Characterizing the neutralizing antibody responses of HIV-1 superinfected individuals

Valerie C. Cortez

A dissertation
submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

University of Washington
2014

Reading Committee:
Julie Overbaugh, Chair
Denise Galloway
Raymond Scott McClelland

Program Authorized to Offer Degree:
Molecular and Cellular Biology

University of Washington

Abstract

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Valerie C. Cortez

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

Eliciting a neutralizing antibody (Nab) response that is protective against diverse HIV-1 variants presents a major challenge to vaccine development. The identification of broad, cross-reactive Nabs from HIV-infected individuals has revealed epitopes on the HIV-1 Envelope that could be exploited by immunogen design. Ideally, presentation of these epitopes would elicit similarly broad and cross-reactive antibodies in vaccinated individuals. However, it is unclear how such immunogens should be constructed since none tested to date have been able to elicit a protective Nab response. Also, broadly Nabs isolated from HIV-infected individuals exhibit unusual characteristics, such as extensive amounts of somatic hypermutation, which is not typically induced by vaccination, but is thought to require years of chronic antigenic stimulation. To improve our understanding of the development of Nabs in response to HIV-1 during natural infection, the three studies in this thesis were initiated to investigate one potential mechanism by which Nab breadth and potency develops by studying cases of HIV-1 superinfection (SI).

We previously showed that Nab breadth is positively associated with viral diversity, which first led us to hypothesize that superinfected individuals would develop broader and more potent Nab responses than singly infected individuals as a result of the increased antigenic stimulation associated with increased viral diversity. In Chapter II we show that superinfected

individuals develop significantly broader and more potent Nab responses compared to singly infected individuals, which was independent of Nab breadth or potency developed prior to SI, CD4+ T cell count and viral load. We also noted two individuals exhibited elite neutralizing activity following SI, with cross-subtype breadth detected within 1.5 years following initial infection, earlier than what is typically seen in singly infected populations.

Next, we sought to determine whether the Nab response that develops following SI is mediated by a dominant monoclonal response to known epitopes or whether it is mediated by a polyclonal response, targeting multiple different epitopes. In Chapter III, we used standard epitope mapping techniques and computational prediction to show that the four main regions on the HIV-1 Envelope commonly targeted by singly infected populations-- the CD4-binding site, V1/V2 glycans, V3 glycans, and the membrane proximal external region of gp41-- are not the principle Nab targets of superinfected individuals. These findings suggest that a broad and potent Nab response following exposure to diverse HIV antigens does not involve a dominant response to any one of these four conserved regions, some of which are conformationally masked or occluded. This may suggest that superinfected individuals primarily generate polyclonal Nab responses and that there are other ways to elicit a broad and potent Nab response beyond what has been uncovered with these four main epitopes.

Finally, Chapter IV describes initial efforts to further characterize the Nab response of one superinfected individual (QB850) who developed the broadest and most potent Nab response in the SI cohort. Individual monoclonals were isolated and characterized, with only a subset demonstrating HIV specificity. The chapter more broadly describes the B cell culture and cloning pipeline in our laboratory and the steps that will require additional optimization with the help of a high-throughput liquid handling system. Identification of additional monoclonal Nabs

from this patient may ultimately help resolve the individual targets of her Nab response, which could lead to the identification of new epitopes on Envelope.

In summary, these studies demonstrate that exposure to diverse antigens during HIV-1 SI augments the Nab response, without targeting conserved epitopes. Thus, future studies should evaluate the possibility of a vaccination strategy that mimics SI using diverse immunogens to elicit a broad and potent polyclonal Nab response in vaccinated individuals.

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AIDS: acquired immunodeficiency syndrome

ADCVI: antibody-dependent cell-mediated virus inhibition

ADCC: antibody-dependent cell-mediated cytotoxicity

ARV: antiretroviral

bNab: broadly neutralizing antibody

CDR: complementarity determining region

HIV: human immunodeficiency virus

IgG: immunoglobulin G

Mab: monoclonal antibody

MPER: membrane proximal external region

Nab: neutralizing antibody

PBMC: peripheral blood mononuclear cell

RT: reverse transcriptase

RSC: resurfaced core

SI: superinfection

SIV: simian immunodeficiency virus

V1/V2: variable regions 1 and 2 of HIV-1 Envelope

V3: variable region 3 of HIV-1 Envelope

Acknowledgements

Many thanks to:

My mentor, Julie Overbaugh, for giving me the opportunity to join her group and for being supremely supportive of my interests both inside and outside the lab. I have truly appreciated her honesty, good humor, and faith in me over the last 6 years. She is an amazing, hard-working scientist and at the same time maintains a good work-life balance, setting an important example for her mentees. I am lucky to have been one of three graduate students that joined her lab in the same year!

To my amazing committee members: Scott McClelland, Denise Galloway, Helen Horton, and Nancy Maizels. I have really appreciated all the sound advice and support I have received year after year. A special thanks to Scott, who supported me in many additional capacities—serving as my Epi mentor, guide/driver in Kenya, as well as career counselor. I also need to extend my appreciation to neighboring labs and PIs both at the University of Washington and Fred Hutchinson Cancer Research Center. Thank you for all the time you have taken to attend seminars, weekly Retrovirus meetings, and journal clubs. I believe you all have helped foster a rich, scientific environment that I have really come to love and am finding hard to leave.

To past and present members of the Overbaugh lab: Stephanie Rainwater, Keshet Ronen, Willi Obenza, Chris Cottrell, John Lynch, Mitchell Chen, Daryl Humes, Caitlin Milligan, Jennifer Maroa, Kate Williams, Sandy Emery, Dylan Peterson, Zahra Lechak, Bingjie Wang, Leslie Goo, Dara Lehman, Cassie Simonich, Ozge Dogan, David Boyd, Maxwel Omenda, Amit Sharma, Julie Weis, Erica Lovelace, Javier Aguilera, Vrasha Chohan, Catherine Blish, Katie Odem-Davis, Helen Pollard, as well as our Kenyan collaborators at the University of Nairobi and Ganjoni Clinic. You all have made the last 6 years fly by and have never failed to help me

celebrate the highs and forget the lows of graduate school. A special thanks to my fellow graduate students for being there, making me laugh, and reminding me not to take myself too seriously. Thank you to the post-doctoral fellows for being my counsel, sharing your experiences, and giving me honest feedback. A huge thanks to the technicians, as well as my undergraduate volunteer, for keeping me in line, and also making my life easier.

To those who made significant contributions to the studies described in this thesis: Stephanie Rainwater, Keshet Ronen, Mitchell Chen, Leslie Goo, Dylan Peterson, Catherine Blish, Katie Odem-Davis, and Ivelin Georgiev. Additional thanks to Johannes Scheid, Penny Moore, and Nancy Haigwood for providing reagents as well as Xueling Wu, Nicole Doria-Rose, and Noah Sather for valuable advice and for sharing protocols.

To my past mentors, most notably John D. Johnson, who mentored me when he was a post-doctoral fellow at the University of Colorado, Boulder—thank you for being such a positive influence for me in the lab. Without the continued support from folks like John throughout college and during summer research programs geared towards the scientific success of underrepresented minorities, I am not sure I would have made it so far down this career path.

Dedication

This thesis is dedicated to the Cortez, Cruz, Ruiz, Mir, Garcia, Brugliera and Boyd families.

Chapter I

Introduction

The global HIV-1 pandemic

The human immunodeficiency virus (HIV) is a product of zoonosis, or the cross-species transmission of pathogens, in this case simian immunodeficiency viruses (SIV) from non-human primates into humans. There are two forms of HIV—HIV-1 and HIV-2, distinguished by origin and geographic distribution, as well as transmissibility and pathogenesis (1). HIV-1 can be further classified into Groups M, N, O and P, that each represents a separate transmission event of SIV into humans. However, it is Group M that is responsible for the global pandemic, and will be the sole focus of this thesis.

Molecular dating supports that Group M entered the human population around the turn of the 20th century (2), and has since infected close to 70 million people worldwide (3). About half of this population has died since the start of the epidemic in the early 1980s, with approximately 1.6 million in the last year reported by UNAIDS in 2012. There were approximately 2.3 million new infections in 2012, with close to 70% of these cases in Sub-Saharan Africa (4). Today, of the 35 million individuals living with HIV-1 or Acquired Immunodeficiency Syndrome (AIDS), two-thirds reside in Sub-Saharan Africa, causing a disproportionate health and economic burden in the countries and communities in this region (5). The global incidence of HIV-1 peaked around 1997 (6), and today people are living with HIV disease for much longer, as evidenced by the steady increase in prevalence resulting from the monumental efforts to improve access and delivery of antiretroviral (ARV) treatment, which can substantially prolong life expectancy. While each year we continue to see a reduction in the incidence of HIV-1, there are a number of barriers in the treatment and prevention cascade that will likely prevent this number from

reaching zero (7-9). To start, only half of all individuals living with HIV know they are infected (6). Of those that are aware of their HIV status, only half are on ARV treatment (6). The number of HIV-infected individuals continues to fall after taking into account whether their ARV drug regimen is effectively achieving viral suppression (6). In short, it has become clear that we cannot treat ourselves out of the epidemic (10). There has also been recent concern that recidivism could eventually impede the HIV prevention and treatment efforts even if some of the barriers described above were overcome (11). For now, the bottom line is that in order to put a full stop on the HIV/AIDS pandemic, our greatest hope lies in developing a safe and effective vaccine.

Basic biology of HIV-1

HIV-1 is a single-stranded positive-sense RNA virus that is classified in the family *Retroviridae* and genus *Lentivirus*. The HIV-1 genome consists of only 9 genes, 3 of which are common to all members of the *Retroviridae* family (*gag*, *pol*, and *env*). In addition, HIV-1 contains 2 genes (*tat* and *rev*) that are involved with regulating viral transcription, while the 4 accessory genes (*nef*, *vif*, *vpr* and *vpu*) each play a significant role in facilitating virus spread and evading host restriction factors (12). *Gag* encodes structural proteins, which together assemble, package, and mediate release of nascent virus particles. *Pol* encodes three enzymes involved with the replication cycle-- reverse transcriptase (RT) generates a DNA copy of the RNA genome, which is then integrated into the host chromosome via the integrase enzyme, while protease is responsible for cleaving viral polyproteins, a critical step in virus maturation.

Env encodes the polyprotein gp160, which is cleaved by the host protease furin into the surface (gp120) and transmembrane subunits (gp41). Together these glycoproteins form a

heterodimer that assembles into a trimer to form the Envelope spike on the surface of the virion (Figure 1.1). Envelope will be a primary focus of this thesis and therefore warrants a lengthier description. As outlined in Figure 1.1, HIV-1 Envelope consists of 5 variable regions interspersed between 5 conserved regions in gp120, and there are 6 major domains within gp41. Envelope undergoes protein-directed glycosylation in the endoplasmic reticulum, which coats the surface of the protein with primarily unprocessed oligomannose glycans (up to 50% by weight) (13, 14). Both the relatively low density of Envelope on the surface of the virion and the extensive glycosylation, especially on the outer domain that includes the aptly named 'glycan shield,' help the virus evade the host's immune response (15, 16). HIV-1 uses Envelope to facilitate entry into a host cell by first docking on the CD4 receptor present on CD4+ T cells, monocytes and macrophages, which then induces a conformational change that triggers binding of the chemokine co-receptor, either CCR5 or CXCR4, and allows for fusion of the virus membrane with the host cell membrane.

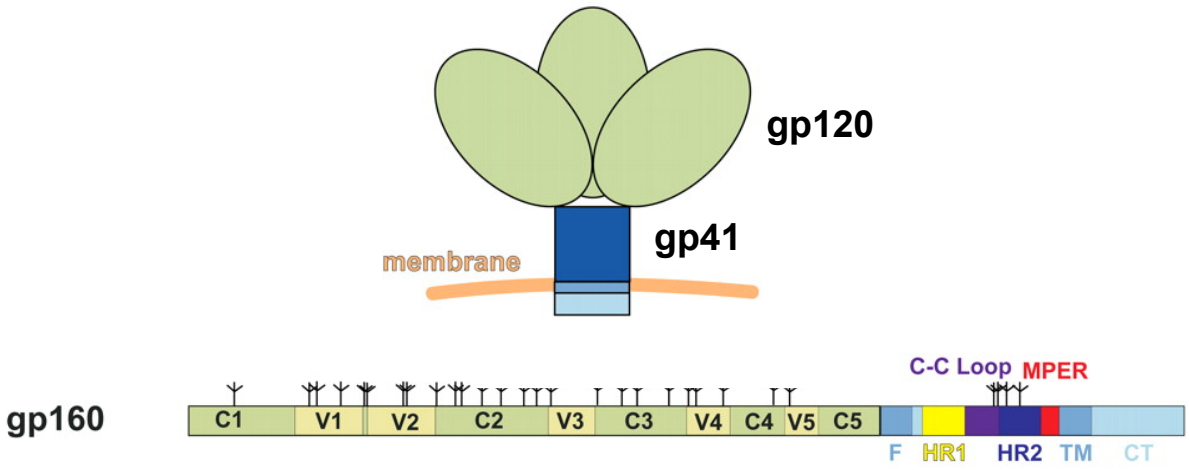

Figure 1.1 HIV-1 Envelope structure and organization.
Figure adapted from (17). The Envelope heterotrimer consists of the surface (gp120) and transmembrane (gp41) domains. Full-length uncleaved gp160 consists of gp120 segments V1-V5, variable regions; C1-C5, conserved regions, and gp41 segments F, fusion peptide; HR1, heptad repeat; HR2, heptad repeat 2; MPER, membrane proximal external region; TM, transmembrane anchor; CT, cytoplasmic tail. Potential N-linked glycosylation sites are shown as tree-like symbols.

HIV-1 diversity

The vast diversity of circulating HIV-1 variants presents a major challenge to developing an effective vaccine, and this in part relates to an inherent property of a retrovirus. Reverse transcription of the virus' RNA genome into a DNA copy is an incredibly error-prone process, as RT is a low-fidelity enzyme that lacks proofreading capability (18). Random changes to the genome can be advantageous for the virus because higher diversity will allow it to more quickly evade host immune pressures. To put this into perspective, let us consider the amount of diversity within a single HIV-infected individual: it is estimated that every possible mutation could occur each day 10,000 times (19). Furthermore, the amount of genetic diversity that exists within a single individual at a single time point during chronic infection is on par with the entire diversity of seasonal influenza during a single year (20).

To account for the vast genetic diversity and demarcate the spread of these variants around the world, a nomenclature system was developed to classify HIV-1 Group M into subtypes, or clades, based on phylogenetic analysis (21). Currently circulating today to varying degrees are subtypes A-K (except no E or I exist), which can vary between 17-35% on the amino acid level, and between 8-17% within a single subtype (20). There are also an expanding number of circulating recombinant forms (CRFs), which are derivatives of these 9 subtypes and now account for almost 20% of HIV-1 infections worldwide (22). CRFs are thought to arise by a process of dual-infection where an individual is infected with two genetically distinct viruses. If these two viruses infect the same cell, both of their RNA genomes could serve as templates for RT to make a new recombinant DNA copy. An early cross-sectional study in Kenya, where subtype A is most prevalent and subtypes C, D, and G co-circulate, suggested there were also a large number of unique intersubtype recombinants in circulation (23). This raised the possibility

that these recombinants were being transmitted or that they arose from co-infection with simultaneous acquisition of two viruses from different subtypes or superinfection (SI), where a second virus is acquired after infection with the first virus. This question motivated subsequent in-depth longitudinal analyses to determine the origin of such recombinants and some of the results from those studies will be discussed in the following section.

HIV-1 superinfection

The first official cases of HIV-1 SI were identified in 2002 by Ramos et al., who described the seemingly rare occurrence in two injection drug users participating in a prospective cohort study in Bangkok, Thailand (24). It was not until years later that the relatively frequent incidence of SI was appreciated, ranging from 0% to 5% annually depending on the cohort (25-38). Indeed, studies have suggested that SI is dependent on a number of host-viral and population-level factors such as ARV use and prevalence of HIV (39, 40). In addition, length of follow up and detection methods can also influence whether cases are found (34, 40, 41). This process typically requires sizable longitudinal cohorts to screen, which is costly and labor-intensive (41). Both intersubtype and intrasubtype SIs can occur, and longitudinal deep sequencing coupled with phylogenetic analyses allow for the most efficient detection (41).

The Overbaugh lab has identified 21 cases of SI from a cohort of female sex workers from Mombasa, Kenya by comparing partial *gag, pol,* and *env* sequences longitudinally beginning with initial infection (31, 33, 34, 38). To date, this represents the largest prospective study of superinfected cases (38). Furthermore, this Kenyan cohort is unique in comparison to other SI cohorts, as it includes individuals initially infected by viruses from multiple subtypes (i.e. A, C, and D), and subsequently reinfected by intra- or intersubtype viruses. The epidemic in

Kenya is still incredibly diverse, with recent studies again detecting numerous unique recombinant viruses in circulation (42).

Superinfection provides a unique scenario to study immune correlates of protection, since the second virus establishes infection in the face of a prior immune response to the initial virus. Multiple studies of the Mombasa SI cohort have examined the pre-SI Nab, antibody-dependent cell-mediated virus inhibition (ADCVI) as well as CD8+ and CD4+ functionality, and have not found any significant differences in comparison to singly infected controls (43-45). Broad CD8+ T cell responses preceding SI have also been reported in a different cohort (46), again suggesting that superinfected individuals do not inherently have weaker immune responses. In contrast, two smaller studies have provided evidence of deficits in the Nab and antibody-dependent cell-mediated cytotoxicity (ADCC) responses in SI cases compared to controls (47-49). While these studies focused on the immune responses pre-SI in order to identify an immune correlate of protection, SI also provides an opportunity to evaluate the affect of harboring two viruses has on the development of the immune response post-SI. Of particular interest would be determining how exposure to diverse antigens during SI can shape the Nab response, a topic explored in Chapter II, as this could provide insight to the development of a Nab-based vaccine.

Virus-antibody evolution

HIV-1 undergoes a genetic bottleneck during transmission (50), but quickly evolves into a large quasispecies, or cloud of genetically related viruses, that diversifies ~0.3 to 1% per year after initial infection (51, 52). At the same time, antibodies that target the Envelope proteins on the surface of the virion are also evolving throughout the course of HIV infection, as depicted in Figure 1.2 (53). The initial antibody response to HIV-1 targets gp41, and then gp120 soon after.

While these antibodies are unable to neutralize the virus, they can mediate other functions such as ADCC, ADCVI, and triggering phagocytosis. Beginning within weeks to months following initial infection, autologous or strain-specific Nabs develop that target the more variable regions of the Envelope spike (54-57). Virus-antibody co-evolution continues throughout chronic infection, but with Nabs always one step behind, as they typically cannot potently neutralize contemporaneous virus (57-59). For this reason and also the waning immunity as CD4+ T cell levels decline during HIV disease/AIDS, developing a robust Nab response does not provide any apparent clinical benefit (60-63). However, autologous virus escape from Nab pressure is thought to drive the development of breadth, or the ability to neutralize diverse HIV-1 isolates (64-68). While it has been estimated that up to 30-50% of individuals develop breadth after years of chronic antigenic stimulation (60, 69-72), only ~1% are estimated to develop elite activity that is both broad and potent (61, 73, 74).

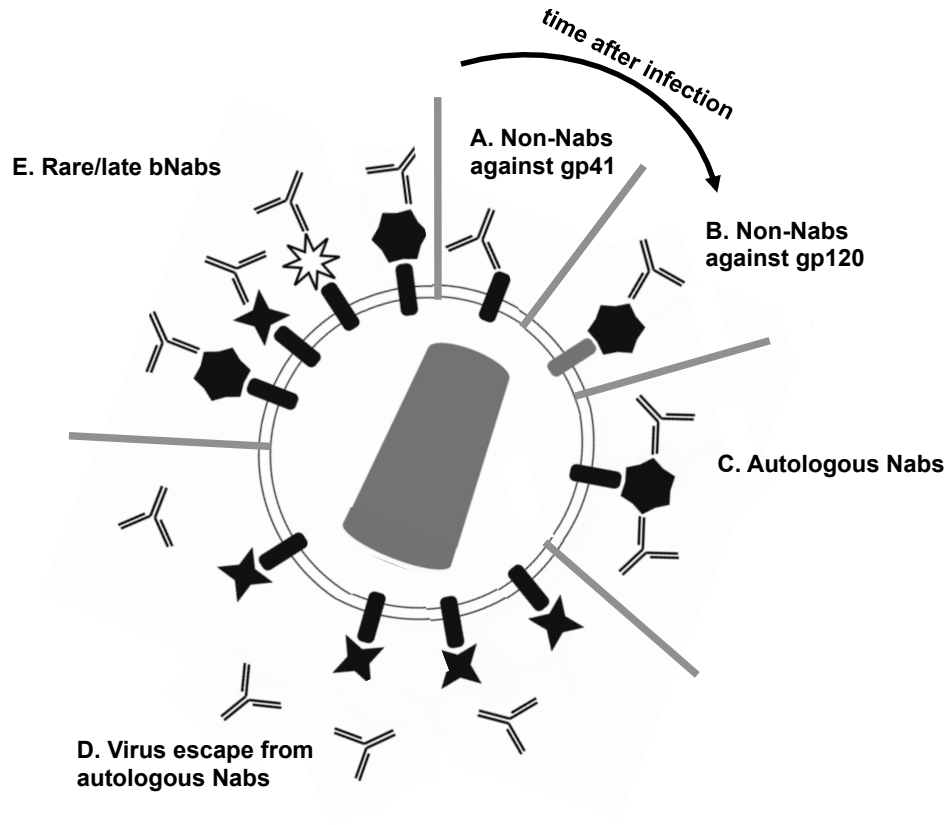

Figure 1.2 The development of the antibody response to HIV-1.
Figure modified from (53). A) Initial response is non-neutralizing, targets gp41, B) Non-Nabs expand to target gp120, C) Between weeks or months, autologous Nabs develop, D) Virus evolution enables it to escape the autologous Nab response, E) Broadly neutralizing antibodies bNabs) capable of neutralizing diverse heterologous viruses arise in a subset of individuals after years of chronic infection.

Along with duration of infection, a number of additional factors including viral load, viral diversity, and CD4+ T cell count have been shown to be critical drivers of Nab breadth (61, 63, 71, 72, 75). By studying these factors in HIV-infected populations, we have increased our understanding of the basic biology surrounding the development of a broad and potent antibody response. However, it was not until the isolation of individual monoclonal antibodies (Mabs) from patients with elite neutralizing activity that led to the more focused study of particular epitopes or targets of Nabs. A brief history of these advances garnered from isolating Mabs will be summarized in the following section.

Monoclonal antibodies reveal potential vulnerabilities on the Envelope spike

Antibodies consist of 2 heavy and 2 light chains that together form the wings of the antibody, or variable portion, that contains 3 complement-determining regions (CDR) interspersed between 4 framework regions (FWR). The stem of the antibody, which is the constant portion, specifies the antibody isotype. Together, the CDRs make up the antigen-binding domain, with the majority of contact with Envelope mediated by the third CDR of the heavy chain. The methods by which HIV-specific Mabs have been isolated have evolved over the years, starting with a group of so-called "first-generation" antibodies that were identified by phage-display, Epstein-Barr Virus transformation of B cells and hybridoma technology (76, 77). These antibodies, including b12, 4E10, 2F5, and 2G12 were isolated before 2009 and in retrospect, were not particularly broad or potent, but for the first time revealed three potential sites of vulnerability on the Envelope spike—the CD4-binding site (b12), glycan shield (2G12), and linear epitopes in the MPER of gp41 (2F5, 4E10) (Figure 1.3) (76, 78-81).

The recent technological advances in single B cell isolation and cloning of antibody genes sparked the identification of "second-generation" antibodies, characterized by significantly greater breadth and potency than what was seen with the previous generation. These technologies, which will be the focus of Chapter IV, include using 'baits' to tag HIV-specific B cells that can be single-cell sorted by flow cytometry as well as culturing methods to take a broader approach in capturing the antibody repertoire (82). In addition, primer sets have been developed to enhance the ability of cloning antibodies that have undergone many rounds of somatic hypermutation (68, 83), an iterative process by which a B cell receptor increases its binding affinity to its cognate antigen. The new group of Nabs isolated using these methods, including VRC01, PG9/PG16, PGT-128, 10E8 and others, further expanded the list of epitope

targets on the Envelope spike to include quaternary epitopes involving glycans in the V1/V2 loop (PG9/16), V3 glycans (PGT-128), and a discontinuous epitope in MPER (10E8) (Figure 1.3) (84). However, it is likely that there are additional epitopes on the HIV-1 Envelope that have yet to be identified, as in some cases, isolated Mabs from a patient do not recapitulate the overall Nab activity found in sera (85). Still, many groups have shown that one or more of these epitopes are most often the main targets of Nab response generated by singly infected individuals during chronic infection (72, 86-89). Whether these are also the main targets in individuals with more than one co-circulating virus, as is the case for superinfected individuals, will be examined in Chapter III.

While the identification of such broad and potent Nabs has provided the field with a template for vaccine design, this excitement was tempered by the realization that these antibodies have unusual characteristics that may make them challenging to elicit with current vaccine technologies (84). Most notably, these antibodies exhibit extensive amounts of somatic hypermutation, as well as additional insertions and deletions in both the CDRs and FWRs (90). A subset of these Mabs also originate from rare germline VDJ gene segments that are underutilized in naïve individuals (91), which may limit their generalizability for a vaccine. Despite these atypical features, the current collection of broadly neutralizing Mabs provide a benchmark to aim for with future vaccination studies and offer significant prospects for immunotherapy, both of which will be highlighted in the following section.

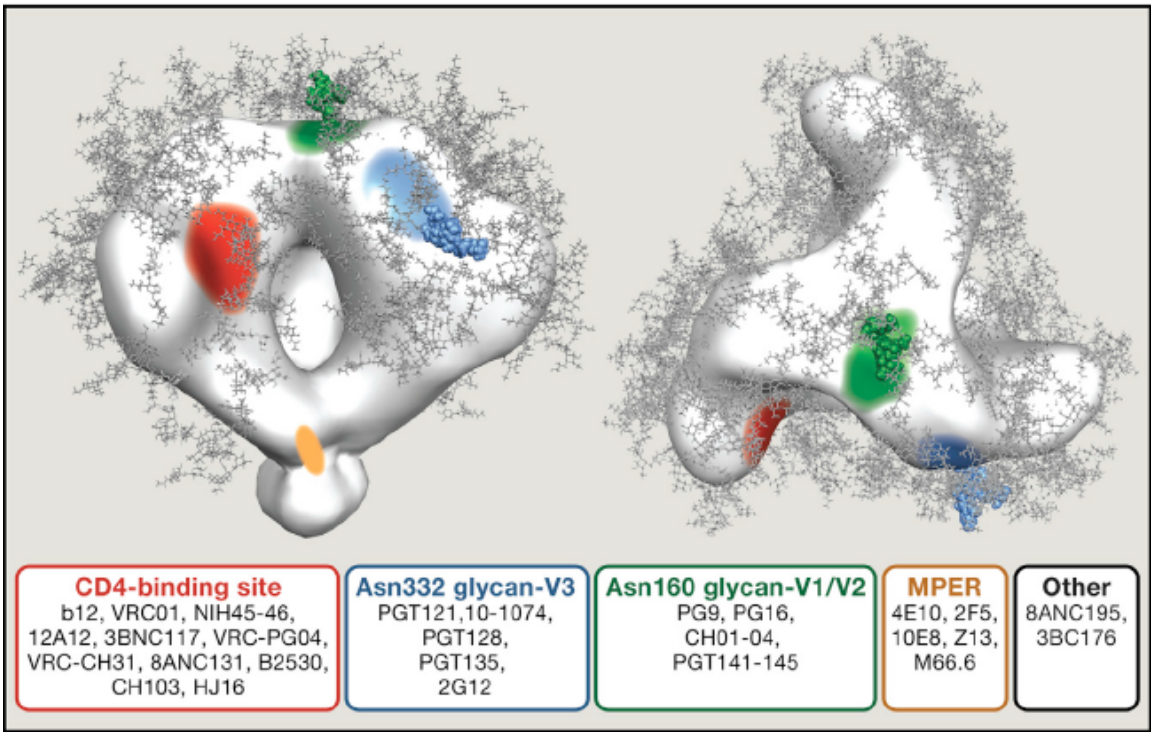

Figure 1.3 Epitopes on HIV-1 Envelope targeted by bNabs.
Figure adapted from (84). The Envelope trimer depicted by a top and side electron microscopy imagine with the four main epitope targets highlighted. Tree-like structures represent glycans. The bNabs that target those regions are listed below.

Protection afforded by neutralizing antibodies in non-human primate models

The rationale behind developing an antibody-based vaccine has largely come from passive immunization studies using both first and second-generation Mabs that have protected non-human primates from intravenous, intrarectal and intravaginal virus challenges (92-99). However, the major caveat to some of these studies was the viruses chosen to challenge the immunized animal were previously deemed sensitive to the very antibodies being tested, bringing to question their relevance to HIV-1 transmission in humans. Furthermore, in many cases the amount of antibody used surpassed physiological levels. Nevertheless, they have provided us with a point of reference for the level of Nab titer that might be needed to provide protection (dilutions of 1:125 to 1:300) (97). So while these experimental results must be

interpreted in the context of these caveats, these studies provide support for the hypothesis that bNabs present prior to exposure could prevent infection. Moreover, two recent studies in non-human primates have shown second-generation antibodies can work as a post-exposure therapeutic, clearing virus from circulation in the weeks following administration, which may provide an exciting prospect for immunotherapy for individuals who are already infected (100, 101).

Unfortunately, vaccine trials in humans have not generated such encouraging results. The following section describes these trials and concludes with a brief summary of future directions for vaccine strategies.

Limited success eliciting a protective immune response in HIV-1 vaccine trials

There have been 5 phase IIb/III clinical vaccine trials since the start of the epidemic, 4 of which failed to show efficacy and one that provided 31% protection from infection in the 6 months following vaccination (102-107). Two of these trials, Vax 003 and Vax 004 attempted to generate a strong antibody response using recombinant gp120 protein (102, 103), whereas the Step/Phambili trials used recombinant Adenovirus serotype 5 (rAd5) and HVTN 505 used a DNA prime with rAd5 to primarily elicit strong cellular responses (104, 105, 107). The moderately efficacious RV144 trial used a canarypox vector containing *gag*, *pol*, and *env* genes that was combined with recombinant gp120 protein boosts aimed at inducing both cellular and humoral immune responses (106). Interestingly, the correlate of protection for the RV144 trial was attributed to non-neutralizing antibody activity, bringing to light the possibility that vaccine-elicited responses need not solely focus on neutralization (108). However, having Nabs as a component of protective vaccine is still thought to be important, given the promising results of

passive immunization studies as well as the fact that many of the effective vaccines in use today provide protection via Nabs (109).

More recent concepts for vaccine design center around rationally designing sequential immunogens to guide the immune response to one of the four main target regions on Envelope (84, 110). In support of this strategy, previous animal studies have shown that sequential immunization strategies are more effective than immunizing with a single antigenic form (111), and more recently, the structure-based vaccine design for respiratory syncytial virus provided a proof of principle for using scaffold-based immunogens in non-human primates (112). Another strategy, termed the B cell lineage immunogen design, essentially aims to emulate what occurs naturally in an HIV-infected patient (90, 113). Recent studies of co-evolving Mabs and virus isolates coupled with longitudinal deep sequencing of the antibody repertoire has allowed two groups to demonstrate the potential of generating such a template for a vaccine (67, 114). In addition, new imaging technologies have further advanced our understanding of the structural features of the antibody-Envelope interactions (84), and will ultimately enhance our ability to design an effective HIV-1 vaccine.

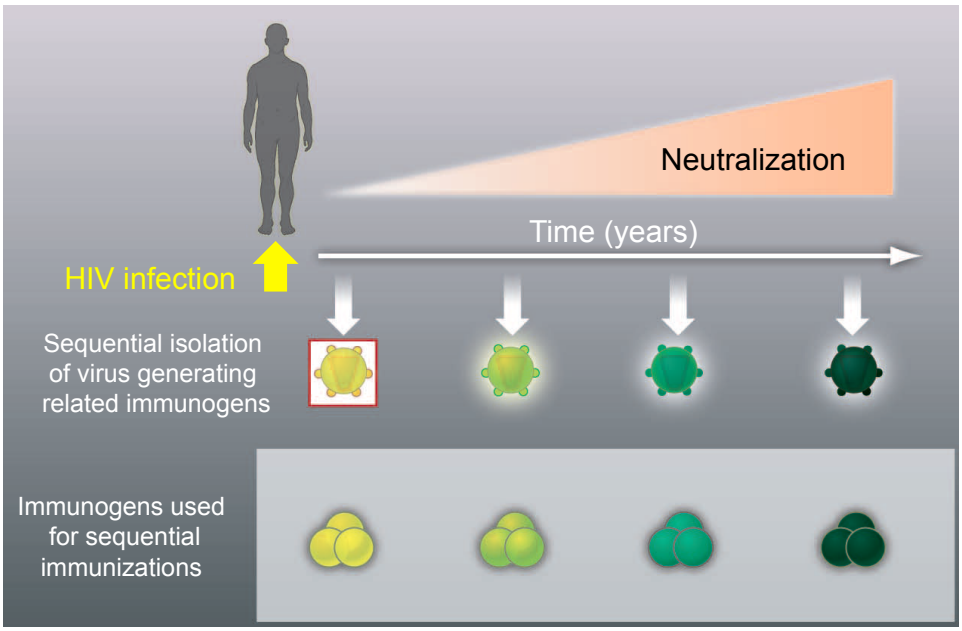

Figure 1.4 B cell lineage immunogen design.
Figure adapted from (90). This vaccine strategy aims to guide the Nab response using Envelope immunogens that were present in a patient from which broadly neutralizing Mabs are isolated.

Goals of this thesis

The overarching goal of this thesis will be to expand our understanding of the mechanisms by which a broad and potent antibody response develops. In Chapter II, I first explore whether SI leads to a broader and more potent Nab response than single infection, as a result of the increased antigenic stimulation provided by two viruses compared to one. Next, in Chapter III I assess whether the Nab responses developed following SI primarily target the four main sites of vulnerability on the Envelope spike. To compliment these studies and further characterize the Nab responses of one superinfected individual who developed the broadest and most potent Nab in the Mombasa cohort, in Chapter IV I will describe the Mabs I have cloned using single B cell culture and cloning techniques. Finally, in Chapter V I will discuss the implications of SI on future strategies for vaccine design.

Chapter II

HIV-1 superinfection in women broadens and strengthens the Nab response

The text in this chapter has been modified slightly from PLoS Pathogens, 8(3): e1002611.doi:10.1371/journal.ppat.1002611

Introduction

As described in Chapter I, multiple studies have demonstrated the potential of HIV-specific Nabs to protect against infection using non-human primate models, but it remains unclear how to elicit a Nab response of sufficient breadth and potency to protect humans against diverse circulating HIV-1 variants, which can differ by several orders of magnitude in neutralization sensitivity (115, 116). Therefore, investigating naturally occurring antibody responses that can neutralize viruses across the major viral subtypes remains a major focus of research (117). In the past few years, multiple HIV-specific broadly neutralizing Mabs have been isolated from HIV-infected individuals with elite neutralizing activity (83, 118-120). This subset of individuals comprise about ~1% of chronically infected individuals and are considered elite neutralizers based on their ability to potently neutralize viruses from multiple subtypes (73). The broad Mabs identified to date, which were isolated more than a decade after initial HIV-1 infection in some cases, have undergone extensive somatic hypermutation, a process that would be difficult to mimic with a HIV-1 vaccine (116, 121). These Mabs have been isolated from individuals who were presumably infected with one HIV strain, although in most cases, the possibility of SI was not addressed. Within singly infected populations, Nab breadth has been positively associated with viral diversity (63). Therefore, individuals infected with multiple

strains as a result of SI by a second source partner may generate broad Nabs in response to stimulation from both viruses.

Initially, it was hypothesized that SI resulted from a weak Nab response that was unable to protect the individual from reinfection. A small study of three SI cases and three viral strains provided some support for this model (47). However, in a larger study using a panel of 16 viruses from a number of different subtypes, Blish et al. showed no significant differences in the Nab breadth or potency in six superinfected cases immediately before acquisition of the second virus compared to 18 singly infected controls at matched time points (43). In this study, where the focus was on correlates of protection from SI, the Nab repertoire and breadth developed in the years following SI were not examined.

In the past year, two studies have provided evidence of a broadening of the Nab response after SI. In a South African individual that became superinfected 13-15 weeks post-initial infection, broad and potent responses were detected 3 years post-infection (32 of 42 heterologous viruses neutralized, some at plasma dilutions >1:10,000) (122). However, it was not possible to determine whether SI is typically associated with a broader Nab response and not merely coincidental in this single case. Powell et al. aimed to address this question by measuring the difference in Nab responses in plasma from four superinfected cases and 23 singly infected controls against seven regional primary isolates from Cameroon and two subtype B viruses (123). They showed that the average change in Nab breadth and potency between pre-SI and post-SI evaluations for superinfected cases was significantly greater than that of non-superinfected controls (123). While this analysis provides some evidence that superinfected individuals develop broader Nab responses than singly infected individuals, the results have to be interpreted in the context of a number of limitations in the cohort studied, including an

imbalanced distribution of cases and controls where matching was based on two groups of controls rather than an equal ratio of individuals. More significantly, since seroconversion dates were unknown for many individuals, the controls were assigned based on the time each individual had participated in the study, rather than how long they had been infected. Therefore, time since infection, which has been strongly associated with the development of Nab breadth (61, 71, 72), could not be accounted for in this analysis. Furthermore, due to the small number of superinfected cases, the investigators were unable to control for potential confounding factors such as CD4+ T cell count and viral load, which also impact Nab breadth (61, 63, 71, 72, 75). Nonetheless, these cases are intriguing and highlight a need for a controlled study with greater numbers of superinfected cases with appropriately matched controls.

Here, prior to the recent use of 454 deep sequencing to identify new cases of SI (38), we designed a nested cohort study in which the original 12 cases of SI identified from the Mombasa cohort (31, 33, 34) were each matched to three non-superinfected women to test the hypothesis that superinfected individuals develop broader and more potent Nab responses compared to non-superinfected individuals as a result of increased antigenic stimulation by two distinct viruses. The results reported here illustrate that SI leads to an augmentation of the Nab response and thus, provides significant support for SI as a useful model for studying the development of the Nab response to diverse HIV variants.

Materials and Methods

Study population and design

The individuals in this study represent a subset of a prospective cohort of HIV-1 negative high-risk women from Mombasa, Kenya (124-126) who have a defined date of infection based

on approximately monthly HIV serology and subsequent retrospective RNA testing of banked plasma from time points prior to seroconversion as described (127). Twelve superinfected individuals were identified from a previous screen of 56 women from this cohort that compared partial *env* and/or *gag* sequences amplified from peripheral blood mononuclear cells (PBMCs) from the first visit after the detection of seroconversion and a visit during chronic infection ~5 years later (31, 33, 34). Briefly, single copy PCR was performed and the sequences from multiple independent PCRs (median of 7) were examined at each time point. Two regions of the HIV-1 genome were examined: envelope V1-V5 (~1.2 kb) and gag p17 (~700 bp). Individuals identified as potential SI cases were further analyzed to verify SI and determine the interval when it occurred. All cases of SI as well as the approximate timing of SI were determined by phylogenetic analysis and allele-specific PCR tailored to the sequences in each individual.

For this study, three singly infected women from the 56 tested for SI in the prior study (31, 33, 34), were matched to each of the 12 cases of SI according to initial infecting viral subtype and sample availability at time points approximately 5 years post-initial infection (post-SI) and 1 year prior to SI (pre-SI). In cases where samples close to 5 years post-initial infection were not available for the SI case, the closest available sample post-SI was selected and the timing of sample selection was similar for the controls. Other than sample availability, the assignment of controls was random, and was performed using random number generation.

Viral loads were determined by Gen-Probe and were available for all women, and CD4+ T cell counts were documented beginning in 1998 and were available for 17 women prior to SI and 45 women post SI (128-130). All women were HIV-1 infected through heterosexual contact (125), and none reported using ARV therapy during follow-up for this study. The University of Washington's, University of Nairobi and Fred Hutchinson Cancer Research Center's

Institutional Review Boards approved the study. Written informed consent was provided by all study participants.

Heterologous pseudovirus panel

The panel of 8 viruses was chosen to include 21 multiple subtypes with varying neutralization sensitivities to a pool of HIV+ plasma from the Mombasa cohort and Mabs (i.e. b12, 4E10 (131, 132)). The viruses used, and their subtypes in parenthesis, were: SF162 (B) (133), Q461d1 (A) (134), Q769b9 (A) (134), Q842.d16 (134), Q259.d2 (A) (134), QC406.70M.F3 (C) (132), DU156 (C) (135), Q435.100M.A4 (D) (132). Plasma was also tested against an envelope from Simian Immunodeficiency Virus (SIV), SIVmne CL8 (136), to ensure that the neutralization observed against the virus panel was HIV-specific. The two elite neutralizers identified in the study were also tested against JR-CSF (B) (137), 6535.3 (B) (138), CAAN5342.A2 (B) (138), and QB857.23I.B3 (D) (132).

Pseudoviruses were made by co-transfecting 293T cells with each of the cloned viral envelopes listed above and a full-length subtype A proviral clone with a partial deletion in envelope (Q23Δenv), as previously described (134). Briefly, an equimolar ratio of envelope-to-provirus plasmid was added to Fugene-6 transfection reagent (Roche) and then incubated with 4 million 293T cells for 12 hours. The media was changed at 10 hours. After a total of ~48 hours post-transfection, supernatants were harvested and filtered through a 0.22um Steriflip Filter Unit (Millipore) to remove cellular debris. The resulting pseudoviruses were screened for infectivity on TZM-bl cells, a HeLa-derived reporter cell line that expresses high levels of CD4, CCR5, and CXCR4 as well as B-galactosidase under the transcriptional control of HIV-LTR (139). Infectious titers were determined by serially diluting viruses 10-fold, adding 20,000 TZM-bl cells in growth media containing 20ug/mL DEAE-dextran per well, and incubating at 37°C for

48 hours. The cells were then fixed and stained for B-galactosidase activity and infected cell foci were enumerated visually.

Neutralization assay

The TZM-bl neutralization assay was used to quantify Nab breadth as previously described (131). Briefly, 500 infectious pseudovirus particles, as determined by the infectious titer described above, were incubated in duplicates with 2- fold serial dilutions of plasma for 1 hour, beginning with an initial concentration of 1:100, before 10,000 TZM-bl reporter cells per well were added. Each plasma-virus combination was tested in duplicate and the assay was repeated twice. Infection levels were determined by B-galactosidase activity after 48hrs using a chemiluminescent readout.

The IC50, or reciprocal plasma dilution at which 50% of the virus is neutralized, for each plasma-virus pair was calculated using linear interpolation from the neutralization curve. In this assay, a plasma sample was considered to be below the detectable limit of neutralization for a given virus if the lowest dilution (1:100) did not show >50% neutralization. With this criterion, plasma samples that showed neutralization below the limit of detection were designated an IC50 value of 50, the midpoint between our starting dilution (1:100) and 0. Each round of assays included HIV-negative plasma and a HIV-positive plasma pool from 30 HIV-1 infected individuals in Kenya between 1998-2000 (130) serving as negative and positive internal controls, respectively. If a run showed neutralization of the negative control virus (SIVmneCL8) greater than the limit of detection, we considered that a failed run and repeated the assay.

Calculation of breadth and potency scores

Results from two independent experiments were averaged on the log scale before calculating breadth and potency scores. A composite breadth score for each plasma-virus pair

was derived for each woman by comparing the IC50 for her plasma to the cohort median IC50 for each virus as described (43). The cohort median IC50 was based on all IC50s from the individuals in the study, and this represented the unique neutralization sensitivity of that virus. If the IC50s for the given plasma-virus pair was greater than the cohort median IC50, then individuals were given a score of 1, while those below were scored as a 0. The overall breadth was then a composite score for each individual against all 8 viruses in the panel, with a maximum score of 8 and a minimum of 0. We also analyzed our dataset using the percent neutralization at the first 3 dilutions in our assay (1:100, 1:200, 1:400) and applied the same breadth scoring method. Potency was calculated by dividing the IC50 value for a given plasma-virus combination by the cohort median IC50 for that virus. The overall potency was then a composite score for each individual against all 8 viruses in the panel.

A method described in Simek et al, was also applied to the data. In this method, for each virus, the average log-transformed titer is calculated for a given sample then scaled to yield a value between 0.0 and 1.0 (73). As a minor modification to take into account the varying sensitivities in our virus panel, we divided the average by the maximum value for the cohort. An average of the scaled averages across viruses was computed as a final breadth score for each individual.

Statistical Analysis

Breadth scores were analyzed using conditional Poisson regression, while log transformed potency scores were analyzed using linear regression generalized estimating equation (GEE). These models were appropriate given the observed mean-variance relationships of the breadth and potency scores. The breadth scoring method described by Simek et al. was analyzed using GEE. All models assessed by GEE used an identity link, exchangeable working

correlation structure, and robust standard errors. We used a paired t-test to compare viral load, CD4+ T cell count and geometric mean IC50s between superinfected and non-superinfected individuals. Spearman's rank correlation was used for comparing the results from our final model and single dilution analysis. Two-tailed P values of 0.05 or less were considered to indicate significance in all statistical tests. Analyses were performed using STATA statistical software (version 11, StataCorp).

Results

Case-cohort characteristics

The 12 cases of SI demonstrated considerable heterogeneity with respect to the temporal occurrence and virologic factors related to their superinfections (Figure. 2.1) (31, 33, 34). Some women were superinfected soon after initial infection, while others became superinfected much later during chronic infection (range: ~2 months to 5 years post-initial infection), with the median occurrence at 1.72 years post-initial infection. Eight individuals (60%) experienced an increase in viral load after SI; in three cases the increase was very small (<0.5 log10 copies/ml), while in the remaining five the mean change was 1.08 $\log_{10}$ copies/ml. Similar numbers of women had inter and intrasubtype SIs based on env and gag sequences, and all women were infected with at least one subtype A virus, the dominant subtype in Kenya (23). Based on the longitudinal analyses previously described (31, 33, 34), the superinfecting strain persisted in combination with the initial virus in seven (58%) of the women, whereas it appeared to have largely replaced the initial virus in the other five (42%) (Figure 2.1).

Three non-superinfected individuals, selected from a pool of women identified as singly infected in prior screens (31, 33, 34), were matched to each superinfected case by the initial

infecting virus subtype, time post-initial infection, and sample availability. Nab breadth and potency were analyzed both pre and post-SI. The pre-SI time point for each superinfected case and her matched controls varied in relation to initial infection depending on the timing of the SI event. In contrast, the post-SI time point was evaluated at a single time point for all individuals an average of 5 years post-initial infection, when all 12 cases had been superinfected for at least 1 year. This post-SI time point enabled us to draw comparisons across the entire cohort after the development of the Nab response to both the initial and superinfecting viruses, yet before the onset of overt immunodeficiency (71, 140-142). Superinfected women had significantly lower mean viral loads than singly infected women pre-SI (Log_{10}VL: 4.24 vs. 4.79, respectively; p=0.034), but were comparable post-SI (Log_{10}VL: 4.78 vs. 4.89, respectively; p=0.699). Both groups also had similar mean CD4+ T cell counts post-SI (370 vs. 380; p=0.886). Insufficient CD4+ T cell count data were available for the cohort at the time points prior to SI for analysis. Superinfected women had similar numbers of partners per week when compared to non-superinfected women (0.66 vs. 0.49, respectively; p=0.069). Superinfected women had unprotected sex on average 22% of the time compared to 33% of the time for non-superinfected women (p=0.175). All individuals in this study were ARV naïve at the time points examined.

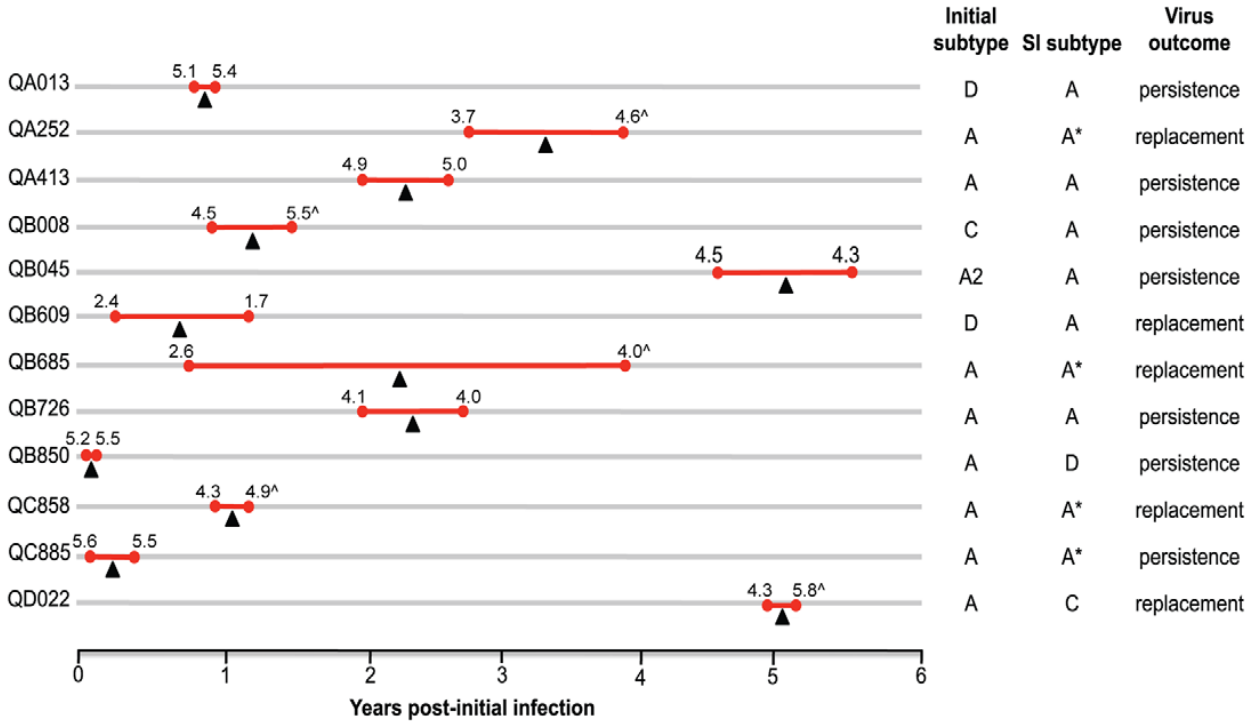

Figure 2.1 Viral characteristics of 12 cases of SI
Subject IDs are shown to the left with time since initial infection plotted at the bottom. Red bars denote the interval during which SI occurred, with closed circles representing the last time point in which SI was last undetected and the time point at which SI was first detected. Black triangles mark the midpoint of this interval, which was used as the estimated time of the SI event. The individual's viral load (log_{10} copies/ml) at the corresponding time points is noted above each red bar, with (^) denoting increases in viral load 0.5 log_{10} copies/ml. Subtyping in the two columns on the right are based on env sequences. However, the data for those individuals with an (*) by their superinfecting virus subtype based on gag sequences alone as the superinfecting env sequence was not detected at time points tested. Virus outcome was based on the longitudinal analyses previously described (31, 33, 34). Persistence was defined as the detection of co-existence of the superinfecting virus with the initial virus following its introduction, while we classified replacement superinfections as the dominance of the superinfecting virus based on lack of detection of the initial virus post-SI (33).

Comparison of Nab breadth and potency in superinfected and singly infected women after SI

Eight viruses from subtypes A, B, C, and D that exhibit varying degrees of neutralization sensitivity to Mabs as well as pooled plasma derived from HIV-1 infected individuals in Kenya were used to measure Nab breadth and potency both pre and post-SI (Table 2.1). Seven of the eight viruses were isolated from individuals during acute infection, and the majority were designated as 'Tier 2' variants, based on a neutralization sensitivity classification system developed by Seaman et al. (143).

Table 2.1 Neutralization sensitivities of 8 HIV-1 envelope variants of different subtypes

Pseudovirus	Subject/transmission route	Time postinfection[a] (days)	Subtype	Tier[b]	IC_{50} for pooled plasma[c]	IC_{50} (ug/ml) for indicated monoclonal				
						VRC01	PG9	b12	2F5	4E10
Q461.d1	female/heterosexual	17	A	1B	489	0.29	1	1	0.18	0.11
Q769.b9	female/heterosexual	56	A	2	52	0.17	0.05	>20	20	>20
Q259.d2.26	female/heterosexual	17	A	2	59	0.55	>1	>20	>20	>20
Q842.d16	female/heterosexual	49	A	2	136	0.51	0.08	>20	>20	14.6
QD435.100M.a4	female/heterosexual	100	D	2	69	0.36	>10	5.74	0.76	2.58
QC406.70M.f3	female/heterosexual	70	C	2	119	>1	0.15	>20	>20	>20
DU156.12	female/heterosexual	28	C	2	195	0.31	0.16	3.13	>20	1.57
SF162	male/MSM[d]	Chronic	B	1A	583	0.6	>1	0.01	4.5	8

[a]This estimate is based on published data and methods for estimating time of infection as described (129).
[b]Tier designation is based on available data (143) or, in some cases, on extrapolation using the IC50 values shown here.
[c]Pooled plasma was from 30 HIV-positive Kenyan individuals from the cohort.
[d]MSM, men who have sex with men

To evaluate whether harboring two viruses compared to a single virus influences the development of Nab breadth and potency, we first tested all superinfected cases and matched control at a time point post-SI, approximately ~5 years post-initial infection (median time after initial infection: 5.01 years, Range: 2.8-8.1 years) (Figure 2.2). Overall, the Tier 1 viruses that were neutralization sensitive (SF162 and Q461d1) had the highest median IC50 values (606 and 583, respectively) for the cohort as a whole; the median IC50s for all six Tier 2 viruses were below 300. Geometric mean IC50s averaged across the entire panel were significantly different between superinfected cases and singly infected individuals (326.19 vs. 193.33, respectively; p=0.038). Furthermore, differences in neutralization potency between individual superinfected cases and matched controls are evident, most notably with the more neutralization resistant viruses such as Q769.b9 and Q259.d2.26 (Figure 2.2). Breadth and potency scores were calculated by normalizing the IC50 of each plasma-virus pair to the cohort median IC50, as described (43). The mean Nab breadth score of the 12 superinfected women was 5.75, while the mean breadth score of the 36 non-superinfected women was 3.42 (Figure 2.3A). Superinfected

women had, on average, 1.68 (CI: 1.23-2.30, p=0.001) times greater breath than non-superinfected women (Table 2.2). Similarly, the mean potency score in superinfected women was higher than singly infected women (17.25 vs. 11.84, respectively) (Figure 2.3B), and superinfected women had 1.46 (CI: 1.03-2.06, p=0.033) times greater potency than the non-superinfected group (Table 2.2).

				Neutralization (IC50)									
			SIVmne	SF162	Q461.d1	Q769.b9	Q842.d16	Q259.d2.26	QD435.100M.a4	QC406.70M.f3	DU156.12		
ID	YPI*	YPSI#	CL8	B	A	A	A	A	D	C	C	Breadth	Potency
SI QB850	4.8	4.7	50	4637	2723	70	556	908	1173	509	684	8	37.02
QC152	4.7		50	861	824	50	707	1676	594	1536	905	7	42.03
QA874	4.9		50	235	361	88	78	50	153	50	50	1	4.58
QB585	4.6		50	896	430	50	328	50	345	50	189	3	8.00
SI QA013	4.9	4.2	50	774	951	224	395	267	1311	1184	394	8	25.84
QC048	4.5		50	165	497	73	72	117	565	50	202	3	7.14
QC370	4.6		50	340	328	293	75	72	133	144	225	1	9.52
QB670	5.2		50	1717	492	86	149	50	101	85	353	3	8.83
SI QB685	4.9	2.5	50	221	879	535	696	209	683	180	367	7	22.62
QA520	4.8		50	2680	2060	50	118	50	472	1442	896	5	24.28
QA028	4.8		50	3533	3956	50	540	50	288	50	50	4	19.10
QA538	4.9		50	102	89	50	100	155	50	50	50	1	4.20
SI QB726	5.5	3.0	50	817	1410	248	397	267	618	280	202	7	18.18
QA779	5.4		50	740	232	50	92	50	50	50	50	1	4.29
QD470	5.9		50	2079	624	99	186	50	459	158	140	6	11.07
QC440	5.5		50	610	359	50	249	50	50	239	150	3	6.92
SI QC885	5.0	4.7	50	564	529	349	659	542	972	662	385	6	27.10
QB765	5.0		50	50	1445	50	152	50	731	88	534	4	10.00
QA261	5.0		50	553	1753	50	726	50	92	50	50	2	10.96
QA078	5.1		50	451	970	50	508	74	133	81	199	2	9.14
SI QD022	7.8	2.7	50	1044	1943	78	314	87	282	256	416	6	13.54
QA268	7.7		50	1445	560	209	333	285	188	715	472	6	19.14
QA966	8.1		50	1175	2373	50	200	1669	50	1149	342	6	35.32
QD121	8.0		50	1533	584	204	144	158	419	295	797	7	15.87
SI QA252	5.2	1.8	50	968	166	110	101	135	1103	268	508	6	13.32
QC036	5.2		50	773	819	50	132	192	104	79	419	4	8.89
QA465	5.2		50	2104	521	50	75	50	131	50	50	1	7.21
QB216	5.4		50	89	50	50	50	50	100	50	50	0	2.80
SI QC858	5.1	4.0	50	540	733	99	137	123	694	509	176	5	12.41
QB857	5.7		50	468	1170	50	355	152	991	279	473	6	14.66
QA284	4.5		50	566	456	126	360	168	183	776	607	5	15.94
QA281	5.2		50	374	393	139	276	200	736	165	765	6	14.05
SI QA413	3.7	1.3	50	926	543	82	261	89	348	237	279	6	10.31
QD729	3.9		50	1387	671	81	1553	124	50	84	514	6	19.06
QD076	3.6		50	293	364	50	50	50	137	50	77	0	3.90
QA750	3.7		50	287	208	50	50	93	118	100	208	1	4.82
SI QB609	3.5	2.7	50	483	659	200	102	147	525	227	276	5	11.87
QB680	3.4		50	602	665	50	95	84	300	153	235	2	7.41
QA560	2.8		50	330	372	254	255	380	503	537	763	6	19.27
QC888	3.2		50	446	878	50	94	50	896	50	255	2	8.64
SI QB008	4.9	3.7	50	1489	755	50	455	80	307	49	92	4	10.22
QD370	5.1		50	88	104	84	50	50	75	86	262	1	4.38
QA638	5.3		50	718	370	50	50	50	176	50	115	1	4.89
QD408	5.0		50	1609	356	85	50	50	104	86	385	3	7.88
SI QB045	6.4	1.8	50	168	98	50	50	50	71	50	517	1	4.59
QA039	6.3		50	81	733	50	128	227	50	205	1021	4	10.75
QA203	6.4		50	318	582	50	98	106	84	243	378	3	7.39
QA918	6.3		50	1456	814	104	161	295	551	251	212	7	14.16
Cohort median:			50	606	583	60	150	91	285	155	278		

Increasing neutralization sensitivity

Figure 2.2 Post-SI neutralization profiles for superinfected and non-superinfected plasmas tested against heterologous HIV-1 variants.
Subject IDs are shown to in the first column. Superinfected individuals denoted with borders and ‘‘SI’’ before ID number, with the three matched controls listed below. The next columns lists the years post initial infection (YPI*) that was tested, which was used to match cases and controls. The following columns list the years post-SI (YPSI#) for each superinfected case. All samples were chosen near 5 years post-initial infection. However, some samples were taken 1–2 years before or after this time point because of sample availability. Subsequent columns contain the IC50 for each plasma-virus pair, which is the reciprocal dilution of plasma that led to a 50% reduction in infectivity. Plasma samples that showed neutralization below the limit of detection were designated an IC50 value of 50, the midpoint between our starting dilution (1:100) and 0. IC50s are shown as a heat map to represent increasing neutralization sensitivity, with white boxes for values below 100, light blue boxes for values between 101 and 300, darker blue boxes for values between 301 and 500, and the darkest blue boxes for values greater than 501. The Nab response breadth and potency scores that are shown here were calculated after taking the average $\log_2$ IC50s from

the two experiments.

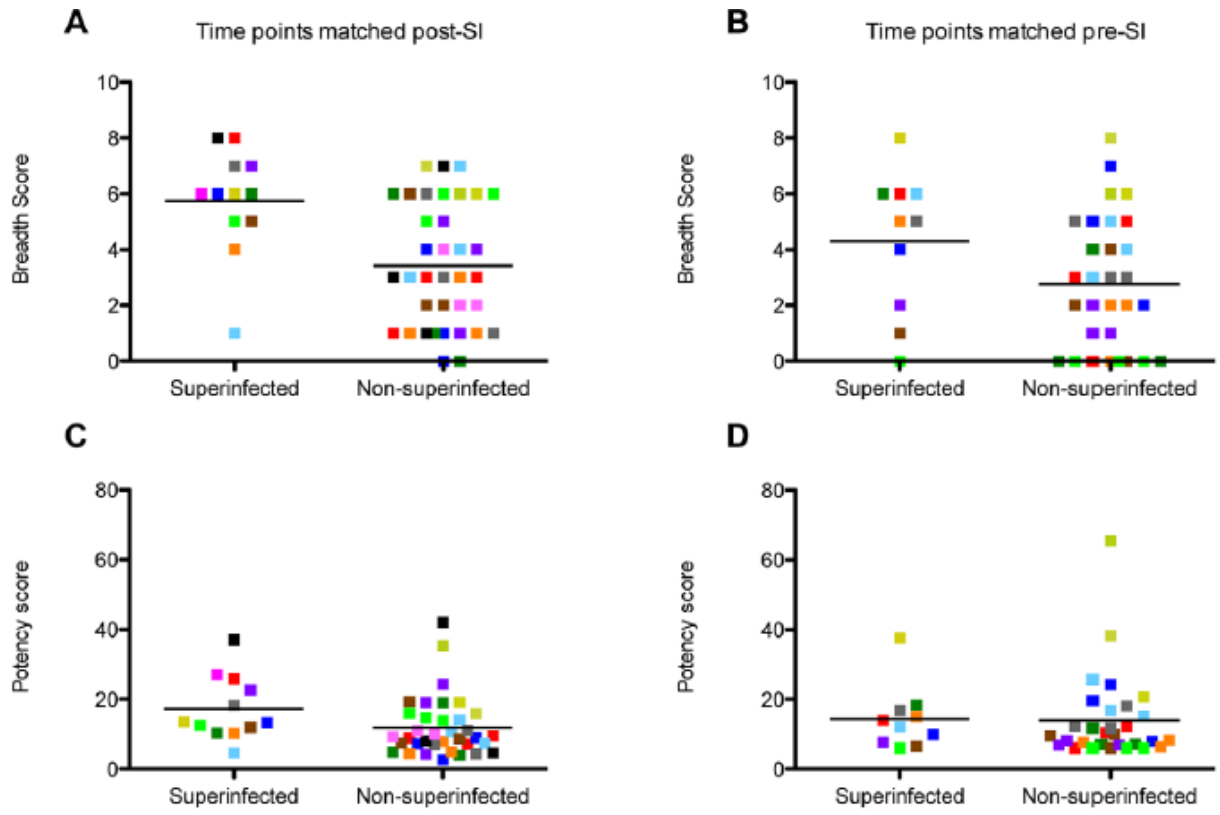

Figure 2.3 Summary of differences in Nab breadth and potency scores between superinfected and non-superinfected women.
Each case and the three matched controls are denoted by a single color. Mean scores shown with horizontal bar. Breadth and potency comparisons post-SI are shown in panels (A) and (C), respectively. Breadth and potency comparisons pre-SI are shown in panels (B) and (D), respectively.

Comparison of Nab breadth and potency in superinfected and singly infected women before SI

In order to determine whether the greater Nab breadth exhibited by superinfected women was independent of any effect of the Nab breadth developed prior to the SI event, we assessed the Nab responses elicited by the initial virus prior to SI in each individual (Figure 2.4). Viruses that were neutralization sensitive (SF162 and Q461d1) had the highest median IC50 values (158 and 130, respectively), followed by two Tier 2 viruses, DU156.12 and QD435.100M.A4 (108 and 61, respectively). The cohort median IC50s was 50 for the other four Tier 2 viruses, with

only a few individuals able to neutralize these viruses at greater than 50% at the lowest dilution tested. Overall, the geometric mean IC50s averaged across the entire panel were not significantly different between superinfected cases and singly infected controls (98.16 vs. 86.02, respectively; p=0.378). The mean Nab breadth score for superinfected women was 4.30, but only 2.80 for non-superinfected women (Figure 2.3C). Superinfected women had, on average, 1.50 (CI: 1.05-2.14, p=0.03) times greater breath than non-superinfected women at the matched pre-SI time points. However, upon adjusting for contemporaneous viral load, which differed between the two groups and is a correlate of Nab breadth (71, 75), the difference in breadth at this time point was attenuated and no longer statistically significant (RR=1.45, CI: 0.97-2.16, p=0.067). We observed comparable mean potency scores between superinfected and singly infected women (14.39 vs. 13.93, respectively) (Figure 2.3D), which were not significantly different in our univariate analysis (p=0.447), or after adjusting for viral load (p=0.195).

		Neutralization (IC50)										
		SIVmne	SF162	Q461.d1	Q769.b9	Q842.d16	Q259.d2.26	QD435.100M.a4	QC406.70M.f3	DU156.12		
ID	YPI	CL8	B	A	A	A	A	D	C	C	Breadth	Potency
SI QB850	N/A	-	-	-	-	-	-	-	-	-	-	-
QC152	N/A	-	-	-	-	-	-	-	-	-	-	-
QA874	N/A	-	-	-	-	-	-	-	-	-	-	-
QB585	N/A	-	-	-	-	-	-	-	-	-	-	-
SI QA013	0.7	50	196	308	50	71	50	104	129	268	5	14.09
QC048	0.5	50	50	50	50	50	50	50	50	50	0	6.04
QC370	0.8	50	270	358	50	50	50	91	94	121	5	12.08
QB670	0.6	50	511	157	50	81	50	50	50	50	3	10.40
SI QB685	0.8	50	50	72	50	50	50	88	50	136	2	7.71
QA520	0.9	50	50	50	50	50	50	50	50	126	1	6.83
QA028	0.8	50	247	136	50	50	50	50	50	71	2	8.17
QA538	1.1	50	50	50	50	50	50	80	50	82	1	6.87
SI QB726	1.6	50	147	859	90	127	77	95	50	89	5	16.87
QA779	1.7	50	624	305	50	75	50	50	50	50	3	12.14
QD470	1.4	50	813	229	50	231	50	72	114	99	5	18.02
QC440	1.6	50	557	150	50	50	50	133	50	82	3	11.71
SI QC885	N/A	-	-	-	-	-	-	-	-	-	-	-
QB765	N/A	-	-	-	-	-	-	-	-	-	-	-
QA261	N/A	-	-	-	-	-	-	-	-	-	-	-
QA078	N/A	-	-	-	-	-	-	-	-	-	-	-
SI QD022	4.6	50	1538	471	146	193	367	381	128	118	8	37.51
QA268	4.7	50	1102	420	50	78	74	50	198	157	6	20.66
QA966	4.5	50	2498	992	50		1636	84	219	94	6	65.40
QD121	4.7	50	2060	508	74	79	79	154	364	653	8	38.16
SI QA252	2.7	50	179	50	50	50	50	84	137	119	4	9.87
QC036	2.7	50	687	740	50	107	107	89	211	312	7	24.22
QA465	3.2	50	663	569	50	86	75	327	50	50	5	19.68
QB216	2.5	50	140	50	50	50	50	74	80	84	2	7.97
SI QC858	0.9	50	50	50	50	50	50	50	50	50	0	6.04
QB857	0.6	50	50	50	50	50	50	50	50	50	0	6.04
QA284	0.8	50	50	50	50	50	50	50	50	50	0	6.04
QA281	0.9	50	50	50	50	50	50	50	50	50	0	6.04
SI QA413	1.7	50	50	50	121	134	140	157	233	241	6	18.33
QD729	1.8	50	81	124	50	50	50	50	50	71	0	7.03
QD076	1.6	50	91	117	50	50	50	50	50	84	0	7.16
QA750	1.5	50	463	74	50	50	50	85	114	153	4	11.75
SI QB609	0.3	50	50	50	50	50	50	50	50	154	1	6.43
QB680	0.3	50	50	50	50	50	50	50	50	50	0	6.04
QA560	0.5	50	166	204	50	50	50	50	73	157	4	9.52
QC888	0.3	50	101	50	50	50	50	170	50	198	2	9.87
SI QB008	0.6	50	236	359	50	50	50	175	182	119	5	14.99
QD370	0.5	50	50	50	50	50	50	50	99	183	2	8.40
QA638	0.7	50	164	75	50	50	50	50	50	121	2	7.69
QD408	0.7	50	50	50	50	50	50	50	50	75	0	6.30
SI QB045	4.2	50	177	237	74	50	50	78	103	220	6	12.03
QA039	4.3	50	151	1114	50	104	71	50	77	50	4	16.89
QA203	4.2	50	87	696	50	50	50	144	50	272	3	15.08
QA918	4.3	50	1201	931	50	196	50	50	128	154	5	25.65
Cohort median:		50	158	130	50	50	50	61	50	108		

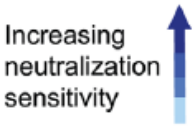

Figure 2.4 Pre-SI neutralization profiles for superinfected and non-superinfected plasmas tested against heterologous HIV-1 variants.
The layout for this figure is as described in the legend for Figure 2.2. Heterologous HIV-1 variants tested were the same as in the post-SI screen. IC50s are similarly shown with darker colors denoting greater neutralization sensitivity. Plasmas not tested are indicated by a pair of dashes.

Multivariate modeling of the Nab breadth and potency after SI

We performed a multivariate analysis to examine the relationship between SI status and the Nab response while adjusting for breadth/potency scores pre-SI as well as contemporaneous viral load and CD4+ T cell count. We found that none of these factors individually had a major impact on the association between SI and Nab breadth post-SI, as our estimate of 1.68 remained statistically significant with estimates ranging between 1.56 and 1.70 (Table 2.2). Adjusting simultaneously for all three variables similarly did not substantially change the original estimate (RR=1.51, CI: 1.01-2.25, p=0.040) (Table 2.2). The multivariate analysis for Nab potency post-

SI after adjusting for these same three variables yielded a higher estimate, changing from 1.46 to 1.68, illustrating that the association between SI and potency is stronger once these variables are accounted for.

Table 2.2 Association between Nab breadth and superinfection

	Breadth			Potency		
Univariate	**RR**	**95% CI**	**P value**	**Ratio**	**95% CI**	**P value**
	1.68	1.27, 2.30	0.001	1.46	1.03, 2.06	0.033
Multivariate adjusting for	**aRR**	**95% CI**	**P value**	**aRatio**	**95% CI**	**P value**
Breadth/potency pre-SI	1.56	1.11, 2.18	0.009	1.40	1.05, 1.86	0.020
Contemporaneous viral load	1.68	1.22, 2.31	0.001	1.54	1.07, 2.25	0.022
Contemporaneous CD4+ T cell count	1.70	1.28, 2.25	<0.001	1.63	1.10, 2.43	0.016
All of the above	1.51	1.01, 2.25	0.040	1.68	1.28, 2.20	<0.001

We next performed the same analysis with a modified breadth scoring method previously used by Simek et al. and the Center for HIV-1 AIDS Vaccine Immunology for Protocol 008 (73). Using this method, we similarly found that superinfected individuals demonstrated greater breadth than singly infected individuals post-SI (Mean scores: 0.75 vs. 0.57, respectively; p=0.002) (data not shown). Pre-SI breadth similarly showed differences between superinfected and non-superinfected individuals (Mean scores: 0.46 vs. 0.34, respectively; p=0.019), but this difference did not remain significant after adjusting for contemporaneous viral load (p=0.435). To determine if our results were sensitive to the exclusion of a particular virus in the panel, we performed a stepwise sensitivity analysis to assess breadth by removing one virus at a time and by using various combinations of viruses from the original 8-virus panel (e.g. only subtype As, only viruses with a particular neutralization profile, such as resistance to b12 or pooled plasma). Overall, the results were similar to our original finding, with estimates from regression analysis ranging from 1.36 to 1.91, all of which represent statistically significant differences between the

two groups (data not shown). In addition, estimates using percent neutralization at a fixed dilution of plasma (1:100, 1:200 or 1:400), as was used in prior studies (73, 123, 144), yielded similar results with point estimates ranging from 1.55 to 1.70 (data not shown). Moreover, breadth scores calculated by the percent neutralization at a single dilution were also highly correlated with the breadth scores using IC50s calculated from full neutralization curves made with all six serial dilutions (Spearman's rho range: 0.76-0.88, all with p=<0.0005) (data not shown).

Longitudinal analysis shows Nab breadth developed early after SI in women with the broadest responses

To determine whether SI led to a rapid enhancement of the Nab response, we analyzed longitudinally banked plasma samples from the two women with the broadest responses post-SI against the same 8-virus panel described above, beginning with the time point available following the initial detection of SI (Figure 2.5). We found that QA013, who was superinfected ~11 months after her first infection, experienced a boost in Nab activity immediately following SI (pre-SI GM IC50=118, range: 50-308, ~0.6 years post- SI GM IC50=299, range: 79-1073). Her response continued to increase in potency, with a GM IC50 of 567 at ~4.2 years post-SI (range: 224-1311). QB850, who was superinfected ~2 months after her first infection, at first displayed modest cross-subtype activity ~1 year post-SI (GM IC50=146, range: 65-428) before gradually developing elite activity around ~2.2 years post-SI (GM IC50=451, range: 168-2268), which was ~2.3 years post-initial infection.

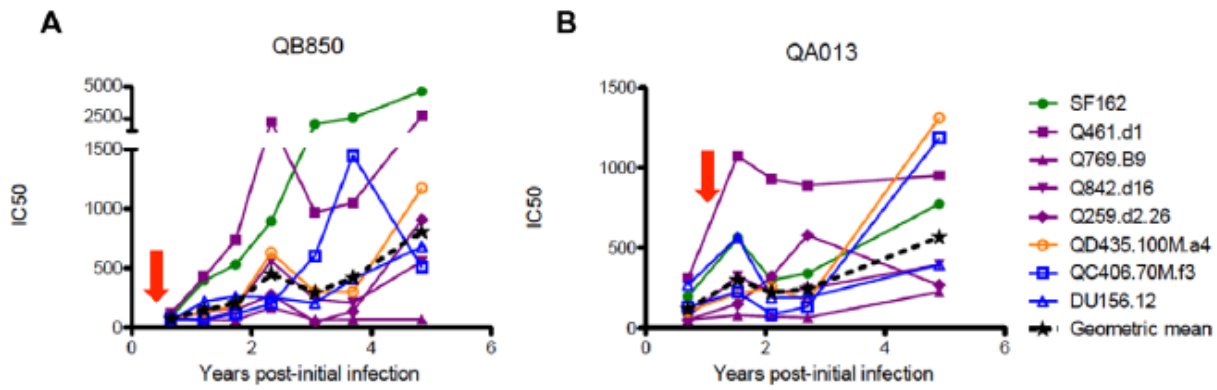

Figure 2.5 Elite neutralizers QB850 and QA013 develop breadth within 1 year following SI.
The kinetics of the Nab responses for QB850 (A) and QA013 (B) are shown versus years post-initial infection. Viruses of the same subtype are shown with the same line color. Geometric mean IC50s for the entire 8-virus panel are shown in the dashed black lines. The approximate time of SI, calculated as described in Figure 2.1, is indicated by the corresponding red arrows for each individual.

Overall, these data demonstrate that QB850 and QA013 developed cross-subtype neutralization within 1 year following SI, with two different trajectories that both led to elite Nab activity. To further assess the breadth of the response of these two individuals, additional viruses were tested, with an emphasis on subtypes that were represented by a single virus in our screen (subtypes B and D). There are limited options for subtype D viruses, but we chose a neutralization resistant variant from the Mombasa cohort, QB857. The subtype B viruses (JR-CSF, 6535.3 and CAAN5342.A2) were chosen based on their use in prior screens for elite neutralizing activity (73). Both QA013 and QB850 plasma samples from 5 years post-initial infection neutralized all of these 4 viruses, with IC50s ranging from 72-810. The IC50 values we observed for QA013 and QB850 against viruses used in the prior screen to define elite neutralizers (JR-CSF: 207 and 163, 6523.3: 322 and 810, CAAN5342.A2: 127 and 106, respectively) were all comparable or above the cohort geometric mean IC50s from the prior screen (73). However, it was not possible to directly compare all of the IC50s to the top 1% of elite neutralizers identified in that study because the IC50 values were not presented.

Discussion

In this study, we tested the ability of antibodies present in superinfected and singly infected women to neutralize a spectrum of circulating HIV-1 variants and thus, discern whether antigenic stimulation by two viruses compared to one has an effect on the subsequent Nab response. Our results demonstrate that the Nab response of superinfected women is significantly broader and more potent than that of singly infected women when compared at matched time points. These data suggest that SI elicits a substantial enhancement of the Nab response with regards to cross-reactivity in the years following reinfection. This conclusion is supported by our analysis controlling for Nab breadth/potency prior to SI and clinical measures that are associated with Nab breadth such as CD4+ T cell count and viral load (61, 63, 71, 72, 75). Thus, the study of superinfected individuals may yield insight into the development of broad and potent Nab responses to diverse HIV-1 antigens.

The majority of superinfected individuals exhibited breadth and potency scores that were greater than the average of their matched controls post-SI. Among the SI cases, the three women with the most potent responses (QA013, QC885, QB850) experienced intersubtype superinfections that were characterized by persistence of both the initial and superinfecting viruses. While we attempted to parse out which factors relating to SI may be influencing the generation of a broad response, we were unable to do so conclusively due to a limited sampling of 12 cases (data not shown). However, the characteristics of these three women suggests that continuous stimulation by two distinct viruses from different subtypes may be critical to the induction of broad Nabs. Still, other factors, including the antigenic nature and replication potential of the infecting viruses, may also be important. Conversely, we found a minority of superinfected individuals (QB045, QC858, QD022) exhibited breadth and potency scores that

were lower than that of the average from their matched controls. As all three had a CD4+ T cell counts >400 cells/ul post-SI, it is unlikely that lower breadth and potency simply reflected a lack of T cell help that could arise at terminal stages of infection. Some potentially informative features of these cases are that two of the three (QB045 and QD022) are the only individuals who became superinfected late after initial infection (>4 years post-initial infection), suggesting that perhaps the timing or duration of either or both infections may be critical to the development of Nab breadth. Moreover, all three cases had just one predominant virus detected post-SI: in one case (QD045) the initial variant persisted, while in two other cases (QD022 and QC858) the superinfecting variant became dominant and the initial virus could not be detected, at least at the levels captured by Sanger sequencing, suggesting that continued antigenic stimulation by both infecting viruses may be important in augmenting the Nab response. Additional studies that include deep sequencing of viruses in tissue and plasma would be one starting point to further explore this hypothesis.

In one individual, QA413, multiple Tier 2 viruses were neutralized prior to SI, while the Tier 1 viruses (SF162 and Q461d1) were not. This may suggest that there are unique epitopes common to the two Tier 1 viruses that are not present in the Tier 2 viruses or vice versa. In the case of Q461d1, the envelope is highly sensitive to neutralization because of conformational changes that expose multiple epitopes (145). However, we have found that Q461d1 is relatively insensitive to neutralization by the PGT-type antibodies that recognize a quaternary structure that includes an Asn at amino acid position 160 (data not shown). SF162 is also not recognized by PG9 and certain PGT antibodies because it encodes a Lys at position 160 (118, 119). Interestingly, the virus that initially infected QA413 encodes an Asn at position 160, while the superinfecting virus encodes a Lys, perhaps suggesting that PGT-like antibodies could contribute

to the antibody response in this woman. Mapping studies to define the epitope specificity will be needed to understand the unusual pattern of virus neutralization observed in QA413 pre- and post- SI.

In this study, we examined breadth and potency after SI against a panel of heterologous viruses. In a prior study, the responses to autologous viruses were examined in five of these individuals (43). Interestingly, one of the elite neutralizers, QA013, developed very high titers against her superinfecting virus, with IC50 values >1,800 (range: 1,000- 25,000+) to all four SI variants cloned at ~ 5 years post-SI (6.3 years post-initial infection). The other four superinfected individuals, who did not develop elite responses, had autologous responses that ranged from <100 to 10,000. There was insufficient data to determine if there was an association between autologous and heterologous responses.

It is difficult to directly compare the Nab responses of the superinfected women to broad neutralizers identified in other studies because there is no standard for quantifying Nab breadth against HIV-1. We addressed this issue by using a diverse panel of viruses weighted towards Tier 2 variants and several alternate breadth scoring methods, which showed that no single virus drove the association observed between SI and Nab breadth and that our conclusion is the same irrespective of the scoring method used. Simek et al. previously defined elite activity as "the ability to neutralize, on average, more than one pseudovirus at an IC50 titer of 300 within a clade group and across at least four clade groups (73)." While we were unable to completely satisfy these criteria since our 8-virus panel included only single variants of clades B and D, we still found that the two superinfected individuals (QA013 and QB850) with the broadest responses could neutralize at least two viruses in clades A and C, as well as both single viruses tested from clades B and D at an IC50 titer greater than 300, supporting the characterization of these

individuals as elite neutralizers. Notably, plasma antibodies from QB850 neutralized viruses from all four subtypes tested at IC50 values greater than 600, more than 2-fold higher than the bar set for elite neutralizers (73). Furthermore, the responses of these two women were greater than those found in a similar screen of 70 singly infected women in this cohort at ~5 years post-initial infection, with some observed IC50 titers against Tier 2 viruses 6-fold more potent than those of the top 10% of the singly infected women (63). Although we had limited opportunity to directly compare the response of the SI individuals studied here and individuals identified as elite neutralizers by Simek et al. in the IAVI cohort, we did find that the SI cases had IC50 values above the cohort geometric mean IC50 for the three viruses tested in common between the studies. Together, these findings suggest that 2 (QA013 and QB850) of the 12 superinfected individuals developed Nabs that exhibit elite activity. This is a remarkable fraction (17%) of individuals with elite activity, although because of differences between screening methods, it is difficult to directly compare this to the 1% of presumably singly infected elite cases reported previously (73).

In a prior study that examined four cases of SI, a greater increase in Nab breadth post-SI among superinfected individuals compared to singly infected individuals was also observed (123). However, it is hard to compare their data with our own, as the previous study used randomly chosen primary isolates for measuring breadth in a cohort with unknown seroconversion dates. It is also interesting to note that this previous study observed a decrease in viral load in three of the four superinfected cases studied at a time point post-SI, two of which had undetectable viral loads. Such a significant drop in viral load has not been previously reported for cases of SI (39). In contrast, we observed an increase or no change in viral load in the majority of superinfected women examined. This is consistent with the observation that viral

load is highly correlated with Nab breadth (63, 71). However, after adjusting our breadth score analysis for contemporaneous viral load, the estimate of 1.68 was unchanged, while adjusting for this variable caused an increase in our estimate for differences in potency. This would imply that breadth in these superinfected cases alone cannot be explained entirely by an increase in viral load following SI, but rather suggests that stimulation from antigenically distinct viruses may contribute to the development of potency.

Finally, we found evidence of cross-subtype breadth, including detectable neutralization of viruses from four different subtypes in the two women with elite Nab responses within 1 year of their SI. Because both of these individuals were superinfected soon after their initial infection, these cross-subtype responses arose relatively soon after HIV-1 seroconversion. Indeed, by 2 years after their initial infection, both women had antibodies capable of neutralizing 7 of the 8 primarily Tier 2 viruses tested. Recent studies suggest that cross subtype breadth is rare before 2 years post-infection in individuals who are presumably singly infected (72, 146). Our findings raise the interesting possibility that some of the individuals identified as having broad responses in prior screens may have been superinfected.

A few caveats to these findings must be considered. First, there may have been potential for misclassification of singly infected women, due to our limit of detection or if recombination occurred between the initial and superinfecting strains in HIV-1 genomic regions outside of *gag* and *env* (34). However, this misclassification would be expected to decrease our ability to detect differences between superinfected and singly infected women, making it more likely that the true association between SI and Nab breadth is stronger than what we observed. Also, we cannot exclude the possibility that there are other factors involved with the development of the broad responses in some of the superinfected women.

This study reveals an unexplored source of naturally-occurring broadly Nabs, and represents a highly relevant approach to inform vaccine strategies in three key ways. First, studies on this and other SI cohorts may provide additional support for immunizing with particular combinations of different HIV-1 strains could be an effective vaccine approach (147-151). Given that the greatest breadth was observed in cases of intersubtype SI, the use of Envelope immunogens from different subtypes may be optimal. Second, the Nabs and viruses isolated from members of this cohort may hold important clues to antigenic determinants capable of eliciting cross-reactive antibodies that can protect against multiple subtypes of HIV-1. Third, longitudinal studies of superinfected individuals that develop broad and potent Nab responses, such as those identified here, may foster an understanding of the mechanism leading to the elicitation of breadth. Of particular interest is the observation that two elite neutralizers developed broad and potent responses soon after infection by a second HIV-1 strain, suggesting that the processes that lead to the development of broad HIV-specific Nabs are accelerated by a second infection. If SI ultimately leads to the rapid capacity of the overall Nab response to recognize diverse circulating HIV-1 variants, a successful vaccination strategy that mimics natural SI may lead to the development of broad Nab in immunized individuals.

Chapter III

HIV-1 superinfection does not focus the Nab response on known epitopes

Introduction

Developing a Nab-based vaccine that is protective against globally diverse HIV-1 variants remains a major challenge today (152), but the identification of broad, cross-reactive Nabs from HIV-infected individuals has led to the prospect that the HIV-1 Envelope epitopes they target could be exploited for immunogen design (85). Characterization of the precise contact residues that mediate neutralization has led to the identification of four main target regions on the HIV-1 Envelope trimer: 1) the CD4-binding site (i.e. VRC01) (120), 2) V1/V2 glycans (i.e. PG9, PG16) (119), 3) V3 glycans (i.e. PGT121, PGT128) (118), and 4) the MPER of gp41 (ie. 10E8) (153-155). Presentation of one or more of these epitopes to the immune system would ideally elicit similarly potent and cross-reactive antibodies in vaccinated individuals, but it is unclear how such immunogens should be constructed since many antigenic and immunogenic barriers exist that may impede their development (85, 113, 155). As discussed in Chapter I, many of the bNabs exhibit unusual characteristics that are not typically seen in vaccine-elicited responses and Envelope uses many strategies to occlude the epitopes targeted by these bNabs, which will be discussed further in Chapter V. In addition, it is unclear if there are additional sites on Envelope that should be considered and whether such a targeted approach is feasible for providing protection in diverse populations.

Another vaccine strategy under investigation aims to take a wider approach to provide protection against circulating HIV-1 variants by eliciting a highly diverse immune response (156). In support of this concept, previous *in vitro* studies have shown that combinations of

bNabs are needed to block infection by HIV-1 transmitted variants from different subtypes (157-159). Treating chronically-infected macaques with combinations of broadly neutralizing Mabs has also proven to be highly effective at clearing virus from circulation in the weeks following administration (100, 101). There is also evidence that sequential immunization strategies using multiple HIV-1 Envelope proteins may provide a more effective way of eliciting a broad Nab response than presentation of a single antigenic form (111, 148, 160-162). Likewise, we showed in Chapter 2 and that SI leads to a broader and more potent Nab response when compared to single infection (163), that was also observed in a previous study (123, 163). This augmentation of the antibody response is presumably due to the increased antigenic stimulation provided by two distinct viral variants, as Envelope diversity is positively correlated with Nab breadth (61, 63). However, it is unclear whether these responses developed following SI are mediated by Nabs that target a single antigenic site or whether they are mediated by a polyclonal response, targeting multiple different epitopes. Distinguishing between the two possibilities would be informative for vaccine design, as it may support the development of immunogens to elicit either a more targeted or diverse antibody response.

In singly infected individuals with similarly broad and potent Nab activity as the SI cases studied here, the four main epitope targets described above often predominate their responses (72, 86-89, 142, 164). Meanwhile, the epitope targets of only a single case of SI have been described in the literature (65, 165). Longitudinal analyses of plasma samples revealed that this individual's antibody response predominately targeted overlapping epitopes in the V2 region of Envelope, including the conserved N-linked glycan at position 160 (65, 165). However, the contribution of SI on these responses is unclear, as the main antigenic targets that appeared to drive this individual's Nab breadth originated in the superinfecting virus, and not both the initial

and superinfecting variants (68, 165). Given this prior work, we hypothesized that SI focuses the Nab response on one or more of the four main target regions on Envelope.

In Chapter 2, we examined the Nab responses after SI in 12 cases that were identified using Sanger sequencing. Here, we extended this analysis to an additional 9 cases identified by 454 deep sequencing before mapping the antibody targets of all 21 SI women. A combination of standard epitope mapping and computational analyses revealed that the four main sites of Envelope vulnerability are not the principle targets of the women's Nab responses. In conclusion, this study illustrates that exposure to diverse HIV-1 antigens following SI can drive a broad and potent Nab response without strongly targeting any one of these four main epitopes.

Materials and Methods

Study cohort

The individuals in this study represent a subset of a prospectively followed seroincident cohort of HIV-1 negative high-risk women from Mombasa, Kenya as described in Chapter II. Among 129 women in the Mombasa cohort, 21 SI women were identified in a variety of studies using a combination of Sanger sequencing and 454 deep sequencing of at least 2 genome regions (31, 33, 34, 38). All women were presumably infected with HIV-1 through heterosexual contact (125), and none reported using ARV therapy during follow-up for this study.

Cloning of Envelope variants from QB850

Single genome amplification of HIV-1 *env* from plasma obtained 73 days post-infection (dpi), 324 dpi, and 632 dpi was performed as previously described (166), with a few modifications. Briefly, 140ul of plasma was used to isolate virus particles by μMACS VitalVirus HIV Isolation Kit (Miltenyi Biotec) for subsequent RNA extraction with QIAamp Viral RNA

Mini Spin Kit (Qiagen). HIV-1 *env* genes were amplified by nested PCR (TaqPlus Precision; Stratagene) from cDNA obtained by reverse transcription (SuperScript III; Invitrogen) and subsequently cloned into pCI-neo mammalian expression vectors (Promega). Point mutations N332A and N301A were generated by site-directed mutagenesis (QuikChange II; Agilent Technologies) and verified by sequencing.

Pseudovirus production

Pseudoviruses were produced by co-transfecting 293T cells with cloned viral Envelopes and a full-length subtype A proviral clone with a partial deletion in envelope (Q23Δenv), as described in Chapter II. To assess the Nab breadth in the newly identified SI cases, the same 8-virus panel used in Chapter 2 was generated. A 21-virus panel was also generated to test plasma from all 21 SI cases. This panel included 6101.10, Bal.01, BG1168.01, CAAN.A2, DU156.12, DU422.01, JRCSF.JB, JRFL.JB, KER2018.11, PVO.04, Q168.A2, Q23.17, Q769.H6, RW020.2, THRO.18, TRJO.58, TRO.11, YU2.DG, ZA012.29, ZM106.9, and ZM55.28a. Data from these experiments was analyzed by Ivelin Georgiev at the Vaccine Research Center, National Institutes of Health, for computational predictions to delineate antibody specificities as previously described (167).

Neutralization assay

The TZM-bl neutralization assay was used as described in Chapter II. The IC50, or reciprocal plasma dilution at which 50% of the virus was neutralized, for each plasma-virus pair was calculated using linear interpolation from the neutralization curve. A plasma sample was considered to be below the detectable limit of neutralization if the starting dilution (1:100) did not show >50% neutralization. Two independent experiments were performed and an average was calculated for replicates. Any plasma-virus pairs that demonstrated >2-fold difference in

IC50 across experiments was repeated in a third experiment and the two most concordant IC50's were used as a final average.

CD4-binding site mapping

Resurfaced core protein (RSC3) and CD4-binding site defective mutant (RSC3Δ371I) constructs were obtained though the AIDS Research and Reference Reagent Program. These proteins were produced as previously described (120), with a few modifications. Briefly, 293F cells were transfected using 293fectin (Invitrogen) and 4-5 days later supernatants were clarified by 0.45um filtration and concentrated by centrifugation in Centricon-Plus 70 filter tubes (Millipore) whilst buffer-exchanged into PBS. Proteins were purified by DEAE sepharose (GE) ion exchange chromatography, followed by His-Select Nickel (Sigma) affinity chromatography. Immulon 2HB 96-well ELISA plates (Thermo) were coated with 200ng/ml of protein in sodium bicarbonate overnight at 4°C. The plates were blocked in 10% non-fat milk with 0.3% Tween-20 in PBS and incubated with 2-fold dilutions of heat-inactivated plasma starting at a dilution of 1:100, or in some cases 1:400 to obtain an endpoint titer, before horseradish peroxidase-conjugated goat anti-human IgG antibody (BioRad) was added at a 1:3000 dilution. Plates were washed with 0.05% Tween-20 in PBS and incubations were all for 1 hour at 37°C. 1-Step Ultra TMB-ELISA substrate (Pierce Biotech) was used to develop the plate for 3 min in the dark before the reaction was stopped using 1N H_2SO_4 and plates were read at 450nm. Optical densities (OD) were analyzed as previously described (164). Briefly, background-corrected OD values for the reciprocal plasma dilution greater than or equal to 0.1 were used to summarize endpoint titers. Plasma samples with endpoint titers below 1:200 were considered non-reactive. Two independent experiments were performed and an average was taken across replicates. The ratio of endpoint titers for RSC3/RSC3Δ371I were calculated, and plasma samples with a ratio

>2.5 were further tested in a protein competition neutralization assay, as previously described (164). Briefly, 25ul of RSC3/RSC3Δ371I protein was added at a final concentration of 25 ug/ml to an equal volume of serially diluted plasma and incubated at 37°C for 30 min before the addition of pseudovirus. The neutralization assay was then performed as described above, after a further 1 hr incubation of protein, virus and plasma.

V1/V2 and V3 glycan mapping

N160K and N332A point mutants in the backgrounds of Q23-17, a subtype A virus (168), and DU156, a subtype C virus (56), were used in neutralization assays alongside their corresponding wildtype viruses. Q23.N160K, Q23.N332A, and DU156.N160K were made by site-directed mutagenesis (QuikChange II, Agilent Technologies) and verified by sequencing. The ratio of wildtype IC50 to mutant virus IC50 (fold-change) was averaged across two independent experiments and used as a summary measure.

MPER mapping

Neutralization profiles against HIV-2 (7312A) and a chimera made from HIV-2 and the HIV-1 subtype B YU-2 MPER engrafted (7312C1) were first compared (169). Plasma samples exhibiting a greater than 3-fold change in IC50 between the HIV-2 and HIV-2/HIV-1 MPER chimera were considered candidates for further testing using a YU-2 MPER peptide (KKKNEQELLELDKWASLWNWFDITNWLWYIRKKK, GenScript) competition neutralization assay as previously described (153). Briefly, 25ul of MPER peptide was added at a final concentration of 25 ug/ml to an equal volume of serially diluted plasma and incubated at 37°C for 30 min before the addition of pseudovirus. Neutralization assays were then performed as described above. Plasma samples exhibiting a greater than 3-fold change in IC50 with the addition of MPER peptide were considered as having evidence of MPER reactivity (153).

Results

Similarly broad and potent responses developed in newly identified cases of SI

The 21 cases of SI studied here consist of an original group of 12 cases that were identified by Sanger sequencing (31, 33, 34) and an additional 9 cases identified by 454 deep sequencing (38). Combining all 21 cases, the timing of SI ranged from 63 to 1,895 days post-initial infection (Table 3.1). Plasma from the 9 recently identified cases of SI were each tested against the same cross-subtype panel of 8 viruses representing 4 different subtypes from Chapter II. We chose the same time point studied previously, approximately 5 years post-initial infection, at which point all of the women had been superinfected for at least 1 year. However, a few cases (n=3) were tested at the last time point available, which was before 5 years, as noted in Table 3.1. There was no statistically significant difference in the geometric mean IC50s across the 8-virus panel between the newly identified cases and the previously examined 12 SI cases ($p=0.455$, Mann-Whitney), demonstrating that they have similar Nab responses.

There were 10 cases of intersubtype SI based on analysis of at least one HIV-1 genomic region (*gag, pol,* or *env*). In the prior study of 12 SI cases and in this analysis of all 21, the two individuals with the broadest responses and highest geometric mean IC50 across the 8-virus panel, QB850 and QA013, were cases of intersubtype SI that occurred within the first year following initial infection. The two women with the lowest geometric mean IC50 were cases of intrasubtype SI that occurred after 1 year following initial infection (QD149 and QB045).

Table 3.1 Superinfection cohort, sorted by geometric mean IC50 across 8-virus panel

Patient ID	Estimated window of SI (dpi)	Estimated timing of SI (dpi)*	Type of SI^	Time at which Nabs were examined: dpi	Time at which Nabs were examined: dpSI	Geometric mean IC50
QB850	52-73	63	Inter	1768	1705	808
QA013	264-385	325	Inter	1790	1465	566
QC885	58-152	105	Inter	1808	1703	559
QF564	17-1270	644	Inter	1722	1078	444
QC369	29-143	86	Intra	451	365	431
QB726	749-1031	890	Intra	2002	1112	425
QF441	255-444	350	Inter	1341	991	411
QB685	303-1453	878	Intra	1800	922	402
QD022	1832-1957	1895	Inter	2859	964	327
QD151	241-801	521	Intra	1701	1180	312
QC858	341-440	391	Intra	1866	1475	283
QG262	59-144	102	Intra	1787	1685	279
QA252	1046-1487	1267	Inter	1912	645	279
QB609	101-485	293	Inter	1262	969	274
QA413	714-1007	861	Intra	1346	485	262
QD696	49-174	112	Intra	1517	1405	251
QG284	155-260	208	Intra	765	557	240
QB210	17-63	90	Inter	800	710	218
QB008	303-591	397	Inter	1803	1406	203
QD149	996-1086	1041	Intra	1367	326	128
QB045	1680-2048	1864	Intra	2334	470	88

*Midpoint between estimated window

^Classified as inter- or intrasubtype SI, based on at least one genomic region (*gag, pol, env*)

dpi, days post-infection; dpsi, days post-superinfection

CD4-binding site is not a target for neutralization

To probe for the presence of CD4-binding site antibodies, the level of binding was compared between the wildtype RSC3 protein and the point mutant, RSC3Δ371I, that abrogates binding of CD4-binding site-specific antibodies. Only 1 of the 21 women (QA013) developed antibodies at ~5 years post-initial infection that bound RSC3 (Endpoint titer: 1:256,000) stronger than the RSC3Δ371I mutant (Non-reactive, endpoint titer: 1:100). The plasmas from the other 20 women were either non-reactive (endpoint titer <1:200) or did not bind these proteins differentially (>2.5-fold) (Figure 3.1A).

To determine when these antibodies arose in QA013, plasma was tested from time points pre-SI (264 dpi) and post-SI (559 dpi, 234 dpSI). Figure 3.1B shows that this individual's CD4-binding site-specific antibodies did not arise until post-SI, as evidenced by strong binding to the RSC3 wildtype protein (Endpoint titer: 1:6,400). Plasma samples from pre and post-SI time points were non-reactive to the RSC3Δ371I mutant. Next, to determine whether QA013's CD4-binding site-specific antibodies were capable of neutralizing HIV-1, RSC3 proteins were used to compete for binding of these antibodies in a standard neutralization assay. Using Q461d1 as a test virus, neither RSC3 nor RSC3Δ371I proteins altered the neutralization profiles post-SI (559 dpi) or at 5 years post-initial infection (Figure 3.1C), demonstrating that QA013 developed binding, but not neutralizing CD4-binding site-specific antibodies in the year(s) following SI.

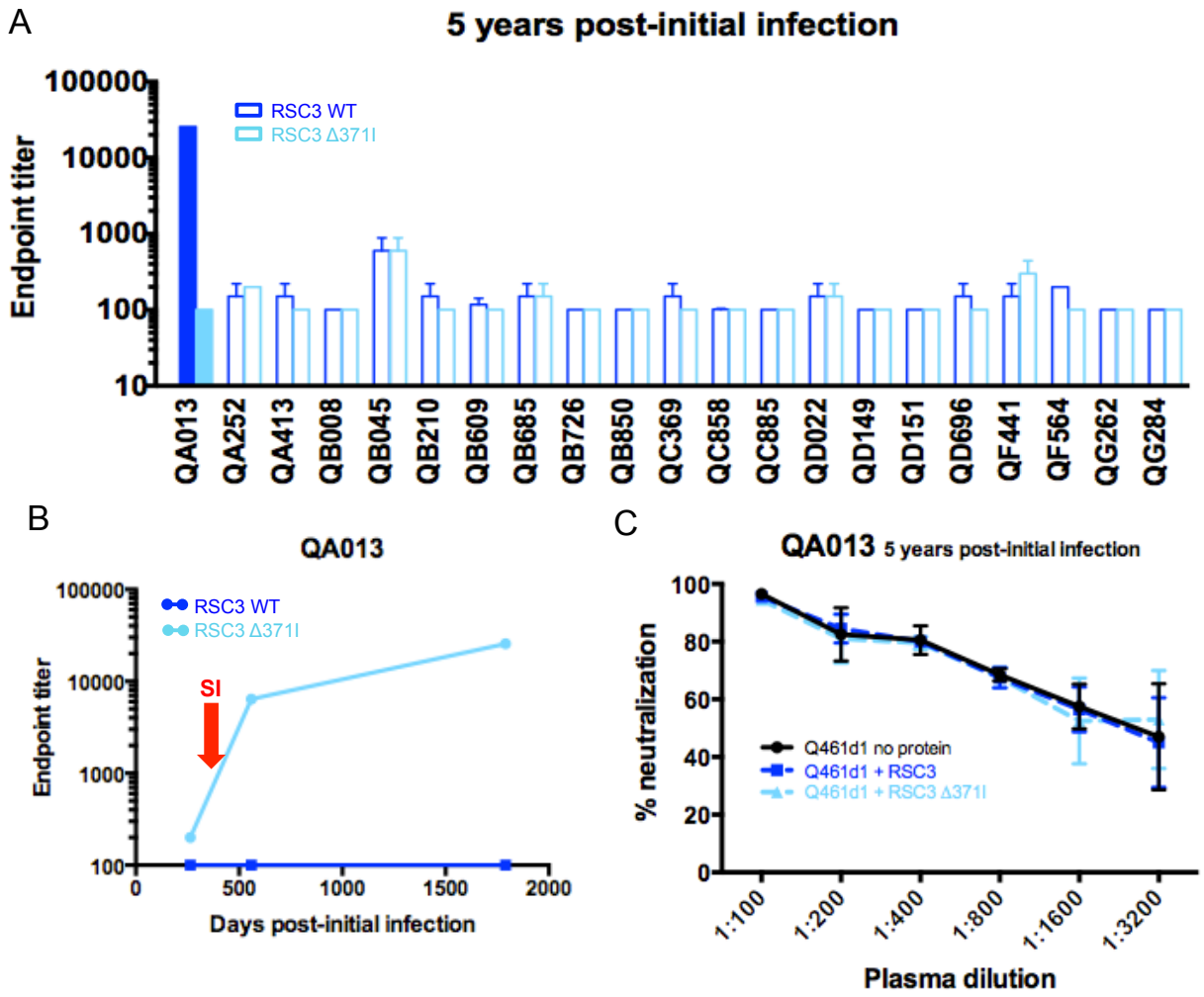

Figure 3.1 Probing for CD4-binding site-specific antibodies
(A) Plasma from all 21 SI cases ~5 years post-initial infection were tested for binding to RSC3 wildtype (dark blue) and mutant proteins (light blue). The one individual (QA013) demonstrating >2.5-fold difference in endpoint titer for the wildtype compared to the mutant is noted with shaded bars. (B) Longitudinal plasma samples from QA013 were for binding to RSC3 wildtype and mutant proteins. The estimated timing of SI for this patient is denoted with red arrow. (C) Neutralization curves for QA013 ~5 years post-initial infection plasma tested against Q461d1 with the addition of RSC3 wildtype or mutant proteins.

<u>No evidence for the development of V1/V2 or V3 glycan-specific Nabs</u>

Point mutants lacking the N-linked glycan residues at positions 160 and 332 in the backgrounds of Q23 and DU156 viruses were used to probe for the presence of PG and PGT-like antibodies, respectively (118, 119). As expected, both N160K mutants completely abrogated

neutralization by PG9, as did the N332A mutants against PGT128 (Figure 3.2A). To first determine an appropriate background cutoff, VRC01, which is a CD4-binding site-specific antibody that does not target N160 or N332 (120), was tested against these wildtype and mutant viruses. Surprisingly, 0.7 to 2.75-fold changes in IC50 were observed when comparing the wildtype to mutant, demonstrating that the removal of these N-linked glycosylation sites alter VRC01's neutralization capability. Figure 3.2B graphically depicts the shifts in neutralization curves that were observed in a representative experiment with VRC01. This reduction in IC50 was also seen with other Mabs that do not target the N160 or N332 glycans (i.e. 2F5 (79) and NIH 45-46W (170), Figure 3.2C) further indicating that these changes have some conformational effects that are unrelated to PG and PGT specificities. To account for these non-specific effects, a 3-fold change in IC50 was used as a cutoff for plasma samples in order to determine the presence of N160 and N332 glycan-specific antibodies.

Only two individuals (QC369 and QG262) came close to demonstrating a >3-fold change in IC50 between Q23 and Q23.N160K mutant viruses, similar to the 2.75-fold change in IC50 observed for VRC01 (Figure 3.3A). In contrast, none of the SI cases demonstrated a >3-fold change in IC50 between DU156 and DU156.N160K viruses, and only one individual came close to demonstrating a similar 1.5-fold change in IC50 that was observed for VRC01 (Figure 3.3A). None of the 21 individuals' plasma samples demonstrated a >3-fold change in IC50 between either sets of wildtype and N332A mutant viruses, but approximately half showed similar fold-changes in IC50 that was observed for VRC01 (Figure 3.3B). These data suggest that these women's responses did not strongly target the glycans at positions 160 and 332 following SI, ~5 years post-initial infection.

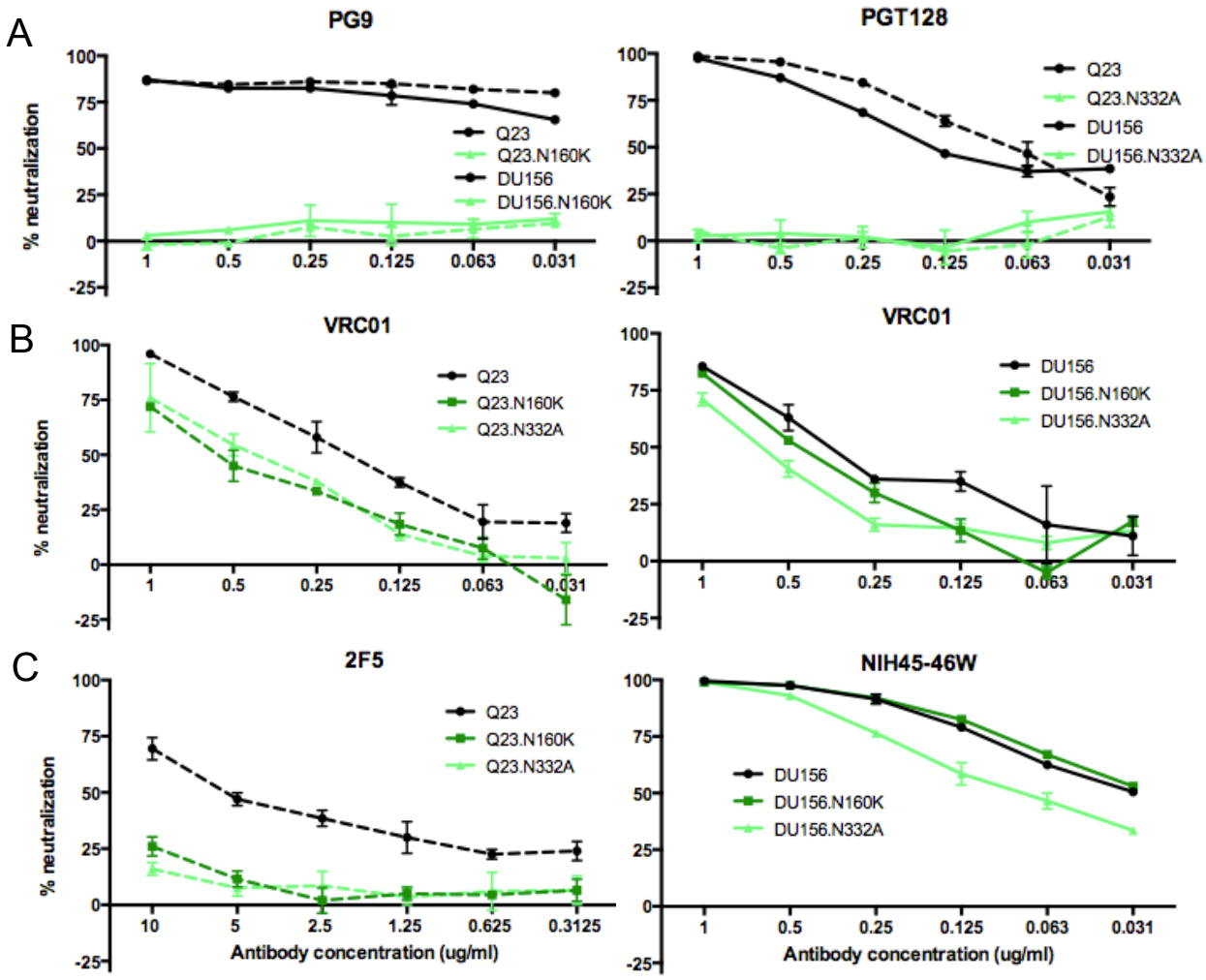

Figure 3.2 Control experiments for probing V1/V2 and V3 glycan-specific Nabs
Representative neutralization curves for positive controls, PG9 and PGT128, tested against wildtype viruses, Q23 (dotted black) and DU156 (solid black) alongside N160K and N332A mutants (dotted/solid green). Panel (B) depicts VRC01, a CD4-binding site-specific Mab that should be unaffected by the removal of N-linked glycans in V1/V2 and V3, tested against Q23 and DU156 alongside mutant viruses. Panel (C) depicts additional control experiments using 2F5, an MPER-specific Mab, tested against Q23 alongside mutant viruses and NIH45-46W, another CD4-binding site-specific Mab, tested against DU156 alongside mutant viruses.

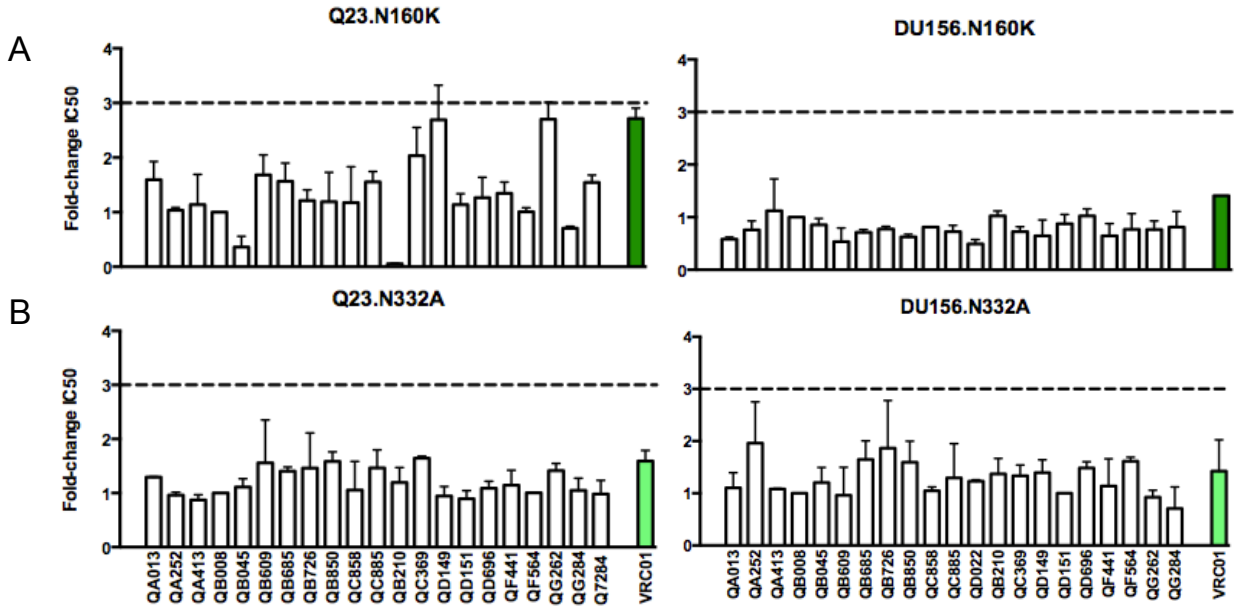

Figure 3.3 Probing for V1/V2 and V3 glycan-specific Nabs
Fold change in IC50 (WT:Mutant) for all 21 SI cases against both Q23 and DU156 and the corresponding N160K (A) and N332A mutant viruses (B). VRC01 control is highlighted in green at the far right of the graph. The 3-fold cutoff is denoted by a dotted line in each plot. Averaged fold-change in IC50 and standard deviation from two independent experiments is shown.

MPER-specific antibodies developed in a subset of superinfected women

Neutralization of an HIV-2/HIV-1 MPER chimeric virus (7312C1) was used as an initial screen for MPER-specific antibodies. Neutralization of the MPER chimera at a level >3-fold than what is observed for HIV-2 (7312A) indicates the potential presence of MPER-specific antibodies (153). Four of the 21 superinfected women demonstrated MPER reactivity, with >3-fold changes in IC50 between HIV-2 and the MPER chimera ranging between 3.2 and 28.2 (Figure 3.4A). To determine when MPER-specific antibody responses arose in these 4 patients, plasma samples were tested at time points pre- and post-SI, except for QG262, who was only tested post-SI because she was superinfected early after initial infection (102 dpi) before Nabs typically develop (53). QA013 and QF441 did not have detectable MPER-specific Nab responses pre-SI, but showed 3.5–fold and 8.4–fold changes in IC50 between the HIV-2 and chimeric

viruses post-SI, respectively (Figure 3.4B). Plasma from QD022 also did not have detectable MPER reactivity pre-SI. Both QD022 and QG262 did not immediately demonstrate evidence of MPER activity following SI (32 dpSI and 246 dpSI, respectively). However, QD022 demonstrated a 9.1-fold change in IC50 between HIV-2 and the MPER chimera at 742 dpSI, and this MPER activity later peaked at 964 dpSI (28.2-fold change in IC50 HIV-2:MPER chimera). QG262 demonstrated a >3-fold change in IC50 much later following SI, approximately 1719 dpSI. Together these data suggest that these MPER-specific responses occurred *de novo* post-SI, but 3 of the 4 individuals did not develop these responses until >1 year following SI.

While HIV-2/HIV-1 MPER chimera neutralization may indicate MPER-reactivity, it may not represent the natural context of the HIV-1 MPER and therefore demonstrate whether these MPER-specific antibodies are mediating the neutralizing activity of patient plasma (153). As a more stringent test to probe for the presence of MPER-specific Nabs, MPER peptides were used to absorb MPER-specific antibodies prior to a neutralization assay with HIV-1 variants. To this end, MPER peptides matching the sequence of the MPER chimera were designed. As a first test, Figure 3.4C illustrates the results from neutralization assays using these MPER peptides to absorb MPER-specific antibodies from plasma prior to the addition of the MPER chimera. Pre-incubation with MPER peptide did not have a large affect on the neutralization of the MPER chimera for any of the four patients at time points pre-SI or immediately post-SI, or for QF441 and QG262 at ~5 years post-initial infection. In contrast, both ~5 year plasma from patients QA013 and QD022 demonstrated >3-fold reductions in IC50 with the addition of MPER peptide, whereas MPER activity for QF441 and QG262 was not diminished. However, none of the plasma samples from the 4 patients at time points pre and post-SI demonstrated >3-fold reductions in IC50 against HIV-1 variant Q461d1 after the addition of MPER peptide (Figure

3.4D). The neutralization capacities of these 3 patients' plasma against the subtype C virus, DU156, were also unchanged upon the addition of peptide (data not shown). As expected, neutralization of the MPER chimera, Q461d1, and DU156 by MPER-specific Mabs 4E10 and 10E8 was substantially decreased with the addition of MPER peptide (average: >4-fold change), while VRC01 neutralization of Q461d1 and DU156 was unaffected (average: 1.02-fold change). Figure 3.4E graphically depicts the shifts in neutralization curves that were observed in a representative experiment testing VRC01 and 4E10 against Q461d1. Since neutralization was unaffected in these 4 patients' plasma when tested against HIV-1 isolates Q461d1 and DU156, it is unlikely that MPER-specific Nabs are mediating their cross-neutralization neutralization.

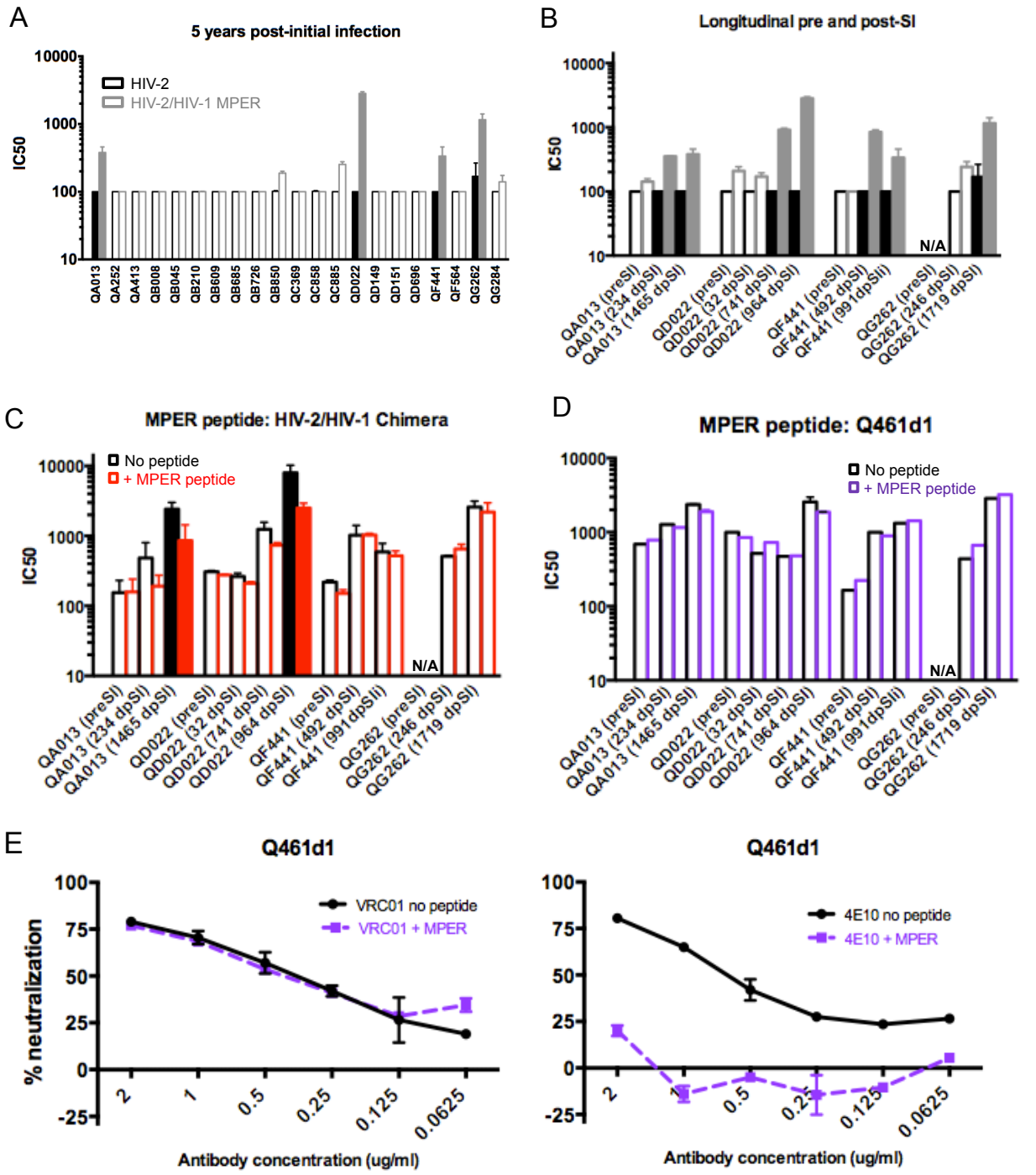

Figure 3.4 Probing for MPER-specific Nabs
In all panels, individuals demonstrating >3-fold difference in IC50 comparing the wildtype to the mutant or absence/presence of MPER peptide is noted with shaded bars. (A) Plasma from all 21 SI cases ~5 years post-initial infection tested for neutralization with HIV-2 (black) and HIV-2/HIV-1 MPER chimera (gray). Longitudinal plasma pre- and post-SI was tested to determine when the MPER activity arose in 4 SI cases (B). Neutralization of the HIV-2/HIV-1 MPER chimera (C) or Q461d1 (D) in the presence (red or purple, respectively) or absence (black) of MPER peptide. Control experiments in panel (E) show VRC01, a CD4-binding site-specific Mab, neutralization is unaffected while 4E10, an MPER-specific Mab, neutralization is dramatically decreased by the addition of MPER peptide when tested against Q461d1.

Plasma delineation analysis

Plasma from each of the 21 women's ~5 year post-initial infection time point was tested against a panel of 21 viruses previously used to predict antibody specificities (167). Nab breadth was calculated as the percent of viruses neutralized by plasma samples, which ranged from 10-100% across cases (Figure 3.5). Two women (QB008 and QD151) were unable to neutralize >25% of the viruses and therefore had too little data to perform the plasma delineation analysis. While most of the women's antibody responses did not correlate well with a single specificity, 4 patients had neutralization profiles that demonstrated a relatively high correlation (>0.5) with a known antibody specificity: QA252 had HJ16-like activity (171), QB850 had PGT-like activity, QB210 had 2F5-like activity (79), and QC369 had PG9-like activity. However, the target function level, which provides a measure of confidence for these predictions, showed that only QB850's PGT-like activity could be strongly supported.

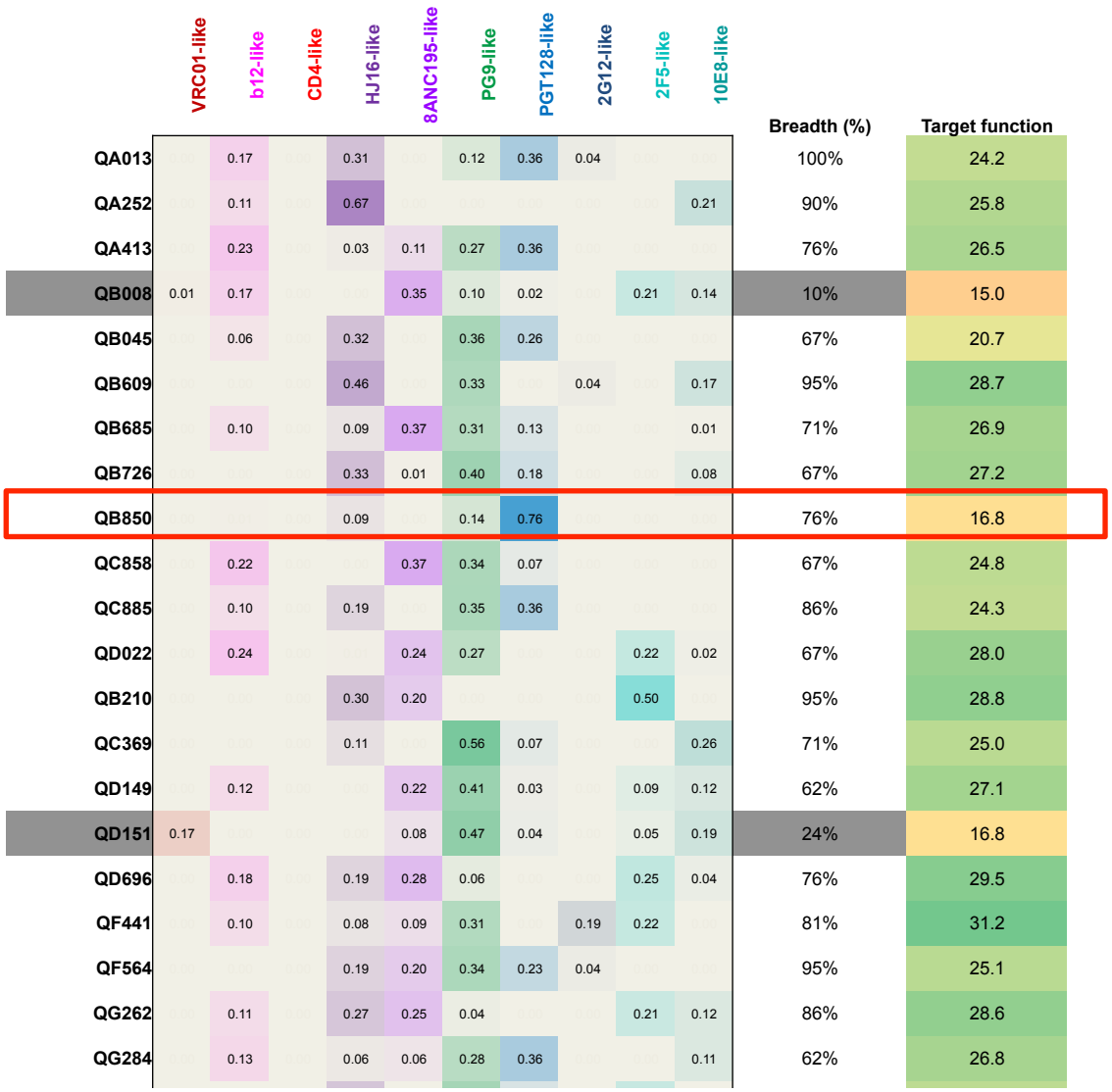

Figure 3.5 Plasma delineation analysis
All 21 women were tested against a 21-virus panel to assess computationally if they demonstrated similar neutralization signatures as those Mabs listed across the top of the table. Correlation scores for each specificity are listed in the columns, with a higher number and darker coloring denoting a greater likelihood that a particular specificity is present in plasma. Percent breadth, calculated as the number of viruses in the panel that were neutralized by the individual, is shown in the following column. The final column lists the target function, or relative confidence of the correlation scores, with a lower score denoting higher confidence. QB850 is highlighted in the red box, to show both a high correlation score with PGT-like activity and low target function.

Autologous N332 and N301 mapping of QB850

To further examine the presence of PGT-like antibody activity in QB850, which was not detected using the N332A mutants in the context of heterologous viruses Q23 and DU156,

autologous clones were isolated and used to screen for this specificity. Three time points were chosen for cloning—632 dpi, 324 dpi, and 73 dpi, which was the earliest time point that SI was first detected. Of the 9 functional clones isolated from 73 dpi, 3 were recombinant variants consisting of superinfecting subtype D regions flanking the initial subtype A virus (C2-HR2) and 6 were subtype A initial variants (Figure 3.6A). Three recombinant variants consisting primarily of the initial A (C1-HR2) and superinfecting D (HR2-CT) were isolated form 324 dpi as well as one initial subtype A variant and two recombinants, similar in structure to those from 324 dpi, were isolated from 632 dpi. The full-length subtype D superinfecting virus was not detected at 73 dpi or any other time point following SI (33), suggesting that recombination between the SI and initial variants took place in the 21-day window during which the SI event occurred. Testing the 9 functional 73 dpi clones against longitudinal plasma showed that potent Nabs began to target the SI recombinants ~443 dpi, but then subsided ~632 dpi, right around the time that cross-reactive Nab responses developed (Figure 3.6B). These autologous neutralization data are summarized for the initial and SI variants in Table 3.2, along with longitudinal neutralization profiles for the clones isolated 324 dpi and 632 dpi.

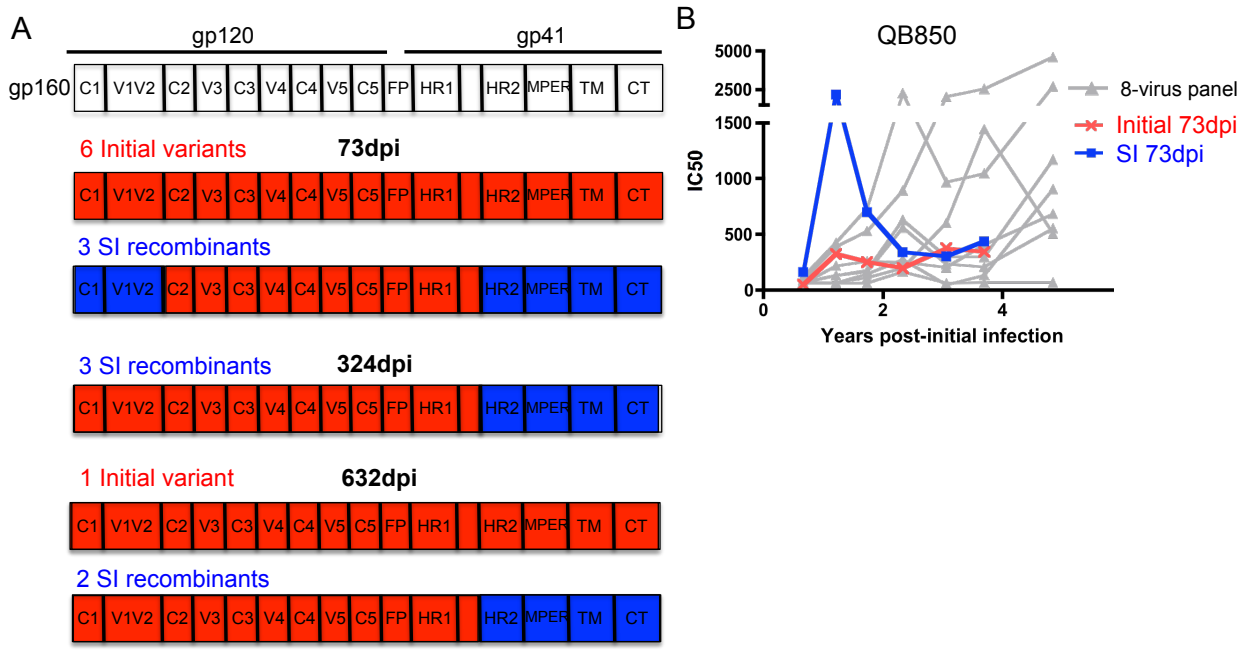

Figure 3.6 Autologous clones from QB850 isolated and tested longitudinally for neutralization
(A) Schematic of the initial and SI recombinants isolated from QB850 plasma collected 73 dpi, 324 dpi, and 632 dpi. (B) Temporal relation of neutralization of the initial and SI recombinants from 73dpi, represented here as the geometric mean, as compared to the development of cross-reactive responses against the 8-virus heterologous panel.

Table 3.2 Longitudinal neutralization of QB850 autologous clones

	Clone:	73p.A1	73p.B1	73p.F1	73p.C14	73p.E3	73p.G5	73p.I15	73p.J18	73p.K12	324p.A4	324p.B1	324p.D2	632.A1	632.C3	632.D4
ID	**DPI**	**D/A/D**	**D/A/D**	**D/A/D**	**A**	**A**	**A**	**A**	**A**	**A**	**A/D**	**A/D**	**A/D**	**A**	**A/D**	**A/D**
QB850	240	133	178	194	<1:100	<1:100	<1:100	<1:100	<1:100	<1:100	-	-	-	-	-	-
QB850	324	115	126	163	<1:100	<1:100	<1:100	103	<1:100	<1:100	88	67	75	-	-	-
QB850	443	2251	1895	2481	1020	106	235	617	154	238	262	194	<1:100	-	-	-
QB850	632	673	671	764	552	117	198	279	268	201	494	284	235	82	<1:100	<1:100
QB850	850	298	373	361	468	85	84	189	288	96	372	204	128	74	<1:100	<1:100
QB850	1117	269	253	416	1087	132	227	189	281	<1:100	837	318	266	203	139	1725
QB850	1768	473	439	412	764	156	317	232	271	149	877	478	439	444	394	2760

Light blue (101-300)
Medium blue (301-500)
Dark blue (>501)

Three autologous clones from 73 dpi that were of varying neutralization sensitivities to longitudinal autologous plasma were chosen to generate N332A and N301A mutations: 73p.A1 (SI recombinant, sensitive), 73p.C14 (initial variant, sensitive), and 73p.E3 (initial variant, resistant). The removal of the N-linked glycans at positions 332 and 301 from the 3 autologous clones abrogated neutralization by PGT128 (data not shown), but a decrease in neutralization was also observed with N332A mutants when using VRC01 as a control (Figure 3.7A), similar to what was observed in the backgrounds of heterologous viruses Q23 and DU156 (Figure 3.2B). In contrast, the N301A mutation had no effect on VRC01's neutralization in the background of 73p.C14, but caused neutralization enhancement for mutants in the backgrounds of 73p.A1 and 73p.E3. Using plasma from QB850 collected 5 years post-initial infection (1,705dpSI), a similar enhancement of neutralization was seen with 73p.E3.N301A, suggesting that this mutation causes the virus to become globally sensitive to neutralization. QB850's neutralization was unaffected by the N332A in the background of 73p.E3 and only slightly reduced in the background of 73p.A1 (Figure 3.7B). N301A and N332A mutations in the background of 73p.C14 as well as the N301A mutation in 73p.A1 caused close to 3-fold reductions in the IC50. Given that neutralization was not completely abrogated upon introducing these N332A mutations suggests that V3 glycan-specific Nabs may only constitute a minority fraction of QB850's overall Nab response.

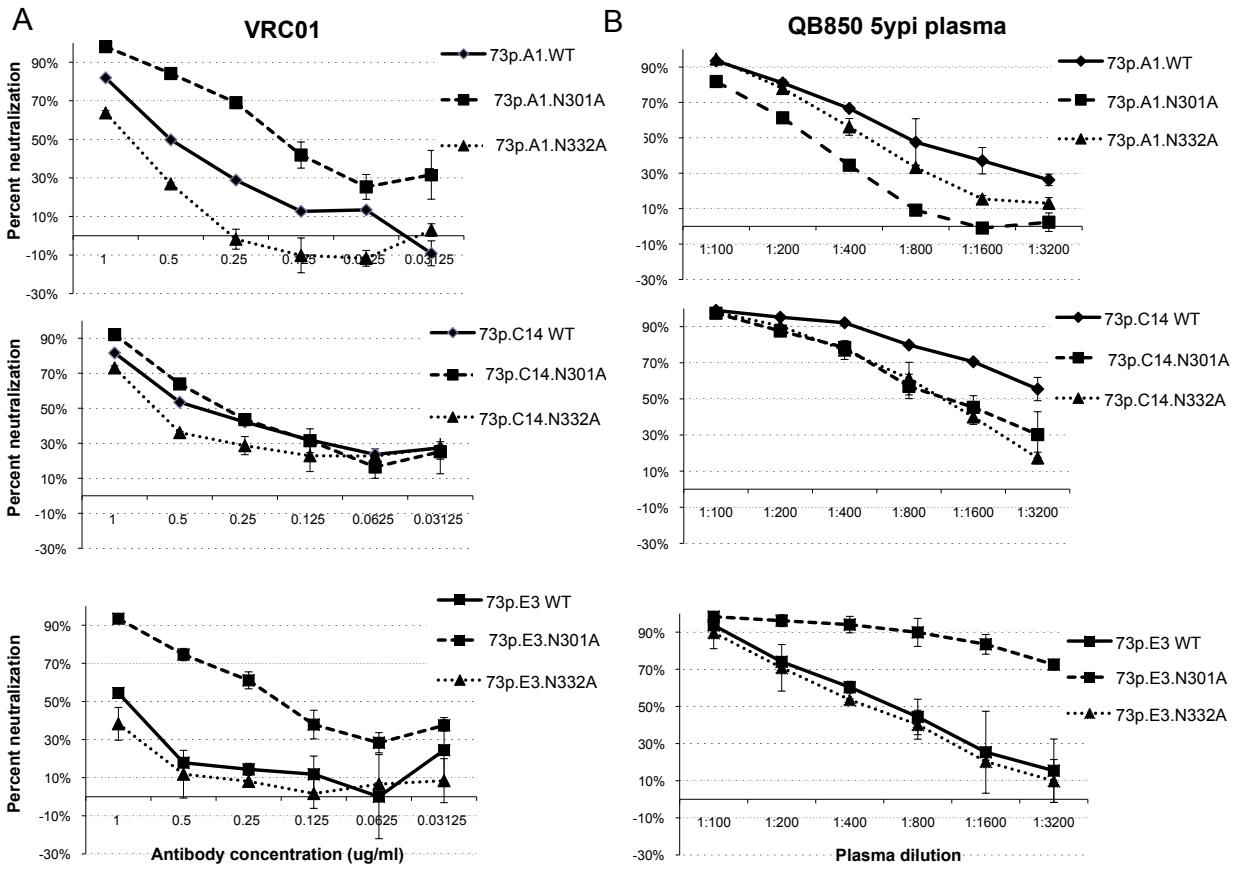

Figure 3.7 Probing for N332 and N302 glycan-specific Nabs using autologous clones from QB850
(A) Neutralization curves for VRC01 and QB850 5ypi plasma (B) tested against wildtype viruses (solid), alongside N332A (dashed triangle) and N301A (dashed square) mutants.

Discussion

Designing immunogens to elicit a broad and potent Nab response that protects against diverse HIV-1 variants remains the current focus in the vaccine field. The most recent advances in this area of study have stemmed from isolating and characterizing the epitope targets of bNabs from chronically infected individuals, which have aided in developing a template for vaccine design. We have shown both here and in Chapter II that SI individuals develop broad and potent Nab responses, and therefore may provide insight to how these responses develop. Before this study, it was unclear whether exposure to diverse HIV-1 antigens following SI would focus the Nab responses on the four known epitopes on the HIV-1 Envelope frequently targeted by singly

infected individuals or if their response would be more polyclonal in nature. Both standard and computational approaches used in this study suggest that the Nab breadth that develops following SI is not due to a dominant Nab response of known specificity, illustrating that a broad and potent antibody response can develop without strongly targeting any one of these four conserved epitopes.

Experiments probing for CD4-binding site-specific antibodies demonstrated that one individual, QA013, developed binding, but not neutralizing antibodies to this site. Also, functional assays using the HIV-2/HIV-1 MPER chimera suggested that 4 individuals (QA013, QD022, QG262, QF441) showed some MPER reactivity. However, closer examination with MPER peptides used in competition neutralization assays with HIV-1 variants Q461d1 and DU156 did not affect neutralization, suggesting that while this specificity may be present in whole plasma, it is unlikely that it is significantly contributing to the patients' ability to neutralize diverse HIV-1 isolates. One potential caveat to these experiments is that only a single MPER peptide matching the subtype B HIV-1 variant YU-2 was used. It is possible that using autologous MPER peptides to absorb MPER-specific antibodies would have resulted in a reduction in neutralization activity. However, computational analyses supported these functional assays, as the results suggested that most of these women's responses could not be assigned to any known specificity with high confidence. While previous reports have shown that plasma delineation analyses are concordant with standard mapping techniques with patients who have a single virus infection (68, 167), the one SI case described here (QB850) that was strongly predicted to have PGT-like antibodies targeting V3 glycans could not be fully confirmed by additional functional assays using autologous viruses. This discrepancy could be explained by the fact that antibody delineation analyses have so far only been validated with patients infected

with HIV-1 subtypes C and B (68, 167). The SI cases described here were primarily infected with subtype A, which could also explain the low level of confidence in the predictions for many of the women for which a specificity could not be determined.

We observed variable levels of background with the assays used to define the CD4-binding site, V1/V2 glycan, V3 glycan and MPER specificities. The CD4-binding site assays using the RSC3 WT and mutant proteins had very little non-specific background effects and thus yielded the clearest assessment. In contrast, the assays for glycan specificities displayed considerably high levels of background, indicating that these specificities may be harder to delineate with functional assays expect in cases where they dominate the antibody response. Minor Nab fractions that map to these specificities may be harder to distinguish with these assays, as was potentially observed with the additional mapping with QB850, where removing N-linked glycans from only 2 of the 3 autologous viruses resulted in a significant decrease shift in neutralization. Overall, it would appear that the initial variants were not the main focus of the Nab response, as evidenced by their moderate neutralization sensitivity (average IC50s ~300) even with plasma from later time points (1,117 and 1,768 dpi). Also, there was no evidence that the clones isolated later after initial infection changed in their neutralization sensitivity to PGT128 (data not shown), and all clones contained both N332 and N301 residues, suggesting a lack of PGT-like antibody escape.

It is interesting to note that the high levels of background observed using VRC01 as a control in assays probing for V1/V2 and V3 glycan-specificities is the first to be reported in an epitope mapping study. However, it has been suggested that the CD4-binding site epitope may overlap with the V3 base epitope cluster (85), potentially explaining the reduction in VRC01's ability to neutralize N332A mutants (Figure 3.2B). Also, removal of the N301 glycan has

previously been shown to cause a global enhancement of neutralization both to pooled plasma and monoclonals (172, 173). Although the PG9 epitope involving the glycan at position 160 is thought to be completely distinct from the CD4-binding site (85), it is possible that the N160 mutation affects the quaternary nature of the CD4-binding site epitope in a context-dependent manner, and could thus explain VRC01's higher background with Q23 in comparison to DU156 (174). To avoid the potential of misclassifying an individual's antibody specificity, a 3-fold cutoff was applied in experiments testing mutant variants vs. wildtype viruses. As a result, this decreased our ability to observe smaller fractions of the Nab response that may target these regions. However, even if a 2-fold cutoff had been used, this would have only changed the results for a few individuals, and none were comparable to the abrogation of neutralization observed with PGT-128. Since our primary question was to determine if any of the four main regions of Envelope vulnerability dominated the Nab responses that developed in this cohort of women post-SI, this conservative approach did not impede our ability to draw conclusions from this study.

In conclusion, we were unable to identify a single epitope specificity that predominated the Nab responses in 21 cases of SI, which may suggest that many more epitope targets can mediate a broad and potent response, and that future immunogen design may not have to be limited to just the 4 known sites. These data suggest that the Nab responses that develop post-SI are likely mediated by a robust polyclonal response, which in some cases like QB850 may include one of these 4 known sites, or that they target unknown epitopes that will need to be identified. Deep molecular and functional characterization of the virus-antibody interplay in these superinfected individuals may help pave a new way for a vaccine that elicits a broad and

potent polyclonal response, a method that could prove to be more feasible with current vaccination technologies.

Chapter IV

Isolating monoclonal antibodies from an HIV-1 superinfected woman

Introduction

Characterizing the epitope targets and ontogeny of broadly cross-reactive Nabs isolated from HIV-infected individuals has greatly informed vaccine design, and more recent longitudinal analyses of the development of these patients' responses has provided a potential 'roadmap' to follow with an immunization strategy (154). Prior to 2009, there were only a handful of antibodies capable of moderate cross-subtype neutralization, but advances in B cell isolating and antibody cloning techniques have led to the recent explosion of newly identified monoclonals with extraordinary broad and potent activity (82, 115). Two approaches have been largely used to support this endeavor. The first approach has utilized a number of different "baits" to tag HIV-specific B cells, including resurfaced gp120 probes that have been engineered to display only certain epitopes, such as the CD4-binding site (83, 120), recombinant gp140 protein (175, 176), virus-like particles (177, 178), as well as transiently transfected cells expressing Envelope protein (179). A second approach involves culturing all IgG+ B cells from a PBMC sample to produce antibodies that can be screened for HIV-1 specificity and functionality (68, 118, 119, 153). This latter approach, although costly and labor-intensive, is incredibly feasible with the use of high-throughput liquid handling. While neither approach guarantees capturing the entire antibody repertoire in a given sample, the bait method may be favorable if the patient sera primarily targets a single epitope. In contrast, if the patient's serum targets either unknown epitopes that are not easily defined or perhaps more than one epitope, using the bait method may be too selective, as it could limit the ability to capture key monoclonals responsible for mediating

the overall Nab response. However, culturing the IgG+ B cell population from PBMCs is not without limitations, as the lengthy process can be riddled with potential pitfalls at each step, and is highly dependent on sample viability.

Thus far, the most broad and potent monoclonal antibodies isolated by these methods display a number of unusual features that could make them hard to elicit with current vaccination technologies. As discussed in Chapter I, many of the antibodies are highly somatically mutated and have insertions and deletions that are not typically found in antibodies that develop in response to vaccination or to other pathogens (84). It is generally thought that these modifications arise after years of antigen stimulation, which is supported by the fact that many of these antibodies were cloned from chronically-infected patients (154, 180, 181). For example, VRC01, the prototypic CD4-binding site-directed antibody, was not isolated until ~15 years following initial infection from a slow progressor (120). Other monoclonals arose from rare germline precursors (91), which may make them hard to stimulate in naïve individuals. Recognizing these potential barriers, there has been a new focus to identify patients who develop cross-reactive responses by a more direct pathway, with the idea that the Nabs elicited in shorter amounts of time may not exhibit such unusual characteristics.

We previously demonstrated in Chapter II that SI women develop broader and more portent responses than singly infected individuals. Further examination of longitudinal samples from elite neutralizer, QB850, showed that she developed cross-subtype breadth within ~1.2 years following initial infection, which is faster than what is typically seen in cohorts of singly infected individuals with broad and potent responses (71, 72, 74, 146). Since QB850 was superinfected approximately 2 months post-initial infection, this cross-reactivity would have developed within only 1 year following SI. To characterize the Nabs from this patient and

determine whether they exhibit the same unusual characteristics as other HIV-specific monoclonals, we sought to isolate and clone individual antibodies for closer examination. Since we previously showed in Chapter III that QB850's Nab response could not map to any of the four main epitope targets on Envelope, we decided to culture all IgG+ B cells from a PBMC sample collected ~2 years following initial infection (738dpi), a time point at which QB850 could neutralize viruses from 4 different subtypes (Figure 2.5). The following describes the group of monoclonals isolated from this time point and more broadly explains the B cell culture and cloning methods adapted in our laboratory, schematized in Figure 4.1.

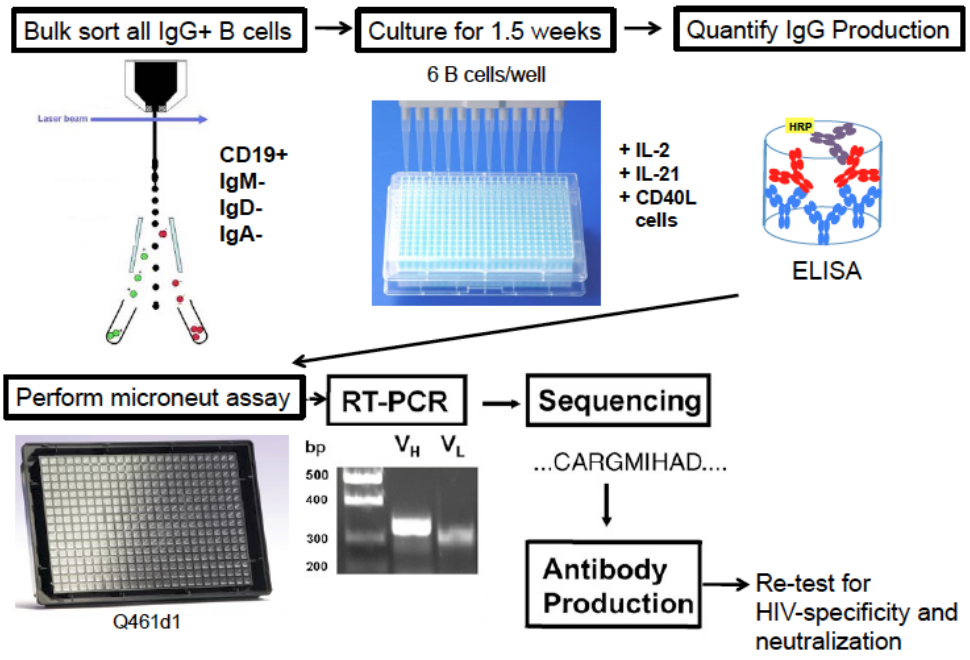

Figure 4.1 B cell isolation and cloning pipeline
Adapted from (177), this schematic shows the process by which individual monoclonal antibodies can be cloned from PBMCs.

Materials and Methods

B cell isolation and culture

Staining and single-cell sorting of B cells was performed as previously described with a few modifications (68, 182). CD19+ B cells were first enriched by negative depletion using magnetic particles coated with antibody complexes recognizing CD2, CD3, CD14, CD16, CD36, CD43, CD56, CD66b, glycophorin A and dextran according to the manufacturer's instructions (StemCell) before they were stained using a cocktail of anti-CD19–PE–Cy7 (BD Bioscience), IgA–APC (Jackson ImmunoResearch Laboratories), IgD–FITC (BD Pharmingen) and IgM–PE (Jackson ImmunoResearch Laboratories) antibodies at 4 °C in the dark for 30 min. The cells were washed with 1.5 ml FACS wash (2% FBS in PBS) and re-suspended in 1 ml FACS wash. Cells were sorted based on gates drawn for $CD19^{+}IgA^{-}IgD^{-}IgM^{-}$ B cells and sorted into media using a FACSAria III cell sorter (Becton Dickinson). Collected cells were further diluted to a density of ~144/ml in IMDM medium (Gibco) with 10% FBS containing MycoZap Plus-PR (Lonza), 100 U/ml IL-2 (Roche), 50 ng/ml IL-21 (Invitrogen) and 1×10^5/ml irradiated 3T3-msCD40L feeder cells. B cells were seeded onto 384-well plate in 55 µl/well to yield approximately 6 cells/well. One row without B cells was also plated as a negative control for downstream assays.

After 10 days in culture, 10 ul of supernatant was removed from a quarter of wells from one plate and tested for antibody content by total IgG ELISA at a 1:10 dilution (Immunology Consultants Laboratory). Plates were harvested after 12 days by collecting 40 ul of culture supernatants and lysing cells with 20 µl lysis buffer containing 0.25 µl of RNase inhibitor (New England Biolabs), 0.3 µl of 1 M Tris pH 8 and 19.45 µl DEPC-treated H_2O and stored immediately. All plates were stored at −80 °C.

Neutralization assays and ELISA

B cell supernatants were screened for neutralization by the TZM-bl assay as described in Chapter II, with a few modifications for the 384-well format. Briefly, 20 ul of supernatant was pre-incubated with 20 ul of virus (150 infectious particles/well, Q461d1) for one hour before the addition of 10 ul TZMs (3,000 cells/well). Infection levels were determined by B-galactosidase activity after 48hrs (Britelite Plus, PerkinElmer). Plates were tested in replicate experiments, with exception to portions of three plates where only one neutralization assay was performed due to limited sample availability as a result of screening those subset of wells by ELISA at a 1:10 starting dilution for HIV-1 specificity using Q461d1 gp140 (kindly provided by Nancy Haigwood) as described in the methods section Chapter III. Wells were selected for antibody cloning if the supernatant displayed >50% neutralization in either replicate, showed >40% neutralization across both replicates, or were reactive against gp140 for a subset tested. Monoclonal antibodies cloned from these wells were later screened for neutralization in the standard 96-well TZM-bl assay as described in Chapter II.

Cloning and production of monoclonal antibodies

The variable region of the heavy chain and the light chain immunoglobulin genes were amplified by RT–PCR as previously described (83, 183) and from personal communications with Nicole Doria-Rose and Xueling Wu. Primer sets used for the heavy chain are summarized in Table 4.1, and primer sets used for the light chain have been previously described in Tiller et al. (183). Briefly, 10ul of lysed B cell culture supernatant was used to generate cDNA using Superscript III with random hexamers (Invitrogen), which provided templates for three independent semi-nested PCRs to amplify the Igγ variable genes and two independent nested PCRs to amplify Igκ and Igλ variable genes. Products from positive PCR reactions were

sequenced before cloning into the corresponding Igγ1, Igκ and Igλ expression vectors (kindly provided by Michel Nussenzweig). For wells that either amplified both Igκ and Igλ gene fragments or where clean sequence was not obtained, TOPO TA cloning (Invitrogen) was employed using bulk PCR products. Cloned products were verified by sequencing. Paired heavy and light chain plasmids cloned from the same well were combined in all possible pairings if there was more than one heavy or light chain isolated and co-transfected into 293T cells using Fugene-6 (Roche) with a 1:1 ratio of plasmid DNA for each chain (1.5 ug total/well in 6-well plate). Full-length IgG produced after 2 days was purified using Protein-A columns (Pierce). Final sequences were characterized by gene family, percent mutated from germline and CDR3 length using the IMGT database (www.imgt.org).

Table 4.1 First and second found primers used for amplifying variable heavy gene fragments

First round heavy chain primer mix G1: anneal at 54°C		
pool, 50 uM	VH1 LEADER-A	ATGGACTGGACCTGGAGGAT
	VH1 LEADER-B	ATGGACTGGACCTGGAGCAT
	VH1 LEADER-C	ATGGACTGGACCTGGAGAAT
	VH1 LEADER-D	GGTTCCTCTTTGTGGTGGC
	VH1 LEADER-E	ATGGACTGGACCTGGAGGGT
	VH1-LEADER-F	ATGGACTGGATTTGGAGGAT
	VH1-LEADER-G	AGGTTCCTCTTTGTGGTGGCAG
single, 25 uM	3'Cg CH1	GGA AGG TGT GCA CGC CGC TGG TC
First round heavy chain primer mix G2: anneal at 48°C		
pool, 50 uM	VH3 LEADER-A	TAAAAGGTGTCCAGTGT
	VH3 LEADER-B	TAAGAGGTGTCCAGTGT
	VH3 LEADER-C	TAGAAGGTGTCCAGTGT
	VH3 LEADER-E	TACAAGGTGTCCAGTGT
	VH3 LEADER-F	TTAAAGCTGTCCAGTGT
	VH4 LEADER-D	ATGAAACATCTGTGGTTCTT
	VH5 LEADER-A*	TTCTCCAAGGAGTCTGT
single, 25 uM	3'Cg CH1	GGA AGG TGT GCA CGC CGC TGG TC
First round heavy chain primer mix G3: anneal at 52°C		
pool, 50 uM	VH3 LEADER-D	GCTATTTTTAAAGGTGTCCAGTGT
	VH4 LEADER-A	ATGAAACACCTGTGGTTCTTCC
	VH4 LEADER-B	ATGAAACACCTGTGGTTCTT
	VH4 LEADER-C	ATGAAGCACCTGTGGTTCTT
	VH5 LEADER-B*	CCTCCACAGTGAGAGTCTG
	VH6 LEADER-A	ATGTCTGTCTCCTTCCTCATC
	VH7 LEADER-A	GGCAGCAGCAACAGGTGCCCA
single, 25 uM	3'Cg CH1	GGA AGG TGT GCA CGC CGC TGG TC
Second round heavy chain primer mix: anneal at 58°C		
pool, 25 uM	5'L-VH 1	ACA GGT GCC CAC TCC CAG GTG CAG
	5'L-VH 3	AAG GTG TCC AGT GTG ARG TGC AG
	5'L-VH 4/6	CCC AGA TGG GTC CTG TCC CAG GTG CAG
	5'L-VH 5	CAA GGA GTC TGT TCC GAG GTG CAG
pool, 25 uM	5xwL-VH1	GCA GCC ACA GGT GCC CAC TCC
	5xwL-VH1-24	CAG CAG CTA CAG GCA CCC ACG C
	5xwL-VH1-69	GGC AGC AGC TAC AGG TGT CCA GTC C
	VH3-L1-MP	GCT ATT TTA AAA GGT GTC CAA TGT
	VH3/4-L1-MP	GTG GCA GCT CCC AGA TGG GTC CTG TC
	VH3/4-L3-MP	GTT GCA GTT TTA AAA GGT GTC CAG TG
	VH5-L1-MP	GCT GTT CTC CAA GGA GTC TGT TCC
single, 25 uM	3'IgGint	GTT CGG GGA AGT AGT CCT TGA C

*Use 2X in primer pool.

Results

Single-cell isolation and culture of IgG+ B cells

In Chapter II, we showed that QB850 developed cross-reactive Nabs after ~1.2 years post-initial infection, which incrementally broadened over the next 4 years (Figure 2.5). To capture monoclonals from a time at which her Nab breadth began to develop, B cells were sorted from a PBMC sample obtained 738dpi or (~2 years post-initial infection). From 6 million viable PBMCs, CD19+ enriched cells were sorted by negative staining for IgD, IgA and IgM and yielded 15,000 IgG+ B cells (~7.7% of the total viable CD19+ B cell population) (Figure 4.2). Culturing at a density of 6 cells/well, this amounted to 8.5 plates. After 10 days in culture, quantification of total IgG levels from a subset of B cell culture supernatants showed that 48 out of 88 wells (55%) were positive for IgG (range 20-2000 ng/ml), with 16 our of 48 wells (33%) overall generating concentrations above 100ng/ml.

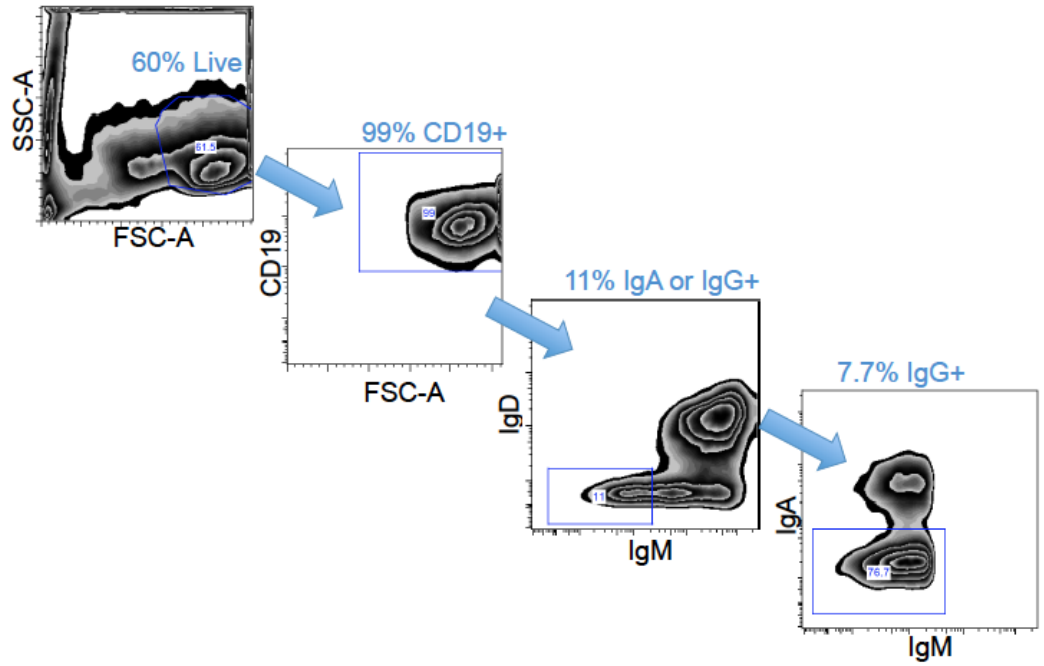

Figure 4.2 Gating strategy to isolate IgG+ B cells from QB850 at 738dpi
Cells were first gated for live cells by FCS-A/SSC-A, and then positive selection for CD19 followed by negative selection for IgD, IgM and IgA.

Identifying wells containing HIV-specific B cells

To identify B cell culture wells that had generated HIV-specific antibodies, all plates were initially screened by microneutralization against a subtype A virus that is sensitive to neutralization (Q461d1) (131). There was a wide range of neutralization observed across plates (0 to 100%), with minimal amounts of background, as control wells lacking B cells neutralized between 0 and 15%. Using a cut-off of 50% neutralization, 3 candidate wells were identified for cloning (Pl6.B20, 100%; Pl6.M20, 100%; Pl5.O20, 52%). To confirm whether these 3 wells would also demonstrate HIV specificity by ELISA, supernatants from these wells were tested alongside a subset of the surrounding wells for Q461d1 gp140 reactivity. Culture supernatant from 1 of the 3 neutralization positive wells (Pl5.O20) was able to bind to gp140. Five out of the 352 additional wells from which neutralization was not previously evident also showed gp140 reactivity (summarized in Table 4.2). A second round of replicate microneutralization assays were used to identify additional wells that showed lower but repeatable neutralization activity across experiments. Given the variability of these data, a positive hit was defined as having >30% neutralization in both experiments, which resulted in 3 additional candidate wells for cloning (Table 4.2).

Table 4.2 Candidate wells cloned from QB850 738dpi

	Well name:	Pl6.B20*	Pl6.M20	Pl5.O20	Pl2.B8	Pl2.D7	Pl2.G11*	Pl2.H11*	Pl5.K12*	Pl8.D13*	Pl8.D16*	Pl8.G10
	Identified by	uNeut	uNeut	uNeut +ELISA	ELISA	ELISA	ELISA	ELISA	ELISA	Double hit uNeut	Double hit uNeut	Double hit uNeut
	% neut and/or OD value	100%, NR	100%, NR	52%, 0.131	0.191	0.141	0.220	1.35	0.185	32%, 34%	35%, 45%	30%, 46%
Clone name	**Igγ**	B20	Did not amplify	O20	B8	D7	G11	H11.1 H11.2 H11.3	K12	D13	D16.1 D16.2	Did not amplify
	Igκ	B20	M20	O20	B8	-	G11.1 G11.2	H11.1 H11.2 H11.3	K12	D13	D16.1	Did not amplify
	Igλ	B20	M20	-	-	D7	-	H11.1	K12	D13	-	Did not amplify

*Topo TA cloned

uNeut, microneutralization assay; OD, optical density; NR, non-reactive against Q461d1 gp140

Monoclonal generation and molecular characterization.

IgG heavy and light chain variable regions were amplified using 5 separate round 1 PCRs—3 for the heavy chain and 1 for each of the light chains—before round 2 PCRs were performed. For 2 of the 11 candidate wells, one or both chains did not amplify (Pl6.M20 and Pl8.G10) (Table 4.2). Of the remaining 9 wells, 7 required TOPO TA cloning as 5 wells amplified both Igκ and Igλ variable genes and 2 others did not generate clean sequence from round 2 PCR product.

In total, 12 variable heavy and 16 variable light gene fragments from 9 wells were cloned into full-length antibody vectors. IMGT query results for antibody gene family, percent identify with putative germline, and CDR3 length are summarized in Table 4.3. A single gene family did not dominate either group of heavy or light chain clones. Also antibodies that shared a variable heavy gene family did not appear to be clonally related, as light chain genes amplified from their respective wells were not from the same family. Percent identity with the putative germline sequence was high for both antibody chains, ranging from 81-96% for variable heavy and 88-97% for variable light. CDRH3 regions ranged between 9 and 19 nucleotides in length.

Table 4.3 Monoclonal antibody variable gene characteristics

	Heavy Chain				Light Chain		
Well	Clone	Family	% Identity	CDR3 length	Clone	Family	% Identity
Pl2.B8	γB8	IGHV1-69*04	89	12	κB8	IGKV1-39*01	92
Pl2.G11	γG11	IGHV1-69*06	90	18	κG11.1	IGKV4-1*01	97
					κG11.2	IGKV1-39*01	96
Pl2.H11	γH11.1	IGHV1-69*06	89	19	κH11.1	IGKV3-15*01	97
	γH11.2	IGHV3-33*01	94	18	κH11.2	IGKV1-39*01	90
	γH11.3	IGHV4-39*01		14	κH11.3	IGKV3-15*01	93
					λH11.1	IGVL1-47*01	94
Pl5.K12	γK12	IGHV4-4*07	94	15	κK12	IGKV3-11*01	94
					λK12	IGLV1-40*01	96
Pl5.O20	γO20	IGHV4-39*01	86	14	κO20	IGKV1-9*01	88
Pl6.B20	γB20	IGHV3-7*03	89	13	κB20	IGKV3-11*01	95
					λB20	IGVL2-14*01	90
Pl8.D7	γD7	IGHV3-23*01	92	16	λD7	IGVL2-14*01	92
Pl8.D13	γD13	IGHV4-34*02	96	17	κD13	IGKV4-1*01	97
					λD13	IGVL2-14*01	96
Pl8.D16	γD16.1	IGHV1-2*02	89	9	κD16	IGKV1-29*39	91
	γD16.2	IGHV3-15*02	93	19			

Functional characterization of monoclonal Nabs

Each combination of paired heavy and light chain genes cloned from a single well was produced in culture, generating 25 monoclonal antibodies. Small-scale transfections of 293T cells produced antibody concentrations between 8 and 500ug/ml. All antibody pairings were first tested for neutralization against the neutralization sensitive HIV-1 variant Q461d1. The majority of the monoclonals were unable to neutralize the virus at the highest concentration tested (50ug/ml or neat if <50ug/ml) (Figure 4.3A). Only one antibody (γG11/kG11.2) was able to neutralize Q461d1 (IC50=0.64ug/ml), and further testing with other HIV-1 variants revealed that the breadth of this antibody was limited to this single isolate (Figure 4.3B). However, γG11/κG11.2's neutralization activity appeared to be HIV-specific as SIV was not neutralized.

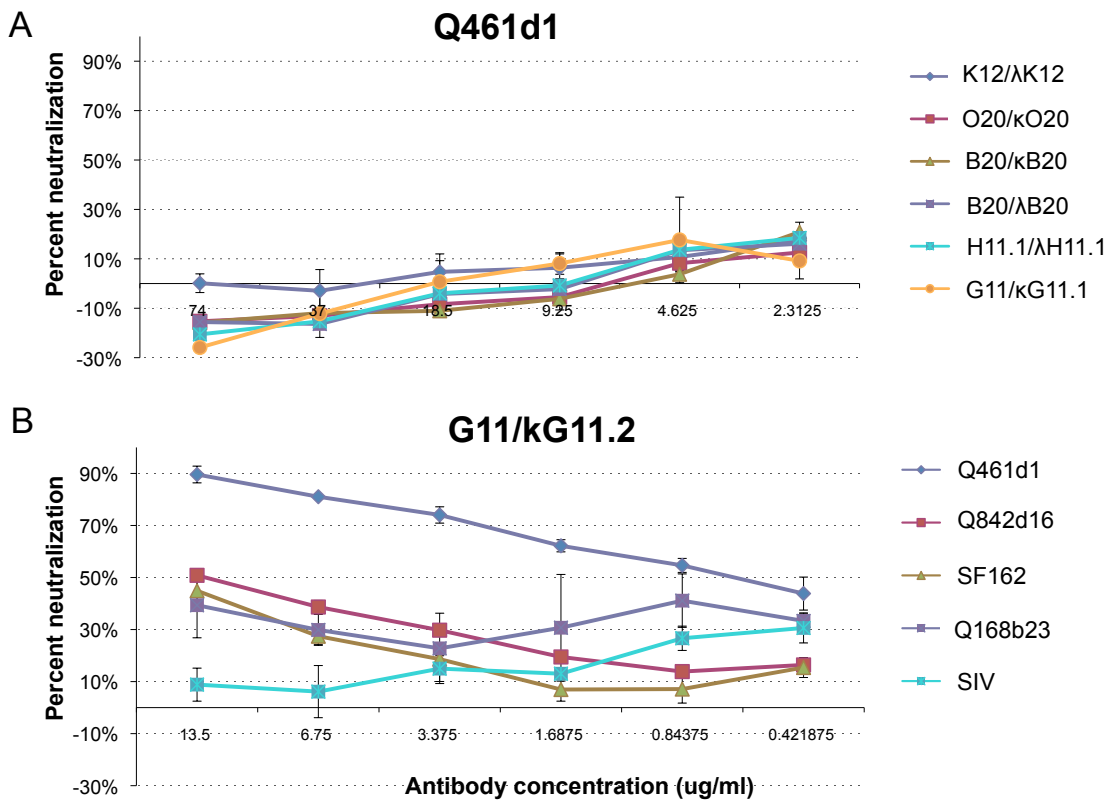

Figure 4.3 Neutralization activity of monoclonal antibodies isolated from QB850 738dpi. (A) Representative data of the initial screen testing against Q461d1 showed no neutralization for most Mabs. (B) γG11/κG11.2 displayed neutralization against Q461d1, but not other isolates or against negative control, SIV.

Discussion

We isolated 12 variable heavy and 16 variable light antibody genes from 9 B cell culture wells that were sorted from PBMCs collected 738 days post-initial infection from SI case QB850. This time point represented the start of this patient's incremental development of breadth over the course of 4 years following SI (163). Only 1 of the 25 antibody pairings made by combining the heavy and light chains produced an antibody that was capable of neutralization. This single antibody (γG11/κG11.2) was able to neutralize 1 of the 6 HIV-1 variants tested with an IC50 of 0.64ug/ml, suggesting it may have been strain-specific. These data demonstrate that the monoclonals isolated in this B cell sort did not recapitulate the neutralizing activity previously observed with QB850's plasma in Chapter II.

A number of potential pitfalls may have occurred throughout the sorting process that could explain the limited success in isolating monoclonals capable of neutralization, and should be considered for future sorts. First, only half as many IgG+ B cells (15,000 cells) were isolated than was expected from the 6 million viable PBMCs. Since typical B cell populations constitute ~10% of total circulating PBMCs and 5% will be IgG+, we would have expected to capture ~30,000 B cells. While this could be attributed to normal person-to-person variability, it is also possible that cells were lost during the magnetic bead separation process or during the multiple wash steps following separation. Despite this smaller starting population of IgG+ cells, the culturing process was fairly effective, with 33% of the wells with 6 cells/well producing >100ng/ml of IgG after 10 days in culture. According to Huang et al., ~60% of wells plated at 2.5 cells/well would be expected to produce >100ng/ml of IgG after 13 days, but it should be noted that these data are highly dependent on the condition and viability of the PBMC sample (182). An additional caveat is that plating 6 cells/well could have decreased the certainty that the correct heavy and light chain pairings were obtained. However, given that the cultures produced levels of IgG that would be expected for a lower seed density, and previous experiments seeding cells at a lower density (2 cells/well) from PBMC samples from other individuals from the same Mombasa cohort resulted in fewer IgG+ wells after 13 days, it is likely that the number of cells that survive and proliferate is on par with previous studies using more viable samples. Finally, our resolution for identifying HIV-specific B cell culture wells was low, as we observed wide ranges of neutralization across plates and little consistency in neutralization data across experiments. This assay would be greatly improved by a more streamlined process using high-throughput liquid handling.

It is interesting to note that we identified almost equal numbers of candidate wells by screening a small subset of wells by ELISA (n=5) as compared to screening all the plates by microneutralization (n=6). ELISA activity, and not microneutralization, was what led to the identification of the one antibody capable of neutralization (γG11/κG11.2). Developing a micro-ELISA may be worth considering for future experiments, as these assays would capture binding antibodies that may not only neutralize, but could also exhibit other antibody-mediated functions such as ADCC and ADCVI.

A number of monoclonal antibodies were recently isolated by B cell culture from CAP256, a South African patient who became superinfected 15 weeks following initial-infection and subsequently developed a similarly broad response as QB850 (72, 122). Examination of this patient's virus-antibody evolution revealed that the autologous superinfecting virus escaped Nab pressure and in the process, drove the development of cross-subtype Nabs after 1 year post-initial infection that targeted overlapping epitopes in V1/V2 involving the glycan at position 160 and residue K169 (65, 165). Unlike our findings, the isolated monoclonals from CAP256 collectively recapitulated the overall Nab response observed with whole sera (68). While no individual monoclonal was particularly broad, some were fairly potent against certain isolates and importantly, CAP256's antibodies were not highly mutated (68). However, all were derived from rare germline precursors (68). The virus-antibody dynamics in QB850 would appear to be more complex, as described at the end of Chapter III. Longitudinal single genome amplification from her plasma showed that the superinfecting virus quickly recombined with the initial virus within ~1.5 weeks following superinfection, and only recombinants were detected at subsequent time points (Figure 3.5A) (33). Similar rapid recombination of both SI and initial strains was also seen in CAP256 (65, 68, 165). However, CAP256's broad and potent Nab responses were solely

generated by antigenic stimulation from the SI virus, and not the initial virus (65, 68). In contrast, autologous mapping studies using chimeric viruses of the SI and initial viruses did not identify particular residues on either virus that were critically involved with QB850's broad response (data not shown). Without a suitable bait to attempt a more directed approach at identifying Nabs from QB850, it is likely that several more PBMC samples from other time points will be needed to isolate the Nabs mediating her overall response. Indeed, multiple samples were sorted before the most recent MPER-specific antibody, 10E8 (153), was identified (personal communication with Nicole Doria-Rose). In addition, deep sequencing of the antibody repertoire from the same time point from which the monoclonal Nab VRC01 was isolated did not produce its cognate variable heavy gene sequence, perhaps best highlighting the needle in the haystack nature of these endeavors (184).

In summary, we sought to identify the monoclonal Nabs responsible for QB850's broad and potent response by isolating and cloning individual antibody gene fragments from B cell cultures. While some steps of this approach proved successful, others will require additional optimization, potentially in the form of upgrading to high-throughput liquid handling in order to fine tune the resolution by which we can identify candidate HIV-specific B cell wells for cloning. Identification of these monoclonal Nabs will ultimately help resolve the neutralization targets from this patient, which could lead to the identification of new epitopes on Envelope and potentially a novel template for eliciting a broad and potent response using genetically diverse antigens.

Chapter V

Gaining insight to the development of an effective Nab-based HIV-1 vaccine

A broad and potent Nab response will likely be a necessary component for the development of a protective HIV-1 vaccine, but Phase IIb/III clinical trials thus far have failed to elicit such responses. To increase our understanding of the development of Nabs in response to HIV-1 during natural infection, the three studies in this thesis investigated one potential mechanism by which Nab breadth and potency develops. Beginning in Chapter II, we demonstrated that SI broadens and strengthens the Nab response, providing support for SI as a relevant model for sequential immunization strategies. Second, in Chapter III we showed that superinfected individuals' Nab responses do not focus on the four main conserved regions on Envelope targeted by the current collection of bNabs, suggesting that vaccine design efforts may not be limited to this small group of occluded and in some cases, conformationally masked epitopes (155). Third, preliminary efforts to isolate and characterize monoclonal antibodies that are elicited following SI were initiated in Chapter IV, but will need to be expanded in order to define the individual epitope targets that constitute the overall Nab response. Together, these studies support the prospect of a feasible template for vaccine design that mimics SI with the continued investigation of the virus-antibody evolution in superinfected individuals. The following chapter will outline avenues of research working towards this goal, while considering outstanding questions in the HIV-1 vaccine field.

Delineating the factors that drive Nab breadth and potency following SI

In Chapter II we showed that superinfected cases developed broader and more potent responses than singly infected controls. This initial discovery led to considering both how and why these responses arose, as these answers may help to inform vaccine design. SI individuals experience an increase in viral diversity, particularly in cases where the superinfecting virus is of a different subtype. This additional antigenic stimulation could be the critical driver of the development of a broader Nab response. In support of this concept, we showed that the two individuals with the broadest and most potent responses in the cohort were cases of intersubtype SI. However, this is likely only half the story since 3 of the 12 SI cases studied in Chapter II did not develop stronger responses compared to their matched controls, including one individual who was superinfected with a virus from a different subtype. There was also one individual with a intersubtype SI from a cohort in Cameroon who did not show greater breadth and potency compared to controls (123). However, this Cameroonian study only contained 4 superinfected cases, making it hard to know whether this was merely a coincidence. We also had too few in our study to perform a formal analysis comparing the Nab responses between inter- versus intrasubtype cases. Still, the fact that a study of 10 intrasubtype C dually-infected individuals from South Africa did not detect significant differences between cases and controls, potentially suggests that the increased viral diversity resulting from intrasubtype SI may not be as efficient at eliciting broader and more potent responses (185). Together, these data suggest a role for diverse antigens enhancing the Nab response, but additional factors, such as timing and antigen context, should also be considered.

Given that the HIV-specific Nab repertoire evolves throughout the course of infection (57, 59, 146, 186-189), the timing at which the SI event occurs could impact the subsequent

development of breadth and potency. Indeed, among with 21 superinfected women studied in Chapter III, the individuals with the most potent Nab responses had all been superinfected within the first year following initial infection, suggesting there may be a window of time during which the Nab response can be strengthened. Studies have shown that there may be a limit to the amount of mutations Envelope can tolerate in order to escape Nabs (75, 190, 191), which could in turn limit the expansion of the overall response if SI occurs multiple years after initial infection. Perhaps more importantly, studies investigating the role of follicular helper CD4+ T cells in B cell responses show that they exhibit diminished function and capacity to provide adequate B cell help after years of chronic HIV-1 infection (192, 193). To date no study has examined this unique population of T cells in superinfected individuals, but it is likely that they could be playing a key role in the development of their Nab responses (194).

Analyzing virus evolution in superinfected cases has also led to a greater understanding of the path by which Nabs develop in response to two distinct viruses. Testing the longitudinal neutralization activity of plasma against initial and superinfecting early/transmitted viruses has shown that in a subset of cases from this cohort and others, that the superinfecting virus shifts the focus of the Nab response. Indeed, high titers against the SI virus developed and were sustained in the years following SI (43, 165, 195), whereas titers against the initial virus remained stagnant (165). From a vaccine perspective, this type of immune 'distraction' may not be as promising as a scenario where the immune response is 'built up' by sequential stimulations. This is in contrast to what was observed with QB850 in Chapter III, where the autologous Nab response in this patient was initially distracted from the initial variant after SI ~443dpi, but then the strain-specific response to the SI virus also diminished a little over a year later ~850 dpi (Figure 3.5B). These data may suggest that the virus evolution in QB850 re-focused the response in the years

following SI, with Nabs evolving to target the recombinant variants that dominate the quasispecies thereafter. The importance of recombination versus co-circulation or replacement of the initial virus as the dominant species on the development of the Nab response has not been formally explored. However, in a small study of 7 SI cases, Chaillon et al. observed one individual with the most potent autologous Nab response was also the only individual whose initial and superinfecting viruses recombined and become the dominate species in the years following SI (195). Further characterizing the virus evolution in the years following SI and testing additional isolates from these time points against longitudinal plasma from QB850 as well as other SI cases may help decipher the virus-antibody interplay that shaped the Nab response, and could potentially inform whether particular combinations of bivalent or polyvalent immunogens would be more likely to distract or enhance the Nab response.

Identifying new epitope targets

A more targeted approach for vaccine design involves eliciting Nabs to 1 or more of the 4 main epitopes defined by bNabs (90). While these bNabs could serve as templates for such a vaccine, it is unclear whether this would be feasible given their unusual characteristics outlined in Chapter I. In Chapter III, we showed that the broad and potent responses that develop following SI do not solely rely on these four regions on Envelope. These data are encouraging for two reasons—1) this may provide additional evidence of unknown epitopes on Envelope that mediate a broad and potent response and 2) this could mean that their responses target more diverse epitopes, which could be easier to mimic with current vaccination technologies.

A number of studies suggest there are unknown epitopes on Envelope that have yet to be defined (66, 83, 179, 196). Also, while the majority of singly infected individuals with broad and

potent responses target the 4 main regions on Envelope, these studies also show that a small subset cannot be defined (86-88, 142). A number of functional and computational techniques exist to define the epitope specificity of a given plasma samples (167), but the data generated are limited by only focusing on known epitopes as we showed in Chapter III, and may not be sensitive enough for cases where the Nab response is highly polyclonal (197). For these reasons, isolating monoclonals from SI patients with techniques adapted in Chapter IV will ultimately help to define the epitope specificities and confirm the polyclonal or monoclonal nature of their Nab responses.

Creating a vaccine to mimic natural infection

Many of the recent advances in the HIV-1 vaccine field have come from better understanding antibody-Envelope interactions, which have largely been facilitated by detailed structural analyses. Overall these studies have shown that in order to generate vaccine-elicited antibodies similar to the current collection of bNabs, a number of immunological barriers will need to be surmounted. First, multiple groups have shown that many of the unmutated germline precursors of these monoclonal antibodies are unable to bind to HIV-1 Envelope (198-201). It is therefore unclear what antigen first engaged these naïve B cell precursors, as it's possible that these cells pre-existed HIV-1 infection. This poses a significant hurdle for a vaccine, since immunogens will need to be specifically designed to initiate interactions with circulating naïve B cells expressing the germline precursors (202, 203). Second, navigating the immunodominate features of Envelope will require many iterations of structural analyses as well as immunization studies in animals to screen potential immunogens and ensure that Nabs and not just binding antibodies are elicited. Masking epitopes (204, 205) or using protein scaffolds (206, 207) to

focus the response could also be used to circumvent this issue, as well as identifying trimers that bind preferentially to Nabs instead of non-neutralizing antibodies (208).

More recent studies coupling the characterization of monoclonals with longitudinal deep sequencing of the antibody repertoire side-by-side with virus evolution has allowed two groups to demonstrate the feasibility of generating a 'B cell lineage vaccine' (67, 68). In essence, this vaccine design aims to guide the Nab response using Envelope immunogens that were present in the patient from which the monoclonals were isolated, including those that can engage the germline precursors (113). This approach would also be amenable to mimic SI, especially for cases like QB850 and QA013, where we have evidence that both viruses contributed to the development of their broad and potent responses (Figure 2.5, Figure 3.5B). Therefore, the initial and superinfecting Envelopes would be candidate immunogens, as well as other longitudinal isolates, including recombinants. As a proof-of-concept, some of the Envelope isolates from QB850 described in Chapter III will be used in a sequential immunization strategy in non-human primates by collaborators at Oregon Health and Science University [personal communication Ann Hessell]. Since both developed cross-reactive responses earlier than what is typically seen in singly infected patients, monoclonal antibodies identified from these patients may display fewer of the unusual characteristics described in Chapter I (i.e. extensive somatic mutation, products of rare germline precursors). While it is unclear how long it takes for Nabs to accumulate these features, recently isolated monoclonal antibodies from CAP256, a superinfected individual from South Africa, showed modest levels of somatic hypermutation (8.3-15.3%) in the heavy chain CDR3, however, the earliest Nabs cloned after 59 weeks were only capable of moderate neutralizing activity and were products of rare germline precursors (68).

While generating a ‘B cell lineage vaccine’ shows incredible promise, it is unclear whether guiding the immune response will be effective on the population level (209), much less programmatically feasible. Still, broadly Nabs identified from different donors exhibit target redundancy and deep sequencing studies have shown that multiple gene families can be stimulated to target the same epitope specificity, provides some evidence that the approach is possible (83, 210). It may be informative to perform similar deep sequencing analyses of the antibody repertoire across multiple superinfected cases to determine whether there are similarities in antibody gene usage and also whether they correlate with either intra- or intersubtype SI. Also, one could hypothesize that superinfected individuals utilize a greater number of antibody gene families compared to singly infected individuals, which contributes to their broad and potent responses and potentially may be indicative of a strong polyclonal response. Given all the exciting possibilities that would from carrying out such studies, the only foreseeable limitation will be the samples available to perform them, which highlights the importance of sustainable funding to maintain longitudinal cohorts and also the tremendous gratitude for the individuals who choose to participate in these studies.

Conclusion

Together, these studies have increased our knowledge base of how a broad and potent Nab response develops and has also laid the foundation for a new vaccine template to be explored. The results from this body of work support the sustained efforts to investigate and identify superinfected cases, as the knowledge we gain continues to have significant implications for vaccine design. It remains to be seen how a protective antibody-based vaccine will ultimately be developed, but the headway made in the field during my graduate tenure has been incredible to watch and I am grateful for the opportunity to contribute to this endeavor.

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epitope-scaffolds allows induction of antibodies specific for a poorly immunogenic HIV vaccine epitope. Structure 18:1116–1126.

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Valerie C. Cortez
2336 Minor Ave E #1, Seattle, WA 98102
vccortez@uw.edu

RESEARCH INTERESTS

Antibody development, epidemiology of infectious diseases, surveillance of emerging pathogens

EDUCATION

University of Washington; Seattle, WA USA **2008-2014**
Concurrent Ph.D. in Molecular & Cellular Biology / M.S. in Public Health Epidemiology
Certificate in Molecular Medicine
GPA: 3.52
Advisors: Julie Overbaugh, PhD and R. Scott McClelland, MD MPH
Dissertation: *Characterizing the neutralizing antibody responses of HIV-1 superinfected individuals*
Thesis: *The effect of HIV-1 superinfection on the neutralizing antibody breadth among female sex workers in Mombasa, Kenya*

Texas A&M University; College Station, TX USA **2003-2007**
B.S. in Biology, Minor in Psychology
GPA: 3.86, *Magna Cum Laude*

FUNDING AND AWARDS

Fogarty Global Health Fellowship	**2014-**
National Institutes of Health STD/HIV Training Grant	**2012-2014**
UW Graduate School Fund for Excellence and Innovation Travel Award	2013
National Institutes of Health Viral Pathogenesis Training Grant	**2010-2012**
American Society of Virology Travel Grant	2012
Howard Hughes Medical Institute Molecular Medicine Scholarship	**2010, 2011**
AIDS Vaccine Conference Scholarship	2011
National Institutes of Health Interdisciplinary Training Grant	**2009-2010**
National Science Foundation Graduate Research Fellowship Honorable Mention	2009

PUBLICATIONS

Cortez V, Chen M, and Overbaugh J. (2014) “HIV-1 superinfection does not focus the neutralizing antibody response on known epitopes.” *In preparation*

Cortez V, Odem-Davis K, Lehman D, Mabuka J and Overbaugh J. (2014) “Quotidian changes of genital tract cytokines in HIV-1 infected women during the menstrual cycle.” *Open Forum Infect Dis. (In press)*

Cortez V, Odem-Davis K, McClelland RS, Jaoko W, and Overbaugh J. (2012) "HIV-1 superinfection broadens and strengthens the neutralizing antibody response." *PLoS Pathogens.* 8(3): e1002611

Provine NM, **Cortez V**, Chohan V, and Overbaugh J. (2012) “The neutralization sensitivity of viruses representing human immunodeficiency virus type 1 variants of diverse subtypes from early in infection is dependent on producer cell, as well as characteristics of the specific antibody and envelope variant.” *Virology.* 427:25-33

Johnson JD, **Cortez V**, Kennedy SL, Foley TE, Hanson H, and Fleshner M. (2008) “Role of central β-adrenergic receptors in regulating proinflammatory cytokine responses to a peripheral bacterial challenge.” *Brain, Behavior and Immunity.* 22.7: 1078-1086.

CONFERENCE PRESENTATIONS

Williams KL, **Cortez V**, et al. (2014) "Virus-like particles identify both binding and ADCC-mediating antibodies from HIV-infected B cells." *Keystone Symposia: HIV Vaccines (X3)*; Banff, Canada. Poster presentation

Cortez V, Ronen K, Goo L, Peterson D, and Overbaugh J. (2013) "Broad neutralizing antibody responses in HIV-1 superinfected women do not target known epitope specificities." *AIDS Vaccine*; Barcelona, Spain. Poster presentation

Cortez V, McClelland RS, Overbaugh J. (2013) "Defining the host and viral factors that contribute to a subset of HIV-1-infected women developing elite neutralizing antibody activity soon after superinfection." *University of Nairobi STD/AIDS Collaborative Meeting*; Nairobi, Kenya. Oral presentation

Cortez V, McClelland RS, Overbaugh J. (2012) "A subset of HIV-1-infected individuals develop elite neutralizing antibody activity soon after superinfection." *American Society of Virology*; Madison, WI USA. Oral presentation

Cortez V, Odem-Davis K, Goo L, McClelland RS, Overbaugh J. (2012) "Characterizing the neutralizing antibody responses of HIV-1 superinfected women." *St. Jude Children's Research Hospital National Graduate Student Symposium*; Memphis, TN USA, Oral and poster presentations

Cortez V, Odem-Davis K, McClelland RS, Jaoko W, Overbaugh J. (2011) "The effect of HIV-1 superinfection on the development of neutralizing antibody breadth among female sex workers in Mombasa, Kenya." *AIDS Vaccine*; Bangkok, Thailand. Oral presentation

RESEARCH

Postdoctoral Fogarty Global Health Fellowship in the laboratory of Daniel Bausch, MD MPH **2014-**
Virology and Emerging Infections Department, U.S. Naval Medical Research Unit-6
Project focus: Prevalence, transmission dynamics and phylogeny of *Leptospira* in Madre de Dios, Peru

Doctoral Research in the laboratory of Julie Overbaugh, PhD **2009-2014**
Human Biology Division, Fred Hutchinson Cancer Research Center
Project focus: Neutralizing antibody responses in HIV-1 superinfected women in Mombasa, Kenya, defining epitope targets, cloning monoclonal antibodies by single B cell isolation

Technician in the laboratory of Lori Bernstein, PhD **2005-2007**
Department of Molecular and Cellular Medicine, Texas A&M University
Project focus: Counter effects of Y box-binding protein-1 on matrix metalloproteinase-1 promoter activity upregulated by low levels of arsenic

Summer research student in the laboratory of Monika Fleshner, PhD **2006**
Department of Integrative Physiology, University of Colorado, Boulder
Project focus: Central β-adrenergic receptors and cytokine production in rats during a peripheral bacterial immune challenge

Summer research student in the laboratory of Ana Tari, PhD **2005**
Department of Experimental Therapeutics, University of Texas M.D. Anderson Cancer Center
Project focus: Measured the efficacy of a panel of nitric oxide pro-drugs on breast cancer cells

Summer research student in the group of Nora Janjan, MD **2004**
Department of Radiation Oncology, University of Texas M.D. Anderson Cancer Center

Project focus: Using symptom management and co-morbidity data to predict the outcomes of gastrointestinal cancer patients undergoing chemoradiation

TEACHING AND MENTORING

Lab Mentor, *Fred Hutchinson Cancer Research Center* **2012-2014**
Supervised and guided research project with UW undergraduate student, Mitchell Chen
Project focus: Cloning Envelope genes from HIV-1 superinfected individuals, constructing chimeras

Guest Lecturer, *Microbiology 301: General Microbiology, University of Washington* **2011-2013**
Introduced basic concepts of HIV pathogenesis, public health issues surrounding HIV/AIDS epidemic for non-microbiology majors in course taught by Professors Denise Anderson and Mira Beins

Teaching Assistant, *Biochemistry 442: Molecular & Cellular Biology, University of Washington* **2011**
Presented lecture material weekly in smaller sections for advanced biochemistry course taught by Professors Richard Palmiter and David Kimmelman
Prepared weekly quizzes, graded exams, and moderated online forum

Teaching Assistant, *BioQuest Academy*, *Seattle Biomedical Research Institute* **2009**
Introduced high school students to standard lab techniques, lectured on HIV/AIDS pathogenesis
Organized and taught SAT II Biology preparatory course

Mentor, *Undergraduate Research Program, Fred Hutchinson Cancer Research Center* **2008-2013**
Participated in panel discussions for underrepresented minority summer research students
Provided constructive one-on-one critiques of students' graduate/medical school admissions essays

Psychiatric Treatment Counselor & Admissions Coordinator, *Alaska Children's Services* **2007-2008**
Counseled severely emotionally disturbed teenage boys in a residential treatment facility
Managed the admissions and transition of patients into the program

SCIENCE OUTREACH

Member, *Hutch United, Fred Hutchinson Cancer Research Center* **2013-2014**
Participated in organization to foster a community that increases the success of underrepresented and self-identified minority scientists through close mentorship

Volunteer, *Molecular & Cellular Biology Program Recruitment, University of Washington* **2008-2014**
Served as panelist and attended lunchtime gatherings with new recruits

Member, *Northwest Association for Biomedical Research* **2008-2014**
Volunteer speaker, mentor and science fair judge at local schools at the elementary, junior high and high school levels

Workshop Presenter, *Seattle Expanding Your Horizons, Seattle University* **2012**
Prepared workshop on antibody-virus interactions specifically geared towards middle school girls as part of a program to encourage them to explore math and science related occupations