGT130	IAVI36	>3 years	Culture	V4-39 D3-16 J5	19	20.62	LV2-8 LJ2or3	11.8	none			(123)
PGT131	IAVI36	>3 years	Culture	V4-39 D3-16 J5	19	21.31	LV2-8 LJ2or3	12.8	none			(123)
PGT135	IAVI39	>3 years	Culture	V4-39 D3-9 J5	18	18.9	KV3-15 KJ1	17.6	VH +15 CDRH1			(123)
PGT136	IAVI39	>3 years	Culture	V4-39 D3-9 J5	18	18.21	KV3-15 KJ1	13.6	VH +15 CDRH1 and +3 CDRH2			(123)
PGT137	IAVI39	>3 years	Culture	V4-39 D3-9 J5	18	23.1	KV3-15 KJ1	12.2	VH +15 CDRH1			(123)
2G12		not stated	Hybridoma	V3-21 D1-26 J3	16	21.2	KV1-5 KJ1	14.3	none		No	(119, 208)

Pediatric broadly neutralizing antibody responses

Mother-to-child transmission of HIV occurs in ~30-45% of infants in the absence of intervention (209). Untreated vertically infected infants progress to disease faster than adults, with rapidly declining CD4 T cells and recurrent infections, failure to thrive, and delayed neurological development (210). Over 50% of HIV-infected infants die by 2 years of age (211), whereas the median survival time in untreated adult infection is approximately 11 years (212).

A study by Richardson et al. compared viral loads between ART-naïve Kenyan adults and infants and found that HIV-infected infants reach higher peak and set-point viral loads by about one log (213). Many other studies have also described high viral loads in vertically infected children, which is predictive of disease progression (214-224), and the decline to set-point is slower in some children (217, 221, 225-227). Furthermore, earlier infection is associated with higher viral loads suggesting that there may be improved viral control by the more mature immune system (213, 214, 228). A number of factors likely contribute to high viral loads and failure of the infant immune system to control viral replication, including an abundance of target CD4+ T cells (229, 230) and inadequate innate and adaptive immunity, reviewed in (210, 228, 231).

The infant immune system must transition from immunological tolerance *in utero* to responding to pathogens after birth. There is an age-dependent increase in maturation and development of the immune system, particularly in the ability to effectively mount responses to intracellular pathogens such as HIV (228, 232-237). Because this thesis characterizes infant HIV neutralizing antibodies, differences in infant and adult humoral immunity will be discussed briefly.

In general, B cell responses of infants (≤ 12 months of age) tend to be delayed in onset with lower peak levels of antibody and shorter antibody persistence. Germinal center reactions are limited and naïve B cells and helper CD4+ T cells in infants have lower levels of surface co-stimulatory molecules resulting in less help for B cell function. Thus, affinity maturation is relatively limited under 4-6 months of age (234). Size and numbers of germinal centers and immunoglobulin somatic mutation increase in an age-dependent manner (238-240). IgG production (IgG1 and IgG3) only reaches about 60% of adult levels by one year of age (234, 235). Another important consideration in infants is the presence of passively acquired maternal antibodies, which gradually wane over a period of about 6 months after birth (200, 241-244). Maternal antibodies play a critical role protecting infants against infection by pathogens the mother has immunity against. These passively acquired antibodies have also been shown to inhibit seroconversion in response to a number of vaccines, reviewed in (245-247).

In the setting of HIV infection, the presence of maternal antibodies may play a different role in influencing infant antibody development against the virus. While maternal neutralizing antibody responses are not associated with protection against vertical transmission of HIV (248), they do exert selective pressure on the virus and variants that escape maternal nAbs are transmitted to infants (200, 249). Escape from maternal nAbs by vertically transmitted variants is mediated by conformational masking of targeted sites (250), therefore maternal antibodies influence the epitopes exposed on transmitted Envs. Passively acquired HIV-specific maternal antibodies may augment developing infant antibody responses. This is suggested by studies of infant macaques where passively administered antibodies enhance endogenous neutralizing antibody responses (251, 252). This effect may not be unique to infants as augmentation of de novo neutralizing antibody responses occurred in juvenile macaques (253) and with bnAb

passive administration to HIV-infected human adults (165). The effect is thought to be mediated by immune complexes formed by passive antibody and HIV that activate germinal center reactions (254-256). Recently, immune complexes enriched with sialylated IgGs were shown to promote affinity maturation of flu-specific antibodies (257). Interestingly, pregnancy is known to be associated with an increase in sialylation of IgG Fc N-glycans (258-260) and these sialylated maternal IgGs are transported across the placenta to fetal circulation (261).

While adult Env-specific antibody responses have been extensively characterized, relatively little is known about infant antibody responses. Studying the development of infant HIV-specific antibody responses is challenging because of the presence of maternal HIV-specific antibodies in infant circulation. To get around this, Pollack et al. isolated lymphocytes from infants at longitudinal time-points and found that gp160-specific antibodies appeared around 6 months of age followed by gp120- and gp41-directed antibodies (262). This is distinct from the specificities of early Env-specific antibodies from HIV-infected adults, which first target gp41 (22). Another study demonstrated waning maternal antibodies and increasing infant antibodies at a median of 6 months (263).

Despite the limitations of infant humoral responses and the seeming delay in Env-specific antibody development, our lab was the first to identify pediatric broadly neutralizing antibody responses (23). Goo et al. screened plasma samples from 28 infants between 1 and 2.5 years of age and identified cross-clade neutralizing antibodies in 20/28 infants at a median of 22 months post-infection. Seven of the infants developed neutralizing activity against tier 2 viruses in four different clades at a median of 20 months, and these responses were defined as broadly neutralizing. Remarkably four of the infants developed broadly neutralizing plasma activity within a year of infection, including infant BF520 who mounted a broad response by 11.2

months post-infection. Infant BG505 developed one of the most impressive responses, neutralizing 91% of viruses in an expanded panel of tier 2 viruses. The kinetics of the bnAb responses for two infants who are the focus of this thesis, BF520 and BG505, are shown in **Figure 1.8**. Interestingly, there was a significant correlation between passively acquired nAb titers and *de novo* infant responses when infant BG505 was excluded. Goo et al. suggest this could indicate shared antigenic features between maternal and infant viruses. It may also suggest that maternal antibodies are modulating infant responses, perhaps similar to what is observed when HIV Abs are passively administered to infant macaques (251, 252). Factors that correlated with the development of neutralization breadth were set-point viral load and Env-specific IgG. This study used two different methods to map the epitope specificities of infant responses and did not identify dominant responses targeting known bnAb epitopes, which led the authors to suggest that infant responses are mediated by bnAbs directed to novel epitopes or perhaps that infant responses are mediated by polyclonal antibodies targeting multiple epitopes.

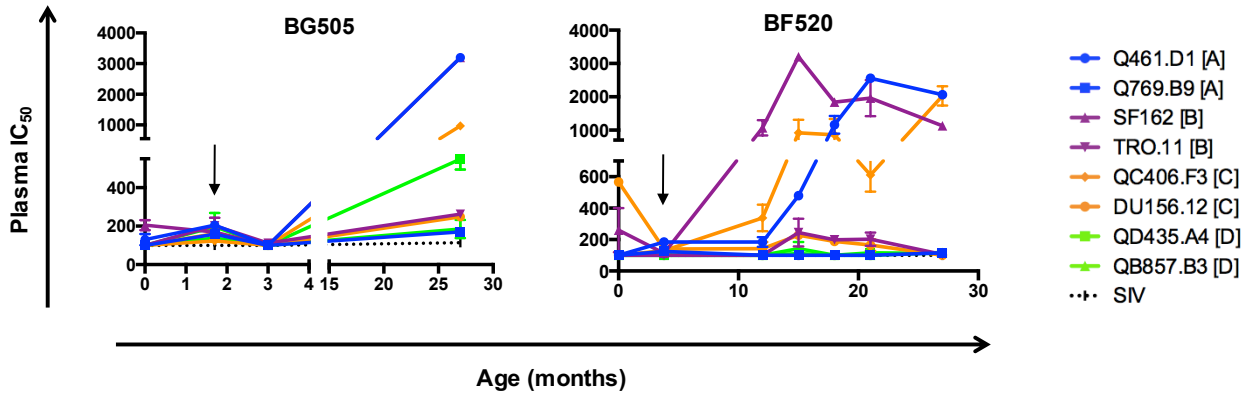

Figure 1.8 Kinetics of bnAb development in infants BG505 and BF520

Figure from (23). Neutralization of eight viruses, two from each clade A-D, by longitudinal plasma from infant BG505 (left) and infant BF520 (right). Black arrows denote when HIV-1 was first detected.

The identification of early broadly neutralizing antibody responses in infants is exciting because it suggests that bnAb responses can develop over a shorter period of time than previously thought based on studies of adult bnAbs (23).

Interestingly, the transmitted Env from infant BG505 has been extensively studied. It first came in to the spotlight when a group identified it as an Env of interest for immunogen design as it was recognized by many bnAbs (264). At the same time, a separate group found that it was unique amongst a large panel of Envs because the infant transmitted Env variant was successfully used to generate the first native-like, soluble Env trimer (47). The BG505 SOSIP trimer has proven to be a useful tool, first to solve a high-resolution structure of the trimer (33, 34), and subsequently to understand glycosylation of the Env trimer (50), the antigenic landscape of Env and epitope targets of bnAbs (114, 116). Many groups have used the trimer as bait to isolate adult-derived bnAbs (114). The BG505 Env trimer also been used as an immunogen in a number of pre-clinical studies, which induced tier 2 neutralizing antibody responses in rabbits, macaques and guinea pigs (199) and bnAbs in cows (189).

Recently, another group identified pediatric broadly neutralizing antibody responses (265). This study examined plasma neutralizing antibody responses of a cohort of children over 5 years of age including nonprogressors and progressors, importantly both groups have high viral loads. Broadly neutralizing antibody responses (>50% neutralization of a panel of tier 2 and 3 clade A, B and C viruses) were identified in 75% of the children compared to 19% of adults. The epitope specificities haven't been mapped for these pediatric bnAb responses. Similar to the findings by Goo et al., viral load was positively associated with bnAb development.

Pediatric bnAbs have now been identified in two cohorts (265). Although adult HIV-1-neutralizing Abs have been extensively characterized, little is known about infant HIV-specific

antibody responses. These two studies describing pediatric broadly neutralizing antibody responses demonstrate that pediatric responses develop with rapid kinetics and more commonly compared to adult responses. The rapid development of plasma neutralization breadth in infants suggests there might be a shorter pathway toward the development of bnAbs and that infant nAbs exhibit less SHM or that SHM occurs more rapidly in infants.

Chapters II, III, and IV will present the first isolation and characterization of infant HIV-specific nAbs. Prior to the studies presented in this thesis, nothing was known about the nAbs contributing to broad plasma responses in infants. The isolation and characterization of HIV-1 specific nAbs from infant samples presented in Chapters II, III and IV has allowed detailed studies of infant Ab sequence characteristics, neutralization breadth and potency and epitope specificities. Chapter III defines a unique pathway to the development of HIV-specific plasma neutralization breadth for infant BF520.

Nairobi Breastfeeding Clinical Trial

To characterize infant neutralizing antibody responses, these studies utilized a unique set of samples from the Nairobi Breastfeeding Clinical Trial (266). This trial was conducted in Nairobi, Kenya with a goal of examining rates of HIV-1 transmission in formula-fed vs. breastfed infants. The results of the study demonstrated that breastfeeding increases the risk of mother-to-child transmission (MTCT). Because the study ran from 1992 to 1998, before the use of antiretrovirals to prevent MTCT, none of the mothers or their infants received antiretroviral therapy. HIV-infected mothers and their infants were followed with regular blood sample collections, which allowed for accurate estimation of the timing of infection, and longitudinal plasma and PBMC samples were available for the studies presented in this thesis.

Goals of this thesis

Overall, the goal for this thesis is to explore infant broadly neutralizing antibody responses. Characterizing infant nAbs contributing to broadly neutralizing plasma responses will inform vaccine strategies aimed at eliciting broad responses over a reasonable time frame. Chapter II will describe the isolation and characteristics of the first infant-derived nAbs from BF520 at ~1 year post-infection, including an infant-derived bnAb. Chapter III will explore the ontogeny of the infant-derived bnAb, BF520.1. Chapter IV examines nAbs isolated from infant BG505 at 2 years post-infection as well as longitudinal Env isolates, which provides insights for optimizing preclinical vaccine studies using the BG505 trimer as an immunogen. Finally, Chapter V discusses the implications of the characterization of infant nAbs in the HIV vaccine development field.

Chapter II

HIV-1 neutralizing antibodies with limited hypermutation from an infant

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Introduction

bnAbs are thought to be an important component of a protective HIV-1 vaccine but eliciting such responses remains elusive. Indeed, broad and potent neutralizing antibody responses are relatively rare even in HIV-infected individuals, and typically take several years to develop, at least in adults where they have been most extensively studied (267). There have now been several detailed studies of adults who develop broad neutralizing antibody responses, with the goal of trying to reproduce this process with a vaccine, and a number of bnAbs have been isolated from chronic infection (116, 117). Two recent studies showed these bnAbs can bind to virus that was transmitted, suggesting that an interaction with the infecting virus may have stimulated the germline B cell receptors (BCRs) to initiate development of the bnAb lineage (82, 84).

Adult-derived bnAbs exhibit features reflective of long-term affinity maturation including high levels of SHM and rare insertions and deletions (indels) (116, 171). Longitudinal studies of bnAb development as well as studies examining predicted intermediates in this process demonstrated that the high degree of mutations and many indels are important for neutralization breadth and potency (82, 84, 131, 150, 176-180). The unusual features of these bnAbs may be the result of a process of iterative rounds of affinity maturation in response to viral escape over years of infection before developing neutralization breadth (116, 171). While studies are underway to develop strategies to mimic this long-term process and guide affinity maturation (268), this will

undoubtedly be a challenging task.

HIV-1-infected infants were recently shown to produce plasma antibody responses that potently neutralize a diverse panel of HIV-1 isolates including more difficult to neutralize variants from across clades and these responses developed as early as 1-2 years post-infection (pi) (23). While adult HIV-1 bnAbs have been extensively characterized, nothing is known about infant bnAbs contributing to broad plasma responses. The relatively rapid development of infant plasma neutralization breadth may suggest that the bnAbs responsible for breadth have distinct features relative to adult HIV-1-specific bnAbs, including lower SHM. Furthermore, whether infant bnAbs target similar or novel epitopes on HIV-1 envelope (Env) compared to adult bnAbs is not known. To better understand the early development of bnAbs in natural infection, we isolated and characterized infant HIV-1-specific neutralizing monoclonal antibodies contributing to plasma breadth within the first year of infection.

Materials and methods

Infant plasma and PBMC samples

Plasma and PBMC samples were from infant BF520 enrolled in the Nairobi Breastfeeding Clinical Trial (266), which was conducted prior to the use of antiretrovirals for prevention of mother-to-child transmission. Approval to conduct the Nairobi Breastfeeding Clinical Trial was provided by the ethical review committee of the Kenyatta National Hospital Institutional Review Board, the Fred Hutchinson Cancer Research Center Institutional Review Board, and the University of Washington Institutional Review Board.

BF520 was HIV-1 DNA negative by PCR and HIV-1 RNA negative using the Gen-Probe HIV-1 Viral Load assay at 8 days of age and was HIV-1 DNA and RNA positive by 3.8 months

of age. Time pi was defined as the time from the infant's first HIV-1-positive nucleic acid test (3.8 months of age).

Sorting of B cells and reconstruction of antibodies

HIV-specific B cells were identified using culture of IgG^+ B cells with subsequent neutralization assays of individual culture supernatants (129). A PBMC sample from BF520 from 15 months of age, 11.2 months pi, was thawed at 37°C and re-suspended in 10 ml B cell media (IMDM medium, Gibco; 10% low IgG FBS, Life Technologies; 5 ml GlutaMAX, Life Technolgoies; 1 ml MycoZap plus PR, Lonza) plus 20 µl benzonase followed by centrifugation at 300 x g for 10 minutes. Cells were washed in FACS wash (1X PBS, 2% FBS) and stained on ice for 30 minutes using a cocktail of anti-CD19-BV510, anti-IgD-FITC, anti-IgM-FITC, anti-IgA-FITC, anti-CD3-BV711, anti-CD14-BV711 and anti-CD16-BV711. Cells were then washed once and resuspended in FACS wash. Cells were loaded onto a BD FACS Aria II cell sorter, and IgG expressing B cells were identified as $CD3^-CD14^-CD16^-CD19^+IgD^-IgM^-IgA^-$ cells. The PBMC sample contained approximately 10 million cells with 72% viability. In total, ~100,000 IgG^+ B cells were sorted into B cell media. Cells were plated using a Tecan automated liquid handling system at a density of 6 B cells in 60 µL per well into 55 x 384-well plates in B cell media containing 100 U ml^{-1} IL-2 (Roche), 0.05 µg ml^{-1} IL-21 (Invitrogen), and $8.85x10^5$ ml^{-1} irradiated 3T3/CD40L feeder cells (kindly provided by S. Riddell, FHCRC). After 14 days of incubation, IgG was detected by ELISA in 81% of a random sample of wells at a concentration of >10 ng ml^{-1} and 54% at >100 ng ml^{-1}.

B cell culture supernatant from each well was divided into 2 x 384 well plates at 20 µL each for neutralization assays using a Tecan automated liquid handling system. B cells were frozen at -80°C in 20 µl RNA storage buffer per well (15 mM Tris and 10 U murine RNase

inhibitor, NEB). For each well, 20 µl of culture supernatant was incubated for 1 hour at 37°C with approximately 325 infectious pseudovirus particles in 20 µl. Next, 3000 TZM-bl cells in 20 µl DMEM plus 10% FBS and 1X PSF, Gibco ($1.5x10^5$ cells ml^{-1}) and DEAE dextran (10 µg ml^{-1} final concentration) were added to each well and cultured 37°C in a CO_2 incubator for 48 hours. β-galactosidase levels were measured using the Gal-Screen system (ThermoScientific). Briefly, 30 µl was removed from each well, 25 µl of substrate diluted 1:25 was added, incubated for 40 minutes at RT, and read using a luminometer. Wells demonstrating >40% neutralization of one or both viruses were selected for RT-PCR amplification of the variable regions of the IgG heavy chain and kappa and lambda light chains, which were cloned into IgG expression vectors as previously described (269) with a modified RT step (144).

Paired heavy and light chain plasmids cloned from the same well were co-transfected in equal ratios into 293F cells using the FreeStyle MAX system (Invitrogen). IgG was purified as described (144). We screened 82 individual wells, from which 22 functional antibodies were produced.

Pseudovirus production and neutralization assays

Methods for making pseudoviruses and performing neutralization assays using the TZM-bl system were as previously described (250). Plasma IC_{50} values are the reciprocal plasma dilution resulting in 50% reduction of virus infectivity. mAb IC_{50} values represent the mAb concentration in µg ml^{-1} at which 50% of the virus was neutralized. Reported IC_{50} values are the average of two or three independent experiments performed in duplicate.

Epitope mapping

To screen for N332 nAbs, we compared neutralization of a clade A wild-type virus Q23.17 and a clade C wild-type virus DU156.12 to that of N332A mutants (156) and a clade A

wild-type virus BG505.W6.C2 (200) to that of a T332N mutant (47). PGT128 and VRC01 were used as positive and negative controls, respectively (123, 130). Reported IC_{50} values are the average of two independent experiments performed in duplicate.

Comparison of infant nAb activity to adult bnAbs

Adult bnAbs b12, 2G12, 2F5 PG16, PGT151, and 10E8 were obtained from the NIH AIDS Reagent Program (116, 128). All adult and infant antibody concentrations were determined by protein absorbance at 280 nm (Nanodrop). Heavy and light chain sequences for adult bnAbs VRC01, PGT121, PGT145 (116) were codon-optimized, and synthesized (https://www.idtdna.com/site) and then cloned into the corresponding Igγ1, Igκ and Igλ expression vectors and expressed and purified by the same method used for infant nAbs.

Somatic hypermutation analysis

Heavy and light chain sequences for adult bnAbs were obtained from bnaber.org (270) or GenBank (271). Sequences were analyzed using IMGT V-QUEST (272) with percent SHM calculated as the VH mutation frequency at the nucleotide level. All sequences for adult nAbs with limited tier 2 neutralizing activity (273) were obtained from GenBank. Sequences for recently published CAP256-VRC26 lineage antibodies (CAP256-VRC26.13-33) (142) were not available and published values were used for percent mutation from germline at the nucleotide level. Groups were compared using the Mann Whitney U test performed using GraphPad Prism 9.0.

Cell-surface binding assays

Binding to cell-surface Env was measured using a flow cytometry-based assay (274). 293T cells ($3x10^6$ cells) were transfected with 4 μg of HIV-1 env DNA using Fugene6 (Promega), harvested 48 hours post-transfection, and incubated with 20 μg ml^{-1} mAb. Next, cells

were incubated with a 1:100 dilution of goat-anti-human IgG-PE (Santa Cruz Biotech, US), subsequently fixed with 1% paraformaldehyde and processed by flow cytometry using a BD FACSCanto II. Data was analyzed using FlowJo software. Percent binding was calculated as the percentage of PE positive cells with background (mAb binding to mock transfected cells) subtracted. PGT128, BF520.3, BF520.4 and BF520.6 demonstrated >10% binding to mock transfected cells. Analyses were performed in GraphPad Prism 9.0.

SOSIP production and purification

Constructs encoding codon optimized BG505.W6.C2 T332N gp120 and SOSIP trimer (47) as well as MG505.W0.E1 D295N I297T T332N were kindly provided by John Moore and colleagues and reverted to wild-type sequences using site-directed mutagenesis (Agilent) while retaining SOSIP modification (tPa signal peptide, furin cleavage site, I559P, A501C, and T605C). A new SOSIP trimer was designed based on the Env sequence of BF520.W14.E3 and synthesized as a codon-optimized gene. A mAb-independent approach for purifying native-like SOSIP trimers was employed (275). In brief, following production in 293F cells, soluble envelope oligomers were separated from the extracellular mileu using *Galanthus nivalis* lectin (Vector Labs). This mixture was then subjected to DEAE cation-exchange chromatography and trimer was resolved from aberrantly disulfide-bonded dimer and gp140 monomer using hydrophobic interaction chromatography and preparative grade size exclusion chromatography. Purity was assessed by SDS-PAGE, BN-PAGE, and dynamic light scattering and found to be >95% native-like trimer. Presence of native-like SOSIP trimer was further confirmed by negative-stain electron microscopy.

Fab fragment preparation

Fab fragments were generated from 500 μg of IgG antibody using a papain digestion kit (Pierce) and separated from Fc fragments and undigested IgG using a Protein A column (Pierce). Purity and size of Fab fragments was confirmed for 6/10 antibodies by SDS-PAGE and mass spectrometry. The remaining 4 antibodies appeared to have been incompatible with papain digestion for Fab isolation from IgG and were not used for subsequent studies.

Kinetic antibody binding assay by Octet biolayer interferometry (BLI)

Binding kinetics of infant antibodies and Fab fragments with SOSIP trimers were determined using biolayer interferometry (BLI) on an Octet RED system (FortéBio). Hydrated anti-human IgG Fc Capture (AHC) or anti-human Fab-CH1 biosensors were immobilized for 4 minutes with purified infant antibodies diluted to 10 μg mL^{-1} in PBS (pH 7.4) supplemented with 1% BSA, 0.01%TWEEN 20, 0.02% Sodium Azide. After a stable baseline signal was established, antibody-immobilized tips were moved to wells containing a 2-fold dilution series of Env SOSIP trimer to monitor association for 4 minutes. Tips were then moved back to wells containing buffer to monitor dissociation for 15 minutes. Kinetics data were analyzed using FortéBio's Data Analysis 7.0. Average measurements from reference wells were subtracted and data were processed by Savitzky-Golay filtering prior to fitting using a 1:1 binding model. Reported kinetic constants are the average of 2 or 3 experiments using independent Env dilution series except in cases where dissociation was too minimal to be fit by the software.

Electron microscopy

A 3 μl aliquot of BG505.W6.C2.T332N-BF520.1 complex, diluted to 20 μg ml^{-1} in PBS was applied for 60 seconds to glow discharged C-Flat, 300 mesh, Cu grids (Electron Microscopy Sciences) and stained for an additional 60 seconds using Nano-W (Nanoprobes). Data were

collected using a FEI Tecnai T12 transmission electron microscope operating at 120 keV. Images were taken using a Gatan 4k × 4k CCD at a magnification of 52,000× and defocus range of 0.5 μm – 3.0 μm corresponding to a pixel size of 2.07 Å. Single particle reconstruction was performed using EMAN2.1 image processing suite (276). In short, particles were selected using interactive particle picking from 392 micrographs. A 2× binned, phase-flipped, CTF-corrected stack of 35,914 particles were created and subjected to reference free 2D classification and clustering to generate 200 2D classes. Classes containing free BF520.1-Fab, or sub-stoichiometric populations were omitted and the remaining 26,013 particles were reclassified to generate 150 2D classes. Again, classes containing sub-stoichiometric and free BF520.1-Fab populations were removed and a 2× binned particle stack containing 18,325 particles was used for 3D refinement using the coordinates from the 5ACO.pdb cryo-EM structure of BG505 SOSIP.664 HIV-1 Env trimer bound by PGT128 Fab (277). The model was low-pass filtered to 60 Å and used as an initial model for refinement with C3 symmetry imposed. Notably the 5ACO coordinates only include the Fv and not the constant region of the PGT128 Fab; thus the density we observe for the complete Fab emerged in the course of the reconstruction. The BG505 SOSIP-664 HIV-1 trimer (pdb:4zmj) (278) and PGT128-Fab crystal structure (pdb:3tv3) (152) were docked into the negative-stained 3D map using UCSF Chimera package from the Computer Graphics Laboratory, University of California, San Francisco (279).

HIV-1 *env* amplification and cloning

Full-length *envs* were cloned from DNA isolated from uncultured PBMCs for the BF520 14-week (3.8 month) time-point as previously described (280). For the 6-month time-point, *envs* were cloned total RNA that was extracted from 50 μL of plasma as described in (281). cDNA

synthesis and nested PCR of full-length *env* was performed as previously described with minor modifications to the primers, which are available upon request (200, 280).

Phylogenetic tree analysis

Maternal (280) and infant *env* sequences were aligned using MacClade version 4.01. A maximum likelihood phylogenetic tree was constructed using the HIV LANL HIV tools database PHYML interface (282) http://www.hiv.lanl.gov/content/sequence/PHYML/interface.html.

Results

Neutralizing activity of infant plasma and isolated nAbs

Infant BF520 was HIV RNA and DNA negative at 8 days of age then subsequently detected positive at 114 days (3.8 months) of age, suggesting transmission likely occurred via breastfeeding. Plasma from this HIV-1 clade A infected infant demonstrated cross-clade tier 2 neutralizing activity by as early as 12 months of age (23). IgG^+ memory B cells from 15 months of age, 11.2 months pi, were isolated and cultured. B-cell culture supernatants were tested for neutralizing activity using a tier 1 clade B virus (SF162) and a tier 2 clade C virus (QC406.F3). These viruses were potently neutralized by BF520 plasma from the contemporaneous time-point (IC_{50} >3200 and 922, respectively) (23). Ten antibodies with HIV-specific neutralizing activity were isolated and tested for neutralization against the cross-clade virus panel originally used to define the breadth of the infant plasma nAb response (**Figure 2.1**) (23). All isolated antibodies neutralized SF162; eight also neutralized either clade A heterologous tier 1 variant Q461.d1 or another clade A heterologous tier 2 virus Q842.d16 or both, indicating modest heterologous breadth specific to the clade of the infecting virus.

One infant antibody, BF520.1, neutralized tier 2 variants from clades A, B and C and one tier 3 variant from clade B. Interestingly, this cross-clade bnAb did not neutralize some clade A

variants that were neutralized by other isolated antibodies. Plasma neutralized 8 viruses that were not neutralized by the isolated nAbs. However, plasma neutralization potency was low (IC_{50}<200) for these 8 viruses, suggesting the antibodies that drive this neutralization may be less potent and thus hard to identify with the functional screening approach used here. Thus, while antibody BF520.1 accounts for much of the plasma neutralization activity, the other nine nAbs we identified as well as additional unidentified antibodies contribute to overall breadth observed with BF520 plasma.

			BF520 Plasma	BF520.1	BF520.2	BF520.3	BF520.4	BF520.5	BF520.6	BF520.7	BF520.8	BF520.9	BF520.10
		SIV	<100	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
Tier 1	Clade B	SF162	>3200	0.22	0.65	1.47	6.96	2.67	1.32	0.75	1.07	3.41	1.08
Tier 2	Clade A	Q461.d1	480	>20	1.72	1.76	6.23	>20	0.69	1.97	2.23	>20	>20
		Q23.17	339	0.29	>20	>20	>20	>20	>20	>20	>20	>20	>20
		Q842.d16	306	>50	25.7	19.5	26.7	11.4	>50	23	>50	42.1	>50
		Q769.B9	<100	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
		Q259.d2.26	121	>50	>50	>50	>40	>50	>50	>50	>50	>50	>50
		BJ613.E1	188	>50	>50	>50	>40	>50	>50	>50	>50	>50	>50
		Q168.a2	<100	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
		Q842.d12	115	>50	>50	>50	>40	>50	>50	>50	>50	>50	>50
	Clade A/D	BF535.A1	128	>50	>50	>50	>40	>50	>50	>50	>50	>50	>50
	Clade B	TRO.11	244	5.26	>20	>20	>20	>20	>20	>20	>20	>20	>20
		THRO4156.18	<100	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
		CAAN.A2	124	42.2	>50	>50	>20	>50	>50	>50	>50	>50	>50
Tier 3		TRJO4551.58	124	>20	>20	>20	>40	>20	>20	>20	>20	>20	>20
		PVO.4	119	38.1	>50	>50	>20	>50	>50	>50	>50	>50	>50
Tier 2	Clade C	ZMN109F.PB4	108	>20	>50	>50	>40	>50	>50	>50	>50	>50	>50
		QC406.F3	922	0.2	>20	>20	>20	>20	>20	>20	>20	>20	>20
		DU156.12	228	5.33	>20	>20	>20	>20	>20	>20	>20	>20	>20
		DU422.1	159	6.81	>50	>50	>20	>50	>50	>50	>50	>50	>50
		DU172.17	159	20.6	>50	>50	>20	>50	>50	>50	>50	>50	>50
		CAP210.E8	186	>20	>50	>50	>40	>50	>50	>50	>50	>50	>50
	Clade D	QB857.B3	142	>20	>50	>50	>40	>50	>50	>50	>50	>50	>50
		QD435.A4	110	>20	>50	>50	>40	>50	>50	>50	>50	>50	>50

Plasma IC_{50}	mAb IC_{50} (μg ml^{-1})
IC_{50} >1000	IC_{50} < 0.2
500 ≤ IC_{50} < 1000	0.2 ≤ IC_{50} < 2
200 ≤ IC_{50} < 500	2 ≤ IC_{50} < 10
100 ≤ IC_{50} < 200	10 ≤ IC_{50} < 20-50
IC_{50} < 100	IC_{50} >20-50

Figure 2.1 Neutralization of panel viruses with BF520 plasma from 15 months of age and isolated nAbs

IC_{50} values (μg ml^{-1}) are color coded with darker shading indicating greater neutralization potency. Gray shading indicates 50% neutralization was not achieved at the highest mAb concentration or lowest plasma dilution tested. SIV was a negative control. Values are an average of at least three independent experiments performed in duplicate.

Breadth and potency of adult and infant bnAbs

To compare the neutralizing activity of BF520.1 to adult bnAbs, we tested for neutralization of a global panel of viruses designed for standardized assessments of nAbs (**Figure 2.2**) (283). BF520 plasma from 15 months of age neutralized 10/12 of the virus panel. BF520.1 neutralized 7/10 viruses neutralized by the corresponding plasma. Only one of the other nine nAbs neutralized a virus from this panel (virus 398F1, nAb BF520.4; IC_{50} = 14 μg ml^{-1}). We compared BF520.1 to first-generation adult bnAbs, which have moderate breadth and potency, and a selection of broad and potent second-generation adult bnAbs (116, 128). The neutralization breadth of BF520.1 (58%; **Figure 2.2**) is greater than that of the first-generation adult bnAbs, which range from 8-50%, and falls within the range for the second-generation adult bnAbs (42-100%). BF520.1 also demonstrates greater neutralization potency (geometric mean IC_{50} for viruses neutralized = 1.95 μg ml^{-1}; **Figure 2.2**) compared to first-generation adult bnAbs (2.4-10.3 μg ml^{-1}) and has comparable potency to the CD4 binding site directed bnAb VRC01 (2.13 μg ml^{-1}) (130), which is actively being pursued in rational vaccine design and tested for efficacy in human trials (162, 284). Overall, these data from the global reference panel show that the infant bnAb BF520.1 demonstrates generally similar neutralization breadth to many adult bnAbs but with lower potency.

			V3 glycan	Outer domain glycan	V3 glycans	CD4bs		V1/V2 glycans			gp120-gp41 interface	MPER		
		BF520 Plasma	**BF520.1**	2G12	**PGT121**	b12	**VRC01**	**PG16**	**PGT145**	**PGT151**	4E10	2F5	**10E8**
Clade A	398F1	527	0.47	>20	<0.02	2.4	0.44	>20	>20	<0.02	>20	4.76	0.81
Clade B	TRO11	175	4.92	2.75	0.03	>20	3.08	16	0.37	>20	6.74	>20	0.08
	X2278	183	1.19	2.14	<0.02	>20	0.63	<0.02	<0.02	2.21	>20	16.0	1.37
CRF07_BC	BJOX002000	159	0.62	>20	<0.02	>20	>20	<0.02	>20	<0.02	16.8	>20	1.37
	CH119	122	5.48	>20	<0.02	>20	7.15	10.3	2.94	>20	>20	>20	3.84
	CE1176	118	4.59	>20	<0.02	>20	17.8	<0.02	>20	<0.02	>20	>20	0.99
Clade C	CE0217	107	2.49	>20	<0.02	>20	1.68	<0.02	0.29	>20	12.9	>20	2.09
	25710	120	>20	>20	<0.02	>20	5.37	<0.02	<0.02	>20	4.98	>20	0.09
Clade G	X1632	116	>20	>20	>20	>20	0.35	0.26	2.24	>20	15.7	8.76	2.29
CRF01_AE	CNE55	113	>20	>20	>20	>20	2.2	17.3	<0.02	>20	>20	6.29	0.28
	CNE8	>100	>20	>20	>20	>20	3.86	3.16	1.29	>20	10.6	4.46	1.01
AC recomb	246F3	>100	>20	>20	>20	>20	1.44	<0.02	>20	8.56	>20	5.58	1.91
% Breadth		83	58	17	67	8	92	92	67	42	50	50	100
geometric mean IC_{50} (μg ml^{-1}) viruses neutralized			1.95	2.43	0.02	2.4	2.13	0.24	0.23	0.17	10.3	6.86	0.82
geometric mean IC_{50} (μg ml^{-1}) all viruses			5.14	14.1	0.35	16.8	2.57	0.35	1.01	2.76	14.4	11.7	0.82

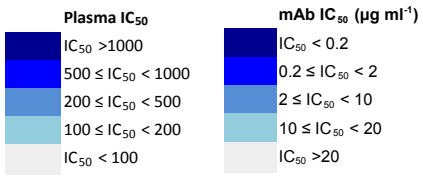


Figure 2.2 Comparison of BF520.1 to adult bnAbs

Neutralization of global panel tier 2 viruses. mAb IC_{50} values are an average of two-three independent experiments performed in duplicate. First-generation bnAbs indicated in gray.

Epitope specificity of BF520.1

The infant cross-clade bnAb BF520.1 exhibited a similar neutralization profile to the PGT-class of bnAbs (285), which target the N332 glycan in V3 (123). Thus, we examined the effect of N332 on BF520.1 neutralization (**Figure 2.3 A and B**). Neutralization was disrupted by removal of the N332 glycan for two viruses tested (93 and 5 fold increase IC_{50}), similar to what was observed for a prototype N332-directed bnAb, PGT128 (32 and 17 fold increase IC_{50}). Adding the N332 glycan to an infant clade A heterologous virus BG505.W6.C2 (200) resulted in increased neutralization sensitivity (17-fold decrease IC_{50}). These data indicate that this infant antibody targets a similar site on Env as the N332-dependent adult nAbs such as 2G12 and the PGT class of adult bnAbs (51, 123, 145, 150, 152, 286). Single-particle negative-stain EM analysis of Fab from the BF520.1 bnAb complexed with BG505.W6.C2.T332N SOSIP trimers,

which encodes N332 and is structurally similar to Env on virus particles (47), confirmed that the antibody targets the base of the V3 loop **(Figure 2.3 C)**. BF520.1 and PGT128 appear to dock to the trimer at the base of V3 with an overlapping footprint and to be oriented relative to the trimer with a similar angle of approach (152, 277). The BF520.1 Fab, however, is slightly twisted and docked more closely to the gp120 core than PGT128 (**Figure 2.4**). At the resolution of the negative-stain reconstruction we are not able to infer the role of the CDR loops or the specific residues on the paratope or epitope that are involved in this interaction. Thus, while our mutagenesis data implicates the glycan at N332 is an important component of the epitope for BF520.1, additional studies are needed to more directly compare the BF520.1 epitope and paratope to those of other antibodies that target the N332 supersite.

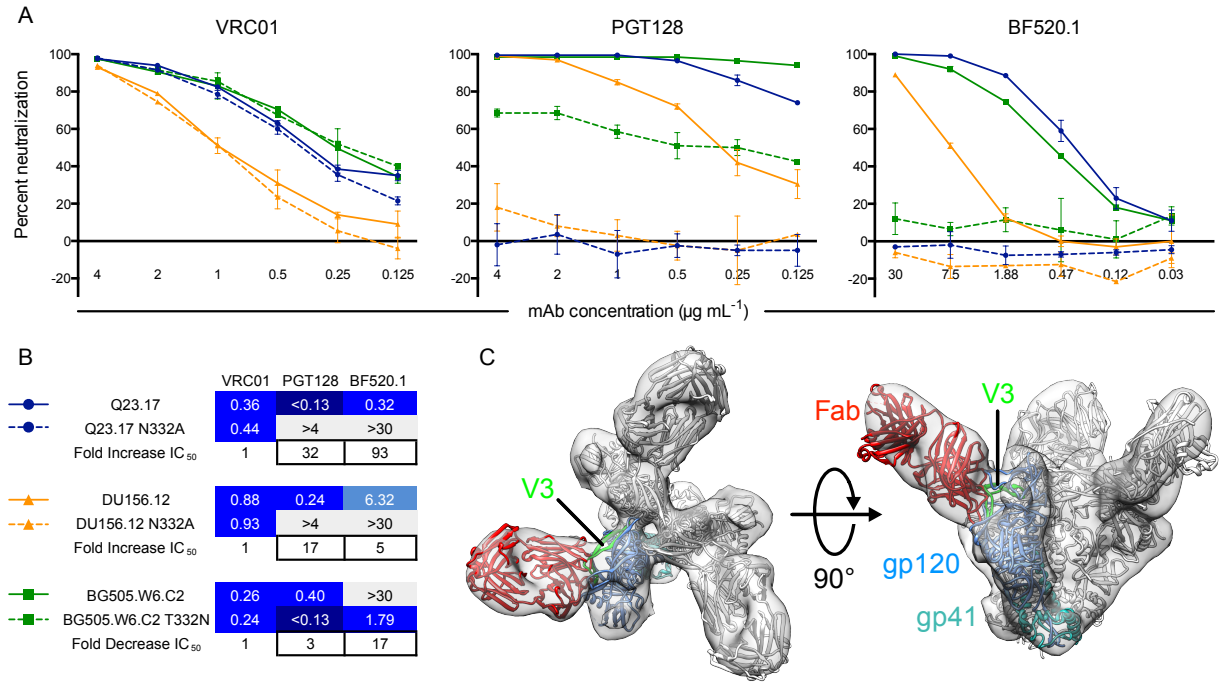


Figure 2.3 Epitope mapping of BF520.1

(A-B) mAb neutralization of Q23.17 and DU156.12 wild-type viruses relative mutant viruses lacking the N332 glycan and BG505.W6.C2 relative to a mutant virus with N332 (T332N). PGT128 and VRC01 are positive and negative controls respectively. Viruses lacking N332 are indicated by dashed lines. (C) Negative-stain EM of the BG505.W6.C2.T332N SOSIP trimer in complex with BF520.1 Fab.

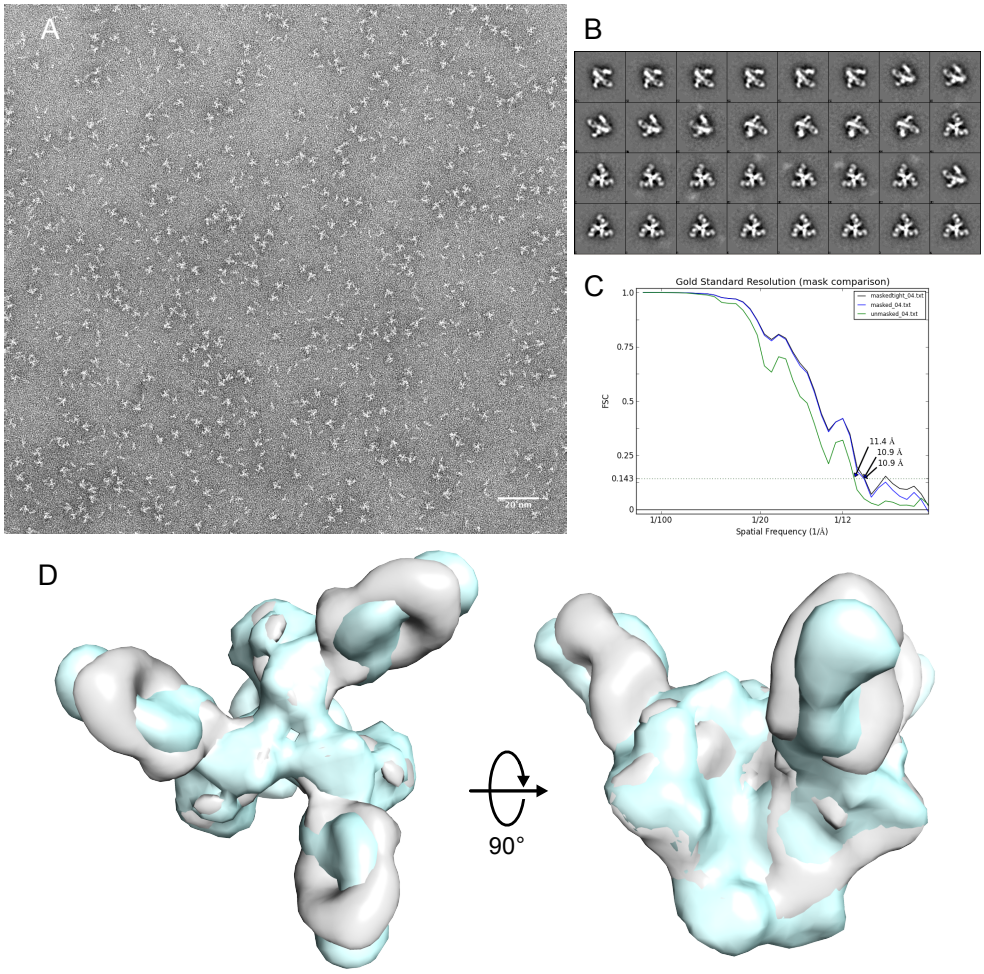


Figure 2.4 Negative-stain EM of BF520.1 Fab bound to HIV Env trimer

(A) Negative-stain image of BG505.W6.C2.T332N SOSIP:BF520.1-Fab complex (scale bar = 20 nm). (B) Reference free 2D class averages showing both top and side views of BG505 SOSIP:BF520.1-Fab complex. 2D classification was not used for 3D refinement but is shown for visual purposes. (C) Fourier-shell correlation (FSC) curve for the BF520.1-BG505 SOSIP trimer negative-stain EM reconstruction obtained by 'gold standard' refinement in EMAN2.1. (D) Surface rendering of BG505 SOSIP:BF520.1-Fab negative-stain EM map, lowpass-filtered to 20 Å, compared with BG505 SOSIP:PGT128 Cryo-EM map (cyan; emd_3121), also low-pass filtered to 20 Å. While epitopes for both antibodies are similar, BF520.1 appears to bind at a slightly different angle of approach relative to PGT128.

Binding to autologous and heterologous HIV-1 envelopes

To gain insight into the development of the isolated infant antibodies, we examined whether the infant nAbs bind and/or neutralize autologous Env variants isolated at first HIV detection (3.8 months/14 weeks of age, designated "W14"). The diversity among these Env variants was low (maximum pairwise distance = 0.0067; **Figure 2.5**). Surprisingly, all 10 nAbs failed to neutralize the 11 early-stage, transmitted variants, despite potent neutralization by contemporaneous plasma (**Figure 2.6**), suggesting our functional screen for heterologous neutralization did not capture the nAbs mediating autologous neutralization by plasma from the 15-month time-point. Despite the lack of neutralization of the earliest isolated variants, BF520.1 and the other 9 infant nAbs bound to the corresponding autologous BF520.W14 cell-surface Env variants (**Figure 2.7**). Each of the 10 infant nAbs also bound SF162, which they all neutralized, with BF520.1 exhibiting the highest level of binding, comparable to that of VRC01 (**Figure 2.7**).

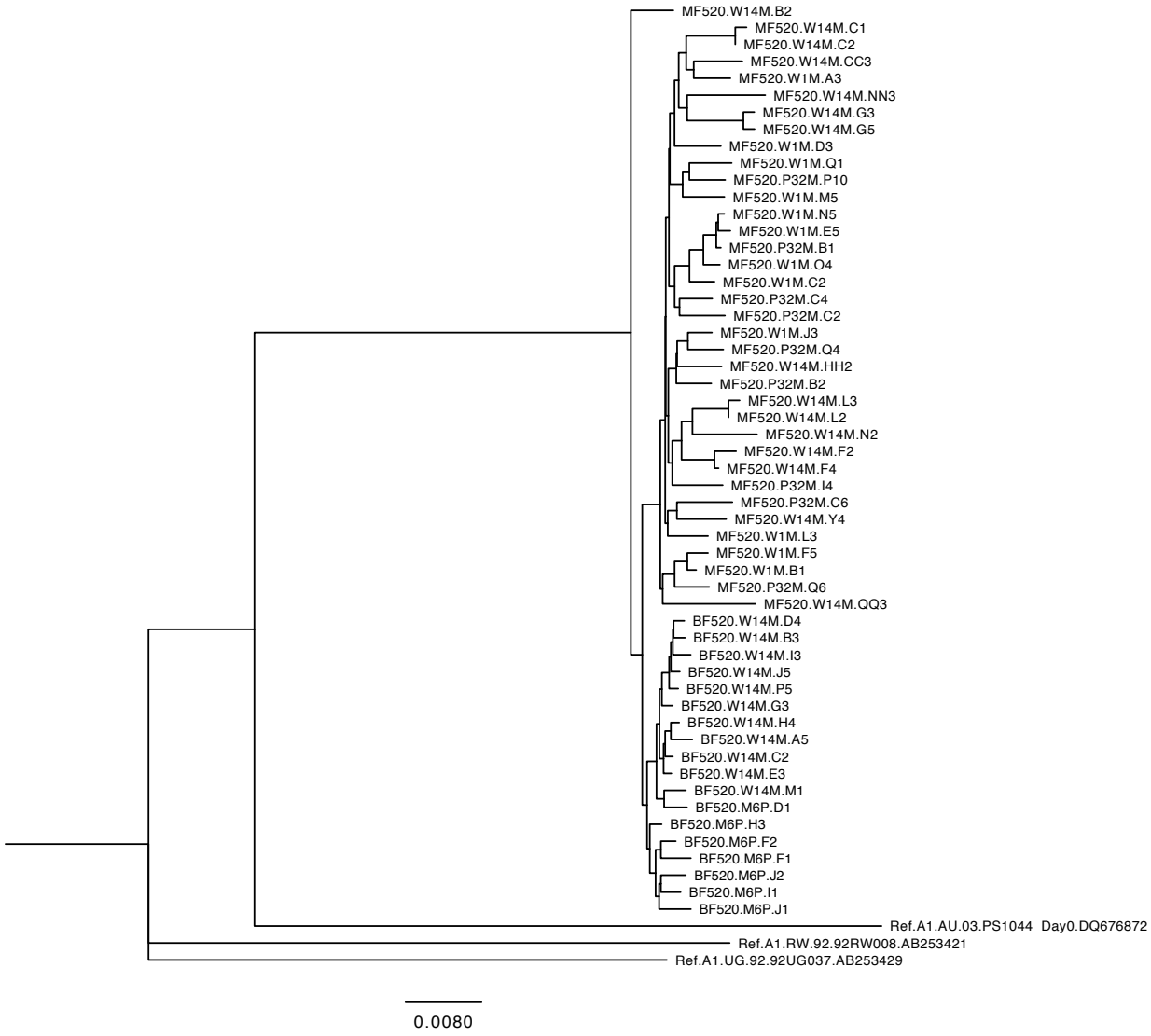


Figure 2.5 Maximum likelihood phylogenetic tree of maternal (MF520) and infant-derived (BF520) envelope variants

Maternal variants were isolated from pregnancy week 32 (P32), 1 week post-delivery (W1), and 14 weeks post-delivery (W14). BF520 infant envelope variants were isolated from 14 weeks of age (W14) when the infant was first detected HIV positive and 6 months of age (M6).

	BF520 M15 Plasma	BF520.1	BF520.2	BF520.3	BF520.4	BF520.5	BF520.6	BF520.7	BF520.8	BF520.9	BF520.10	PGT121
SF162	>3200	0.22	0.65	1.47	6.96	2.67	1.32	0.75	1.07	3.41	1.08	<0.02
BF520.W14.A5	668	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	0.25
BF520.W14.B3	375	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	0.02
BF520.W14.C2	246	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	<0.02
BF520.W14.D4	342	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	<0.02
BF520.W14.E3	450	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	0.13
BF520.W14.G3	260	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	<0.02
BF520.W14.H4	378	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	0.03
BF520.W14.I3	365	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	0.09
BF520.W14.J5	1356	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	<0.02
BF520.W14.M1	382	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	0.07
BF520.W14.P5	172	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	0.27

Plasma IC_{50}

- IC_{50} >1000
- $500 \le IC_{50} < 1000$
- $200 \le IC_{50} < 500$
- $100 \le IC_{50} < 200$
- $IC_{50} < 100$

mAb IC_{50} (µg ml^{-1})

- $IC_{50} < 0.2$
- $0.2 \le IC_{50} < 2$
- $2 \le IC_{50} < 10$
- $10 \le IC_{50} < 20$
- IC_{50} >20

Figure 2.6 BF520 autologous neutralization

Infant envelope variants isolated from the first time point following detection of infection (14 weeks of age, W14, or 3.8 months) are listed in the first column. Neutralization of these autologous variants by BF520 plasma from 15 months of age and mAbs isolated from the contemporaneous time point are shown in columns. IC50 values (ug ml-1) are color coded with darker shading indicating greater neutralization potency. BF520 plasma IC50 is the reciprocal plasma dilution at which 50% neutralization was achieved. Gray shading indicates that 50% neutralization was not achieved at the highest mAb concentration or lowest plasma dilution tested. SF162 was included as a positive control for mAb neutralizing activity and PGT121 was included as a positive mAb control. Values are an average of two independent experiments performed in duplicate.

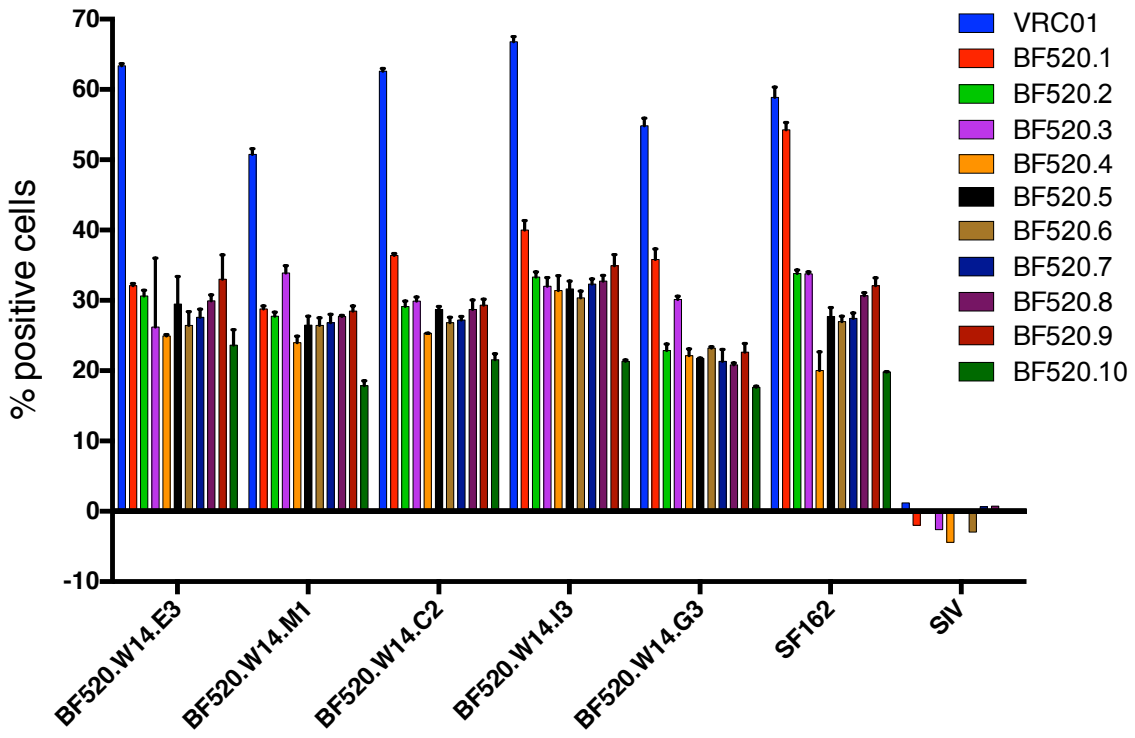


Figure 2.7 mAb binding to cell-surface expressed autologous HIV Envs from the time-point when infection was first detected (14 weeks of age, W14)

Infant nAb binding to representative BF520 cell-surface expressed Env variants detected by flow cytometry as percentage of cells positive for bound antibody with background subtracted (mAb binding to mock transfected cells). VRC01 was included as a positive control for Env expression. SIV was included as a negative control. Data are representative of two independent experiments performed in duplicate. Error bars indicate SD based on duplicates within an experiment.

Because it is unexpected that antibodies that bind Env trimer expressed natively on the cell surface would not neutralize the corresponding virus (287-290), we sought to validate the flow cytometry analysis of antibody binding by measuring binding of the BF520 antibodies to BF520 native-like SOSIP trimer based on the BF520.W14.E3 transmitted Env variant. The purity of the native-like BF520 SOSIP trimer preparation was confirmed by SDS-PAGE, BN-PAGE and dynamic light scattering (**Figure 2.8).** Biolayer interferometry (BLI) demonstrated that all 10 infant nAbs bound tightly to the BF520 SOSIP trimer representing the transmitted virus (**Figure 2.9**). To address whether avidity contributes to the observed binding kinetics, we measured Fab fragment binding to BF520 SOSIP trimer for representative nAbs. Purity and size of Fab fragments was confirmed for 6 of the infant nAbs by SDS-PAGE and mass spectrometry including the infant bnAb BF520.1. These Fabs bound to the autologous trimer with lower affinity compared to IgG for 5/6 Fabs tested and comparable affinity for 1/6 Fabs (**Figure 2.10**).

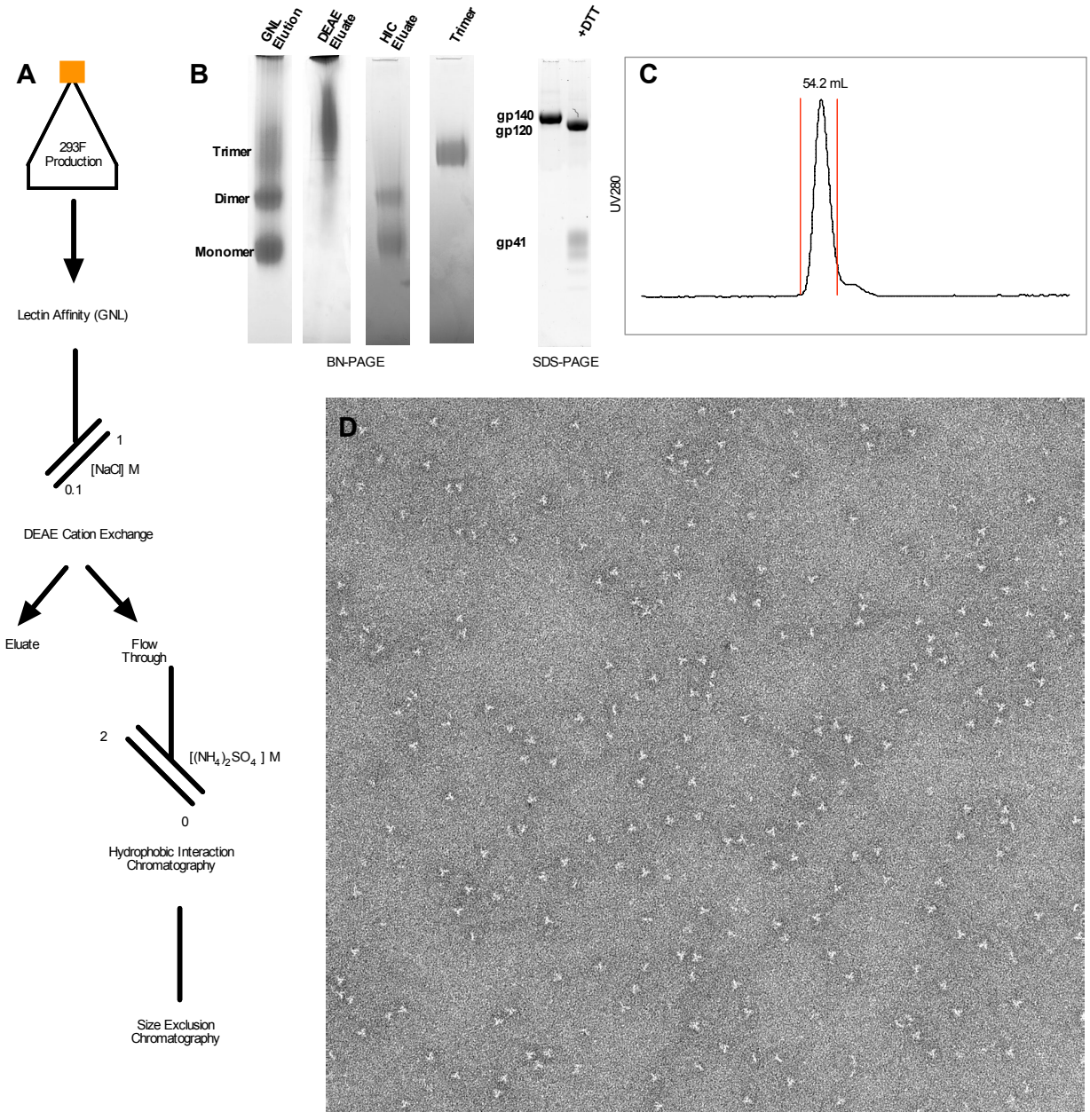


Figure 2.8 Purification scheme for biochemical resolution of BF520.W14.E3 native-like SOSIP trimer from various recombinant soluble Env species

(A) Blue-native polyacrylamide gel and SDS-PAGE analyses of purification. (B) Size exclusion chromatography of the trimeric fraction off of the hydrophobic interaction column on a Superdex S200PG column (C) Peak fractions (indicated by red lines in chromatogram) were collected and concentrated for analysis and binding studies. (D) Negative-stain electron microscopy confirmed the presence of native-like trimers distinguishable by their compact three-fold symmetry.

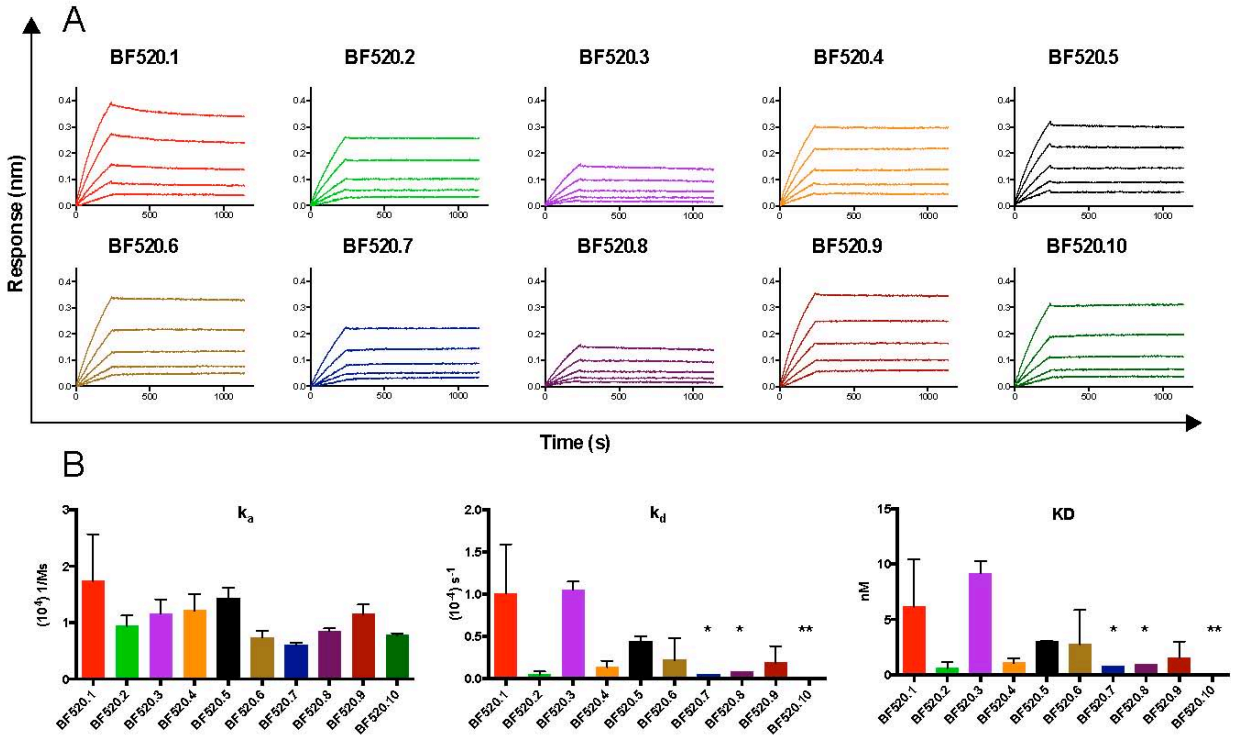


Figure 2.9 Infant nAb binding to BF520.W14.E3 native-like SOSIP trimer

(A) Representative reference-subtracted sensorgrams for each interaction between an infant nAb and the autologous SOSIP. Analyte concentrations range from 500 nM to 31.25 nM. (B) Summarized affinity, association and dissociation parameters (KD, ka, and kd respectively) from best fitting to a 1:1 model of ligand:analyte binding are shown. Each parameter represents the average of 2 or 3 independent experiments unless otherwise indicated. Error bars indicate the standard error of the mean (SEM). *minimal dissociation could only be fit to one replicate **no dissociation after 30 minutes.

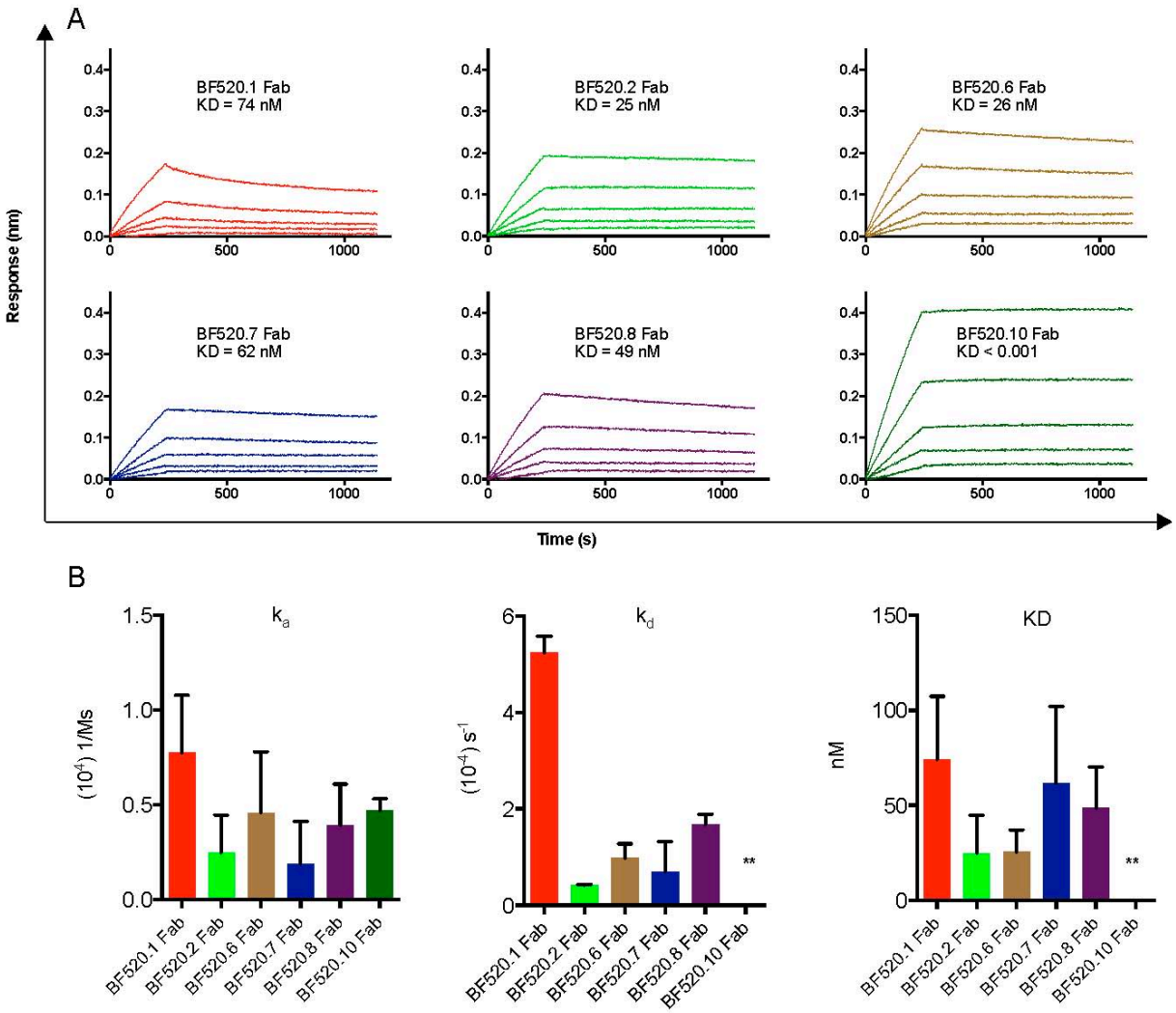


Figure 2.10 Infant nAb Fab fragment binding to BF520.W14.E3 native-like SOSIP trimer

(A) Representative reference-subtracted sensorgrams for each interaction between an infant nAb Fab fragment and the autologous SOSIP. Analyte concentrations range from 500 nM to 31.25 nM. (B) Summarized affinity, association and dissociation parameters (KD, ka, and kd respectively) from best fitting to a 1:1 model of ligand:analyte binding are shown. Each parameter represents the average of 2 independent experiments. Error bars indicate the standard error of the mean (SEM). **no dissociation after 30 minutes

To compare infant bnAb BF520.1 binding to the early autologous variant with binding to heterologous envelope variants, we again assessed binding to cell-surface expressed Env by flow cytometry and to SOSIP trimers by BLI. The envelopes derived from heterologous viruses that were neutralized by the BF520.1 antibody (SF162, Q23.17 and BG505.W6.C2.T332N; **Figure 2.11**) showed strong antibody binding (**Figure 2.11 B**), and those that were not neutralized (BG505.W6.C2 and the related maternal-derived MG505.W0.E1 Env, both of which lack N332 (Wu et al., 2006); **Figure 2.11 A**) did not show binding to cell surface expressed Env (**Figure 2.11 B**). Similar binding results were observed with the soluble SOSIP trimers for BG505.W6.C2, MG505.W0.E1 and BG505.W6.C2.T332N (**Figure 2.11 C**). While binding by BF520.1 to BG505.W6.C2 soluble SOSIP trimer was detected by BLI, a high off-rate was observed (**Figure 2.11 C**). Again BF520.1 binding was detected to both the surface expressed and soluble forms of the BF520.1 Env trimer with a Kd of 8nM.

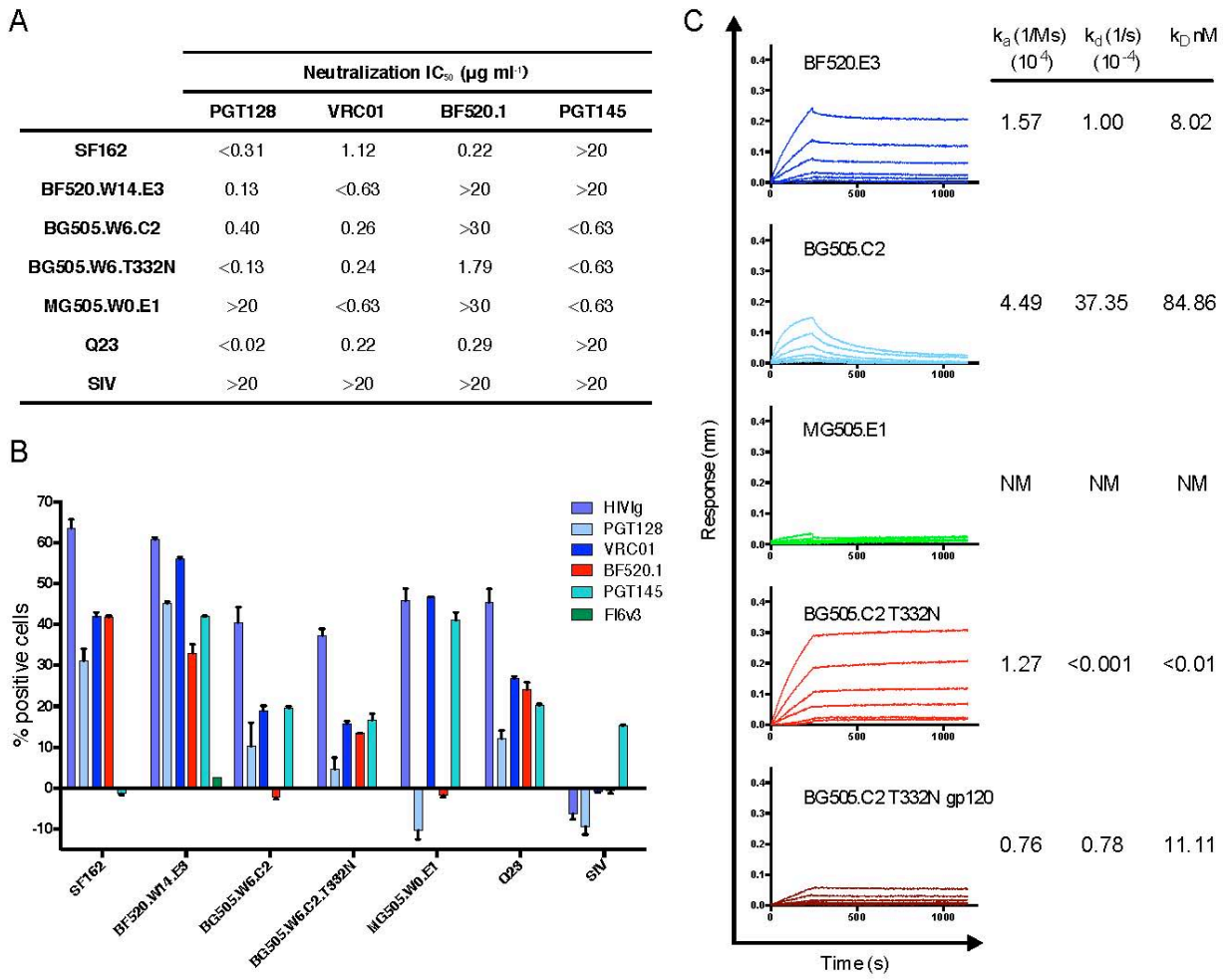


Figure 2.11 Binding to and neutralization of heterologous Envs

(A) Neutralization IC_{50} values (µg ml^{-1}) for mAb neutralization of the corresponding virus. Values are an average of 2 independent experiments. (B) mAb binding to cell-surface Env detected by flow cytometry. FI6v3 was an influenza-specific negative control. HIVIg was a positive control for Env expression. Data are representative of two independent experiments performed in duplicate. Error bars indicate SD based on duplicates within an experiment. (C) BF520.1 binding to native-like soluble SOSIP trimers measured by BLI. NM = not modeled.

To determine whether the infant bnAb, BF520.1 neutralizes later autologous viruses, Env variants were isolated from 2.2 months after the first, early, transmitted viral sequences were obtained (6 months of age, designated "M6"; **Figure 2.5**), which was 9 months prior to the time the nAbs were obtained. BF520.1 demonstrated potent neutralization of 3/7 viruses from this time-point (**Figure 2.12**).

	BF520.1	PGT121
BF520.M6.D1	0.17	<0.01
BF520.M6.F1	>20	0.85
BF520.M6.F2	>20	0.04
BF520.M6.H3	>20	1.40
BF520.M6.I1	0.99	0.02
BF520.M6.J1	1.38	0.03
BF520.M6.J2	>20	1.89

mAb IC_{50} (µg ml^{-1})
$IC_{50} < 0.2$
$0.2 \leq IC_{50} < 2$
$2 \leq IC_{50} < 10$
$10 \leq IC_{50} < 20$
$IC_{50} > 20$

Figure 2.12 BF520.1 autologous neutralization

Infant envelope variants isolated from 2.2 months post-infection (6 months of age; M6) are listed in the first column. Neutralization of these autologous variants by BF520.1 and PGT121 is shown in columns. IC_{50} values (µg ml^{-1}) are color coded with darker shading indicating greater neutralization potency. Gray shading indicates that 50% neutralization was not achieved at the highest mAb concentration tested. PGT121 was included as a positive mAb control. Values are an average of two independent experiments performed in duplicate.

Sequence characteristics of infant versus adult bnAbs

The 10 infant antibodies have different heavy chain gene rearrangements and CDRH3 sequences (**Figure 2.13 A**) suggesting that they are produced from distinct lineages of B cells. Because these antibodies developed within 1 year pi, we were interested in examining the level of SHM of isolated infant-derived nAbs compared to adult bnAbs. All infant nAbs had low SHM (2.0-6.6% at the nucleotide level, nt; **Figure 2.14 A** and **Figure 2.13**), in contrast to adult bnAbs (3.8-32.6% nt) (270, 272) as well as adult nAbs with limited tier 2 neutralizing activity (tier 1 nAbs; 2.4-18.6% nt) (273). The infant nAbs also had lower SHM than adult nAbs, including

those with and without breadth, isolated relatively early pi (1-4 years) from CAP256-VRC26 (4.2-18% nt) (84, 142). Overall, infant HIV-specific nAbs are remarkable for the low level of SHM compared to adult nAbs.

BF520.1 had a much lower level of mutation (6.6% nt) compared to adult N332-dependent bnAbs (15.8-23.1% nt) (**Figure 2.14 A and B**). This infant bnAb was also notable when compared to adult bnAbs targeting the N332 supersite in that it lacks heavy and light chain indels (**Figure 2.14 B**). BF520.1 has a CDRH3 of 20 amino acids, comparable to the PGT bnAbs (20-26 amino acids), but different heavy chain VDJ and light chain VJ gene rearrangements than the adult N332 bnAbs (**Figure 2.14 B**) (123, 145, 170, 291).

A

BF520 nAb Heavy Chain Sequence Characteristics								
Ab	**CDRH3 AA sequence**	**CDRH3 length (AA)**	**V_H % identity**	**V_H mut freq (nt)**	**VH mut freq (AA)**	**V gene**	**D gene**	**J gene**
BF520.1	ARGPFPNYYGPGSYWGGLDF	20	93.4	6.6	11.2	V1-2	D3-10	J4
BF520.2	ARDERSVPTTIRSGMDI	17	97.2	2.8	2.1	V1-69	D2-2	J6
BF520.3	ARDCSSTGCAPWVYYNYYMDV	21	95.8	4.2	8.2	V1-18	D2-2	J6
BF520.4	TRDEAPYVVQFLTWLPPNVFDI	22	98.0	2.0	2.0	V3-49	D3-3	J3
BF520.5	ARGGYPGMTIFGVVLPFFDY	20	94.7	5.3	8.2	V4-59	D3-3	J4
BF520.6	ASGSSVTIFGVVTPYFDY	18	96.9	3.1	6.2	V1-69	D3-3	J4
BF520.7	ARPYSVVGPLQLLRINDAFDI	21	96.2	3.8	5.1	V1-69	D3-16	J3
BF520.8	ARGRHAVVSGASGFDP	16	94.1	5.9	8.2	V1-69	D2-2	J5
BF520.9	ARGEATHDNFWGGPGAYYYYGMDV	24	96.5	3.5	6.1	V1-2	D3-3	J6
BF520.10	ASPLRTFFGVVIGYGMDV	18	97.2	2.8	3.1	V1-24	D3-3	J6

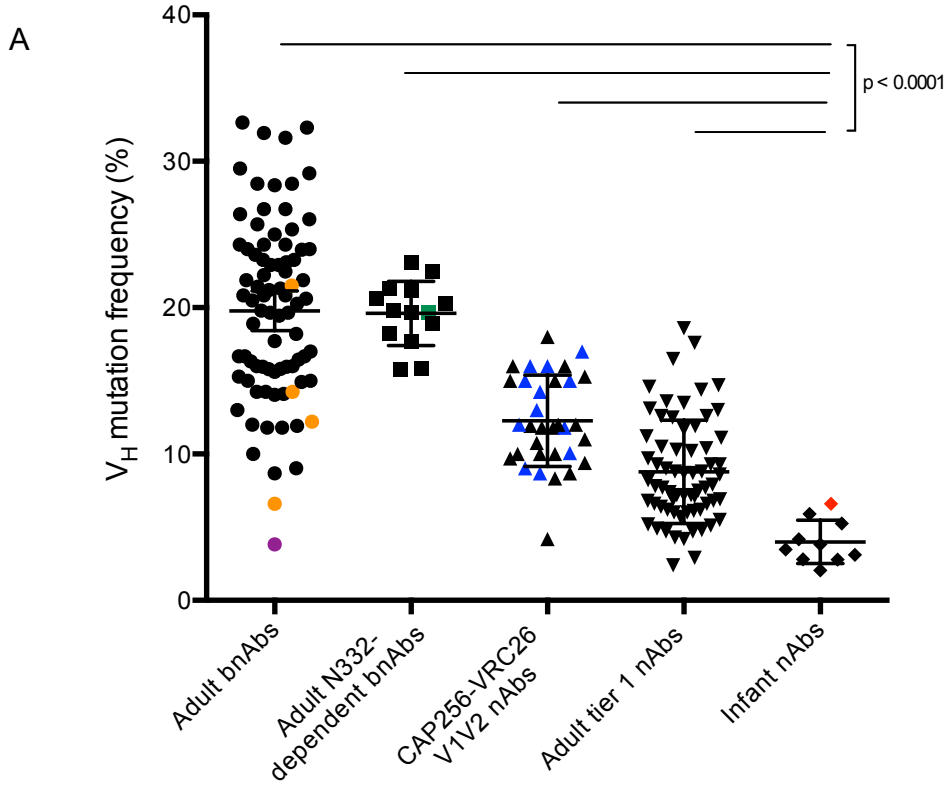
B

BF520 nAb Light Chain Sequence Characteristics							
Ab	**CDRL3 AA sequence**	**CDRL3 length (AA)**	**V_H % identity**	**V_H mut freq (nt)**	**V_H mut freq (AA)**	**V gene**	**J gene**
BF520.1	QQYNNWPSPFT	11	95.0	5.0	11.6	KV3-15	KJ3
BF520.2	QQYANSPRT	9	96.1	3.9	7.4	KV3-20	KJ1
BF520.3	CSYAGSSTYV	10	97.6	2.4	3.1	LV2-23	LJ1
BF520.4	TQATQFPRT	9	97.6	2.4	3.0	KV2-24	KJ1
BF520.5	QQYNNWPPWT	10	97.5	2.5	4.2	KV3-15	KJ1
BF520.6	QQYGSSRT	8	97.5	2.5	4.3	KV3-20	KJ1
BF520.7	QQSYSTPGT	9	97.1	2.9	6.4	KV1-39	KJ4
BF520.8	QQSYSTNT	8	97.5	2.5	6.5	KV1-39	KJ2
BF520.9	MQGTLPFT	8	97.6	2.4	5.2	KV2-30	KJ3
BF520.10	CSYAGSSTFRV	11	95.8	4.2	8.1	LV2-11	LJ2

Figure 2.13 Sequence characteristics of infant nAbs

Infant nAb (A) heavy and (B) light chain sequences were analyzed using IMGT V-QUEST.

A



B

	V-gene	D-gene	J-gene	CDRH3 length (AA)	VH mut frequency (nt)	VH indels (AA)	V-gene	J-gene	VL mut frequency (nt)	VL indels (AA)
PGT121	V4-59	D3-3	J6	26	20		LV3-21	LJ3	19	-7 (FR1)/ +3 (FR3)
PGT128	V4-39	D3-10	J5	21	20	+6 (CDR2)	LV2-8	LJ2	9	-5 (CDR1)
PGT135	V4-39	D3-9	J5	20	19	+5 (CDR1)	KV3-15	KJ1	18	
2G12	V3-21	D1-26	J3	16	21		KV1-5	KJ1	14	
BF520.1	V1-2	D3-10	J4	20	7		KV3-15	KJ3	5	

Figure 2.14 Levels of nAb SHM

Adult bnAbs include all nAbs available from bnaber.org. Yellow indicates 1st generation bnAbs. Purple indicates M66.6. V3 bnAbs include PGT120s, PGT130s and 2G12. Green indicates PGT121. CAP256-VRC26 nAbs with >30% neutralization breadth are shown in blue. Adult tier 1 nAbs have limited tier 2 neutralizing activity. The cross-clade infant nAb BF520.1 is shown in red. Horizontal bars indicate mean and 95% confidence intervals. Mann Whitney U test comparing infant nAb SHM to adult Nabs.

Discussion

Adult bnAbs identified to date were isolated from chronically infected adults from as early as 2 to over 15 years post-infection (84, 130). Longitudinal studies of bnAb development suggest these antibodies undergo iterative rounds of SHM and affinity maturation over years of infection before developing neutralization breadth (116). bnAbs that develop early in natural infection will be important to help design vaccine strategies to elicit these antibodies over a shorter period of time. In this study, we successfully isolated infant nAbs contributing to plasma neutralization breadth at about a year pi. These infant nAbs exhibit low levels of SHM compared to adult nAbs suggesting that HIV-1-specific neutralization breadth can develop without the requirement for several years of antibody affinity maturation. Moreover, they suggest that infants may provide unique insights into optimal pathways to develop HIV-1 specific bnAbs.

Adult bnAbs target a number of conserved sites of vulnerability on the HIV-1 Env trimer (292). Here, we identified an infant bnAb targeting one of these known epitopes, the glycan-dependent N332 supersite. BF520.1 neutralization is dependent on the N332-glycan and the antibody binds the base of the V3 loop. bnAb responses targeting the N332 supersite are of particular interest for vaccine design as they are one of the most commonly found bnAb responses (94). However, the high levels of SHM seen in adult N332-dependent bnAbs present significant challenges as vaccine-elicited HIV-specific antibodies in humans exhibit much lower levels (0 to 8.2% V_H mutation) (175). BF520.1 falls within this range and overall shows better breadth and potency for this level of SHM compared to less mutated lineage variants of adult bnAb PGT121 (179) as well as the MPER-directed adult bnAb M66.6 (181), which has the lowest level of SHM amongst adult bnAbs (**Figure 2.14**; indicated in purple). In addition, the PGT lineage antibodies have indels (123) that are important for neutralization (150, 179) and BF520.1 lacks these rare

insertions and deletions, which may simplify eliciting this response by vaccination. Another characteristic of glycan-targeting bnAbs is a long CDRH3 loop that reaches through the glycan shield and contacts the protein surface of Env (150, 152). While BF520.1 has the critical long loop structure, it utilizes distinct V and J heavy chain genes as well as different heavy and light chain gene rearrangements compared to other N332-dependent bnAbs (170) suggesting there are multiple pathways that can lead to the development of these glycan-dependent bnAbs.

We found that the nAbs isolated at ~ 1 year pi bound to Env trimer of the transmitted virus. However, these nAbs do not neutralize the corresponding virus, although they neutralize heterologous viruses. For HIV-1-specific bnAbs isolated to date, there is usually a strong linkage between trimer binding and neutralization (287-290). Lower affinity binding by Fab fragments compared to IgG for some infant nAbs suggests avidity effects may contribute to the strong binding for IgG and may account for the lack of neutralization of early autologous virus exhibited by some of the isolated nAbs. However, BF520.10 Fab fragments bound with comparable affinity as IgG to native-like soluble trimer of the autologous virus yet it did not neutralize the corresponding virus. Furthermore, all 10 infant nAbs bound to cell-surface expressed Env variants from the early virus. Thus, this seems to represent a case where antibodies were elicited that are capable of binding specifically to native Env of the early autologous virus but lack the ability to neutralize the corresponding virus. The BF520.1 bnAb does neutralize autologous virus from 2.2 months later, which was 9 months prior to when the nAbs were isolated. Thus, one model for these data is that the binding interaction of the BCRs with the autologous transmitted Env antigen may have initiated the maturation of these antibodies and subsequent responses to the evolving virus led to selection of B cells expressing nAbs. Studies of the infant BCR repertoire prior to and over the course of infection will be needed to test this model and to more precisely define the progenitor

BCRs for these nAbs.

It is unclear how infants develop bnAbs, although high viral load has been implicated (23). Furthermore, it is not known whether the lower levels of SHM seen for these infant antibodies is a result of inherent limitations of early-life B cell responses. However, infant B cell responses can exhibit adult-like diversity and SHM before 1 year of age (Siegrist and Aspinall, 2009), and there are unique features of mother-infant transmission that may also contribute to the rapid development of these responses. For example, passively acquired maternal antibodies present in infant circulation at the time of HIV-1 exposure may be playing a role by augmenting de novo nAb responses, as suggested by studies of macaques (252, 253). In addition, passively acquired antibodies may shape the epitopes exposed on vertically transmitted variants as maternal antibody escape variants are transmitted to infants (200, 250). Relevant to this, an infant envelope variant shows promise as an immunogen (293). Given that BF520.1 binds the Env of the transmitted variant, these findings raise the possibility that using Env immunogens based on vertically transmitted variants, possibly along with passively administered HIV-1-specific nAbs, may elicit antibodies similar to those identified here. In addition, this study, while only of a single infant, provides strong rationale to characterize nAbs from additional infants to determine the ontogeny of infant nAb responses and whether infant bnAbs generally have low levels of SHM.

In summary, we have isolated HIV-1-specific neutralizing antibodies from an infant who developed plasma cross-clade neutralization by one-year pi. One of these demonstrates broad neutralization and has unique features compared to adult bnAbs targeting the same N332 supersite including low SHM, a lack of indels, and distinct germline gene usage. Moreover this bnAb can bind the transmitted viral Env trimer, but does not neutralize the virus, another unique feature compared to other described HIV bnAbs. Overall, the identification of an infant bnAb that

developed early and has low SHM is encouraging for vaccine development. Specifically, BF520.1 may provide a template for glycan-dependent bnAbs that require limited SHM and thus may be relevant to studies to define immunization strategies to elicit such bnAbs without the requirement for a long-term maturation pathway.

Chapter III

Rapid development of an infant-derived HIV-1 broadly neutralizing antibody lineage

The text in this chapter was submitted as a manuscript that is currently under revision at Cell. This study was co-led by myself and Laura Noges. The B cell repertoire deep sequencing was performed by Laura Noges and antibody lineage inference was performed by Laura and Erick Matsen's group (Figure 3.1). I led the functional studies including study design and data interpretation (Figures 3.2 – 3.10) and wrote the manuscript.

Introduction

Considerable efforts have been made to define evolutionary pathways of broadly neutralizing antibodies (bnAbs) in HIV-1 infection with the premise that these pathways will help guide effective immunization strategies (294). Particular emphasis has been placed on bnAb epitopes that are common in different individuals (148) such as the V3-glycan region of HIV-1 envelope (Env) (94), and much progress has been made toward characterizing the development of V3-glycan bnAbs in adults (85, 133, 179, 196). However, significant challenges remain to be addressed for inducing bnAb responses by vaccination. One such challenge is that most adult bnAbs take years to develop as a result of a complex interplay between viral escape and antibody maturation (148) which often leads to extensive somatic hypermutation (SHM), ranging from ~6-29% (averaging ~18%) for adult-derived V3-glycan bnAbs (51, 85, 119, 123, 133, 134, 143, 202). In addition, the inferred germline precursors of many bnAbs lack detectable binding to recombinant HIV envelope and thus require the design of germline-targeting immunogens for vaccines (85, 134, 178, 179, 195, 196). Furthermore, some bnAbs are limited by autoreactivity (133, 134, 295).

To date, there has not been success in eliciting bnAbs in humans using vaccine candidates, although there has been recent progress in animal models towards eliciting

autologous tier 2 neutralizing antibodies in response to immunization with HIV Env trimers, often a trimer from an infant-derived transmitted virus, BG505 (199, 200). As the evolutionary pathway of adult bnAbs have been dissected (148), immunization strategies that mimic lineage pathways defined for adult-derived bnAbs have been attempted in animal models, and cross-clade nAbs were elicited in knock-in mice carrying the inferred germline precursors of bnAbs (296) and sporadically in rabbits and non-human primates (297).

Our lab discovered that infants can also develop bnAb responses and that they do so rapidly, within 1-2 years post-infection(23). bnAb responses were also identified in another pediatric cohort, and these studies collectively demonstrate that these responses develop at least as commonly, if not more frequently, in children when compared to adults (265). To begin to characterize these early infant cross-clade neutralizing antibody responses, we previously isolated antibodies from an infant with a rapid and broad plasma nAb response at about one year post-infection. One infant-derived bnAb, BF520.1, demonstrated cross-clade neutralization breadth and targets the V3-glycan region of HIV Env. In contrast to most adult-derived bnAbs, BF520.1 has limited SHM (VH = 6.6%, VK = 5% nt) (298). Because nAbs have only been isolated and characterized from a single HIV-infected infant, the differences between infant and adult antibody development is not known. Here we identify the naïve antibody precursor of BF520.1 and describe the subsequent evolution of this antibody lineage for the first and as yet only infant-derived HIV-specific bnAb that has yet been described.

Materials and methods

Sample acquisition and cell preparation

Peripheral blood mononuclear cell (PBMC) samples were obtained from infant BF520 enrolled in the Nairobi Breastfeeding Clinical Trial (266), which was conducted prior to the use of antiretrovirals for prevention of mother-to-child transmission. Approval to conduct the Nairobi Breastfeeding Clinical Trial was provided by the ethical review committee of the Kenyatta National Hospital Institutional Review Board, the Fred Hutchinson Cancer Research Center Institutional Review Board, and the University of Washington Institutional Review Board. PBMCs stored in liquid nitrogen for ~20 years were thawed at 37°C, diluted 10-fold in pre-warmed RPMI and centrifuged for 10 min at 300x*g*. Cells were washed once in phosphate-buffered saline, counted with trypan blue, centrifuged again, and total RNA was extracted from PBMCs using the AllPrep DNA/RNA Mini Kit (Qiagen), according to the manufacturer's recommended protocol. RNA was stored at -80°C until library preparation.

We performed library preparation, sequence analysis, and antibody lineage reconstruction in technical duplicate, using the same RNA isolated from each time-point, week 1 (W1) and month 9 (M9).

Antibody gene deep sequencing

Antibody sequencing was performed as described (187). Briefly, RACE-ready cDNA synthesis was performed using the SMARTer RACE 5'/3' Kit (Takara Bio USA) using primers with specificity to IgG, IgK and IgL. The cDNA was diluted in Tricine-EDTA according to the manufacturer's recommended protocol. First-round Ig-encoding sequence amplification (20 cycles) was performed using Q5 High-Fidelity Master Mix (New England BioLabs) and nested gene-specific primers (**Table 3.1**). Amplicons were directly used as templates for MiSeq

adaption by second-round PCR amplification (20 cycles). Amplicons were then purified and analyzed by gel electrophoresis, and indexed using Nextera XT P5 and P7 index sequences for Illumina sequencing according to the manufacturer's instructions (10 cycles). Gel-purified, indexed libraries were quantitated using the KAPA library quantification kit (Kapa Biosystems) performed on an Applied Biosystems 7500 Fast real-time PCR machine.

Libraries were denatured and loaded onto Illumina 600-cycle V3 cartridges, according to the manufacturer's suggested workflow

Table 3.1 Illumina Miseq library preparation primers

Conc. (nM)	Primer ID	Sequence
	RT-PCR	
500	3'IgM (OUTER)	CCACTTCGTTTGTATCCAACG
500	3'IgG (OUTER)	GCCGGGAAGGTGTGCACGCCGCTGGTC
500	3'IgK (OUTER)	GTCCTGCTCTGTGACACTCTC
500	3'IgL (OUTER)	TGTTGCTCTGTTTGGAGGG
	PCR 1	
800	3'IgM (INNER)	GCATTCTCACAGGAGACGAGG
800	3'IgG (INNER)	CCGGTTCAGGGAAGTAGTCCTTGAC
800	3'IgK (INNER)	ATTCAGCAGGCACACAACAGAGGC
800	3'IgL (INNER)	AGACACACTAGTGTGGCCTTG
800	vv535: Step out primer	GACAAGCAGTGGTATCAACGCAG
	PCR 2	
400	MiSeq HsRh IgM CH1 R	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG* GGGTTGGGGCGGATGCACT
400	MiSeq HsRh IgG CH1 R	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG* GGGGGAAGACCGATGGGCCCTT
400	MiSeq HsRh IgK CL1 R	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG* GAAGACAGATGGTGCAGCC
400	MiSeq HsRh IgL CL1 R	*GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG* GGAACAGAGTGACCGTGGG
400	vv539: MiSeq-adapted step out primer	TCGTCGGCAGCGTC AGATGTGTATAAGAGACAG CACTCTATCCGACAAGCAGTGGTATC

Sequence analysis and clonal family clustering

Sequences were preprocessed as previously described (187). Briefly, amplicons were reconstructed from forward and reverse MiSeq reads using FLASH (299) and the amplification primers were trimmed using cutadapt (300). Sequences that contained low-confidence base calls (N's) were removed (FASTX-toolkit). Filtered sequences from both time-points (W1 and M9) were combined and annotated with partis (https://github.com/psathyrella/partis) using the default options (including per-sample germline inference, Ralph in review at PLOS Comp Biol http://arxiv.org/abs/1711.05843). Sequences with internal stop codons, or with CDR3 regions out

of frame were removed during this step. Sequences were then clustered into clonal families using the seed clustering method (301, 302) and the previously-identified BF520.1-IgG and BF520.1-IgK sequences (298) as "seeds". This method included the inference of the unmutated common ancestor sequence for each clonal family.

Antibody lineage reconstruction

Initial phylogenetic trees of BF520.1 heavy and light chain clonal families were inferred using FastTree (http://dx.plos.org/10.1371/journal.pone.0009490.g003) and pared down to 100 sequences based on proximity to nodes within the "seed lineage" extending from the inferred naïve ancestor (root) to the mature BF520.1 heavy or light sequence (prune.py, https://git.io/vA7wH). A formal description of this method for determining antibody lineages is in progress, including benchmarking comparisons. The 100 clonal sequences were then analyzed by BEAST (303). BEAST output was summarized for internal node sequences using the approach presented by Gong et al. (304). In the resulting summary graphic, each red oval represents a unique inferred sequence, with color intensity proportional to the relative confidence that the true lineage included that intermediate. Arrows correspond to amino acid substitutions, with color intensity proportional to the relative confidence that this specific substitution occurred. The most probable BF520.1-IgG lineage paths were consistent between both technical replicates. For BF520.1-IgK, replicates agreed on probable lineage paths but each replicate offered higher resolution at a different part of the lineage (early mutations vs. later mutations). These data were combined to estimate the M0-M9 BF520.1-IgK lineage.

In addition to Bayesian lineage analysis, maximum likelihood (dnaml) and parsimonious (dnapars) phylogenetic trees were inferred from the list of 100 clonal sequences, including ancestral sequence reconstruction using PHYLIP tools

(http://evolution.genetics.washington.edu/phylip/getme-new1.html) (Felsenstein, J. 2017. PHYLIP (Phylogeny Inference Package) version 3.696. *Distributed by the author. Department of Genome Sciences, University of Washington, Seattle*). Trees were rooted on the inferred naïve sequence and we identified intermediate sequences that were found on lineage paths between naïve to seed sequence. For dnaml and dnapars analyses, filtered IgK sequences were down sampled to 100,000 randomly-selected sequences prior to partis seed partitioning and FastTrees were pared down using an earlier version of prune.py (https://git.io/vA7ww).

mAb preparation

Antibody heavy and light chain variable regions were synthesized as "gBlocks" by Integrated DNA Technologies (www.idtdna.com) and subsequently cloned into IgG expression vectors as previously described (269). Equal ratios of heavy and light chain plasmids were co-transfected into 293F cells using FreeStyle MAX (Invitrogen) according to the manufacturer's instructions. Protein G columns were used to purify IgG as previously described (144).

Pseudovirus production and neutralization assays

Pseudovirus production and neutralization assays were performed as previously described (250)with an alternative cell lysis and B-galactosidase detection system (Gal-Screen; ThermoFisher). Specifically, 85uL of the 150uL total volume was removed from each well (50uL remaining) and 50uL of Gal-Screen substrate (diluted 1:25 in "Buffer A") was added to each well. Luminescence was measured after 40 minutes incubation at room temperature. The mAbs were diluted 2-fold from 20 to 0.6 μg ml^{-1}. IC_{50} values represent the concentration (μg ml^{-1}) at which 50% of the virus was neutralized and are the average of two or three independent experiments performed in duplicate.

Biolayer interferometry assays

Binding of mAbs to HIV Env SOSIP trimers was measured using biolayer interferometry on an Octet RED instrument (ForteBio). For the qualitative comparison of lineage intermediate binding, IgG antibodies diluted to 10 μg ml^{-1} in PBS plus 1% BSA, 0.01% TWEEN20, and 0.02% sodium azide were immobilized onto anti-human IgG Fc capture (AHC) biosensors and BG505.SOSIP.664 (diluted to 1μM in the same buffer) was flowed as analyte in solution. For mAb kinetic determination, BG505.SOSIP.664-AviB (25 μg ml^{-1}) was immobilized onto Streptavidin (SA) biosensors. Varying concentrations of IgG were flowed as analyte in solution. A series of six, two-fold dilutions of naïve$_{VH}$ mature$_{VK}$ and mature$_{VH}$ naïve$_{VK}$ mAbs (667 to 21 nM) as well as 0 nM IgG were tested. The BF520.1 naïve mAb concentrations ranged from 2.67 to 0.33 μM (series of four, two-fold dilutions) and 0 μM. Association was monitored for 6 minutes and dissociation for 10 minutes. Binding-affinity constants (K_D; on-rate, K_{on}; off-rate, k_{dis}) were determined using ForteBio's Data Analysis 7.0. Average measurements from reference wells were subtracted and data were processed by Savitzky-Golay filtering prior to fitting using a 1:2 (bivalent analyte) model of binding.

Results

Ontogeny of the infant-derived bnAb BF520.1

HIV infection of infant BF520 with a clade A virus was detected at 3.8 months of age and the V3 glycan-directed monoclonal antibody (mAb) BF520.1 was isolated at 15 months of age or about 1 year post-infection (pi) (298). To explore the ontogeny of this bnAb, we performed next generation sequencing of the infant's B cell repertoire both pre-infection (1 week of age) and post-infection (6 months pi or 9 months of age). We then used partis, a computational tool specifically developed for analyzing antibody lineages, to infer the naïve heavy and light chain

ancestors of the BF520.1 mAb and to identify clonally-related sequences that derived from the same inferred naïve ancestor (301, 302) (manuscript by Ralph and Matsen, "Per-sample immunoglobulin germline inference from B cell receptor deep sequencing data" (https://arxiv.org/abs/1711.05843)). Maximum likelihood phylogenetic trees were inferred with dnaml (Felsenstein, J. 2017. PHYLIP (Phylogeny Inference Package) version 3.696. *Distributed by the author. Department of Genome Sciences, University of Washington, Seattle*) for the heavy and light chain clonal families, including the inferred naïve and mature BF520.1 sequences, to illustrate where BF520.1 falls within the context of its clonal family (**Figure 3.1 A and C**). To reconstruct the most likely developmental routes taken by BF520.1 heavy and light chain sequences within the first 6 months of infection, we performed Bayesian phylogenetic and ancestral sequence inference analyses for each antibody chain. Specifically, we used BEAST (303) to sample BF520.1 clonal family phylogenies from an associated posterior distribution and then summarized the BEAST output to display relative confidences for internal node sequences using an approach developed by Gong et al. (304). The resulting Bayesian antibody heavy and light chain lineage graphics display multiple possible lineages of amino acid transitions and their relative confidences (**Figure 3.1 B and D**). Using two replicates of NGS data, we selected the most probable routes of development for the BF520.1 heavy and light chains between infection and M6 pi and produced the appropriate lineage intermediates for further study: heavy chain (VH) intermediates 1-3 (Int1-3_{VH}) and light chain (VK) intermediates 1-4 (Int1-4_{VK}). Selection of these lineages relied on considering the most confident transitions (Fig 1B and D; darkest blue arrows) from the naïve sequence to the mature BF520.1 sequence in both NGS replicates. For the heavy chain, both replicates indicated that the G57D substitution occurred first and, thus, $Int1_{VH}$ included this change. The Y32N amino acid substitution occurred next in both replicates and was

therefore incorporated in $Int2_{VH}$. The M34I and F114L substitutions were inferred in one of the two replicates, but the order was unclear, so both were added to $Int3_{VH}$. For the kappa chain, both replicates supported L78M as the first mutation, so this substitution was added to create $Int1_{VK}$. However, there was less agreement between replicates on the chronological order of the substitutions that followed. Both agreed that two substitutions (S30A and S67F) occurred prior to two later substitutions (S28N and T53S), so these steps were incorporated as $Int2_{VK}$ and $Int3_{VK}$. Finally, the A25T substitution was incorporated into $Int4_{VK}$ because one of the two replicates indicated that this was a possible late substitution. Using this approach, we reconstructed highly probable routes of heavy and light chain antibody development within the first 6 months of HIV infection.

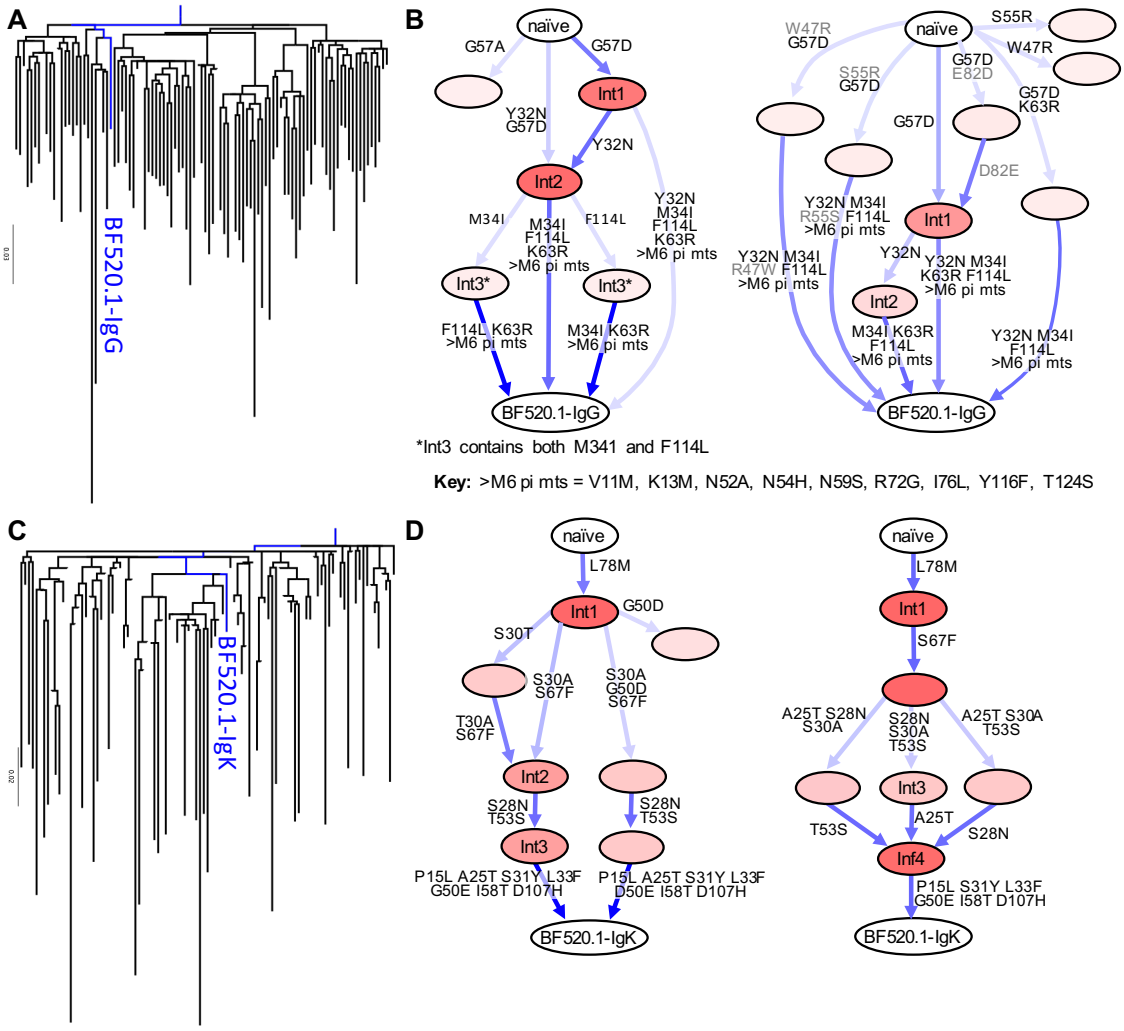


Figure 3.1 Ontogeny of the infant-derived bnAb BF520.1

(A, C) maximum likelihood phylogenetic relationships of (A) heavy and (C) light chain antibody gene variable regions. Trees display the inferred naïve ancestor (root), BF520.1 from M12 pi (blue), and representative clonal family member next generation sequencing (NGS) reads from M6 pi (black). Units for branch length estimates are nucleotide substitutions per site. (B, D) Most probable routes of BF520.1 (B) heavy and (D) light chain development determined from two NGS technical replicates (left, right). Amino acid substitutions (arrows) connect the inferred naïve sequence to the mature BF520.1 sequence via reconstructed ancestral intermediate sequences (nodes). Labeled nodes were chosen as the most probable Bayesian lineage intermediate sequences. Darker colors indicate higher relative confidence. The red shading of nodes is proportional to the posterior probability that this ancestral sequence was present in the lineage. For a given node, the blue shading across arrows arising from that node is proportional to the corresponding transition probability. Transient mutations are labeled in grey.

Increasing heterologous neutralization by the maturing BF520.1 heavy chain lineage

Antibodies were tested for HIV neutralizing activity against a panel of heterologous viruses that were selected from both the original virus panel used to describe infant plasma neutralization breadth (23) and the standardized "global panel" (283) based on their ability to be neutralized by the mature BF520.1 antibody with an IC_{50} <20 μg ml^{-1} (298). The inferred naïve mAb derived by pairing the inferred naïve heavy and light chains did not demonstrate neutralizing activity against any viruses in this panel (**Figure 3.2 A**). To identify the heavy chain substitutions gained within the first 6 months pi that were important for neutralization breadth, the naïve gamma chain and Bayesian lineage intermediates (Int1-3_{VH}) were paired with the mature kappa light chain and tested for HIV neutralizing activity. In contrast to the naïve mAb, the naïve heavy chain paired with the mature kappa light chain (naïve$_{VH}$ mature$_{VK}$) demonstrated cross-clade tier 2 neutralizing activity (**Figure 3.2 A**). The Int1_{VH} mature$_{VK}$ mAb had comparable activity to the naïve$_{VH}$ mature$_{VK}$ mAb suggesting that the CDRH2 G57D substitution in this Int1_{VH} did not confer increased neutralizing activity (Figures 2A and 2B). Subsequent heavy chain intermediates demonstrated increasing heterologous, cross-clade neutralizing activity with affinity maturation. Notably, a Y32N substitution in the CDRH1 conferred increased neutralization breadth for Int2_{VH} and further cross-clade breadth and potency was observed in Int3_{VH} with FR2 M34I and CDRH3 F114L substitutions (**Figure 3.2 A and B**).

Because the BF520.1 clonal family sequences were collected from a midpoint sample (M6 pi), these sequences only informed the first 6 months of BF520.1 antibody development and thus we could not infer the chronology of mutations that occurred in the mature BF520.1 between M6 and M12 pi, when BF520.1 was isolated. Therefore, for mutations that occurred in BF520.1 between M6 and M12pi, we rationally incorporated amino acid substitutions according

to their position in the variable region. In order of precedence, we added CDR mutations, CDR-adjacent framework (FR) mutations, and non-conservative FR mutations (Int4-6_{VH}) (**Figure 3.2 B**). With the addition of N52A in the CDRH2 of $Int4_{VH}$, the antibody gained breadth and potency comparable to the mature mAb BF520.1 (**Figure 3.2 A and B**).

Previous studies of antibody lineages inferred intermediate sequences from single maximum likelihood and parsimony phylogenies (82, 84, 86, 133) and thus did not consider relative confidence in lineage steps used in our Bayesian approach. Given this precedent, we also used single maximum likelihood (ML) (**Figures 3.3 A**) and parsimony (Pars) phylogenies (**Figures 3.3 C**) to infer BF520.1 heavy and light chain lineage intermediates. Interestingly, the heavy chain intermediates inferred from the maximum likelihood phylogeny included substitutions that were not found in either the naïve or mature mAbs, indicating that they were likely erroneous. For comparison, some Bayesian lineages included substitution reversions as well, but these paths consistently had lower posterior probabilities than paths lacking these artifacts (**Figure 3.1 B and D**). When present, these substitutions decreased neutralizing activity, which is apparent when comparing ML $Int3_{VH}$ and ML $Int4_{VH}$ (**Figure 3.3 A and B**). These findings suggest that the Bayesian antibody lineage determination that incorporated relative confidence over a number of possible lineage pathways prevented the inference of artifactual, transient mutations in antibody intermediates. Regardless, our studies of the maximum likelihood and parsimony lineage intermediates led to similar conclusions about the importance of heavy chain substitutions: CDRH1 Y32N and CDRH2 N52A substitutions resulted in increased neutralization breadth (**Figure 3.3 A-D**). The contribution of the Y32N CDRH1 substitution was particularly apparent when comparing Pars $Int3_{VH}$, which lacks Y32N and Pars $Int4_{VH}$ (contains Y32N) (**Figure 3.3 C and D**).

We next examined whether or not the heavy chain substitutions that led to neutralization breadth occurred in AID hotspots; a recent study demonstrated that an adult-derived V3-glycan bnAb with limited SHM took years to develop because it required an unusual AID mutation (85). For BF520.1 VH, the Y32N substitution was encoded by a T94A nucleotide mutation (**Figure 3.4 A**) which occurred at an AID hotspot defined as TW (W = A or T) (272). The N52A substitution resulted from two adjacent nucleotide mutations A154G and A155C (aac>gcc) (**Figure 3.4 A**). The A155C nucleotide mutation is in an AID hotspot (WA) whereas the A154G is not, though it is not in a canonical coldspot.

Overall, these data demonstrate increasing heterologous neutralizing activity with affinity maturation and show that the heavy chain CDRH1 Y32N and CDHR2 N52A substitutions are important for the neutralization breadth of BF520.1.

A

Tier	Clade		BF520.1 lineage heavy chain intermediates								
		VH	naïve $_{VH}$	naïve $_{VH}$	Int1 $_{VH}$	Int2 $_{VH}$	Int3 $_{VH}$	Int4 $_{VH}$	Int5 $_{VH}$	Int6 $_{VH}$	mature $_{VH}$
		VK	naïve $_{VK}$	mature $_{VK}$							
1A	B	SF162	>20	0.7	0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6
1B		Q23.17	>20	1.1	2.2	0.9	<0.6	<0.6	<0.6	<0.6	<0.6
2	A	BG505.W6M.C2_T332	>20	>20	>20	7.0	3.1	1.7	1.0	1.3	1.1
		398F1	>20	5.5	4.9	3.7	1.3	0.6	<0.6	<0.6	<0.6
	B	X2278	>20	19.5	>20	3.8	2.3	1.2	0.7	0.9	1.0
		TRO.11	>20	>20	>20	>20	>20	6.5	4.8	7.9	5.9
	BC	BJOX002000	>20	>20	>20	15.8	3.4	1.0	0.8	1.3	0.9
		CH119	>20	>20	>20	>20	14.7	6.1	3.7	6.1	6.0
	C	CE1176	>20	17.0	17.9	13.5	8.7	6.1	5.6	7.0	5.1
		QC406.F3	>20	<0.6	0.7	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6
		CE0217	>20	>20	>20	>20	12.3	2.1	2.1	4.4	2.8
		DU156.12	>20	>20	>20	16.9	9.8	2.0	1.8	2.3	2.2
		DU422.1	>20	5.7	12.9	3.2	4.4	2.2	1.4	2.9	2.6
		SIV	>20	>20	>20	>20	>20	>20	>20	>20	>20

B

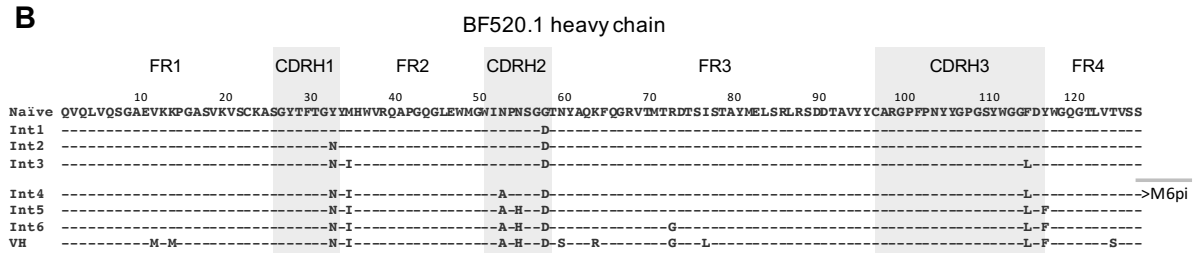


Figure 3.2 BF520.1 heavy chain lineage heterologous neutralization

(A) Neutralization of panel viruses by BF520.1 inferred lineage intermediates. The top rows of the table (VH and VK) show the origin of the antibody chain sequence used. For VH this included the naïve, Bayesian (0-6 months pi) and rationally inferred (6-12 months pi) lineage intermediates, indicated by Int#$_{VH}$ with # indicating the progression of intermediates in the lineage. These were paired with the indicated kappa chain, in most cases the mature kappa chain (mature$_{VK}$). The panel viruses are shown to the left, with the tier, clade and name indicated. SIV was included as a negative control. IC_{50} values (µg ml^{-1}) represent an average of two to three independent experiments performed in duplicate. IC_{50} values are color-coded with darker shades of blue indicating more potent neutralization. Grey indicates that 50% neutralization was not achieved at the highest mAb concentration tested. (B) Amino acid alignment of BF520.1 naïve, Bayesian (0-6 months pi) and rationally inferred (6-12 months pi) lineage intermediates and mature heavy chain sequences. Intermediates designated as >M6 pi were rationally inferred.

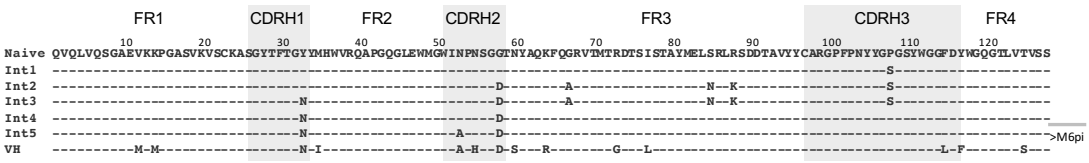
A

BF520.1 VH maximum likelihood lineage intermediates

Tier	Clade		VH / VK	naïve$_{VH}$	ML Int1$_{VH}$	ML Int2$_{VH}$	ML Int3$_{VH}$	ML Int4$_{VH}$	ML Int5$_{VH}$	mature$_{VH}$
			mature $_{VK}$							
1A	B	SF162		0.7	0.7	<0.6	<0.6	<0.6	<0.6	<0.6
1B	A	Q23.17		1.1	2.0	3.6	1.2	<0.6	<0.6	<0.6
2	A	BG505.W6M.C2_T332		>20	>20	>20	>20	6.3	2.5	1.1
		398F1		5.5	>20	>20	19.6	2.4	1.3	<0.6
	B	X2278		19.5	18.8	19.9	7.1	2.8	1.3	1.0
		TRO.11		>20	>20	>20	>20	>20	15.3	5.9
	BC	BJOX002000		>20	>20	>20	>20	11.9	2.8	0.9
		CH119		>20	>20	>20	>20	>20	8.8	6.0
	C	CE1176		17.0	>20	>20	>20	8.3	7.0	5.1
		QC406.F3		<0.6	<0.6	1.3	0.8	<0.6	<0.6	<0.6
		CE0217		>20	>20	>20	>20	14.6	6.0	2.8

B

BF520.1 heavy chain (maximum likelihood)



BF520.1 VH parsimony lineage intermediates

C

Tier	Clade		VH / VK	naïve $_{VH}$	Pars Int1 $_{VH}$	Pars Int2 $_{VH}$	Pars Int3 $_{VH}$	Pars Int4 $_{VH}$	Pars Int5 $_{VH}$*	mature $_{VH}$
			mature$_{VK}$							
1A	B	SF162		0.7	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6
1B	A	Q23.17		1.1	0.9	1.4	1.2	<0.6	<0.6	<0.6
2	A	BG505.W6M.C2_T332N		>20	>20	>20	>20	3.0	1.7	1.1
		398F1		5.5	5.2	15.7	11.7	1.4	0.6	<0.6
	B	X2278		19.5	14.1	19.7	15.8	1.7	1.2	1.0
		TRO.11		>20	>20	>20	>20	15.1	6.5	5.9
	BC	BJOX002000		>20	>20	>20	>20	3.1	1.0	0.9
		CH119		>20	>20	>20	>20	10.6	6.1	6.0
	C	CE1176		17.0	17.9	>20	>20	6.3	6.1	5.1
		QC406.F3		<0.6	<0.6	0.7	0.9	<0.6	<0.6	<0.6
		CE0217		>20	>20	>20	>20	11.6	2.1	2.8

* same mAb as the Bayesian lineage VH Int4

D

BF520.1 heavy chain (parsimony)

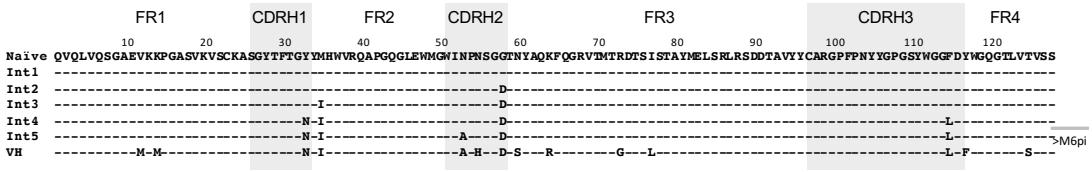


Figure 3.3 BF520.1 heavy chain ML and Pars lineage heterologous neutralization

BF520.1 (A) maximum likelihood and (C) parsimony lineage heavy chains paired with the mature kappa light chain, mAb neutralization of viruses. IC_{50} values (μg ml^{-1}) are an average of two to three independent experiments performed in duplicate. IC_{50} values are color-coded with darker shades of blue indicating more potent neutralization. Grey indicates that 50% neutralization was not achieved at the highest mAb concentration tested. Amino acid alignments of (B) maximum likelihood and (D) parsimony heavy chain lineage intermediates. Intermediates designated as >M6 pi were rationally inferred.

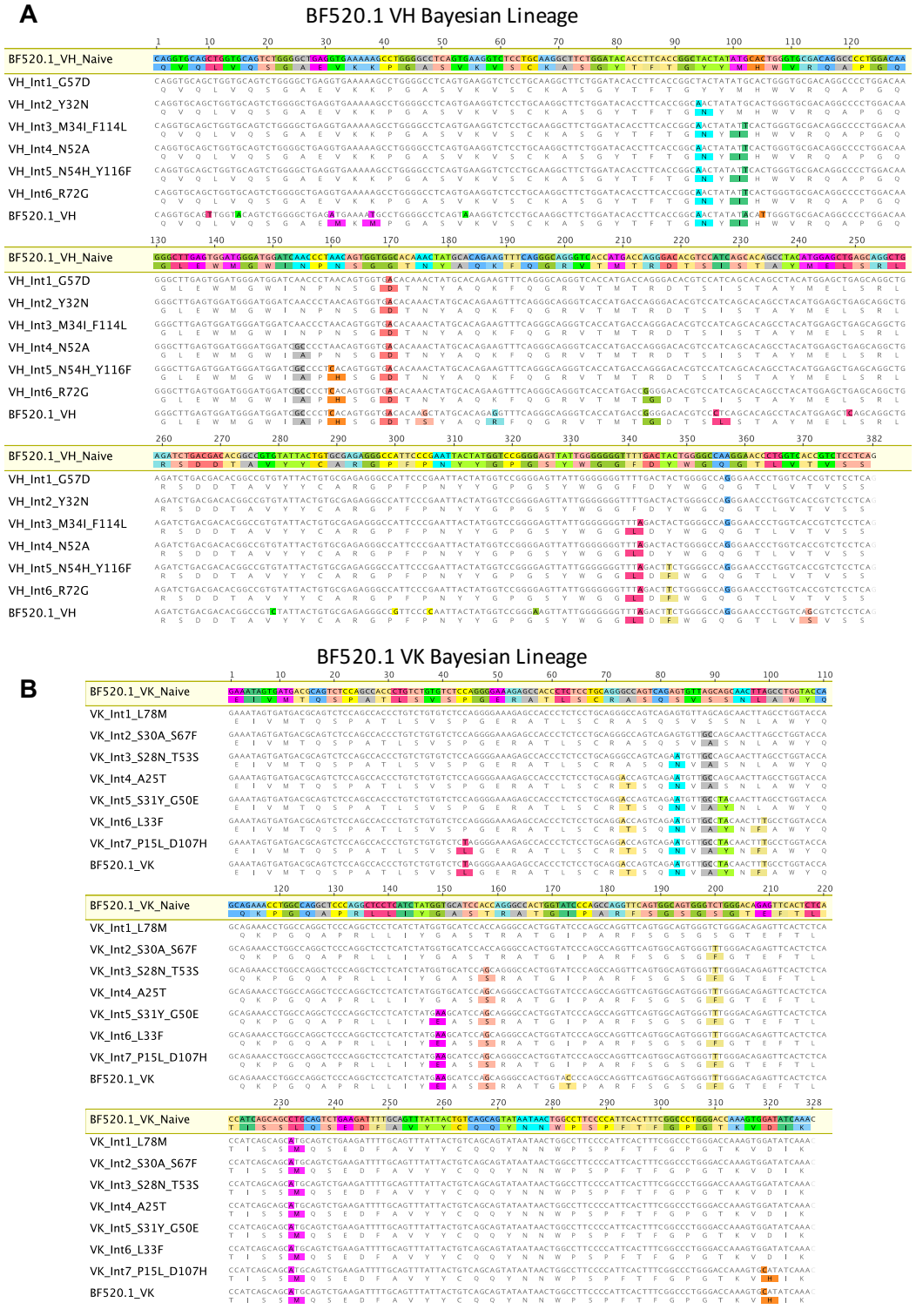


Figure 3.4 BF520.1 lineage nucleotide and amino acid alignments

BF520.1 naïve, Bayesian (0-6 months pi) and rationally inferred (M6-12 pi) lineage intermediates and mature sequences. Amino acid and nucleotide alignments of the BF520.1 (A) heavy chain and (B) kappa chain naïve, intermediate and mature sequences.

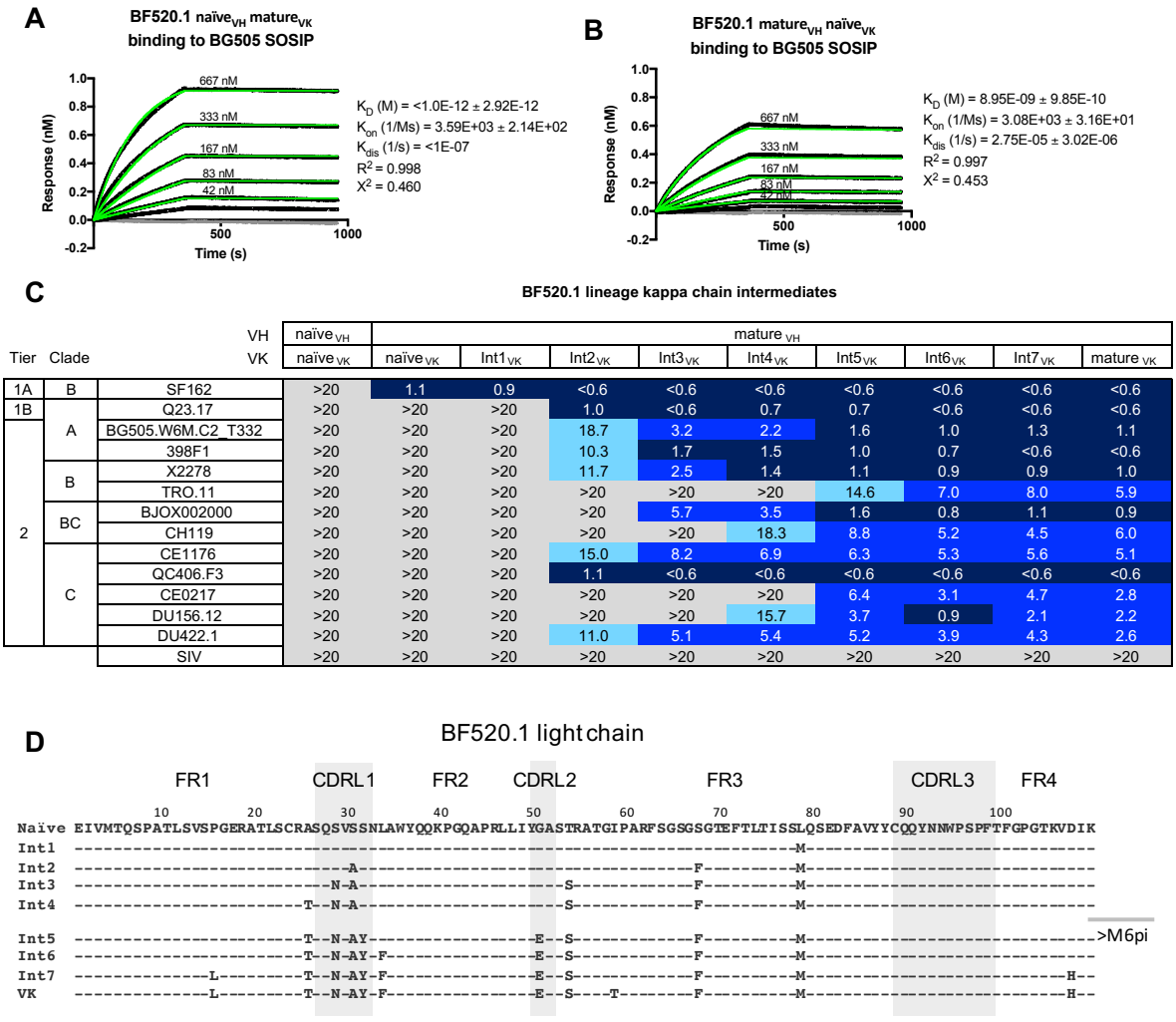
Contribution of kappa light chain maturation to HIV binding and neutralization

Given the surprising finding that the naïve heavy chain paired with the mature kappa light chain (naïve$_{VH}$ mature$_{VK}$) demonstrated cross-clade tier 2 HIV neutralizing activity and the naïve mAb did not (**Figure 3.2 A**), we also examined the evolution of the kappa chain in relation to binding of HIV Env and neutralization breadth. We cross-paired mature and naïve heavy and light chains (naïve$_{VH}$ mature$_{VK}$ and mature$_{VH}$ naïve$_{VK}$) and tested them for Env binding and neutralization capacity. Both antibodies bound the BG505 SOSIP trimer, which is an Env that is sensitive to BF520.1 neutralization. The naïve$_{VH}$ mature$_{VK}$ mAb demonstrated stronger binding to the BG505.SOSIP.664 trimer (K_D = <0.001nM) (**Figure 3.5 A**) than the converse mature$_{VH}$ naïve$_{VK}$ mAb (K_D = 8.95nM) (**Figure 3.5 B**). In contrast to the heterologous neutralizing activity seen for the naïve$_{VH}$ mature$_{VK}$ mAb, the mature$_{VH}$ naïve$_{VK}$ mAb neutralized only the tier 1A SF162 variant and did not demonstrate tier 2 neutralizing activity (**Figure 3.5 B**). These data suggest that maturation in the kappa light chain is necessary for BF520.1 heterologous neutralization breadth.

To identify which mutations in the kappa chain contributed to increased neutralizing activity, kappa chain Bayesian lineage intermediates (Int1-4$_{VK}$) and subsequent rationally-designed intermediates (Int5-7$_{VK}$) were paired with the mature heavy chain and tested for cross-clade tier 2 neutralizing activity. Overall, kappa chain lineage intermediates demonstrated increasing breadth with maturation (**Figure 3.5 C and D**). A dramatic jump in neutralization breadth was demonstrated by Int2$_{VK}$, which contains CDRL1 S30A and FR3 S67F substitutions (**Figure 3.5 C and D**). Although S67F was observed prior to the S30A substitution in the ML and Pars kappa lineages, it did not increase neutralization breadth (**Figure 3.6 A-D**), suggesting that the S30A CDRL1 substitution alone enables the observed heterologous neutralization

breadth. The S30A CDRL1 substitution that conferred a dramatic increase in neutralization breadth required two nucleotide mutations A88G and G89C (**Figure 3.4 B**), both of which are in canonical AID hotspots defined as WA and RGYW, respectively (W = A or T; R = A or G). Further augmentation of potency and breadth was conferred by substitutions observed in $Int3_{VK}$ (CDRL1 S28N and CDRL2 T53S). The T53S substitution was observed prior to S28N in the ML kappa lineage and it did not increase breadth, but did confer a slight increase in potency. $Int4_{VK}$ (FR1 A25T) had a very modest increase in activity. Importantly, $Int5_{VK}$ (CDRL1 S31Y and CDRL2 G50E) neutralized all viruses that were neutralized by the mature antibody and $Int6_{VK}$ (FR2 L33F) reached potency comparable to BF520.1 (**Figure 3.5 C and D**). Overall, these data suggest that substitutions in and around the CDRL1 are important for neutralization breadth and potency.

The mature BF520.1 kappa chain CDRL1 has three out of six amino acids mutated with two additional amino acid substitutions in close proximity. These CDRL1-proximal substitutions account for 5/12 of the total amino acid substitutions between the naïve and mature kappa chain (Figure 3D). Furthermore, the BF520.1 kappa chain does not contain CDRL3 mutations, based on the inferred naïve sequence. These data suggest that kappa chain mutations in and around the CDRL1 are important for BF520.1 heterologous neutralization breadth. Overall, these data suggest that the BF520.1 kappa chain makes a significant contribution to binding and neutralization of heterologous viruses.



Tier	Clade		VH	naïve$_{VH}$	mature$_{VH}$							
			VK	naïve$_{VK}$	naïve$_{VK}$	Int1$_{VK}$	Int2$_{VK}$	Int3$_{VK}$	Int4$_{VK}$	Int5$_{VK}$	Int6$_{VK}$	Int7$_{VK}$	mature$_{VK}$
1A	B	SF162		>20	1.1	0.9	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6
1B		Q23.17		>20	>20	>20	1.0	<0.6	0.7	0.7	<0.6	<0.6	<0.6
2	A	BG505.W6M.C2_T332		>20	>20	>20	18.7	3.2	2.2	1.6	1.0	1.3	1.1
		398F1		>20	>20	>20	10.3	1.7	1.5	1.0	0.7	<0.6	<0.6
	B	X2278		>20	>20	>20	11.7	2.5	1.4	1.1	0.9	0.9	1.0
		TRO.11		>20	>20	>20	>20	>20	>20	14.6	7.0	8.0	5.9
	BC	BJOX002000		>20	>20	>20	>20	5.7	3.5	1.6	0.8	1.1	0.9
		CH119		>20	>20	>20	>20	>20	18.3	8.8	5.2	4.5	6.0
	C	CE1176		>20	>20	>20	15.0	8.2	6.9	6.3	5.3	5.6	5.1
		QC406.F3		>20	>20	>20	1.1	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6
		CE0217		>20	>20	>20	>20	>20	>20	6.4	3.1	4.7	2.8
		DU156.12		>20	>20	>20	>20	>20	15.7	3.7	0.9	2.1	2.2
		DU422.1		>20	>20	>20	11.0	5.1	5.4	5.2	3.9	4.3	2.6
		SIV		>20	>20	>20	>20	>20	>20	>20	>20	>20	>20

Figure 3.5 Contribution of kappa light chain maturation to HIV binding and neutralization

(A and B) BLI representative reference-subtracted sensorgrams for each interaction between the BG505.SOSIP.664 (ligand) and BF520.1 (A) naïve$_{VH}$ mature$_{VK}$ and (B) mature$_{VH}$ naïve$_{VK}$ (analyte). IgG concentrations ranged from 667 to 42 nM. The gray lines show 0 μM IgG. K_D, K_{on}, and K_{dis} are shown from best fitting (green lines) to a 1:2 bivalent analyte model of binding. (C) Neutralization of panel viruses by BF520.1 inferred lineage intermediates. The top rows of the table (VH and VK) show the origin of the antibody chain sequence used. For VK this included the naïve, Bayesian (0-6 months pi) and rationally inferred (6-12 months pi) lineage intermediates, indicated by Int#$_{VK}$ with # indicating the progression of intermediates in the lineage. These were paired with the indicated heavy chain, in most cases the mature heavy chain (mature$_{VH}$). SIV was included as a negative control. IC_{50} values (μg ml^{-1}) represent an average of two to three independent experiments performed in duplicate and are color-coded with darker shades of blue indicating more potent neutralization. Grey indicates that 50% neutralization was not achieved at the highest mAb concentration tested. (D) Amino acid alignment of BF520.1 naïve, Bayesian (0-6 months pi) and rationally inferred (6-12 months pi) lineage intermediates and mature kappa chain sequences. Intermediates designated as >M6 pi were rationally inferred.

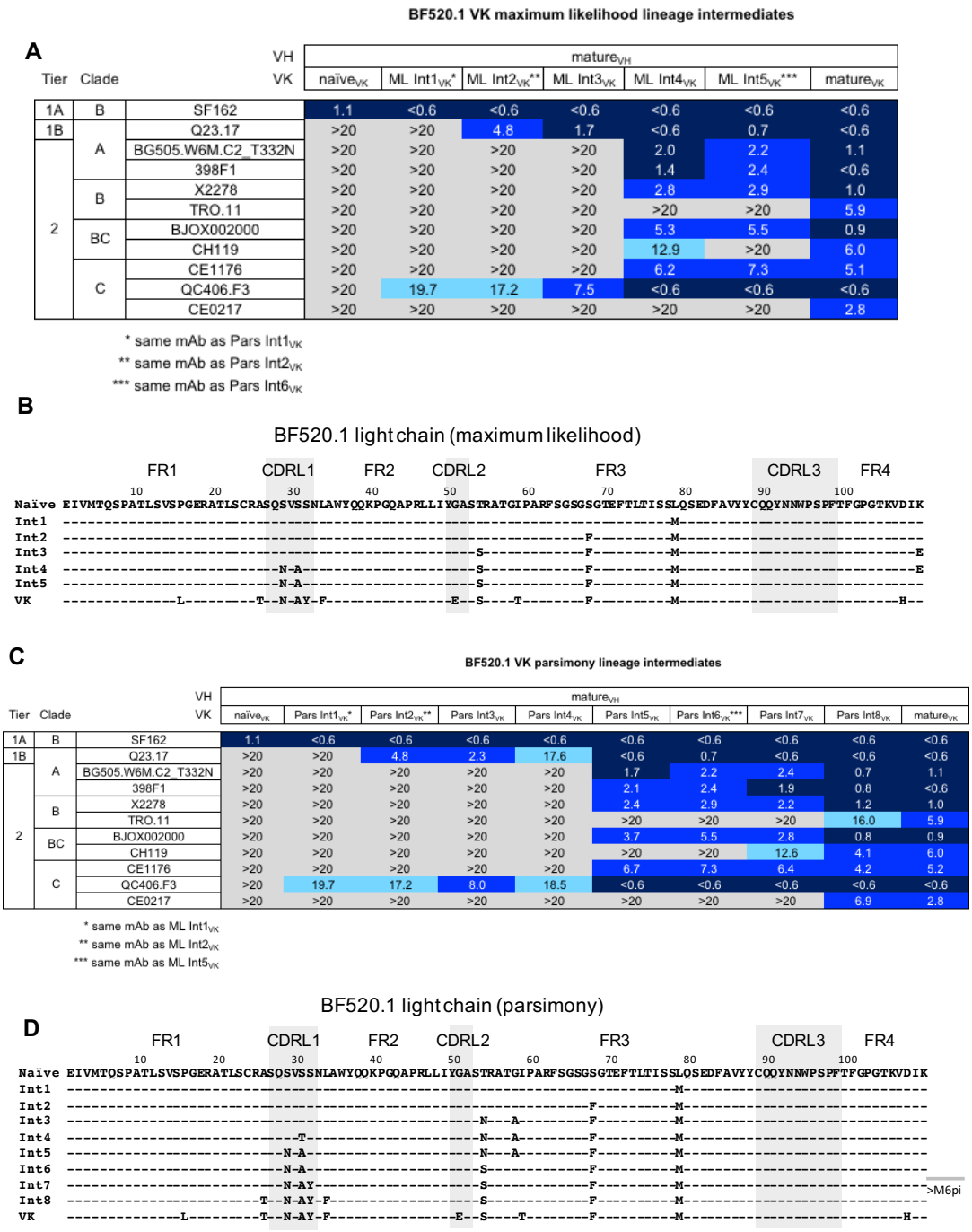


Figure 3.6 Contribution of kappa light chain maturation to HIV neutralization

BF520.1 (A) maximum likelihood and (C) parsimony lineage kappa chains paired with the mature heavy chain, mAb neutralization of viruses. IC_{50} values (μg ml^{-1}) are an average of two to three independent experiments performed in duplicate. IC_{50} values are color-coded with darker shades of blue indicating more potent neutralization. Grey indicates that 50% neutralization was not achieved at the highest mAb concentration tested. Amino acid alignments of (B) maximum likelihood and (D) parsimony kappa chain lineage intermediates. Intermediates designated as >M6 pi were rationally inferred.

Cross-clade neutralization with limited SHM in paired intermediates

To define the minimal combination of changes in VH and VK that are needed for breadth, the M0-M6 pi heavy and light chain intermediates (Bayesian lineage Ints1-3_{VH}; **Figure 3.2** B and Ints1-4_{VK}; **Figure 3.4 D**) were paired together using percentage SHM to assign probable pairings. The remaining post-M6 pi intermediates were paired based on increasing SHM and their incorporation of additional CDR mutations, CDR-adjacent FR mutations and non-conservative FR mutations. Tier 1A neutralizing activity was observed following just two amino acid substitutions in the heavy chain ($Int2_{VH}$) and three in the light chain ($Int2_{Vk}$) (**Figure 3.7**) with the additions of the VH Y32N and VK S30A being important for the neutralizing activity. Cross-clade heterologous neutralization was achieved with the addition of the VH M34I and VK S28N in the $Int3_{VH}$ $Int3_{Vk}$ mAb, which had a total of four heavy chain and five light chain amino acid substitutions or 1.3% and 1.8% SHM at the nucleotide level in the heavy and light chains, respectively. $Int4_{VH}$ paired with $Int4_{VK}$ neutralized the majority of the viruses (9/13; 69%) that are neutralized by mature BF520.1 with only 1.8 VH and 2.1% VK SHM. Further increased neutralization was conferred by single additional substitutions in both the heavy and light chains: N52A ($Int4_{VH}$) and A25T ($Int4_{VK}$). The breadth of BF520.1 was reached with 1.8% VH and 3.4%VK SHM (2.5% overall SHM) for $Int4_{VH}Int5_{VK}$. Finally, the $Int5_{VH}Int6_{VK}$ mAb reached the breadth and potency of mature BF520.1 with only 2.4% VH and 3.7% VK SHM (3.0% overall SHM) (**Figure 3.7**). Similar observations were made using the ML (**Figure 3.8 A**) and Pars (**Figure 3.8 B**) lineages. Because the M6-M12 pi rationally inferred substitutions would be the same for ML and Pars lineages, these additional changes were added to the Pars lineage only. Cross-clade neutralizing activity was seen with 0.8% VH and 2.4% VK SHM for ML and

Pars 1.3% VH and 2.1% VK SHM. Pars $Int5_{VH}Int6_{VK}$ neutralized 73% of the viruses neutralized by the mature BF520.1 with 1.8% SHM in both the heavy and kappa chains.

BF520.1 lineage paired intermediates

			VH	naïve VH	Int1 VH	Int2 VH	Int3 VH	Int3 VH	Int4 VH	Int4 VH	Int4 VH	Int5 VH	Int6 VH	mature VH	
					0.5	0.8	1.3	1.3	1.8	1.8	1.8	2.4	2.6	6.5	% SHM
			VK	naïve VK	Int1 VK	Int2 VK	Int3 VK	Int4 VK	Int4 VK	Int5 VK	Int6 VK	Int6 VK	Int7 VK	mature VK	
Tier	Clade				0.3	1.2	1.8	2.1	2.1	3.4	3.7	3.7	4.3	4.6	% SHM
1A	B	SF162		>20	>20	1.7	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	
1B		Q23.17		>20	>20	>20	2.1	1.2	<0.6	<0.6	<0.6	<0.6	0.7	<0.6	
2	A	BG505.W6M.C2_T332		>20	>20	>20	>20	>20	6.2	2.1	1.4	1.0	1.6	1.1	
		398F1		>20	>20	>20	>20	>20	2.0	1.2	<0.6	<0.6	0.8	<0.6	
	B	X2278		>20	>20	>20	>20	>20	2.2	1.3	0.7	0.7	0.9	1.0	
		TRO.11		>20	>20	>20	>20	>20	>20	18.9	10.3	6.0	6.7	5.9	
	BC	BJOX002000		>20	>20	>20	>20	>20	11.9	1.6	0.8	0.7	0.9	0.9	
		CH119		>20	>20	>20	>20	>20	>20	8.2	4.4	3.0	5.7	6.0	
	C	CE1176		>20	>20	>20	>20	>20	7.5	3.7	4.0	4.1	7.4	5.1	
		QC406.F3		>20	>20	>20	3.1	1.1	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	
		CE0217		>20	>20	>20	>20	>20	>20	15.0	5.4	3.7	4.9	2.8	
		DU156.12		>20	>20	>20	>20	>20	>20	4.1	2.2	2.0	2.2	2.2	
		DU422.1		>20	>20	>20	10.3	8.2	2.8	2.9	2.3	1.5	4.1	2.6	
		SIV		>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	

Figure 3.7 Neutralization properties of paired intermediates of VH and VK

The top rows show the intermediate in the lineage tested for both VH and VK chains as shown in Figs 2B and 3D. Below each the % SHM is indicated. The virus panel and figure layout is as described in the legend to Figure 3.2 A.

BF520.1 maximum likelihood lineage

A

			VH	ML Int1$_{VH}$	ML Int3$_{VH}$	ML Int4$_{VH}$	ML Int5$_{VH}$	mature$_{VH}$	
				1.0	2.1	0.8	1.3	6.5	%SHM
			VK	ML Int1$_{VK}$	ML Int3$_{VK}$	ML Int4$_{VK}$	ML Int5$_{VK}$	mature$_{VK}$	
Tier	Clade			0.3	1.5	2.4	1.8	4.6	%SHM
1A	B	SF162		>20	15.9	<0.6	<0.6	<0.6	
1B	A	Q23.17		>20	>20	7.0	0.9	<0.6	
2	A	BG505.W6M.C2_T332N		>20	>20	>20	>20	1.1	
	A	398F1		>20	>20	>20	13.7	<0.6	
	B	X2278		>20	>20	>20	>20	1.0	
	B	TRO.11		>20	>20	>20	>20	5.9	
	BC	BJOX002000		>20	>20	>20	>20	0.9	
	BC	CH119		>20	>20	>20	>20	6.0	
	C	CE1176		>20	>20	>20	17.5	5.1	
	C	QC406.F3		>20	>20	7.4	1.8	<0.6	
	C	CE0217		>20	>20	>20	>20	2.8	

B

BF520.1 parsimony lineage

			VH	Pars Int1$_{VH}$	Pars Int3$_{VH}$	Pars Int4$_{VH}$	Pars Int4$_{VH}$	Pars Int4$_{VH}$	Pars Int5$_{VH}$	Pars Int5$_{VH}$	Pars Int5$_{VH}$	mature$_{VH}$	
				0.5	0.8	1.3	1.3	1.3	1.8	1.8	1.8	6.5	%SHM
			VK	Pars Int1$_{VK}$	Pars Int3$_{VK}$	Pars Int4$_{VK}$	Pars Int5$_{VK}$	Pars Int6$_{VK}$	Pars Int6$_{VK}$	Pars Int7$_{VK}$	Pars Int8$_{VK}$	mature$_{VK}$	
Tier	Clade			0.3	1.2	1.5	2.1	1.8	1.8	2.4	3.0	4.6	%SHM
1A	B	SF162		>20	>20	13.9	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	
1B	A	Q23.17		>20	>20	>20	2.5	3.8	0.9	0.7	<0.6	<0.6	
2	A	BG505.W6M.C2_T332N		>20	>20	>20	>20	>20	13.1	6.8	1.5	1.1	
	A	398F1		>20	>20	>20	>20	19.6	2.6	2.1	0.8	<0.6	
	B	X2278		>20	>20	>20	>20	>20	8.9	3.7	1.2	1.0	
	B	TRO.11		>20	>20	>20	>20	>20	>20	>20	>20	5.9	
	BC	BJOX002000		>20	>20	>20	>20	20.0	15.8	13.1	1.5	0.9	
	BC	CH119		>20	>20	>20	>20	>20	>20	>20	5.1	6.0	
	C	CE1176		>20	>20	>20	>20	>20	11.4	9.6	4.3	5.1	
	C	QC406.F3		>20	>20	>20	3.0	4.4	<0.6	<0.6	<0.6	<0.6	
	C	CE0217		>20	>20	>20	>20	>20	>20	>20	9.0	2.8	

Figure 3.8 Neutralization properties of ML and Pars paired intermediates of VH and VK

Neutralization of panel viruses by BF520.1 (A) maximum likelihood and (B) parsimony lineage heavy and light chain paired intermediates. IC_{50} values in μg ml^{-1}.

BF520.1 naïve and lineage members bind HIV Env trimer

Many groups have generated inferred naïve or unmutated common ancestors of adult-derived bnAbs and most do not bind to recombinant HIV envelope gp120 and/or trimer, raising the question of how the response was initially stimulated (Stamatatos et al., 2017). Though the inferred naïve ancestor of BF520.1 did not demonstrate neutralizing activity (**Figure 3.2**), we tested its ability to bind to HIV Env using biolayer interferometry (BLI). Specifically, we examined naïve and intermediate antibody binding to BG505.SOSIP.664 trimer, which is a clade A transmitted envelope variant from a Kenyan infant who was in the same cohort as BF520 (Nduati et al., 2000; Wu et al., 2006). The inferred naïve ancestor of BF520.1 weakly bound to the BG505 SOSIP trimer (**Figure 3.9 A**) and this binding was dependent on increasing mAb concentration (**Figure 3.9 B**). Intermediate mAbs that demonstrate neutralization showed stronger binding to the Env trimer (**Figure 3.9 A**). Qualitatively, we observed increased binding with affinity maturation (**Figure 3.9 A**) with the most significant increase in binding demonstrated by $Int2_{VH}$ $Int2_{VK}$ and more subtle increases by later intermediates. Mutations acquired by $Int4_{VH}4_{VK}$ enabled similar binding to Env as the mature BF520.1. Despite clear binding to the BG505 SOSIP trimer by $Int2_{VH}2_{VK}$, $Int3_{VH}3_{VK}$, and $Int3_{VH}4_{VK}$, these intermediates were not able to neutralize the BG505 pseudovirus (**Figures 3.7 and 3.9 A**). These data indicate that the BF520.1 naïve mAb had the potential to recognize HIV Env.

Our approach to inferring the naïve ancestor using replicate sequence data differed from that of previous studies in the field, which only used single replicates of NGS data (Doria-Rose et al., 2014; Liao et al., 2013; MacLeod et al., 2016). To compare the two methods, we also inferred the BF520.1 naïve ancestor using just the first replicate of antibody sequence data. This approach resulted in uncertainty in the naïve nucleotide sequences at one site in the heavy chain

and two in the kappa chain (**Figure 3.10 A and B**). In comparison, naïve inference using replicate datasets yielded sequences without uncertain bases. We synthesized alternative BF520.1 naïve mAbs with sequences that differed from our most probable naïve sequences at the sites of single-replicate uncertainty. When tested, neither of the two alternative inferred naïve mAbs demonstrated neutralization of heterologous or autologous viruses (**Figure 3.10 C**), nor did they bind trimer (**Figure 3.10 D**), which contrasts the ability of the more probable replicate-inferred naïve mAb. We also found differences in the autoreactivity of the inferred naïve mAbs: single dataset-inferred mAbs demonstrated weak autoreactivity (**Figure 3.10 E**) whereas this was not observed for the more probable replicate-inferred naïve mAb. Likewise the mature BF520.1 was not autoreactive. These data demonstrate that uncertainty in the inference of bnAb precursors can result in significant differences in HIV Env binding and autoreactive properties.

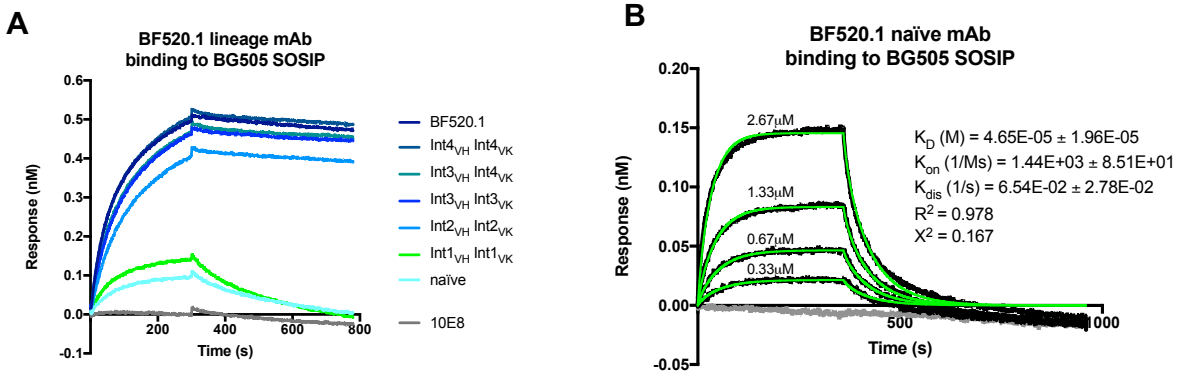


Figure 3.9 BF520.1 naïve and lineage binding to HIV Env trimer

(A) BF520.1 paired lineage intermediates (ligand) binding to the BG505.SOSIP.664 (analyte) measured by BLI. 10E8 is a negative control as the SOSIP trimer does not contain the targeted MPER epitope. Data are representative of two independent experiments. (B) BLI binding analysis of varying concentrations of BF520.1 naïve antibody (analyte) binding to BG505.SOSIP.664 (ligand). The gray line shows 0 μM IgG. K_D, k_{on}, and k_{dis} from best fitting (green lines) to a 1:2 bivalent analyte model of ligand:analyte binding are shown.

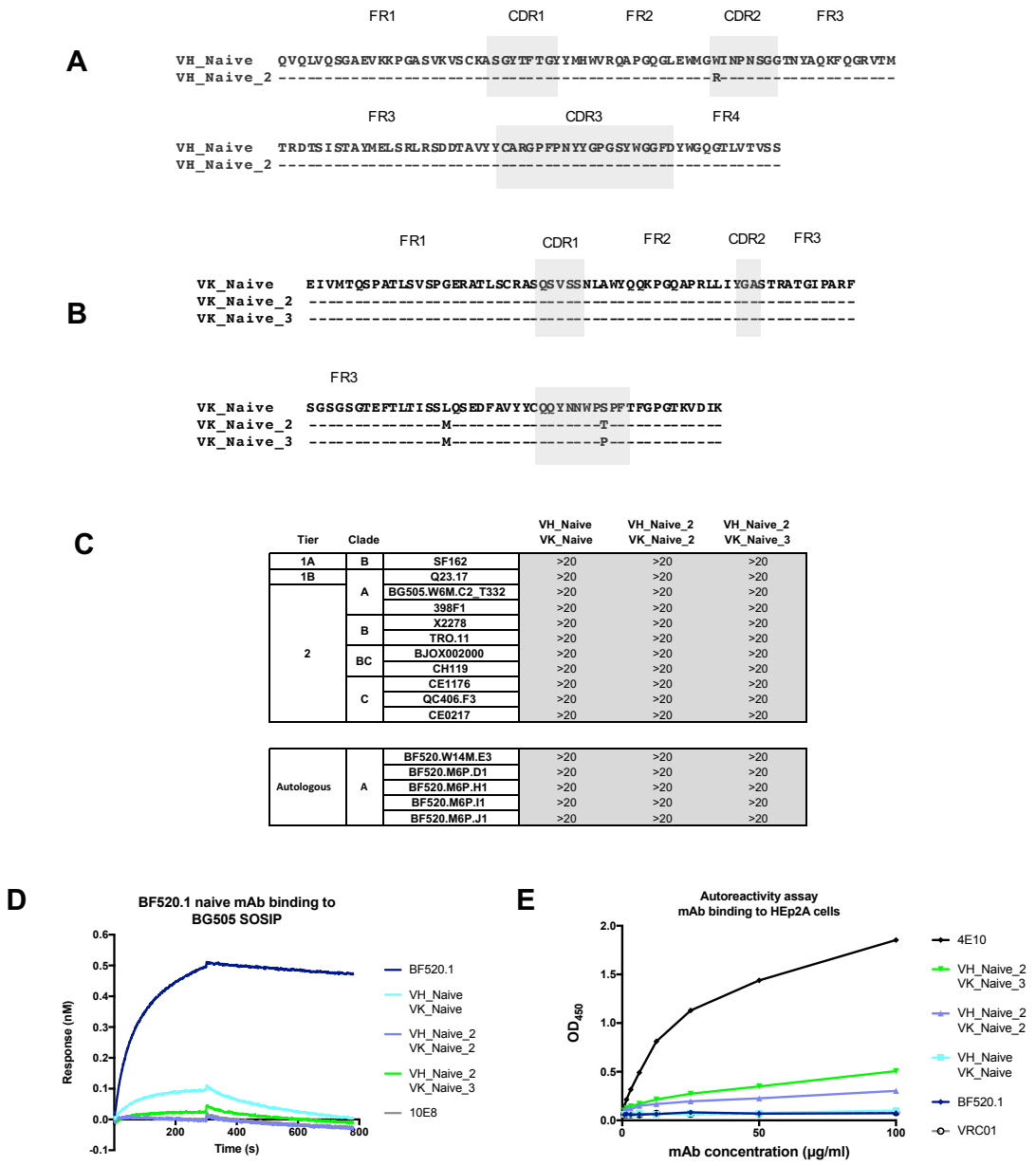


Figure 3.10 BF520.1 inferred naïve mAbs

Amino acid alignment of (A) heavy chain and (B) kappa chain inferred naïve sequences. (C) BF520.1 naïve mAb neutralization of viruses. IC_{50} values (µg ml^{-1}) are an average of two to three independent experiments performed in duplicate. Grey indicates that 50% neutralization was not achieved at the highest mAb concentration tested. (D) mAb (analyte) binding to the BG505.SOSIP.664 (ligand) measured by BLI. 10E8 is a negative control as the SOSIP trimer does not contain the targeted MPER epitope. Data are representative of two independent experiments. (E) mAb binding to HEp2A cells measured by ELISA (AESKULISA ANA-HEp-2; Aesku Diagnostics). The MPER-directed bnAb 4E10 is known to be autoreactive and was included as a positive control. VRC01 was included as a negative control. Data are representative of two independent experiments.

Increasing autologous neutralization by the BF520.1 lineage

We previously observed that BF520.1 does not neutralize any of the 11 HIV Env variants isolated from the first time point following detection of HIV infection at 14 weeks of age (W14). BF520.1 does, however, neutralize variants isolated from 6 months of age (M6), which is ~2.2 months after infection was detected. Similar to the mature mAb BF520.1, the naïve and early lineage intermediates were not able to neutralize a representative W14 early stage transmitted autologous envelope variant (**Figure 3.11**). Likewise, the earliest paired intermediates failed to neutralize the M6 variants. Lineage member $Int4_{VH}4_{VK}$ neutralized BF520.M6P.I1 with lower potency compared to BF520.1 (5.8 vs. 1.1 μg ml^{-1}, respectively). Additionally, $Int4_{VH}5_{VK}$ and $Int4_{VH}6_{VK}$ mAbs demonstrated stepwise increases in neutralization of the autologous viruses from ~2.2 months pi, further highlighting the importance of the kappa chain contribution to autologous neutralizing activity. Substitutions in the CDRL1 and CDRL1-proximal regions were particularly impactful for autologous neutralization: CDRL1 S31Y, CDRL2 G50E and FWR2 L33F (**Figure 3.11**).

BF520.1 VH	$naïve_{VH}$	$Int1_{VH}$	$Int2_{VH}$	$Int3_{VH}$	$Int3_{VH}$	$Int4_{VH}$	$Int4_{VH}$	$Int4_{VH}$	$Int5_{VH}$	$Int6_{VH}$	$mature_{VH}$
BF520.1 VK	$naïve_{VK}$	$Int1_{VK}$	$Int2_{VK}$	$Int3_{VK}$	$Int4_{VK}$	$Int4_{VK}$	$Int5_{VK}$	$Int6_{VK}$	$Int6_{VK}$	$Int7_{VK}$	$mature_{VK}$
BF520.W14M.E3	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
BF520.M6P.D1	>20	>20	>20	>20	>20	>20	6.1	0.7	<0.6	<0.6	<0.6
BF520.M6P.H1	>20	>20	>20	>20	>20	>20	>20	11.6	4.6	2.3	1.4
BF520.M6P.I1	>20	>20	>20	>20	>20	5.8	2.1	1.1	0.7	1.6	1.1
BF520.M6P.J1	>20	>20	>20	>20	>20	>20	>20	3.6	1.8	1.7	1.8

Figure 3.11 Neutralization of autologous virus by BF520.1 paired lineage intermediates

The origin of the VH and VK chains are shown at the top and are described in Fig 2B and 3D. Viruses tested are shown to the left and include a variant at first HIV detection (14 weeks of age) designated ‘‘W14’’ and four variants from ~2.2 months pi (6 months of age, “M6”). IC_{50} values (μg ml^{-1}) are color coded with darker shading indicating greater neutralization potency. Results are an average of 2 independent experiments.

Discussion

The complex developmental pathways of bnAbs in HIV-infected adults have been carefully examined to guide immunogen design targeting key precursors in antibody evolutionary pathways (148). In many cases, this approach is complicated by the observation that the inferred naïve progenitor of the bnAb does not bind HIV Env, presenting challenges to stimulating the lineage (195). Taking advantage of the more rapid development of HIV bnAb responses in infants, here we defined an evolutionary pathway that starts with a naïve antibody that is capable of binding HIV Env trimer and requires as little as 2% SHM to achieve some cross-clade breadth, and 3% to achieve the full cross-clade breadth of this lineage, a level of SHM that is easily within reach by vaccination (175). Remarkably, this antibody lineage relies on changes in both the heavy and light chains, most outside of the CDRH3, to develop HIV specificity and breadth.

The isolation of V3-glycan targeting bnAbs from a number of different individuals, including infant BF520, is encouraging for vaccine development because this class of bnAbs is not restricted by particular germline genes (85, 123, 133, 134, 143, 298) and thus it may be possible to elicit nAbs targeting this epitope in many individuals. The V3-directed mAb BF520.1 is of particular interest because it arose within a year of infection, which is earlier than V3-bnAbs isolated from adults. This study demonstrates that the minimal set of mutations required for cross-clade heterologous neutralizing activity was only four heavy chain and five light chain amino acid substitutions (1.3 and 1.8% SHM at the nucleotide level) and that these mutations arose prior to 6 months pi. With the addition of select mutations that arose between 6-12 months pi, breadth and potency comparable to the mature BF520.1 mAb can be reached with as little as 3.0% SHM (2.4% VH and 3.7%VK SHM). Even this low level of SHM may overestimate the

required level of VK SHM since some of the initial mutations did not appear to contribute to breadth. Some adult-derived V3-glycan bnAbs also demonstrated heterologous breadth with relatively low SHM compared to adult-derived bnAbs targeting other epitopes (85, 133, 179), but required rare mutation events to occur (85, 179) and/or higher levels of SHM compared to BF520.1 (133). While some of the amino acid substitutions required for BF520.1 neutralization breadth resulted from nucleotide mutations that are not associated with canonical AID hotspots, these mutations did not significantly delay the development of BF520.1 cross-clade heterologous neutralizing activity as they naturally occurred within 6 months to 1 year pi. This indicates that vaccines may be able to stimulate similar mutations in reasonable timeframes.

Interestingly, mutations important for increasing BF520.1 functional activity were primarily found in the heavy chain CDRH2 and light chain CDRL1 with little to no contribution from the CDRH3 and CDRL3. This contrasts with many HIV bnAbs, where major determinants of breadth reside in the CDR3 region, particularly in VH (170). The VRC01 class of anti-CD4bs bnAbs rely on unusually short CDRL3 regions as well as extensive SHM in the heavy chain (296). We identified that maturation in the BF520.1 antibody light chain was particularly important for HIV Env binding and neutralization, demonstrated by the increased heterologous breadth observed when the mature kappa light chain was paired with the naïve gamma chain and the five amino acid substitutions in and around CDRL1 that contribute to neutralization breadth. Other V3-glycan bnAb studies have largely focused on the heavy chain's contribution to heterologous neutralizing activity (85, 133), with one exception showing the importance of a light chain FR3 insertion for neutralization breadth (179). Our studies highlight a unique role for the antibody light chain in determining HIV specificity of BF520.1: a role that can potentially be harnessed to develop vaccine approaches that elicit such bnAbs with relatively little SHM.

Much of our understanding of the role of the light chain in HIV Env recognition by V3-glyan targeting bnAbs comes from structural studies of the bnAb in complex with Env. Such studies of adult-derived V3 bnAbs have demonstrated that residues in the CDRH3 are critical for making contacts with both the N332 glycan and conserved GDIR motif at the base of V3. Meanwhile, the CDRL2 can contact the N332 glycan and the CDRL1 and CDRL3 can contribute to GDIR motif binding (203). Another recent study demonstrated that a new V3-glycan bnAb uses the CRL2 to contact the GDIR motif (143). Our study of BF520.1, which points to residues in the CDRH2 and CDRL1 as determining breadth, along with these studies of adult V3-glycan bnAbs highlight the diverse ways the immune system can target this bnAb epitope. Depending on the mAb, it may be important to consider the light chain when designing immunogens to drive bnAb affinity maturation.

While we see neutralization of heterologous Env variants by the early BF520.1 lineage intermediates, autologous neutralization is observed only with later stage intermediates ($Int4_{VH}Int4_{VK}$ and beyond) against BF520 autologous Env variants isolated at ~2 months pi. While CDRH2 and CDRL1 mutations are important for heterologous breadth, autologous neutralization was conferred by additional kappa chain mutations in and around the CDRL1. The observation that the earlier intermediates are HIV-specific but do not neutralize the autologous virus suggests that the virus at ~2 months pi may have escaped neutralization by the early lineage. We do not know whether the naïve or early lineage mAbs were able to bind autologous viruses at the time of infection or at ~2 months pi. The BF520.1 naïve mAb and early lineage intermediates do, however, bind the heterologous BG505.SOSIP.664 trimer. The BG505.SOSIP.664 trimer was derived from the clade A transmitted Env from an infant in the same cohort as BF520, which is the same clade as the infecting virus in BF520 (200, 266). This

binding of the naïve ancestor of BF520.1 to recombinant HIV Env contrasts with many adult bnAb precursors (195), including some V3-glycan bnAbs (85, 179). We cannot be certain that this inferred naïve mAb, when expressed as a BCR, would have the same binding properties or whether the binding affinity would be sufficient for B cell activation. However, the affinity of the BF520.1 naïve mAb for the Env trimer is within the range of affinities observed for VRC01 class naïve precursors binding to a monovalent germline-targeting antigen (305).

This study suggests that an immunization strategy to elicit a BF520.1-like response could use the BG505.SOSIP.664 or an optimized target to initiate the response and then could be boosted with BF520 Env trimer from ~2 months pi to drive affinity maturation.

To identify appropriate immunogens that might stimulate bnAb precursors, it is critical that inferred bnAb precursor heavy and light chain sequences are as accurate as possible. Our use of replicate NGS data resolved uncertainties in the inferred naïve sequences that impacted the overall characterization of our antibody lineage. The inferred naïve mAb containing uncertainties displayed autoreactivity, which is a feature of many adult bnAbs (295) and some V3-glycan bnAbs (133, 134). However, like the mature BF520.1, our more probable replicate-inferred naïve mAb did not demonstrate autoreactivity. Likewise, only our most probable inferred naïve mAb demonstrated measurable HIV binding. These discrepancies highlight the potential imprecision of naïve antibody inference when sequencing data are of insufficient quality or depth, and suggest that these newer approaches designed specifically for antibody lineage analysis should be used.

The finding that infants develop broad and potent HIV bnAb responses has raised the question of whether HIV vaccine efforts should focus on infants (247). Indeed, pediatric immunizations for the prevention of viral infections is a practical strategy because infants and

children have regular contact with the healthcare system. Understanding the ontogeny of HIV neutralizing antibody responses in the pediatric population is necessary to facilitate the rational design of such vaccine strategies. Given that the BF520.1 naïve ancestor binds HIV Env, that very little SHM is required for this V3-directed antibody lineage to demonstrate heterologous cross-clade breadth, and that these antibodies lack unusual or rare features, the BF520.1 lineage should be considered an attractive template for vaccine design. These studies suggest that the infant immune system may be uniquely poised to develop bnAb responses rapidly.

Chapter IV

Isolation of HIV-neutralizing antibodies from infant BG505

The study described in this chapter is being prepared for manuscript submission.

Introduction

Massive efforts have been made to understand broadly neutralizing antibody (bnAb) responses in HIV-infected individuals in an attempt to inform their induction by vaccination (114, 148). bnAb responses take years to develop in HIV-infected adults (93). In contrast, our lab identified bnAb responses in seven infants that developed at a median of 20 months of age, with broadly neutralizing responses detectable within a year after infection for 4 infants (23). A subsequent study also identified pediatric bnAb responses in children over 5 years of age, and demonstrated that pediatric bnAb responses develop more frequently in children (75% of pediatric cases) compared to adults (19% of infected adults). Dozens of adult-derived bnAbs and nAbs have been studied in detail (114, 115), whereas until recently no HIV nAbs had been isolated from infants. We previously isolated the first HIV nAbs from an infant, BF520, and demonstrated that infant HIV-specific nAbs have unique characteristics compared to adult bnAbs including much lower levels of SHM (298) – Chapters II and III. Of the seven infants who developed broad responses, BG505 developed one of the most impressive responses, neutralizing 91% of viruses in an expanded panel of tier 2 viruses - detected at approximately two years post-infection.

The name BG505 is well known throughout the HIV field as it refers to an HIV-1 envelope (Env) trimer. This transmitted Env variant from infant BG505 (200) is famous since it was used to generate the first native-like soluble Env trimer, BG505.SOSIP.664 (47). The

BG505.SOSIP.664 trimer has been the focus of numerous detailed studies of HIV Env trimer including the first high resolution structural characterization of a HIV Env trimer (33, 34). The BG505 trimer has facilitated greater understanding of the conformational states of Env and the function of Env in receptor binding (33, 34, 48, 278, 306-308), trimer glycosylation (36, 48-50, 309, 310), the antigenic landscape of Env and the epitope targets of broadly neutralizing antibodies (48, 84, 125, 128, 139-143, 149, 174, 196, 199, 203, 308, 311, 312). The BG505.SOSIP has been used to generate more biologically relevant SHIVs for macaque models of infection (313-315). Additionally, this trimer has been tested in a number of pre-clinical vaccine trials, eliciting tier 2 nAbs in rabbits, guinea pigs and macaques and bnAbs in cows (189, 199, 293, 308, 316-322).

To identify HIV-specific B cells contributing to the broadly neutralizing plasma response of infant BG505, we utilized every available tool for nAb isolation by combining the two methods commonly used to identify HIV-specific B cells (114). We utilized the autologous clade A BG505 SOSIP trimer and heterologous clade C SOSIP trimer as baits as well as large-scale culture and screening of all remaining IgG B cells. In addition to isolating >70 nAbs from infant BG505, we cloned Env variants from time-points later in infection that will be useful tools for vaccine studies. Characterizing the mAbs that developed in response to this unique transmitted HIV variant will complement studies using the BG505 Env as an immunogen and add to our understanding of cross-clade neutralizing antibody responses.

Materials and methods

Infant plasma and peripheral blood mononuclear cell samples

Plasma and peripheral blood mononuclear cell (PBMC) samples were from infant BG505 enrolled in the Nairobi Breastfeeding Clinical Trial (266), which was conducted prior to the use of antiretrovirals for the prevention of mother-to-child transmission. Approval to conduct the Nairobi Breastfeeding Clinical Trial was provided by the ethical review committee of the Kenyatta National Hospital Institutional Review Board, and the University of Washington Institutional Review Board.

BG505 was HIV-1 DNA negative by PCR and HIV-1 RNA-negative using the Gen-Probe Viral Load assay at birth and was HIV-1 DNA- and RNA-positive at 6 weeks of age. The infecting virus was clade A based on envelope sequence.

B cell sorting

A PBMC sample from BG505 from 27 months of age (M27), a time-point where the plasma demonstrated broadly neutralizing activity (23), was thawed as previously described (298). Biotinylated Env SOSIP.664-aviB trimers kindly provided by Marit van Gils were conjugated to streptavidin-fluorophores (3.75ug trimer and 1.5uL streptavidin-fluorophore) at 4C for 1 hour. BG505.SOSIP.664-aviB was conjugated to streptavidin-PE (Premium Grade, Life Sciences Technologies) and streptavidin-APC (BD Pharmingen), separately. DU422.SOSIP.664-aviB was conjugated to streptavidin-PECy7 (eBioscience) and streptavidin-BV21 (BD Horizon). Cells were stained on ice for 30 minutes using a cocktail of the 4 trimer conjugates, anti-CD19-BV510, anti-IgD-FITC, anti-IgM-FITC, anti-IgA-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). Cells were loaded onto a BD FACS Aria II cell

sorter. The gating strategy was set up such that IgG expressing B cells ($CD3^-CD14^-CD16^-CD19^+IgD^-IgM^-IgA^-$ cells) that were double positive for the BG505.SOSIP.664-aviB were sorted into FACS wash. The remaining IgG B cells that were double positive for the DU422.SOSIP.664-aviB were sorted into B cell media (IMDM medium, GIBCO; 10% low IgG FBS, Life Technologies; 5 ml GlutaMAX, Life Technologies; 1 ml MycoZap plus PR, Lonza). All remaining IgG B cells that were not double positive for either bait were bulk sorted into B cell media. Immediately following the B cell sort, cells that were sorted for binding to the BG505.SOSIP.664 trimer were diluted in RNA storage buffer (15 mM Tris and 10 U murine RNase inhibitor, NEB) to a concentration such that each 20uL aliquot contained approximately a single B cell and cells were frozen at -80C. Cells that bound the DU422.SOSIP.664 trimer were plated at a density of 4 B cells in 60 ul per well into 46 culture wells and IgG-expressing B cells that did not bind bait were plated at 6 B cells in 60ul per well into 130 x 384-well plates in B cell media (100 U/mL IL-2, Roche; 0.05 ug/ml IL-21, Invitrogen, and irradiated 3T3/CD40L feeder cells, 8.85x105/mL). Cultured B cells were incubated for 12 days at 37C in a CO2 incubator based on the protocol by Huang et al. (129).

B cell culture supernatant IgG ELISA

IgG was measured by ELISA on culture days 9-12 from a random sample of wells from multiple culture plates. Immunolon 2HB ELISA plates were coated with 2500 ng/well Sigma goat anti-human Ab in 0.1M sodium bicarbonate, pH 9.4 overnight at 4C. Plates were washed three times with 200uL wash buffer (1X PBS, 0.05% Tween-20) and blocked for 1 hour at room temperature (RT) in blocking buffer (1X PBS with 10% non-fat milk and 0.05% Tween-20). IgG at a known concertation was serially 4-fold diluted 8 times from 3ug/ml for a standard curve. 5uL of B cell culture supernatant was diluted 1:10 into blocking buffer. 50uL of the standard

curve and diluted supernatants were added to each well and incubated at 37C for 1 hour. Plates were washed and incubated with secondary antibody (goat anti-human IgG-HRP, Sigma diluted 1:2500 in blocking buffer) for 1 hour at RT. After washing, 50uL of TMB-ELISA solution (Pierce) was added for 10 minutes at RT and stopped with 50uL 1N sulfuric acid. Absorption was read at 450nM, and concentrations were interpolated based on the standard curve.

B cell culture harvest, microneutralization assay and reconstruction of antibodies

On day 12, B cell culture supernatants were divided into 2 x 384-well plates at 20ul each for neutralization assays using a Tecan automated liquid handling system. B cells were frozen at -80C in 20ul RNA storage buffer per well. Microneutralization assays were performed as previously described in Chapter II(298), with the same 2 viruses used for screening BF520 (tier 1 clade B SF162 and tier 2 clade C QC406.F3). Wells demonstrating >40% neutralization of one or both viruses were selected for antibody cloning. RT-PCR amplification of the IgG heavy and light chain variable regions using our previously described method was performed for B cells identified by the functional assay (this included the B cells that bound the heterologous bait) as well as B cells that were sorted based on binding to BG505.SOSIP.664. Functional heavy and light chain variable region sequences amplified by RT-PCR were determined using IMGT V-QUEST (272) and subsequently cloned into IgG expression vectors as previously described in Chapter II(298). In parallel, for the B cells identified by the functional assay, an aliquot of B cell RNA was sent to Atreca (https://www.atreca.com) for deep sequencing of the antibody heavy and light chain variable regions from each well. In cases where Atreca identified additional heavy and/or light chains, those were synthesized as gene fragments by Integrated DNA Technologies and subsequently cloned into IgG expression vectors. The Freestyle MAX system (Invitrogen) was used to co-transfect paired heavy and light chain plasmids cloned from the same

well, and IgG was purified as described (269). For each well, all possible heavy and light chain pairs were generated.

Pseudovirus production and neutralization assays

Methods for pseudovirus production and neutralization assays were previously described by Goo et al. (250). Plasma IC_{50} values are the reciprocal plasma dilution resulting in 50% reduction of virus infectivity. Monoclonal antibody (mAb) IC_{50} values represent the mAb concentration (ug/mL) at which 50% of the virus was neutralized. Reported IC_{50} values are an average of two independent experiments performed in duplicate.

Neutralizing antibody sequence analysis

Heavy and light chain sequences were analyzed using IMGT V-QUEST (272). Percent SHM was calculated as the VH mutation frequency at the nucleotide level compared to the predicted germline VH allele in the IMGT database. Comparisons between adult bnAbs and infant BF520 antibodies were previously published (298).

HIV-1 *env* amplification and cloning

BG505 week 6 (W6) and MG505 week 0 (W0) Env variants were previously isolated by Wu et al. (200). BG505 week 14 (W14) and month 27 (M27) full-length envelope variants were cloned from total RNA that was extracted from plasma as described in (281). cDNA synthesis and nested PCR of envelope was performed as previously described (200, 280).

Phylogenetic tree construction

MG505 (maternal) and BG505 (infant) *env* sequences were aligned using Geneious version 11.1.2. A maximum likelihood phylogenetic tree was constructed using the LANL HIV tools database PHYML interface (282).

Results

HIV-specific IgG B cells

The BG505 PBMC sample from 27 months of age was ~90% viable and contained ~6 million total live cells. The autologous BG505.SOSIP.664 bait was bound by 192 IgG B cells, and paired heavy and light chains were amplified from 24 wells. None of the 24 reconstructed mAbs demonstrated neutralizing activity against the easy-to-neutralize tier 1 variant SF162 or the autologous BG505.W6M.C2 virus expressing the same Env that the BG505.SOSIP.664 is based on (**Figure 4.1 A and C**). We were interested in HIV-neutralizing antibodies, and therefore did not determine whether these 24 antibodies are able to bind but not neutralize the BG505 transmitted Env variant.

The heterologous bait was bound by 186 B cells. Because performing RT-PCR on the almost 400 total bait-positive B cells would have been time and labor intensive, the B cells sorted based on binding to the heterologous bait were cultured and screened a second time through the microneutralization assay. Additionally, ~220,000 IgG expressing B cells that did not bind bait were bulk sorted and cultured under conditions that promote B cell proliferation and antibody secretion and screened with the microneutralization assay (**Figure 4.1 A**). IgG was detected in 40% of a random sample of wells at day 9 in culture and 58% of wells were IgG positive at day 12 (**Figure 4.1 B**) (limit of detection = 10ng/mL). For the B cells that were double positive for the heterologous DU422.SOSIP.664 bait, 13/46 of the culture wells (4 B cells/well) were positive in the microneutralization assay. Twelve mAbs were produced, and nine demonstrated neutralizing activity against SF162 (**Figure 4.1 A and C**). Of the cultured IgG B cells, an additional 296 wells (6 B cells/well) were identified by the microneutralization assay as

potentially containing HIV-specific nAbs. mAbs were reconstructed from 150/296 wells and 63 antibodies demonstrated neutralization of SF162 (**Figure 4.1 A and C**).

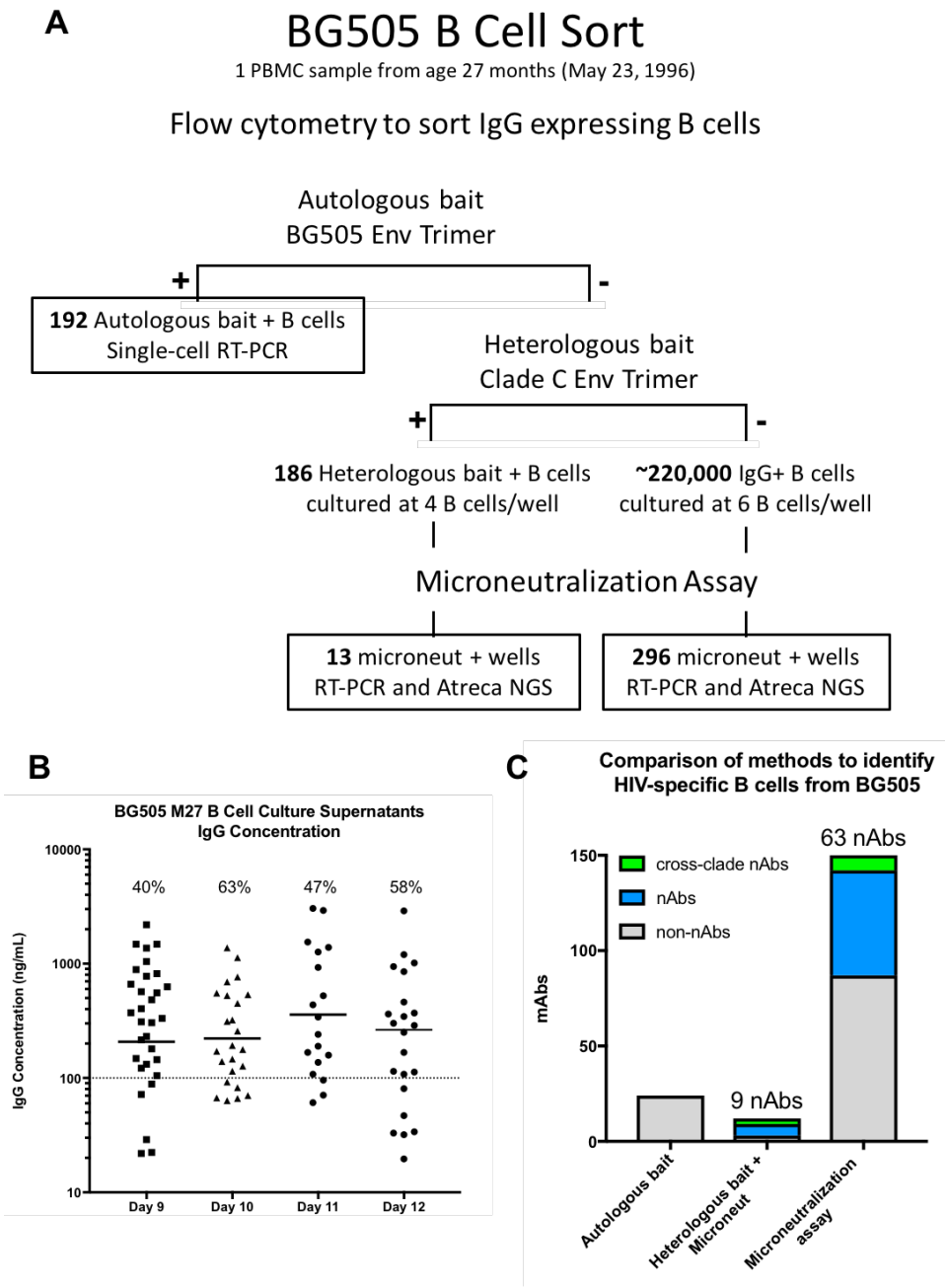


Figure 4.1 Identification of HIV-neutralizing antibodies

(A) Schematic of the BG505 B cell sort and numbers of potentially HIV-specific B cells identified by each method. (B) Concentration (ng/mL) of IgG in a random sample of B cell culture wells at days 9-12. Percent of wells with detectable IgG is indicated above each day. The dashed line at 100ug/ml indicates the IgG concentration needed to detect neutralizing activity. Values below the limit of detection (10ng/uL) are not shown. (C) Each bar shows the number of mAbs generated from the bait-positive and/or microneutralization positive B cells and the number of mAbs with HIV-neutralizing activity (blue and green). Green indicates that the nAb had cross-clade neutralizing activity as shown in Figure 4.3.

BG505 neutralizing antibody clonal lineages

The 72 neutralizing antibodies isolated from BG505 at 27 months of age belong to 19 clonal lineages with up to 13 nAbs in a single lineage. Clonal lineages were defined as antibodies with identical heavy and light chain V and J genes and CDR3 amino acid length. Interestingly, BG505 nAbs utilized only 4 heavy chain germline V-genes, VH1-18, VH1-69, VH3-21 and VH5-51, with a single VH3-21 antibody and >50% composed of VH1-69. Heavy chain CDR3s ranged from 8-26 amino acid residues (**Figure 4.2**).

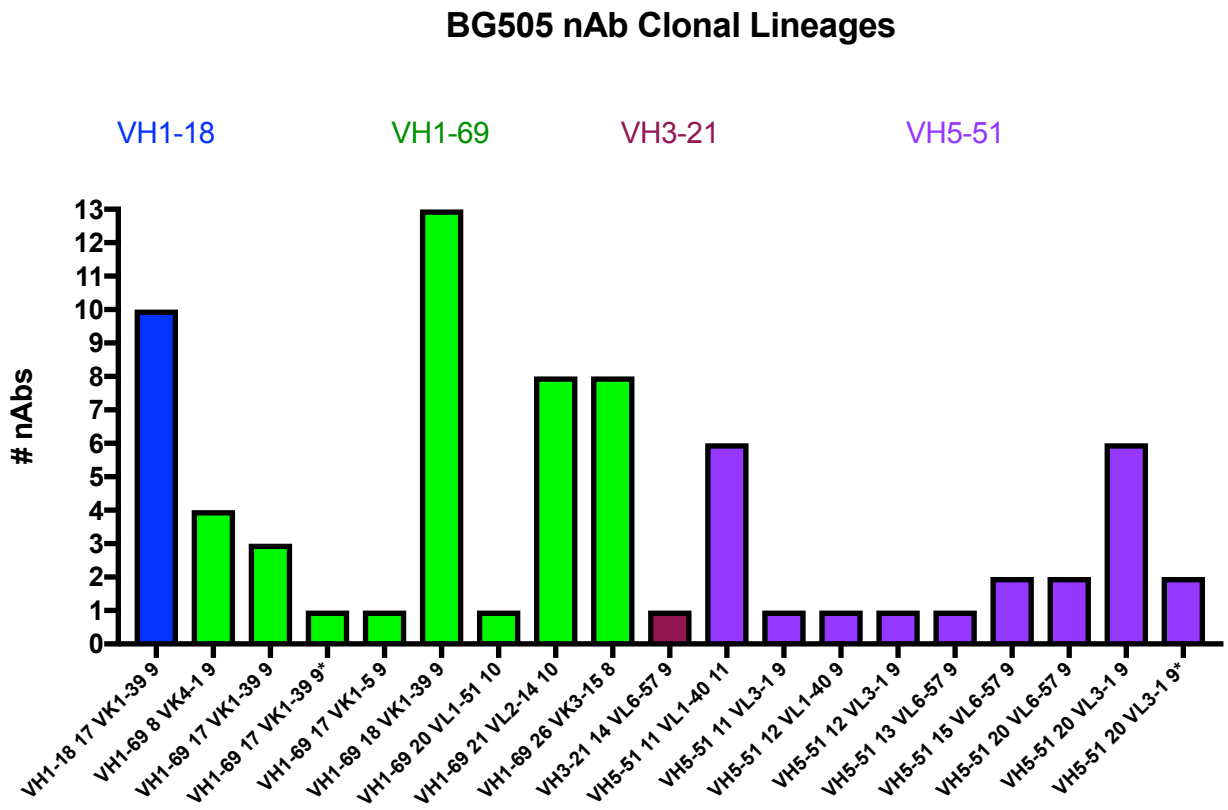


Figure 4.2 BG505 nAb clonal lineages

Each bar represents a unique antibody clonal lineage and the height of the bar corresponds to the number of nAbs identified for a particular lineage. Clonal lineages are distinguished by heavy chain V-gene usage, CDRH3 amino acid length, Light chain V-gene usage, and light chain CDRL3 length. Colors indicate heavy chain V-gene usage (blue = VH1-18, green = VH1-69, purple = VH5-51 and red = VH3-21). An asterisk indicates that the mAb may belong to the clonal family immediately to the left.

Heterologous neutralizing activity of BG505 antibodies

Figure 4.3 shows the heterologous neutralizing activity of the isolated BG505 nAbs. A handful of the BG505 Abs neutralized only SF162, and many neutralized only SF162 and the tier 1 clade A virus Q461.D1. The tier 2 clade A virus Q842.d16, same clade as the infecting virus, is neutralized by 25 of the isolated antibodies. Eleven BG505 neutralizing antibodies demonstrated low potency neutralization of tier 2 viruses from clades B and D with different antibodies mediating neutralization of viruses from different clades suggestive of a polyclonal response. Three of the cross-clade nAbs were identified using the heterologous bait combined with the functional assay (BG505.29, 41 and 42) and the other eight were isolated from cultured IgG-expressing B cells by the microneutralization assay (BG505.18, 29, 20, 22, 25, 65, 68). Five of the QD435-neutralizing antibodies belong to the second largest clonal family identified, defined by VH1-69 and CDRH3 of 18 residues. None of the antibodies recapitulate the full breadth of the plasma.

								Tier 1		Tier 2				
								Clade B	Clade A		Clade B	Clade C		Clade D
Antibody	Heavy Chain	CDRH3 Length	VH SHM (%nt)	Light Chain	CDRL3 Length	VL SHM (%nt)	SIV	SF162	Q461.D1	Q842.d16	6535	QC406.F3	CAP210.E8	QD435.A4
						BG505 M27 Plasma	<1:100	>3200	>3200	482	154	971	590	548
BG505.1	V1-69 D3-10 J1	8	10.4	KV4-1 J1	9	4.7	>50	0.02	5.6	>25	>20	>50	>25	>25
BG505.2	V1-69 D3-10 J1	8	7.6	KV4-1 J1	9	2.7	>20	2.7	>20	>20	>20	>20	>20	>20
BG505.3	V1-69 D5-18 J1	8	9	KV4-1 J1	9	4	>50	0.1	15.8	>25	>20	>50	>25	>25
BG505.4	V1-69 D5-18 J1	8	9	KV4-1 J1	9	4	>20	0.2	5.5	>20	>20	>20	>20	>20
BG505.5	V5-51 D2-2 J4	11	4.9	LV1-40 J3	11	3.3	>50	0.2	0.9	>25	>20	>50	>25	>25
BG505.6	V5-51 D5-24 J4	11	8.7	LV1-40 J3	11	2.9	>50	<0.02	0.8	>25	>20	>50	>25	>25
BG505.7	V5-51 D5-24 J4	11	4.9	LV1-40 J3	11	3.3	>20	<0.02	0.1	>20	>20	>20	>20	>20
BG505.8	V5-51 D5-24 J4	11	6.3	LV1-40 J3	11	4.3	>50	<0.02	0.8	>25	>20	>50	>20	>25
BG505.9	V5-51 D5-24 J4	11	6.9	LV1-40 J3	11	1.8	>20	<0.02	0.2	>20	>20	>20	>20	>20
BG505.10	V5-51 D5-24 J4	11	5.2	LV1-40 J3	11	3.3	>20	0.1	0.8	>20	>20	>20	>20	>20
BG505.11	V5-51 D6-19 J4	11	5.9	LV3-1 J1	9	6.1	>30	<0.02	3.8	3.5	>20	>30	>20	>20
BG505.12	V5-51 D1-26 J3	12	5.2	LV3-1 J2	9	8.2	>50	<0.02	6.8	3.2	>20	>50	>20	>25
BG505.13	V5-51 D5-12 J3	12	8.7	LV1-40 J2	9	6.3	>14	<0.02	2.5	>20	>20	>14	>20	>20
BG505.14	V5-51 D5-24 J4	13	7.3	LV6-57 J3	9	3.8	>20	0.1	4.6	15.6	>20	>20	>20	>20
BG505.15	V3-21 D3-3 J3	14	3.1	LV6-57 J3	9	3.4	>30	1.5	10.0	>20	>20	>30	>20	>20
BG505.16	V5-51 D1-26 J4	15	6.6	LV6-57 J3	9	4.8	>50	0.0	2.7	2.3	>20	>50	>25	>25
BG505.17	V5-51 D1-26 J4	15	8	LV6-57 J3	9	5.2	>50	<0.02	3.1	5.6	>20	>50	>25	>25
BG505.18	V1-18 D1-20 J4	17	10	KV1-39 J4	9	7.9	>20	0.1	2.8	>20	>20	>20	>20	42.2
BG505.19	V1-18 D3-10 J4	17	11.1	KV1-39 J4	9	6.8	>50	0.1	4.4	>25	>20	>50	>25	~25
BG505.20	V1-18 D3-16 J4	17	9.4	KV1-39 J4	9	6.4	>20	0.2	1.4	>20	>20	>20	>20	21.9
BG505.21	V1-18 D4-17 J4	17	10	KV1-39 J4	9	10	>30	0.1	4.6	>20	>20	>30	>20	>20
BG505.23	V1-18 D6-13 J4	17	10.4	KV1-39 J4	9	5.4	>50	0.3	8.3	>25	>20	>50	>20	50.0
BG505.22	V1-18 D6-13 J4	17	11	KV1-39 J4	9	5.7	>20	0.3	1.0	>20	>20	>20	>20	>20
BG505.24	V1-18 D6-13 J4	17	9.4	KV1-39 J4	9	7.5	>11	1.1	8.0	>10	>10	>10	>10	>10
BG505.25	V1-18 D6-13 J4	17	7.6	KV1-39 J4	9	5	>20	0.5	0.9	>20	>20	>20	>20	50.0
BG505.26	V1-18 D6-19 J4	17	8.3	KV1-39 J4	9	3.6	>30	0.4	3.6	>20	>20	>30	>20	>20
BG505.27	V1-18 D6-19 J4	17	7.3	KV1-39 J4	9	3.9	>20	12.4	12.2	>20	ND	>20	ND	ND
BG505.28	V1-69 D3-10 J4	17	5.6	KV1-39 J4	9	ND	>20	13.6	5.4	>20	>20	>20	>20	>20
BG505.29	V1-69 D3-10 J4	17	9.4	KV1-39 J4	9	8.6	>50	15.9	1.0	10.5	>20	>50	>25	22.9
BG505.30	V1-69 D3-10 J4	17	10.8	KV1-39 J4	9	8.2	>50	1.9	3.1	10.6	>20	>50	>20	>25
BG505.31	V1-69 D3-3 J4	17	4.9	KV1-39 J2	9	4.7	>20	3.7	8.9	>20	>20	>20	>20	>20
BG505.32	V1-69 D6-13 J6	17	11.1	KV1-5 J1	9	2.9	>50	50.0	>20	>25	>20	>50	>25	>25
BG505.33	V1-69 D2-2 J4	18	9.7	KV1-39 J1	9	3.6	>30	1.0	1.0	13.6	>20	>30	>20	>20
BG505.34	V1-69 D3-3 J5	18	10.1	KV1-39 J1	9	5.7	>16.5	1.6	4.6	>20	>20	>20	>20	>20
BG505.35	V1-69 D3-3 J5	18	8	KV1-39 J1	9	3.9	>20	2.4	3.4	15.4	>20	>20	>20	>20
BG505.36	V1-69 D3-3 J5	18	9.7	KV1-39 J1	9	5	>20	2.0	1.7	>20	>20	>20	>20	>20
BG505.37	V1-69 D3-3 J5	18	5.7	KV1-39 J1	9	2.5	>20	2.2	3.9	>20	>20	>20	>20	>20
BG505.38	V1-69 D3-3 J5	18	9	KV1-39 J1	9	3.2	>20	1.6	2.2	9.6	>20	>20	>20	>20
BG505.39	V1-69 D3-3 J5	18	8.7	KV1-39 J1	9	2.5	>30	0.8	1.5	14.0	>20	>30	>20	>20
BG505.40	V1-69 D3-3 J5	18	7.3	KV1-39 J1	9	3.6	>20	3.1	3.4	>20	ND	>20	>20	>20
BG505.41	V1-69 D3-3 J5	18	7.6	KV1-39 J1	9	2.9	>30	0.8	0.2	5.5	21.0	>30	>20	>20
BG505.42	V1-69 D3-3 J5	18	6.6	KV1-39 J1	9	3.6	>50	1.0	3.8	13.9	>20	>50	>25	50.0
BG505.43	V1-69 D3-3 J5	18	6.3	KV1-39 J1	9	10	>31	19.8	7.6	>20	>20	>31	>20	>20
BG505.45	V1-69 D3-3 J5	18	7.6	KV1-39 J1	9	2.5	>20	1.0	1.1	6.9	>20	>20	>20	>20
BG505.44	V1-69 D3-3 J5	18	8.7	KV1-39 J1	9	3.6	>20	1.6	1.3	6.3	>20	>20	>20	>20
BG505.46	V1-69 D2-8 J3	20	8.3	LV1-51 J2	10	4.6	>30	0.03	0.5	5.7	9.3	>30	>20	>20
BG505.47	V5-51 D2-15 J4	20	6.3	LV6-57 J3	9	4.1	>50	0.1	2.1	11.0	>20	>50	>20	>25
BG505.48	V5-51 D2-15 J4	20	6.9	LV6-57 J3	9	4.8	>50	<0.02	1.3	10.8	>20	>50	>25	>25
BG505.49	V5-51 D2-15 J3	20	5.2	LV3-1 J2	9	6.4	>9	1.5	8.4	>20	>9	>9	>9	>9
BG505.50	V5-51 D2-15 J3	20	6.3	LV3-1 J2	9	4.7	>50	<0.02	0.6	3.2	>20	>50	>20	>25
BG505.51	V5-51 D2-15 J3	20	3.5	LV3-1 J2	9	3.9	>50	<0.02	0.4	3.0	>20	>50	>25	>25
BG505.52	V5-51 D2-15 J5	20	8.3	LV3-1 J2	9	7.2	>50	0.1	1.7	5.6	>20	>50	>20	>25
BG505.53	V5-51 D2-15 J5	20	5.9	LV3-1 J2	9	4.7	>20	0.1	1.3	3.5	>20	>20	>20	>20
BG505.54	V5-51 D2-15 J5	20	6.6	LV3-1 J2	9	ND	>20	0.1	0.2	3.1	>20	>20	>20	>20
BG505.55	V5-51 D2-8 J5	20	6.6	LV3-1 J3	9	7.5	>20	<0.02	0.3	2.9	>20	>20	>20	>20
BG505.56	V5-51 D2-8 J5	20	6.3	LV3-1 J3	9	6.4	>30	<0.02	0.2	3.4	>20	>30	>20	>20
BG505.57	V1-69 D3-10 J6	21	6.9	LV2-14 J3	10	5.6	>30	0.0	5.1	>20	>20	>30	>20	>20
BG505.58	V1-69 D3-10 J6	21	8.3	LV2-14 J3	10	10	>20	0.2	11.1	>20	>20	>20	>20	>20
BG505.59	V1-69 D3-10 J6	21	6.3	LV2-14 J3	10	ND	>20	0.6	5.9	>20	>20	>20	>20	>20
BG505.60	V1-69 D3-10 J6	21	8	LV2-14 J3	10	4.9	>20	0.2	4.1	>20	>20	>20	>20	>20
BG505.61	V1-69 D3-10 J6	21	8.7	LV2-14 J3	10	5.2	>20	0.7	5.9	>20	>20	>20	>20	>20
BG505.62	V1-69 D5-24 J6	21	8	LV2-14 J2	9	3.8	>20	1.1	9.1	>20	>20	>20	>20	>20
BG505.63	V1-69 D5-24 J6	21	6.3	LV2-14 J3	10	ND	>30	0.5	ND	>20	>20	>30	>20	>20
BG505.64	V1-69 D5-24 J6	21	6.3	LV2-14 J3	10	3.1	>20	0.7	9.7	>20	>20	>20	>20	>20
BG505.65	V1-69 D1-1 J6	26	10.8	KV3-15 J1	8	1.4	>50	0.3	2.7	>25	>20	>50	>25	25.0
BG505.66	V1-69 D1-1 J6	26	9	KV3-15 J1	8	2.5	>30	0.1	3.3	>20	>20	>30	>20	>20
BG505.67	V1-69 D1-1 J6	26	9.7	KV3-15 J1	8	3.6	>30	8.5	9.0	>20	>20	>30	>20	>20
BG505.68	V1-69 D1-1 J6	26	9.7	KV3-15 J1	8	1.4	>30	<0.02	0.1	>20	>20	>30	>20	12.7
BG505.69	V1-69 D5-12 J6	26	9.7	KV3-15 J1	8	1.4	>30	0.1	1.6	>20	>20	>30	>20	>20
BG505.70	V1-69 D5-12 J6	26	9	KV3-15 J1	8	2.2	>20	1.4	ND	>20	ND	>20	ND	ND
BG505.71	V1-69 D5-12 J6	26	7.6	KV3-15 J1	8	1.8	>20	2.2	ND	ND	>20	>20	ND	ND
BG505.72	V1-69 D5-12 J6	26	7.3	KV3-15 J1	8	4.7	>20	0.2	0.1	>20	>20	>20	>20	>20

Figure 4.3 Heterologous neutralization by BG505 antibodies

Neutralization of panel viruses by BG505 M27 plasma and nAbs. Plasma neutralizing activity against this heterologous virus panel is shown at the top. Antibodies are in rows with names and sequence characteristics of each nAb shown on the left. Clonal families are separated by horizontal lines. Heterologous viruses are in columns to the right with the first two rows indicating virus tier and clade. SIV was included as a negative control. IC_{50} values (ug/mL) represent an average of two independent experiments performed in duplicate. Darker blue shading indicates more potent neutralization. Gray indicates that 50% neutralization was not achieved at the highest mAb concentration tested.

BG505 autologous Env variants

We were interested in determining whether these nAbs with limited heterologous neutralizing activity are able to neutralize autologous Env isolates. Env variants from the time-point when HIV was detected, week 6 (W6) were previously cloned (200). Additional Env sequences from early in infection, week 14 (W14) and later in infection, month 27 (M27) were isolated (**Figure 4.4**). Samples were not available from this infant between W14 and M27 limiting our ability to examine autologous neutralization by isolated antibodies.

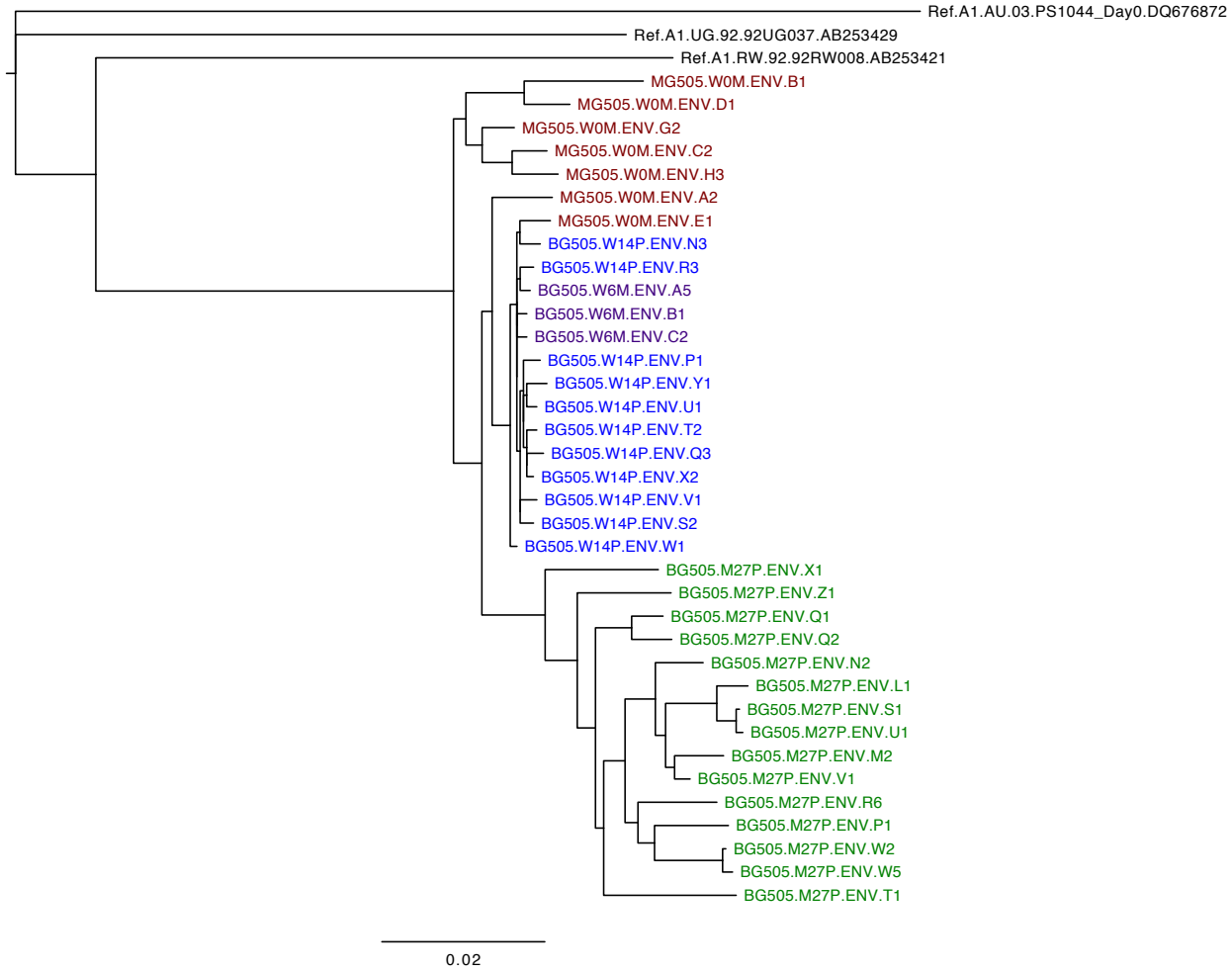


Figure 4.4 Phylogenetic tree of maternal and infant Env variants

Maximum likelihood phylogenetic tree of maternal (MG505) and infant-derived (BG505) envelope variants. Maternal variants were isolated around the time of delivery (W0, red) and are provided as a reference. BG505 Env variants are from 6 weeks (W6, purple), 14 weeks (W14, blue) and 27 months (M27, green) of age.

Earlier Env variants isolated from BG505 at 6 and 14 weeks of age are potently neutralized by plasma antibodies from 27 months of age. Plasma from BG505 at month 27 neutralizes contemporaneous viruses to varying degrees. A few M27 Env variants are potently neutralized by the M27 plasma, but most are neutralized to a lesser extent. None of the isolated M27 isolates are completely resistant to plasma neutralization (**Figure 4.5**).

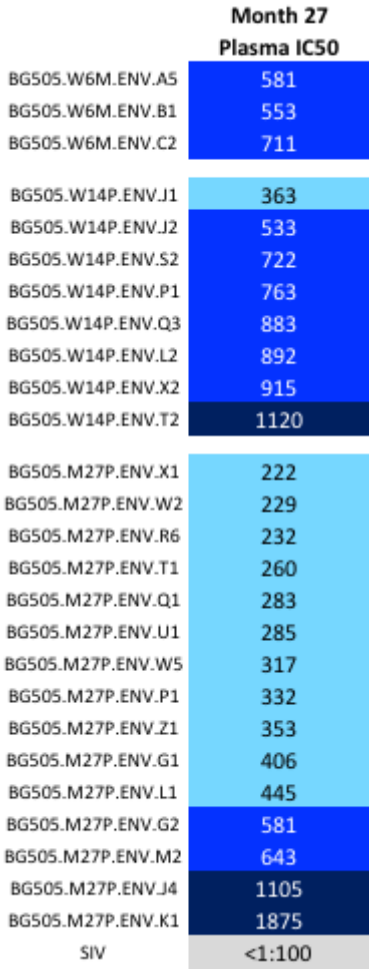


Figure 4.5 Neutralization of autologous variants by BG505 plasma

BG505 M27 plasma neutralization of autologous Env variants in rows that are from 6 weeks (W6), 14 weeks (W14) and 27 months (M27) of age. SIV was included as a negative control.

Selected BG505 nAbs did not demonstrate neutralization of early (W6 and W14) or contemporaneous viruses (**Figure 4.6**). The 24 mAbs isolated using the autologous BG505 Env bait, which is based on the BG505.W6M.C2 Env clone, did not neutralize virus expressing this Env (data not shown). Combined with the heterologous neutralization data, these data show that a substantial portion of the plasma neutralizing activity is not captured by the isolated nAbs.

	Env Isolates								
	Week 6	Week 14				Month 27			
	C2	J1	Q3	X2	P1	J4	M2	T1	Z1
BG505.3	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.5	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.8	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.12	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.14	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.17	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.19	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.20	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.28	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.29	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.30	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.32	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.35	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.42	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.45	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.47	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.48	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.51	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.52	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.53	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.60	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.64	>20	>20	>20	>20	>20	>20	>20	>20	>20
BG505.65	>20	>20	>20	>20	>20	>20	>20	>20	>20

Figure 4.6 Neutralization of autologous variants by BG505 nAbs

BG505 nAbs are in rows and Env isolates in columns. Gray indicates that 50% neutralization was not achieved at the highest mAb concentration tested.

Affinity maturation of BG505 neutralizing antibodies

We next compared the level of somatic hypermutation (SHM) of BG505 nAbs to adult- and infant-derived antibodies including adult antibodies that are broadly neutralizing and Tier 1 neutralizing (**Figure 4.7**). nAbs were also isolated from the mother of BG505 (MG505) from a time-point near delivery by Laura Noges. The MG505 nAbs have Tier 1 and limited tier 2 neutralization of a single clade A virus and the time post-infection is not known. The heavy chain variable region average SHM for BG505 nAbs was 7.8%, which is comparable to adult Tier 1 nAbs (average = 8.7%) and MG505 Tier 1 nAbs (average = 9.8%). BG505 nAbs with low potency, cross-clade neutralization had slightly higher SHM than the overall group of BG505

nAbs (average = 9.2%). BG505 antibodies were isolated at ~2 years post-infection and have approximately double the SHM compared to antibodies isolated from infant BF520 at ~1 year post-infection (average = 4%).

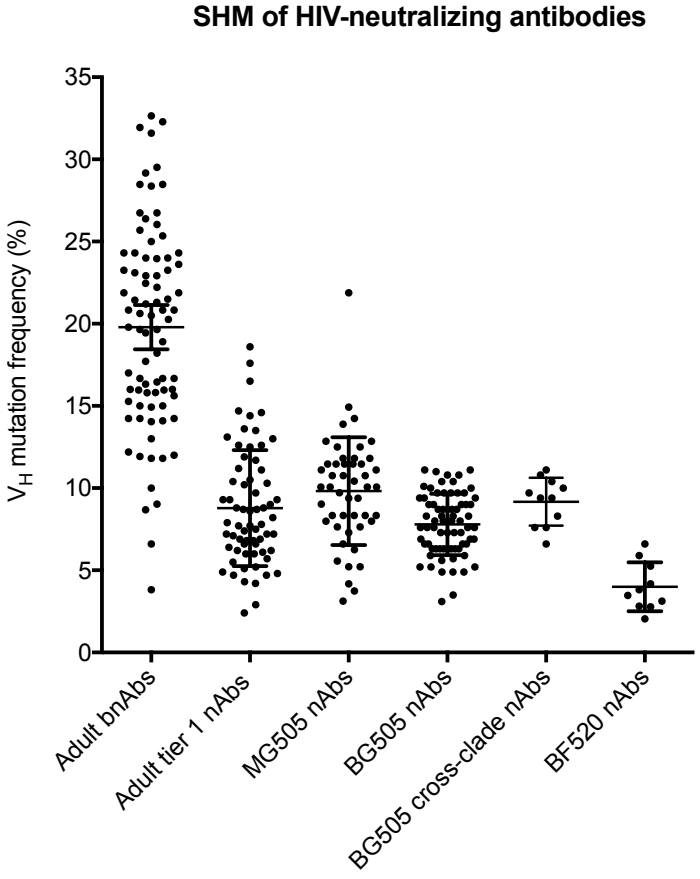


Figure 4.7 BG505 nAb SHM

Adult bnAb and nAb sequences were previously analyzed in Chapter II (298). Neutralizing antibodies from the mother (MG505) of BG505 were isolated by Laura Noges in our lab and sequences were provided for SHM comparison. MG505 nAbs have limited Tier 2 neutralization.

Discussion

The isolation of adult-derived bnAbs has been achieved using either single B-cell culture coupled to high-throughput neutralization screening or sorting of single B cells that bind an HIV Env bait (114). Due to the extensive diversity of HIV Env (14), selecting Env variants that have the potential to identify any B cell expressing a nAb is challenging. The approach of bait alone selects for particular mAbs that bind the Env variant used as bait. Similarly, the microneutralization assay approach identifies nAbs that neutralize the particular selected virus or viruses. For BG505, a single vial of ~6 million cells was available. To maximize our chances of identifying as many HIV-specific neutralizing antibodies as possible, we combined methodologies for HIV-specific B cell identification, which included an autologous Env variant and 3 heterologous Env variants. Infant BG505 mounted a diverse and impressive nAb response. The 72 nAbs identified belong to several expanded clonal families of antibodies utilizing a wide range of CDRH3s with 8-26 amino acid residues. Most of the isolated nAbs demonstrated limited heterologous tier 2 neutralization restricted to the clade of the infecting virus. Eleven antibodies had low potency, cross-clade neutralization of either a clade B or clade D virus, and these cross-clade nAbs were primarily found in the expanded clonal families with the exception of BG505.46. The antibodies targeting the clade B and clade D viruses were distinct. These results suggest a diverse polyclonal response to the virus.

Despite the diversity in CDRH3 length and identification of several clonal families, 71/72 of the nAbs utilize only 3 V-genes in the heavy chain (VH1-18, VH1-69 and VH5-51), with one additional nAb utilizing VH3-21. A surprising number utilize VH5-51, especially considering that VH5 is poorly represented in the IgG BCR repertoires of healthy individuals (187). This raises the possibility that the identification of nAbs was biased; however, we used several

methods including autologous, heterologous and cross-clade baits and viruses to identify HIV-specific B cells. This could also suggest that the nAb repertoire in BG505 is highly enriched for these particular V-genes.

Despite isolating an impressive 72 nAbs, we were not able to recapitulate the breadth and potency of the plasma response. It is possible several antibody lineages with limited heterologous neutralizing activity in a polyclonal combination account for the plasma neutralization breadth. This is supported by the study by Goo et al., which did not identify a dominant plasma response targeting known bnAb epitopes (23). Because we screened the memory B cells for HIV specificity using two different baits (autologous clade A Env and heterologous clade C Env) as well as two different viruses (tier 1 clade B and tier 2 clade C) it would be surprising if we missed a dominant bnAb if it was at a reasonable frequency in the memory B cell population. While synergy in neutralization has not been observed with HIV nAbs, a future experiment will be to combine isolated nAbs, particularly those with heterologous breadth to look for enhanced breadth and/or potency. Another possibility is that we simply didn't catch the bnAb. One piece of evidence supporting this possibility is the VH1-69 clonal family with a CDRH3 of 18 residues, which has a family member that neutralized the clade B virus 6535 and a different clonal family member that neutralized the clade D virus QD435. This suggests that the antibody lineage is targeting a relatively conserved epitope, and that we may not have sampled the lineage member that gained breadth. Overall, we identified an impressive number of independently responding B cell lineages. Because studies of adult bnAbs haven't gone to these lengths to identify HIV-specific B cells, it's not clear if this is unique to infant BG505.

Most of the isolated antibodies are likely strain-specific. The observation that these antibodies isolated from 27 months of age are not able to neutralize the viral isolates from the

same time-point is not surprising considering several other studies that have demonstrated resistance by contemporaneous viruses to autologous nAbs (38, 68, 69, 71, 76). The M27 Env variants likely have already escaped these nAb responses, yet memory B cells expressing these antibodies are still circulating. The isolated M27 nAbs are also unable to neutralize viruses from the time-point when infection was first detected (week 6) and shortly after (week 14), which suggests that these nAbs developed in response to Env variants that evolved between 14 weeks and 27 months of age. Unfortunately, samples are not available between early infection and month 27, so we are unable to examine the interaction between these nAbs and autologous viruses. There is not a precedent for inferring intermediate HIV-1 Env sequences, though such a tool would be useful.

Because many studies have demonstrated that strain-specific nAbs can develop breadth or cooperate with other B cell lineages to drive the development of breadth (77), it may be interesting to examine the epitope specificities of these nAbs in future studies as it may suggest regions of Env being targeted by the humoral immune response. Furthermore, the BG505 SOSIP trimer has been used as an immunogen in rabbits and macaques and several strain-specific nAbs have been isolated and characterized (316). Many of the isolated antibodies target holes in the glycan shield that are easily filled by the virus as an escape mechanism. Because the transmitted virus from BG505 is the variant that was used for those studies and contains those glycan holes, it would be interesting to determine whether these isolated nAbs have any overlap in their epitope targets. It is important to consider, however, that unlike the rabbit and macaque nAbs, these BG505 nAbs do not neutralize the W6 transmitted variant. We don't yet know if they can bind the BG505 trimer from week 6.

Surprisingly, no nAbs were identified using the autologous Env trimer as bait, whereas the heterologous Env trimer identified 9 nAbs. Additionally, BG505 nAbs from M27 don't neutralize the transmitted virus, and this is similar to the observation made with nAbs isolated from infant BF520 (298). This may suggest that the development of infant nAbs may be delayed and rather than developing in response to the vertically transmitted virus, develop in response to later variants. In support of this, previous studies have shown that Env-specific antibody responses in vertically infected infants are delayed until on average until 6 months of age (262, 263). Furthermore, studies utilizing the BG505 SOSIP trimer based on the transmitted Env as an immunogen have only elicited strain-specific responses (199). There is recent emphasis on sequential immunization strategies that mimic viral evolution in individuals who develop bnAbs (148). Thus, the BG505 week 14 and month 27 longitudinal Env variants described here may be useful tools to generate sequential immunogens based on viral evolution in this infant.

The level of SHM in the BG505 nAbs isolated at ~2 years post-infection is comparable to adult Tier 1 nAbs and twice the level of SHM observed in infant BF520 nAbs, which were isolated at ~1 year post-infection. This suggests that the level of SHM in infant nAbs reflects the time since infection and for affinity maturation and not necessarily something intrinsic to the infant immune system. How the bnAb responses of infants are generated rapidly compared to adults, though, is not known and may be unique to infants.

Overall, these results suggest that polyclonal nAbs with relatively limited maturation contribute to BG505 plasma neutralizing activity, and this study provides useful tools for HIV vaccine development, particularly the sequential BG505 Env isolates.

Chapter V

Conclusions and implications for neutralizing antibody-based HIV-1 vaccine design

HIV-1 broadly neutralizing antibodies develop in a subset of infected adults and exhibit high levels of SHM due to years of affinity maturation. There is no precedent for eliciting highly mutated antibodies by vaccination, nor is it practical to wait years for a desired response. HIV-infected infants develop broadly neutralizing plasma responses with more rapid kinetics than adults (23), suggesting the ontogeny of these responses could inform a more straightforward path to achievable vaccine targets. The studies presented in this thesis investigated infant HIV-neutralizing antibodies contributing to broadly neutralizing plasma responses. In Chapter II, we isolated nAbs from infant BF520 from approximately one year post-infection and demonstrated that polyclonal nAbs contribute to plasma breadth and that infant nAbs, including one bnAb, have significantly lower levels of SHM compared to adult HIV-neutralizing antibodies. In Chapter III, we defined the ontogeny of the first and only identified infant bnAb, BF520.1. We show that unlike many adult bnAb precursors, the inferred naïve precursor of BF520.1 recognizes HIV Env, and cross-clade neutralizing activity developed within six months of infection. In Chapter IV, we identified an impressive number of independent clonal lineages of nAbs contributing to a broad plasma response at two years post-infection from infant BG505. Overall, these studies demonstrate that antibodies from multiple B cell lineages requiring limited SHM contribute to broadly neutralizing plasma activity that developed relatively early post-infection for 2 infants. The following chapter will consider the impact of these findings to the HIV prevention field and future studies relevant for HIV-1 vaccine design.

Polyclonal nAb responses

A protective nAb-based vaccine will likely require the elicitation of polyclonal nAbs targeting multiple epitopes as no single bnAb neutralizes all circulating strains of the virus (115). A polyclonal response would provide increased protection against diverse circulating variants and limit the ability of the virus to escape. Many of the studies describing the isolation of adult-derived bnAbs focused on dominant monoclonal responses (82, 84, 85, 89, 122, 123, 130, 131, 133, 134), with a few exceptions (21, 125, 132, 157, 158). In many cases, the use of Env bait proteins limited the identification of bnAbs to those targeting specific epitopes, particularly for bnAbs targeting the V3-glycan (85, 133, 134, 143) and CD4bs (114). The dominant epitope specificities of broad plasma responses can be mapped in >70% of HIV-infected adults (94, 96, 153, 154), though signatures suggestive of polyclonal responses are found in some individuals (80, 94, 96, 154-156). For HIV-infected infants who developed bnAb responses, plasma mapping studies performed by Goo et al. failed to identify dominant responses targeting known bnAb epitopes. In order to generate a vaccine-elicited polyclonal response, a better understanding of these responses in natural infection is needed.

To identify HIV-specific B cells from infant BF520, we used the microneutralization assay approach with two different viruses. For infant BG505, we combined bait and microneutralization approaches including four different Env variants to isolate nAbs. These approaches allowed us to identify as many HIV-specific B cells as possible and capture a more complete picture of the overall nAb responses. For both infants BF520 and BG505, polyclonal nAbs from multiple clonal lineages, which describes antibodies encoded by B cells that originated from a single naïve B cell precursor as a result of proliferation and affinity maturation, were characterized. All infant-derived nAbs had levels of SHM that are within reach by

vaccination. In chapter II we described the isolation of 10 nAbs from distinct clonal lineages, including one bnAb, that contribute to plasma neutralization breadth for infant BF520 at approximately one-year post-infection. In Chapter IV, we identified 72 nAbs from infant BG505 at ~2 years post-infection that belong to 19 clonal lineages with up to 13 nAbs in a single lineage. The 11 nAbs with cross-clade neutralizing activity from infant BG505 belonged to 5 clonal lineages. For both infants, nAbs from multiple clonal lineages neutralized distinct viruses and the isolated nAbs fail to recapitulate the full neutralizing activity of the plasma responses, suggesting there are additional nAb lineages contributing to the overall responses. These studies highlight the potential of infants to generate polyclonal nAbs without the requirement for long-term affinity maturation that in combination provide neutralization coverage of diverse HIV isolates.

Another study from our lab by Williams et al. isolated nAbs from a superinfected adult with a plasma signature suggestive of a polyclonal response. The isolated nAbs were from 4 clonal lineages and recapitulated about 50% of plasma activity (206). Together, these studies underscore the challenge of recapitulating plasma activity with isolated nAbs when the responses appear to be polyclonal (156, 206, 298). The limited availability of PMBC and plasma samples, particularly from pediatric cases where small volumes of blood are drawn, require careful consideration of the most efficient use of samples.

To better understand these unique polyclonal responses, additional methods to comprehensively map polyclonal plasma responses requiring a limited amount of plasma would be useful tools. Computational “neutralization fingerprinting” is one method used to predict the epitope specificities of polyclonal plasma responses by comparing patterns of neutralization of panel viruses between bnAbs targeting known epitopes and plasma samples (136, 155).

However, this method failed to identify dominant responses in infants (23) and requires a large quantity of plasma to screen for neutralization of a panel of viruses. The ability to define neutralization patterns and breadth with a small quantity of plasma is needed. One option for more efficient use of samples would be to develop a method that uses a single plasma dilution (323). An exciting method to define plasma neutralization of multiple viruses with small quantity of plasma is being developed in our lab, and the premise is to neutralize a pool of viruses with plasma and quantify neutralization of each virus by deep sequencing the virus pool before and after neutralization.

In addition to the development of methods to map polyclonal plasma responses, methods to more comprehensively identify HIV-specific nAbs from plasma are being developed. A method to directly identify HIV-specific antibodies from plasma integrates paired deep sequencing of the B cell repertoire with a proteomics-based approach referred to as "IgG Seq," where plasma antibodies that bind to an HIV protein are identified by affinity chromatography and peptide sequences of those antibodies are determined by mass spectrometry. The complete antibody can be reconstructed by identifying clonal relatives of the plasma antibody in the B cell repertoire (324-326). This method was used to identify MPER-directed bnAbs from both memory B cells and plasma (191). For studies where the plasma specificity is not known, the Env bait must be carefully selected to ensure nAbs targeting any epitope can be identified.

For infant BF520, additional plasma and PBMC samples are available, and this approach is one option to identify additional nAbs contributing to overall plasma breadth. If this approach was utilized for infant BF520, bait that has the potential to identify antibodies that recapitulate plasma neutralization of autologous and heterologous viruses should include a combination of trimers. The nAbs isolated thus far from BF520 do not recapitulate plasma autologous

neutralization and heterologous neutralization of clade A and D viruses. Therefore, the bait should include longitudinal autologous trimers such as BF520.W14M.E3.SOSIP and BF520.M6P.D1.SOSIP and cross-clade heterologous trimers of clade A and D Envs.

Development of an infant-derived bnAb

Over 50 bnAbs have been isolated from HIV-infected adults from as early as 2 to over 20 years post-infection, and the longitudinal development of bnAb lineages have been detailed in eight studies (82, 84, 85, 87-89, 127, 133, 191). These antibodies have a number of unusual features detailed in Table 1.1 and 1.2 and required iterative rounds of SHM and affinity maturation over years of infection before developing neutralization breadth. Efforts are ongoing to identify ways to elicit bnAbs despite these immunological barriers (148, 173, 327). Additional studies to identify bnAbs lacking unusual features will provide a more straightforward pathway to bnAb induction. In Chapter II we describe the isolation of a single infant-derived bnAb, BF520.1 from ~1-year post-infection. BF520.1 targets the V3-glycan or N332 supersite on Env, but unlike adult bnAbs targeting this site, has low SHM and lacks rare indels. In Chapter III, we defined the evolutionary pathway for the BF520 infant-derived bnAb. Unlike the naïve progenitors of many adult-derived bnAbs (85, 134, 178, 179, 195, 196), the BF520.1 naïve precursor is capable of binding HIV Env and lineage intermediates demonstrate increasing neutralization breadth with as little as 3% SHM required to achieve the full breadth of this lineage, a level of SHM that is easily within reach by vaccination (175). This is remarkable when compared to adult broadly neutralizing antibodies targeting the same epitope, which have an average of 18% SHM (Table 1.2). Furthermore, both the naïve and mature mAbs lack detectable autoreactivity, which has been observed with many adult bnAbs (111, 172, 173, 295)(Table 1.1 and 1.2). This infant bnAb lacks any of the unusual features observed in adult-derived bnAbs and

demonstrates that HIV-1-specific neutralization breadth can develop without prolonged affinity maturation and extensive SHM.

As V3-glycan bnAbs have different modes of Env recognition (85, 152, 196, 202), high-resolution structural characterization of BF520.1 with the Env trimer will reveal important glycan and peptide contacts. This lineage is unique in the dependence on affinity maturation in the kappa light chain for the development of heterologous breadth, and definition of the paratope may provide insights about critical residues in the antibody sequence. Furthermore, early intermediates of BF520.1 are able to bind but lack neutralizing activity against the BG505 Env. A structural comparison of early lineage intermediates to the mature antibody may reveal potential differences in the modes of binding by developmental intermediates.

The importance of antibody-virus co-evolution has been revealed by studies of adult bnAbs (77, 148). Further studies of the interaction between the developing BF520.1 lineage and the autologous viral evolution could refine our understanding of the development of breadth and how viral evolution shaped the response. Additionally, deep mutational scanning with HIV Env will inform how the virus can escape from this antibody (328) and further fine-map the epitope target of BF520.1.

The BF520.1 lineage should be considered an attractive template for vaccine design. An important question that remains to be addressed is whether the level of binding to Env observed for the naïve antibody is sufficient for B cell activation. The naïve precursor was shown to bind the BG505 SOSIP trimer, which is already being tested in pre-clinical vaccine studies (199). If the level of binding is sufficient to activate the naïve B cell, the availability of a well-characterized initial immunogen further supports this antibody as a possible vaccine target. Some adult-derived V3-glycan bnAbs do not demonstrate detectable binding recombinant Env, but are

able to recognize a synthetic germline-targeting V3 peptide (85, 134). One way to determine if the binding to the Env trimer is sufficient for B cell activation or if this antibody requires the identification of a germline-targeting immunogen would be to measure the activation of B cells expressing the naïve antibody as a BCR as described by McGuire et al. (329). Another important consideration is whether the precursors of BF520.1-like B cells are of sufficient frequency in the B cell repertoire for induction by vaccination (330, 331). Because the naïve precursor of BF520.1 is not autoreactive and N332-directed antibodies in general are not restricted to particular germline gene usage, potential naïve B cell precursors may be at a higher frequency.

A longer-term goal for vaccine elicitation of BF520.1-like antibodies is the design of a sequential immunization strategy or a B-cell lineage immunogen design similar to those described for adult bnAbs (148) and studies in animal models to determine whether a similar nAb lineage can be induced. Saunders et al. recently described the elicitation of cross-clade nAbs in a single macaque following a sequence of six immunizations based on a lineage pathway defined for an adult (198). Presumably BF520.1 would require fewer immunogens to induce a similar response because it developed over a shorter period of time compared to the adult bnAbs that previous sequential immunogens were based on. A potential immunization strategy to elicit a BF520.1-like response could use the BG505.SOSIP.664 to initiate the response with subsequent boosting with BF520 Env trimer from ~2 months pi to drive affinity maturation.

As described above, a protective vaccine will likely need to elicit more than N332-directed bnAbs as the N332 glycan is present on ~67% of isolates (316), and ~78% have the ‘‘GDIR’’ peptide sequence (133). A polyclonal response targeting multiple epitopes will be required. For example, a combination of bnAbs targeting the CD4bs and V3-glycan can provide near complete coverage of diverse viruses (285). Identification of additional targets of infant

bnAb responses through plasma mapping studies or the isolation of additional infant bnAbs could reveal common epitope specificities that may be more easily elicited by vaccination. How to induce polyclonal response remains unknown and warrants further investigation.

Development of neutralization breadth in infants

These studies suggest that the infant immune system may be uniquely poised to develop bnAb responses rapidly. Understanding the factors that contribute to these unique infant responses and whether those factors can be mimicked in a vaccine setting is critical for designing strategies to induce similar responses.

Goo et al. demonstrated that infant bnAb responses develop more rapidly compared to adults and Muenchhoff et al. showed that pediatric responses develop more frequently (in 75% of cases) compared to adults (19% of infected adults). Combined, these studies suggest that something about the unique setting of pediatric HIV infection contributes to responses that are distinct from those in adults. Goo et al. demonstrated that high viral load correlated with bnAb development in infants suggesting that high antigenic stimulation contributes to bnAb emergence (23). A second study describing pediatric bnAb responses also showed a positive association between viral load and bnAb development (265). Furthermore, infants reach higher peak and set-point viral load compared to adults (213). Despite the high level of antigenic stimulation, however, isolated infant nAbs described in this thesis have low levels of SHM. This is not unexpected as the size and numbers of germinal centers and immunoglobulin somatic mutation increase in an age-dependent manner (238-240). Whether high antigenic stimulation is necessary for the development of breadth is not known, though lower levels of SHM doesn't preclude the development of neutralization breadth. Furthermore, antibodies from infant BG505 at two years post-infection had on average twice the level of SHM compared to BF520 antibodies from one

year post-infection, which suggests the level of SHM may simply reflect the length of time since infection. How neutralization breadth develops within 1-2 years of infection is an important unanswered question that this thesis has only begun to touch on.

Factors intrinsic to the developing immune system in infants that might contribute to the rapid development of bnAb responses include a bias toward Th2 responses that could promote B cell function (234), and higher levels of IgG1 and IgG3 (332), which have been shown to have greater neutralizing activity than IgG2 and IgG4 (333-335).

As discussed in the introduction, another important consideration in infants is the presence of passively acquired maternal antibodies, which gradually wane over a period of about 6 months after birth (200, 241-244), and may influence the development of infant nAbs in two ways. First, maternal antibodies may influence the epitopes exposed on vertically transmitted Envs. Vertically transmitted variants escape maternal nAb responses (200) through conformational masking of targeted epitopes (250). It is also possible that maternal nAbs bind and mask more variable regions of Env. The ability of antibodies to bind autologous Env trimer but not neutralize corresponding viruses was demonstrated in Chapter II (298). The vertically transmitted Env variant from BG505 was successfully used to generate the first native-like, soluble Env trimer (47) and induced tier 2 nAb responses in animal models (199) suggesting that vertically transmitted Envs may have unique structural characteristics. Additional studies to characterize vertically transmitted Envs compared to Envs isolated from infected adults are needed.

The second way that passively acquired HIV-specific maternal antibodies may influence infant nAb responses is by augmenting developing infant antibody responses through the formation of germinal-center-activating immune complexes (254). This has been suggested by

studies in macaques where passively administered antibodies enhance endogenous neutralizing antibody responses (251-253) and with bnAb passive administration to HIV-infected human adults (165). The correlation between maternal and infant nAb responses suggests that maternal antibodies may be playing a role (23). Interestingly, infant BG505 was found to be an outlier in the analysis with a broad response despite lower passively acquired maternal antibodies. It was later discovered that BG505 was born 8 weeks premature and therefore received fewer maternal antibodies. Understanding the contribution of maternal antibodies and the development of breadth may be revealed by studies isolating maternal nAbs and maternal and infant Envs near the time of transmission. Additionally, immunization strategies combining active immunization with passive administration of Env-specific Abs, perhaps comparing binding, tier 1 nAbs, and bnAbs, may clarify the role of passively acquired antibodies in augmenting de novo responses. Immunization with IgG immune complexes can increase vaccine responses (255, 256). Influenza vaccination with immune complexes has been shown to enhance the breadth and potency of antibody responses, and sialylated IgGs were shown to elicit higher-affinity antibody responses (257). Pregnancy is known to be associated with an increase in sialylation of IgG Fc N-glycans (258-260). Thus, an approach utilizing immune complexes composed of the HIV Env trimer and bnAbs enriched for sialylated glycoforms could be considered.

Studies utilizing the BG505 SOSIP trimer based on the transmitted Env as an immunogen have only elicited strain-specific responses (199), and there is recent emphasis on sequential immunization strategies that mimic viral evolution in individuals who develop bnAbs (148). Thus, future studies to identify the autologous Env isolates that initiated the development of BF520 and BG505 nAbs and drove the evolution of neutralization breadth are needed. The BG505 week 14 and month 27 longitudinal Env variants described in this thesis may be useful

tools to generate sequential immunogens based on viral evolution in this infant. Interestingly, the nAbs isolated from BF520 and BG505 do not neutralize the earliest vertically transmitted variants. The bnAb BF520.1 is able to neutralize viruses from ~2 months post-infection suggesting it may have developed in response to the later Env variants. Determination of the Env variants that stimulated the development of infant nAbs contributing to plasma breadth can be accomplished by identifying the naïve precursors of the isolated infant nAbs and testing for binding to the transmitted or later Env variants. In addition, recent unpublished work by Bette Korber shows a correlation between complete glycan shields and the development of bnAb responses in adults suggesting that bnAb-stimulating immunogens should contain minimal glycan holes. A similar study examining the amount of glycan holes in vertically transmitted Envs and whether this correlates with plasma breadth may inform the design of immunogens.

Overall, HIV-specific nAbs have only been isolated and characterized from two infants and only a single infant-derived bnAb has been described. Further characterization of bnAb responses in additional infants are needed to better understand these unique infant responses. It's not known whether the rapid development of bnAbs is a result of features intrinsic to the infant immune system or whether these types of responses could be elicited in adults by mimicking certain features such as the use of passive antibodies and vertically transmitted Envs.

Antibody-mediated prevention of MTCT and pediatric immunization

Despite successes in reducing mother-to-child transmission, approximately 160,000 children were newly infected with HIV in 2016 (1). To prevent transmission, infants are treated with ART after birth and during the breastfeeding period (336). Concerns about toxicity and resistance to ART underscore the need for additional interventions. bnAbs have been shown to prevent infection in a number of studies (92) and can even clear early infection and prevent the

establishment of viral reservoirs (167). Whether bnAbs can protect from infection in humans is currently being tested in a proof-of-concept trial (168). Furthermore, IgG can be modified for increased half-life (337, 338). Thus, a combination of the more broad and potent bnAbs isolated to date may provide a novel and safe strategy to prevent mother-to-child transmission of HIV (339-341). Even if infection occurs, nAbs may provide some benefit as they have been shown to reduce early viremia in animal models (92, 166, 252, 342-345).

The finding that infants develop broad and potent HIV bnAb responses has raised the question of whether HIV vaccine efforts should focus on infants. Pediatric immunizations for the prevention of viral infections is a practical strategy because infants and children have regular contact with the healthcare system, which provides an opportunity for high vaccine coverage (247). If and when an effective HIV vaccine is developed, it will probably require multiple boosts (148), and a pediatric immunization would provide a longer time-period before HIV exposure. Furthermore, 22% of new HIV diagnosis in the United States in 2015 were among adolescents aged 13-24 years (346), and earlier vaccination has the potential to reduce infection in this age-group. Despite the differences between infant and adult immune responses discussed in Chapter I, infants can mount higher magnitude responses to HIV Env compared to adults (347). Understanding the ontogeny of HIV neutralizing antibody responses in the pediatric population is necessary to facilitate the rational design of such vaccine strategies.

Conclusion

Eliciting broadly neutralizing antibody responses against HIV-1 is an elusive goal of vaccine efforts. Studying these responses following infection guides strategies to elicit bnAbs by vaccination. The studies presented in this thesis have increased our knowledge of infant broadly neutralizing antibody responses. These are the first HIV-specific nAbs isolated from infants and highlight the potential of infants to generate polyclonal nAbs without the requirement for long-term affinity maturation. The infant immune system may be uniquely poised to develop bnAb responses rapidly, which raises the question of when to immunize if/when we have a protective HIV vaccine.

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