Development and Characteristics of the Earliest Cross-Neutralizing Antibody Response to HIV-1

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Abstract

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A neutralizing antibody response of sufficient potency, magnitude and duration is considered an important part of a successful HIV vaccine. A better understanding of the factors associated with the development of broadly neutralizing antibody responses (effective against a wide range of clinical isolates), and the epitopes they target, will aid in our understanding of how to elicit such responses by vaccination. Cross-sectional studies of chronic HIV-1 infection have demonstrated that approximately 15% of HIV-1-infected subjects develop such responses. We characterized the development and epitope specificities of the earliest serum cross-reactive neutralizing antibody responses in an acute/early HIV infection cohort. We demonstrate that 29% of subjects in that cohort develop such responses within 2-3 years of infection. Our epitope-mapping results indicate that the earliest cross-neutralizing antibody responses target a limited number of regions on the HIV Envelope, often involving the highly conserved CD4-binding site on the HIV Envelope. In a case study of an HIV-1-infected individual we aimed to understand the emergence and evolution of the earliest cross-neutralizing antibody responses, and identified two distinct epitope specificities. Antibodies that targeted the CD4-BS became detectable at around 3 years post infection, and were responsible for the neutralization of most cross-clade viral isolates tested. Another specificity became apparent over a year later, which was due to broadly neutralizing antibodies specific to a carbohydrate at position 160 on the HIV Env. Our findings supports vaccine design efforts that aim to elicit multiple antibody specificities.

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DEDICATION

To my family.

CHAPTER I

INTRODUCTION

More than 30 years have passed since the beginning of the Acquired Immune Deficiency Syndrome (AIDS) epidemic. Although much is known about its etiological agent, Human Immunodificiency Virus (HIV), about the course of infection, and the immune response to the virus, a cure or an effective preventive vaccine are still out of reach. The epidemic officially began in 1981 when clinicians in the US described a few unusual clustered cases of opportunistic infections in previously healthy young homosexual men in Los Angeles and New York (2, 146, 194). Within a year, the epidemic had spread to 27 states with 593 cases and 41% mortality rate (3). In 1983 HIV was first isolated from the blood of AIDS patients (37, 179) and soon thereafter the secretary of Health and Human Services announced that *We hope to have a vaccine ready for testing in about two years*. Three decades later, HIV is still a major global health burden with 29 million people dead, 34 million living with the disease, and 2.7 million incident infections, and no effective vaccine is yet available.

HIV DIVERSITY

The origin of HIV lies in the Simian Immunodeficiency Virus (SIV), which has been shown to naturally infect over 30 African primate species (208). There are two genetically and immunologically distinct types of HIV (HIV-1 and HIV-2), and inferring phylogenetic trees of the primate viruses has demonstrated that the two HIV viruses are related to different SIVs and have different evolutionary origin. The more pathogenic HIV-1 originates from SIVcpz found in some chimpanzee subspecies (180, 447). The much less virulent HIV-2 is most closely related to SIVsm found at high prevalence in African sooty mangabeys (181). The HIV-2 virus is mostly confined to West Africa, and contributes considerably less to global infections as compared to HIV-1. Patients infected with HIV-2 have a lower viral loads than patients infected with HIV-1, and progresses more slowly to AIDS than HIV-1 patients (103). HIV-1 is incredibly genetically diverse and this is demonstrated by the number of groups and classes into which it is categorized **(Figure 1.1)**. Molecular phylogenies show that there have been multiple cross-species transmissions from SIVcpz into humans. It is thought that at least three such ‘jumps’ explain the distinct origins of the M, N, and O groups of HIV-1 (473). Recently, a new group of HIV-1 has been identified in Cameroon, designated group P, which is more closely related to

SIVgor found in gorillas, and most likely resulted from an independent gorilla-human transmission (404, 517). With a near global distribution and the highest virulence, group M (Main) is driving the current pandemic, causing more than 90% of cases, and the bulk of the morbidity and mortality associated with AIDS. Within group M there are at least 9 genetically distinct subtypes or clades (A, B, C, D, E, F, G, H, J, and K), and 43 circulating recombinant forms (CRFs) (498), which form when viruses from two clades infect the same cell within an infected individual and recombine their genetic material. Subtypes A through D are the most prevalent, with A, C and D accounting for 65% of worldwide HIV-1 infections (74, 223). However, subtype B is the most prevalent clade in North America and Europe, and has been most extensively studied. Over the decades, through base substitutions, insertions, deletions and recombination, the genetic diversity of HIV-1 has become such that the surface envelope protein of circulating isolates can have 35% amino acid diversity (184). To put it into perspective, the entire diversity of influenza hemagglutinin in one year is less than the diversity of HIV within one chronically-infected individual (271).

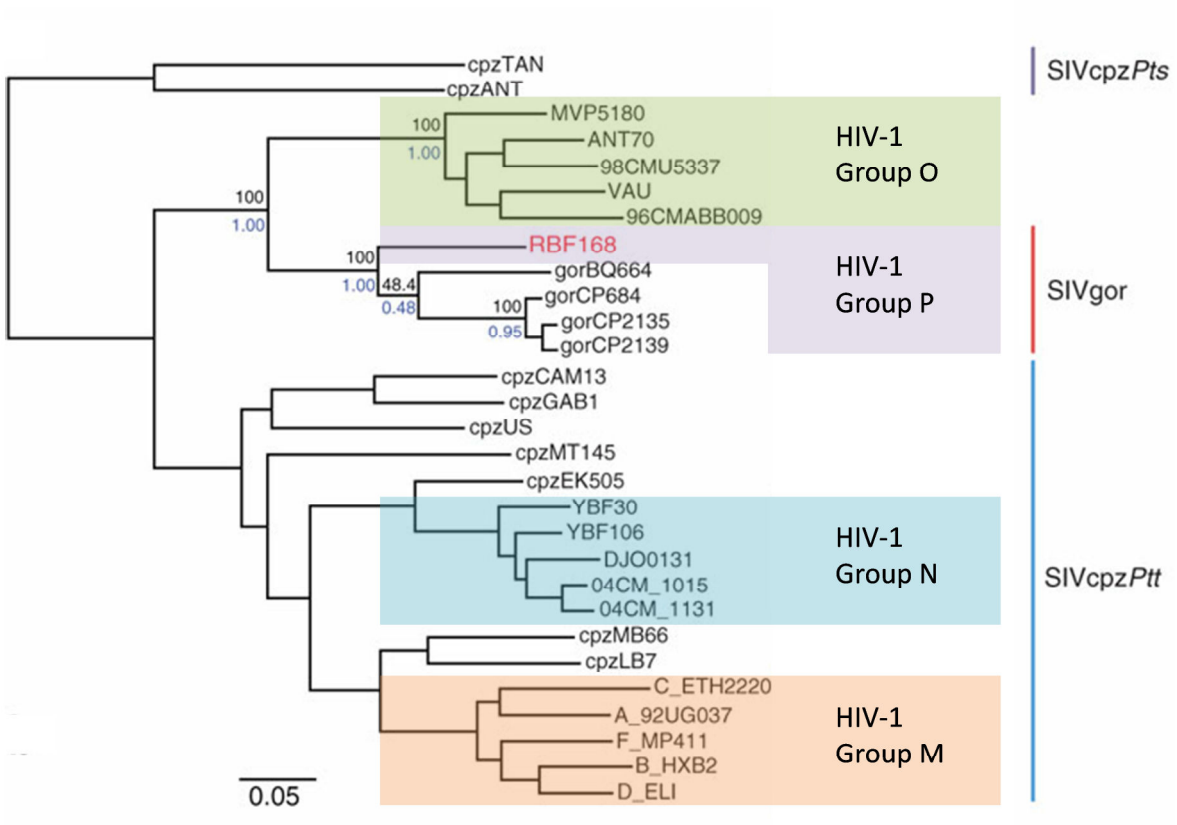

Figure 1.1 HIV diversity. Maximum likelihood phylogeny inferred from amino acid alignments of HIV and SIV sequences. The values in black above the branches are from bootstrap analyses, 1000 maximum likelihood, shown in percentages. The blue values below the branches represent posterior probabilities of amino acid Bayesian analysis, shown as proportions. Adapted from Plantier et al, 2009 (404).

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CLASSIFICATION, LIFE CYCLE, AND TROPISM

HIV is a member of the *Retroviridea* family in the *Lentivirus* genus. The genome is single-stranded positive-sense enveloped RNA virus that is packaged as a dimer into virions. The primary targets of HIV are CD4+ monocytes and T helper cells (263), although it also infects or is transported by dendritic cells, macrophages and brain microglial cells (207, 221). Upon cell entry, the two copies of the RNA genome are transcribed by the virally encoded reverse transcriptase into double stranded DNA. The transcription enzyme is error prone and there is frequent recombination between the two genomic copies, allowing for rapidly increasing genetic diversity and escape from immune responses (416). The viral DNA is transported into the nucleus and is integrated into the host DNA by the virally-encoded integrase. Two major consequences of integration are persistence and latency – factors that make clearance of this infection incredibly problematic. Upon reactivation, the viral genome is transcribed and translated by the host machinery, producing more virions that bud out from the cell wall, incorporating some of the host lipid-bilayer, including host proteins, into the viral envelope.

A major breakthrough in HIV research was the discovery of the receptor and co-receptor molecules that explained the cell tropism of the virus. The main cellular receptor of HIV is the CD4 molecule (118, 264), as suggested by the preferential infection of CD4+ T cells in the periphery and lymph nodes of infected patients. Both resting and activated CD4+ T cells are infected throughout the course of the disease, and although proliferating activated CD4+ T lymphocytes are more efficient at viral production, resting CD4+ T cells are in fact the ones that produce the bulk of HIV virions, and are also the stable latently infected population that allows HIV to persist (576, 578). Resident macrophages, dendritic cells (DCs) and Langerhans cells are also considered important cell targets for HIV, especially because it is thought that these populations present in the mucosa are some of the first cell types infected upon transmission, or are carriers of HIV and help disseminate it to lymph nodes. DCs in particular are very efficient antigen presenting cells, and can transfer HIV virions into the lymph nodes where they can more efficiently infect CD4+ T-cells (19, 186).

Although CD4 was identified as the main receptor for HIV early on, the question remained of why cell lines transfected in vitro with CD4 could not mediate viral entry. A decade later Feng and colleagues were the first to identify the seven-transmembrane G-protein coupled receptor CXCR4, involved in inflammation and hematopoiesis, as a co-receptor for T cell line-tropic HIV

isolates (162). Shortly thereafter, CCR5 was discovered as a major co-receptor for macrophage- and primary cell-tropic HIV (12, 98, 126, 145, 547). Transmitted/founder viruses almost always use CCR5 as a co-receptor (255, 442, 584), while CXCR4-tropic and dual-tropic viruses (ones that can use either co-receptor) emerge later on during chronic HIV infection (236, 371, 471). It is interesting to note that isolates which use CXCR4 as a main co-receptor have higher cytopathicity and enhanced pathogenicity; the emergence of these syncytia-forming isolates is generally associated with higher viral load, accelerated loss of CD4+ T cells, and rapid disease progression (106, 107, 270, 499). This can be partially explained by the differential expression of the two co-receptors: CCR5 is mainly expressed on activated/memory T lymphocytes, while CXCR4 is predominantly expressed on cells with resting/naïve phenotype (49), hence, providing an additional target cell population for the CXCR4-tropic isolates (48), and allowing the new virus population to evolve independently (521). This phenomenon also explains why activated/memory T cells in the gut are preferentially depleted early in infection, and peripheral CD4 T lymphocytes start decreasing substantially during chronic infection (107, 213, 334).

HIV TRANSMISSION AND NATURAL HISTORY OF INFECTION

HIV can be transmitted through three major routes: a) unprotected sexual contact, b) blood and blood byproducts – horizontally as in transfusion or sharing drug needles, or vertically from mother to child prepartum or intrapartum, and c) postpartum through breast milk. The burden of the disease falls predominantly on low- and middle-income countries where 96% of new HIV infections occur (515). Heterosexual transmission is the most common mode of transmission globally and accounts for approximately 85% of all HIV-1 cases, especially in Sub-Saharan Africa where the majority of HIV cases are clustered (258, 478). Globally, populations at increased risk of HIV are injection drug users, men who have sex with men (MSM) and commercial sex workers (115, 164, 513). The increased susceptibility of MSM to HIV infection was underscored in a meta-analysis that demonstrated cumulative odds ratios for MSM of 33.3 in the Americas, and 18.7 in Asia (30). In the United States MSM account for over 60% of new HIV infections and 79% of HIV diagnoses among all men (1); prevalence in this population can range 18-40% in some large metropolitan areas (4).

There are a number of risk factors associated with the rate of HIV transmission including viral load, circumcision, injection drug use, presence of other sexually transmitted diseases, number of sexual partners, biological properties of the virus, and route of transmission. The typical rate of heterosexual transmission during chronic infection is 0.1%, or 1 in 1000 coital acts (91, 203, 234, 541), increasing by roughly an order of magnitude in acute infection (91, 234, 399, 541), while the average risk of transmission following accidental percutaneous injury (needlestick) is estimated at 3% (469). Mother-to-child transmission is the most effective mode of HIV transmission with approximately 1 in 4 babies infected (15%-40%) in the absence of antiretroviral treatment (514). Unsurprisingly, HIV RNA viral load is the most important predictor of infection, with a statistically significant dose response relationship between increased transmission and higher viral load (413). Viral load is highest during acute HIV-1 infection and longitudinal data support the hypothesis that transmission during that, albeit brief, period contributes disproportionately to the global transmission rates (399).

Because of integration into the host genome, transmission of HIV can involve either cell-free or cell-associated virus. At transmission, a bottleneck occurs, whereby a single or very few viral isolates initiate the infection (193, 255, 307, 584). Studies in non-human primates suggest that, at the outset, a small number of T cells, macrophages and DCs are infected in the lamina propia of the mucosa (207), after which the virus is transported to the draining lymph nodes where initial replication occurs. Within an average of 10 days after transmission viral RNA can be detected in the blood **(Figure 1.2)** (167, 503), and when latency is established (100). Ten days is the window of opportunity for the immune response to extinguish HIV infection and provide sterilizing protection.

As infected T lymphocytes and virions circulate the blood stream, large numbers of CD4+ T cells in the GALT, spleen and bone marrow are infected, which is when the initial wave of massive viral production occurs. Typically, peak viremia reaches 10^7 viral RNA copies per ml of plasma, which is accompanied by a rapid depletion of T lymphocytes in the intestine (334, 524) and genital tract (525). At this stage of the infection, clinical flu-like symptoms can be observed in half of the cases, while the rest are clinically asymptomatic. Two to six months after infection, plasma viremia subsides by one to two orders of magnitude to a set-point level, which can be maintained for years without the development of any visible signs of infection, even in the absence of anti-retroviral treatment. The set-point viral load is highly variable between individuals but is typically below 20,000 RNA copies per ml. The decline in viral load is a

consequence of the innate and adaptive immune responses mounted against the virus. Cytotoxic T lymphocytes expand clonally weeks after infection, lyse HIV-infected cells, and exert selection pressure on the virus to escape their action **(Figure 1.2)** (58, 273). The CTL response has also been associated with long-term control of HIV infection (383). Although they appear later on, neutralizing antibodies have also been shown to select for escape mutations in the virus (177, 424). The branches of the immune system that respond to the infection are covered in more detail in the section “Immune response to HIV”

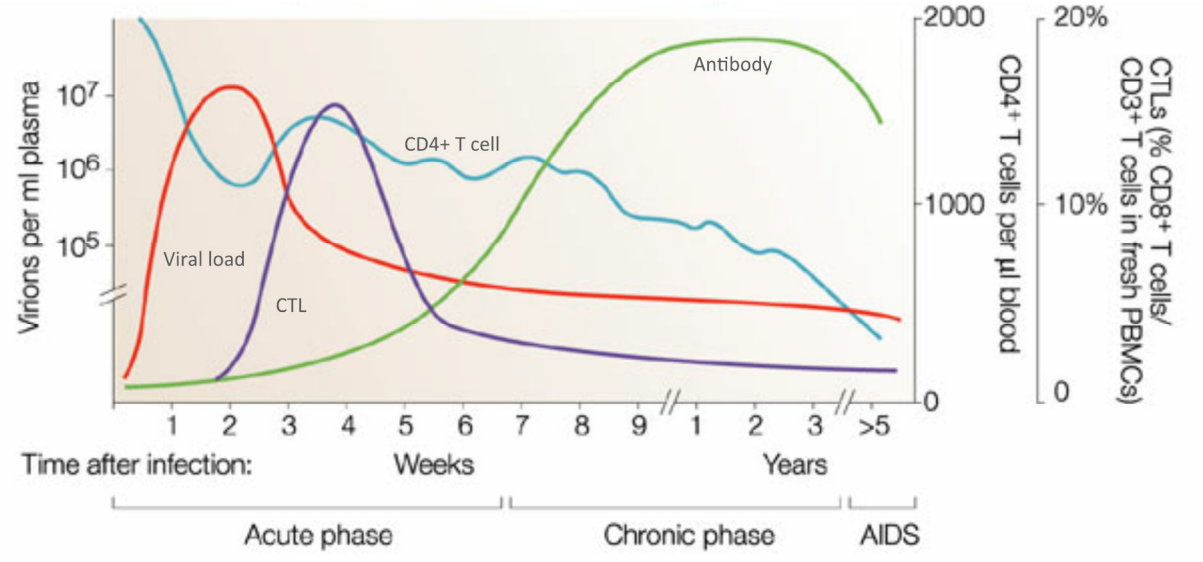

Figure 1.2 HIV infection and disease progression. The acute phase of HIV infection is characterized by a peak in viremia (red line) and a simultaneous decline of CD4+ T cell numbers (blue line). Viral replication is later brought under control by clonally expanded cytotoxic T lymphocytes (purple line), and a lower “set point” viremia is maintained for a number of years. Anti-HIV antibody responses (green line) appear weeks to months later. Adapted from Goulder & Watkins, 2004 (196).

After the acute/early stage of HIV infection, the infected individual enters an asymptomatic, yet progressive, stage of variable duration (average of 10 years) during which, in the absence of antiretroviral therapy, the virus replicates productively and increases in genetic variability. With a half-life of 29 – 100 min, the entire plasma virus population can be replaced very quickly (231, 417). As the major targets of HIV, over time naïve and memory CD4+ T cells are gradually depleted (142), however the number of infected cells does not account for the level of T cell immune dysfunction seen throughout infection. The most plausible explanation for the qualitative loss of CD4+ T cell response first to HIV and later to other pathogens that infect HIV+ subjects (104, 362), is that HIV preferentially infects HIV-specific T lymphocytes, the cells specifically recruited to respond to the infection by providing long term ‘help’ to key immune

cells such as B cells and CTLs (141). Subsequent recruitment and activation of other CD4+ T cells as they respond to their specific antigens, is thought to results it their infection and loss. Other hallmarks of chronic HIV infection are generalized immune activation, hypergammaglobulenemia, and B cell dysfunction (171, 256, 283, 342-344). Without anti-retroviral treatment the majority of infected people progress to AIDS, characterized by immunideficiency and opportunistic infections, and ultimately death.

STRUCTURE AND FUNCTION OF THE HIV ENVELOPE

Like all retroviruses, HIV is enveloped by host cell-derived lipid bilayer and a virus-encoded glycoprotein (13, 428). The 160 kiloDalton (555) envelope glycoprotein (Env) is the most important factor in viral entry as it binds the receptor and co-receptor molecules and mediates fusion of the viral and target cell membranes, ultimately allowing the viral RNA to enter the cytoplasm. Functional, fully processed Env as present on the viral surface is a trimer of noncovalently linked surface subunit gp120 and transmembrane subunit gp41 heterodimers. Being the only virally-encoded protein on the surface of the intact virion, Env is also the only target of neutralizing antibodies. The dual pressures from successful entry into target cells and escape from the host immune response have shaped and continue to shape the structure of Env.

Env biosynthesis and processing

The HIV-1 envelope is a type I integral membrane protein but before it can be incorporated into its mature trimeric structure on the virion surface, Env undergoes a number of co- and post-translational modifications. It is synthesized in the rough endoplasmic reticulum as a 845-870 amino acid polyprotein precursor, called gp160 (556). In the ER the protein is folded into a structure competent to bind CD4, intramolecular disulfide bonds are formed, and asparagine (N)-linked high mannose residues are added (63, 150, 163, 174, 546). The oligomerized protein (403, 458) is transported to the Golgi where it is cleaved by a cellular protease into the gp120 and gp41 subunits, which remain non-covalently linked (174, 546). Also in the Golgi, the high-mannose N-linked oligosaccharide side chains are for the most part trimmed down and can be modified into more complex side chains (288), as discussed in more detail in the section “The gp120 subunit”. Finally, the mature trimer of gp120/gp41 heterodimers is transported to the cell surface where it is incorporated into budding virions.

Env mediates viral entry

Viral entry into the target cell commences with the attachment of gp120 to the CD4 receptor. Understandably, the CD4 binding site **(CD4BS)** on Env is a highly conserved region, with very little sequence variability between circulating strains, and as such, is of great interest in vaccine design. This interaction triggers major conformational changes in gp120, which contribute to the formation or exposure of the high-affinity binding site for the co-receptor molecule (usually CCR5 or CXCR4 as explained above) (451, 500, 509, 547). The interactions with the receptor and co-receptor initiate further conformational changes that lead to the opening up of the trimer to reveal the gp41 machinery. In the unliganded Env spike, gp41 is buried in the oligomer in a non-coiled-coil conformation, and harbors considerable potential energy that is ultimately used to fuse the viral and host membranes (more details below). It is currently thought that gp41 functions similar to the HA protein of influenza – it inserts itself into the host membrane by a "spring-loaded" coiled coil mechanism, and then forms a 6-helix bundle that is responsible for fusing the two membranes together to allow for lipid mixing (92, 93, 544).

The gp120 subunit

The surface envelope glycoprotein gp120 is heavily glycosylated, with host-derived carbohydrates contributing to roughly half of its molecular weight. Typically the number of potential N-linked glycosylation sites on a gp120-gp41 protomer is 30 (288), which means that on average a trimeric spike might have 80-90 carbohydrate moieties on its surface, making it one of the most highly N-glycosylated structures found in nature. Glycans are bulky, flexible structures, over 20 times the size of an amino acid R-group, and as such form a "cloak" around the trimer that effectively shields the underlying protein from neutralizing antibodies (27, 43, 53, 327, 554). The position of the N-linked glycan is encoded by a conserved motif (Asn-X-Ser/Thr, where X is any amino acid excluding Proline). N-linked glycosylation takes place in the endoplasmic reticulum and Golgi apparatus and ultimately results in three categories of structures: oligomannose, hybrid and complex **(Figure 1.3)**.

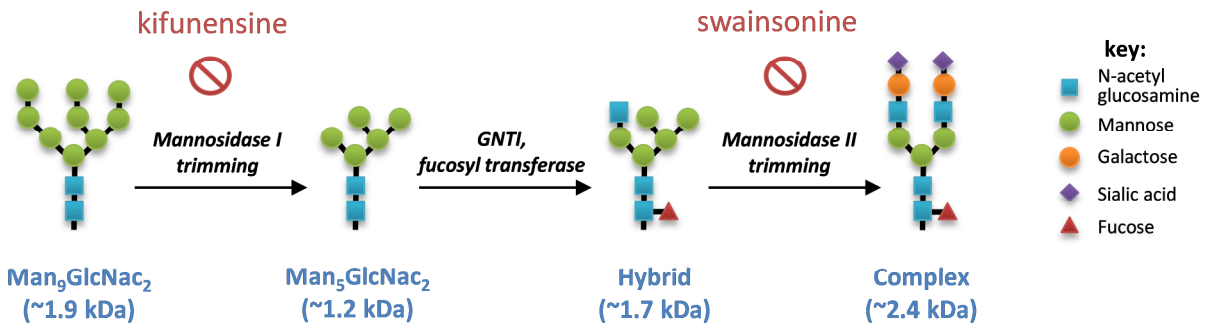

Figure 1.3 Essential steps of glycan biosynthesis. The Man9GlcNac2 precursor is trimmed to Man5 by Mannosidase I, a process that can be inhibited by kifunensine. GnTI then transfers a GlcNAc moiety to the precursor creating a hybrid glycan. Further mannose trimming and addition of more GlcNAc results in multiantennary complex glycans. This process can be inhibited by swainsonine. Galactose and sialic acid are ultimately added. Adapted from Binley et al, 2010 (43).

Gp120 exhibits protein-directed glycosylation, whereby the density of carbohydrates in some regions creates steric hindrance and results in untrimmed oligomanose glycans (585). It is important to note that on recombinant gp120 (as produced in the lab in mammalian cells) N-linked glycans are fully processed into producer cell type-specific complex carbohydrates (188, 341, 585). In contrast, the vast majority of glycans on infectious virus particles are oligomannose glycans (135).

Gp120 is composed of 5 conserved regions (C1 – C5) interspersed with 5 hypervariable regions (V1 – V5), which are bracketed by disulfide bonds and form 4 variable loops **(Figure 1.4)** (288, 492). Not much more than that was known about the structure of gp120 until the last decade when a number of X-ray crystal structures and cryoelectron 3D tomograms of unliganded and liganded gp120 or native trimers were published (94, 216, 238, 279, 303, 332, 384, 555, 583). To obtain crystal with sufficient resolution researchers have had to use a gp120 core protein, which lacks the variable loops V1V2 and V3, and is stripped of most of the carbohydrates, yet still retains important antigenic characteristics such as binding to CD4. The core is formed from the more conserved inner domain, which is important for gp120-gp41 interaction (384), the highly glycosylated outer domain, and connecting them is a conserved 4-stranded bridging sheet. All three of these domains are important for receptor and co-receptor binding (94, 279, 555). The CD4BS is a recessed pocket of approximately 800 Angstrom^2 formed by multiple noncontiguous regions on gp120 (279). A deep hydrophobic cavity, called the “Phe-43 cavity” because of the occupancy by the CD4 Phe 43 residue in that region, is a defining characteristic of the CD4BS (555).

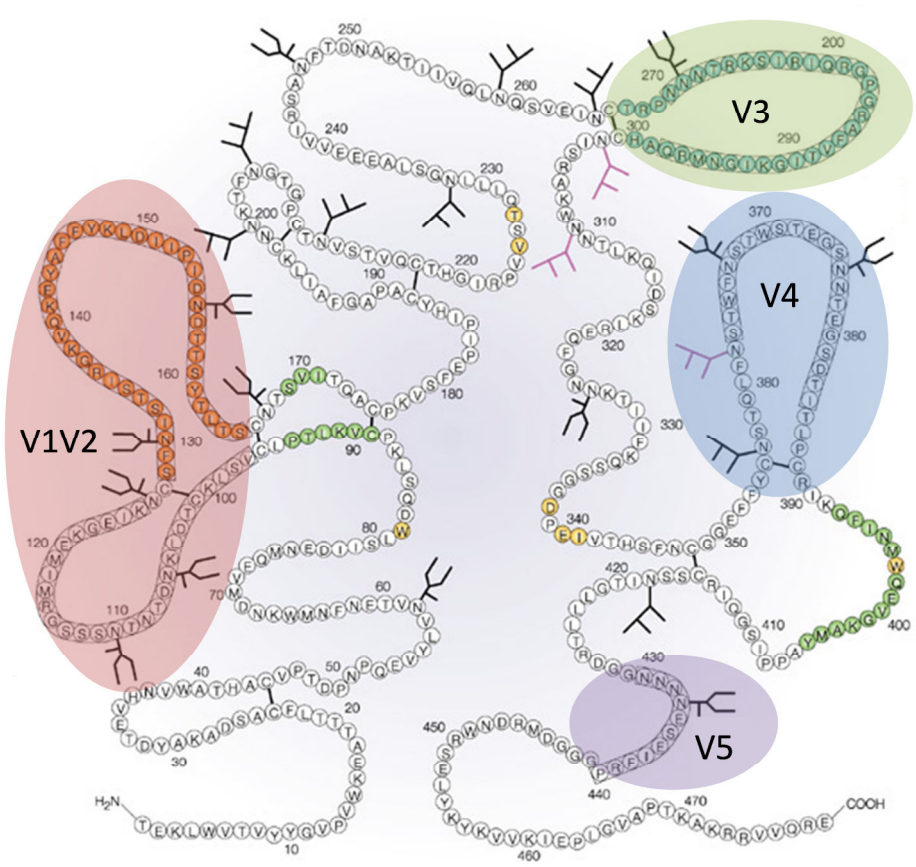

Figure 1.4. Structure of the HIV-1 gp120 glycorprotein. Secondary structure of gp120. The variable regions (V1-V5, outlines in circles) are interspersed with the constant regions (C1-C5, not marked). Potential N-linked glycosylation sites are indicated; mannose and hybrid glycans with branched structures and complex glycans with U-shaped structures. Amino acid residues of some neutralization epitopes are highlighted – CD4BS (yellow), CD4-indiced epitope (green), 2G12 epitope (purple glycans), V2 loop (orange), and V3 loop (blue). Adapted from Zolla-Pazner et al, 2004 (587).

Cryoelectron tomography provides much less resolution than crystallography, however, it allows us to study the structure of the native spike in its membrane-bound trimeric form. These 3D structures can help elucidate how much protection from antibodies is provided by the glycans on the surface of gp120. Schief et al published computer modeling images in which crystal structures of b12-bound core gp120 (b12 is an antibody that binds the CD4BS) were fitted into cryoelectron density of the unliganded trimer **(Figure 1.5)** (464). The images demonstrated the high density of carbohydrates that cover almost every epitope on Env. The CD4BS pocket is one of the few exposed regions, however the bulky variable loops, neighboring carbohydrates, and the deep recessed nature of the CD4BS pocket hinder targeting by neutralizing antibodies (95). In addition, the packing of gp120 within the trimer is such that it creates a very narrow

cavity, big enough for CD4 to bind but very restrictive for antibodies with two immunoglobulin domains (464).

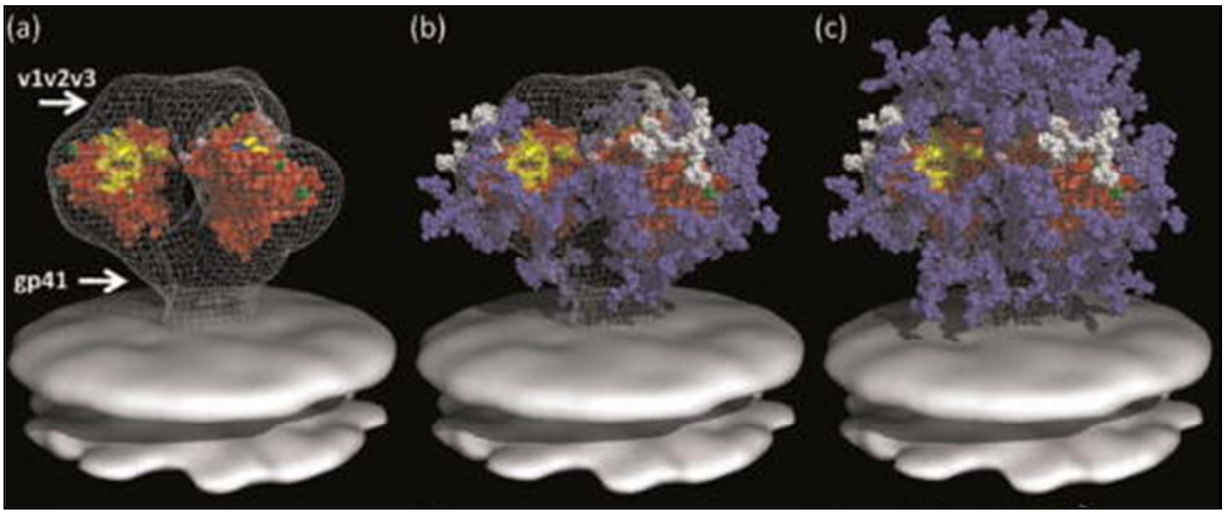

Figure 1.5 Model of the unliganded trimeric HIV-1 spike. The model was obtained by fitting b12-bound gp120 coordinates into a cryo-EM density of membrane bound trimer (mesh), with virion membrane electron density at the bottom of each image. **A)** Unoccupied densities of V1, V2, V3 and gp41 are indicated, as are gp120 core (red) and b12 epitope (yellow). **B-C)** High-mannose and complex gycans are added to the structure with 2G12 epitope in white and the rest of the glycans in light blue. Adapted from Schief et al, 2009 (464).

The gp41 subunit

The gp41 glycoprotein contains three major domains: extracellular or ectodomain, transmembrane domain, and cytoplasmic tail **(Figure 1.6 A)**. The N- and C-terminal portions of the ectodomain contain heptad repeat sequences, which together with the fusion peptide mediate the fusion process (59, 173). Located in this region is the highly conserved hydrophobic membrane proximal external region **(MPER)**, which is the target of the broadly neutralizing antibodies 4E10, 2F5, and Z13. This is discussed in more detail in the section 'Epitope specificities of human monoclonal antibodies'. MPER is thought to also play a role in fusion, however, the exact mechanism has not been fully elucidated (365). Crystal structures of a stable, soluble, truncated gp41 shed light into the fusion process and the postfusion conformation of gp41 (92, 495, 544). Otherwise extended away from each other, post fusion, the N- and C-terminal heptad repeats "jack-knife" or fold back onto one another into a hairpin. **(Figure 1.6 B-D)** Together, three N-terminal and three C-terminal heptide repeats form what is called the 6-helic bundle - a trimeric stable coiled coil structure, which ultimately brings the two membranes together and promotes aggregation **(Figure 1.6 D)**.

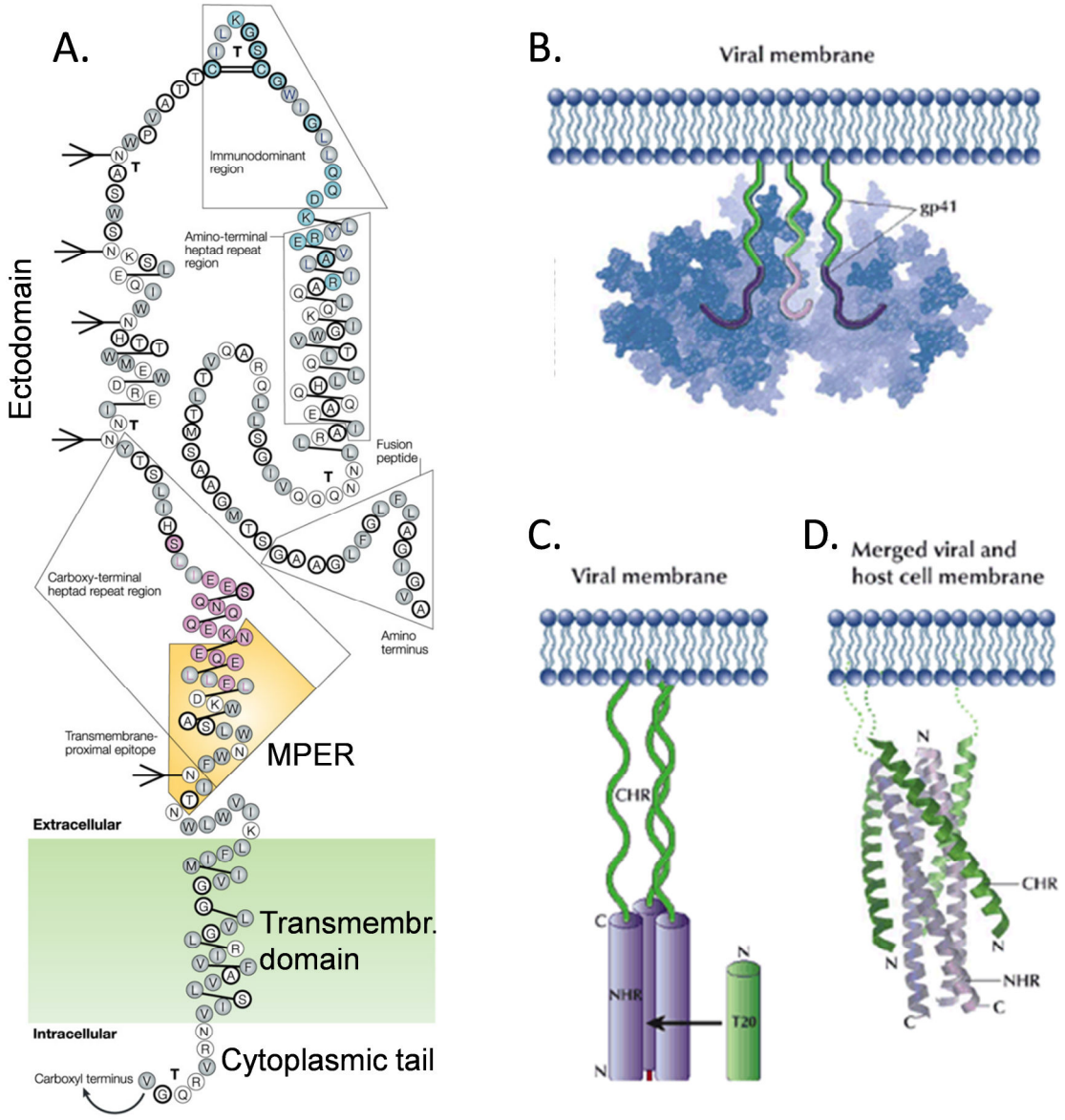

Figure 1.6. Structure of the HIV-1 gp41 glycoprotein. A) Secondary structure of gp41. Shown are α-helices (three- and four-amino acid grouping connected by single lines), hydrophobic residues (grey circles), charged residues (unfilled circles) and neutral residues (heavily outlined circles). Potential N-linked glycosylation sites are indicated by branched structures, and strong turns are marked by 'T'. Important structural regions are boxed or colored as indicated. **B-D) Conformation of gp41 before (B), during (C), and after (D) fusion of the viral and cellular membranes. B)** In the native conformation the gp41 ectodomain is largely occluded by gp120 (transparent). **C)** Upon CD4 and co-receptor binding large conformational changes in gp120 expose gp41 and activate the fusion machinery. The C-terminal and N-terminal heptad repeats (C-HR, N-HR) are thought to be in a more extended arrangement, positioning the fusion peptide for insertion into the host membrane. The fusion inhibitor T20 is indicated. **D)** Fusion is thought to be mediated by 'jackknifing' of gp41 whereby the now antiparallel N-HR and C-HR regions form a six-helix bundle which brings the two membranes together. Adapted from Zolla-Pazner et al, 2004 (587), and Zwick et al, 2004 (591).

As the essential fusion machinery of HIV, gp41 has become an important target for designing therapeutics and vaccines. A number of synthetic peptides have been designed that bind to various regions of gp41 and block entry into target cells, and have been used in successful antiretroviral treatment regimens [reviewed in (386, 480, 527)]. The function of gp41 entails the presence of highly conserved regions – potentially, an excellent target for neutralizing antibodies elicited by a vaccine. However, in its prefusion state, most of gp41 is sequestered away by gp120, and in the postfusion state its conformation is so considerably altered, that antibodies to that region cannot recognize any other gp41 conformations (591). Fusion itself last only minutes, which significantly limits access to the transiently exposed conserved epitopes.

Env immunogenicity in recombinant subunit vaccines

Multiple attempts have been made over the years to elicit neutralizing antibodies by vaccination that would be effective not only against the vaccine strain but also against the wide diversity of circulating isolates. As the only target of neutralizing antibodies, the HIV envelope has been at the forefront of these efforts. After the first failed attempts at using monomeric gp120 (23, 149, 209, 322, 369), the field has turned to two major approaches to designing subunit HIV vaccines – immunizations with trimeric Env oligomers, and with modified Env glycoproteins.

The first approach to HIV-1 immunogen design focuses on the preservation of the Env trimeric spike with the basic idea that mimicking as much as possible the native spike on the viral surface will improve the chances of eliciting neutralizing antibodies. In these cases, soluble gp140 trimers, which comprise the entire gp120 fused to the ectodomain of gp41, have been the prototype immunogen (62, 151, 204, 522, 566). As the two glycoproteins are naturally held together by relatively weak noncovalent bonds, gp140 trimers were stabilized for better purification by removing the cleavage site between gp120 and gp41. Several studies have demonstrated marginal improvement of using trimeric over monomeric glycoproteins in eliciting neutralizing antibodies (39, 149, 151, 296, 377, 566, 573). One possible explanation is that immunogenic epitopes normally occluded on the trimer are exposed on gp120 monomers, and the antibodies elicited to these regions cannot access their epitopes on the native spike (68). Alternatively, trimeric glycoproteins generate larger proportions of antibodies to conformational epitopes than monomeric gp120 (149), which might make them better able to recognize the native spikes on HIV (349, 452).

The second strategy involves the introduction of a number of modifications to even better mimic the native spike, to stabilize the protein, or to divert the antibody response toward more conserved regions. As it was recognized that the lack of cleavage site might interfere with the immunogenicity and antigenicity of gp140 (224), one approach to preserve the gp120-gp41 cleavage site and still maintain stable subunit interaction has been to introduce cysteine residues in gp120 and gp41, which form an intersubunit disulfide bridge (46, 446, 467). Immunizations with such cleaved oligomers have provided marginal improvement in terms of neutralization breadth (39, 40, 251). A way to covalently link gp120 and gp41 has been to incorporate heterologous trimerization motifs, such as GCN4 or the trimeric motif of T4 bacteriophage fibritin (563, 565), and have been shown to be more efficient at generating neutralizing antibodies than gp120 monomer (62, 204, 566). Because of the strong immunogenicity and tolerance for escape mutations of the Env variable regions, soluble uncleaved trimers with variable loop deletions have been evaluated extensively in antigenicity and immunogenicity studies. The goal is to direct the immune response towards conserved epitopes that should be common to many HIV-1 isolates, otherwise shielded by the variable regions. Although proteins with large variable loop deletions indeed had altered immunogenicity, they were same as or worse than their wild type counterparts at inducing neutralizing antibodies (210, 260, 308, 557). It is possible that large deletions alter Env structure too drastically to generate antibodies that recognize the virion spike. Attempts have been made to immunize with gp140 trimers with smaller deletions in the V2 loop, however these only slightly improved the breadth of the neutralizing antibodies (34, 130, 455, 485, 486, 489). Progressive shortenings of the V3 loop abolished the functional activity and immunogenicity of Env, however, a smaller deletion in V3 combined with V1V2 deletion enhanced the ability of the immunogen to elicit neutralizing antibodies (567). The V1 region of Env is highly immunogenic but elicits very type-specific neutralizing antibodies (130, 296, 548). In an attempt to redirect the immune response from that region and to make the elicited antibodies more broadly neutralizing, the V1 loop was either deleted or replaced by that of unrelated virus strains. This altered the immunogenicity of other Env regions, yet provided a marginal improvement in terms of the breadth of the antibody response (97). Finally, modifications have been made on the glycosylation pattern of Env immunogens, which aim to unmask the epitopes hidden beneath the bulky sugars. However, epitopes outside of the modified areas can also be affected. Studies of site-specific or whole molecule deglycosylations have not recorded major differences in the antibody responses elicited by the wild type and mutant immunogens (41, 52, 75, 209, 414). However, removal of the glycans in the V1 region of SIV immunogens redirected the antibody response to the V3

loop in one study (105), and significantly improved their immunogenicity in another (421). On the other end of the spectrum is hyperglycosylation of Env immunogens, which involves the dampening of the immune response to undesired epitopes by the selective incorporation of additional N-linked glycans. While this strategy maintained responses to conserved regions such as the CD4 binding site, it has provided no improvement on the antibody response (183, 388, 468). Together, these data indicate that immunogenicity cannot be readily predicted and that further improvements are needed to existing HIV immunogens (237).

HOST IMMUNE RESPONSE TO HIV-1 INFECTION

Much of the immune system seems to be involved in the fight against HIV infection. However, HIV is a chronic infection that attacks the CD4+ T lymphocyte component of the immune system itself. The combination of the continuous give and take between an effective immune response and escape by the virus, the constant reservoir of latently infected cells, impaired T cell help, and chronic immune activation ultimately results in a losing battle against HIV. Nevertheless a thorough understanding of the immune responses to HIV is imperative if we are to discover correlates of protection against this infection, and characterize the types of immune responses that should be elicited by an effective HIV vaccine.

Innate Immunity

Several lines of evidence have demonstrated the importance of early control of viral replication and the subsequent HIV viral setpoint and disease progression, and the importance the innate immune response can play in those (15, 56, 205, 287, 301). Innate immunity is the first line of defense early to infection and consists of innate immune cells, including natural killer (NK) cells, macrophages, and dendritic cells (DCs), that recognize pathogens through pattern recognition receptors such as the Toll-like receptors (TLRs). The single-stranded RNA of HIV is a ligand for TLRs 7 and 8 and can mediate immune activation in vitro (336). Engagement of these receptors on innate immune cells results in the upregulation of proinflammatory antiviral cytokines, particularly Interferon (IFN) α (60, 487), mostly produced by plasmacytoid DCs. Inhibition of HIV replication by IFN-α has been shown *in vitro* for monocytes and macrophages (26, 312). In addition, IFN-α can affect other immune cell types, including NK cell activation, B cell regulation and antibody production, and CD4+ and CD8+ T cell survival through inhibition of apoptosis (8, 315, 505). Despite its antiviral effects during acute and early HIV infection, continuous

stimulation by IFN-α can have pathologic effects during chronic infection. Expression levels of interferon stimulated genes are elevated in chronic infection and are associated with immune activation and disease progression (532). In addition, during chronic infection CD4+ T cells show diminished responsiveness to the anti-apoptotic effect of IFN-α (431).

NK cells become highly activated and increase in frequency during HIV expansion (18), and their ability to suppress HIV replication has been demonstrated *in vitro* (574). Epidemiologic and functional studies have demonstrated that NK cells can recognize and kill virally infected cells through a number of inhibitory and activating KIRs (killer immunoglobulin-like receptors) (17, 316, 317), and the immune pressure they exercise *in vivo* has been shown through KIR-related polymorphisms in HIV-1 sequences from chronically infected subjects (16).

CTL response

Cytotoxic T lymphocytes, or CD8+ T cells kill virally-infected cells and as such are important responders to HIV infection. Even though they cannot prevent infection, multiple lines of evidence suggest that they have a significant role in controlling HIV replication. CTLs have been associated with decrease in the RNA viral load, particularly those with specificity to the conserved HIV structural protein p24 (257, 383). Furthermore, the clonal expansion of HIV-specific CTLs during acute and early infection coincides temporally with the drop in viral replication and the establishment of a viral load set point **(Figure 1.1)** (57, 190, 273, 361, 387). CD8+ T cells recognize infected cells through T cell receptor contact with viral peptides presented by HLA Class I on the surface of the infected cell. A number of HLA Class I alleles have been associated with successful long-term control of HIV infection or with disease progression (86, 182, 253, 338, 496). A very important indication for the cytotoxic effect of CTLs on HIV infected cells came with studies on viral escape. Furthermore, sequence variations in HIV that result in loss of, or diminished, CTL recognition are indicative of the selection pressure these cells exert on the virus. CTL escape has been unequivocally demonstrated to happen frequently in both acute and chronic HIV and SIV infections (14, 35, 158, 159, 176, 195, 347). The case has also been made that acute CTL escape by the virus and the subsequent loss of viral control can lead to rapid disease progression (58, 411), however, the location of the escape mutations and the fitness cost to the virus have to be considered (196). Finally, the important role of CD8+ T cells on the control of infection was further underlined in studies of SIV-infected macaques, where CD8-specific monoclonal antibodies where used to deplete CTLs. There was a profound increase in viral load when acutely infected macaques were

treated with an anti-CD8 MAb, which was also marked by accelerated disease progression (246, 324, 465). Suppression of viremia correlated with the reappearance of CD8 T lymphocytes. Collectively, these data suggest that CTLs responses exert substantial selection pressure on the replicating virus and play an important role in viral suppression starting in acute infection.

During the late stages of the infection, as the immune system collapses, CD8 T cells also become dysfunctional and exhausted with impaired cytokine production and TCR zeta chain expression (272, 508), and a loss of proliferative capacity (337, 470, 571). Engagement of programmed death (PD-1) receptor on T lymphocytes inhibits their proliferation and cytokine production, and blocking that pathway in chronically LCMV-infected mice restores the capacity of these exhausted cells back to their fully functional cytotoxic activity (32). This led researchers to consider a role of PD-1 in the impairment of the CTL response during HIV infection. Two seminal studies demonstrated that PD-1 is significantly overexpressed on HIV-specific CTLs during chronic infection and correlated with viral load and with impaired cytokine production and proliferative capacity of HIV-specific CD8+ T cells (121, 507). A detrimental consequence of the accumulation of dysfunctional CD8+ T cells could be preventing the renewal of a functional and effective anti-HIV CD8 repertoire.

The substantial correlative evidence that HIV-specific CTLs play a role in viral control in HIV and SIV infection, and the inability to induce neutralizing antibodies against diverse circulating isolates led to a shift in the HIV field towards T-cell based vaccines. The basic concept is that, although unable to protect an individual from becoming infected, a vaccine that induces robust cellular immunity to HIV would dramatically lower the viral load, delay progression to AIDS, and reduce transmission rates (534). Strong evidence for the benefit of exploring such vaccines came from studies that showed robust protection by T-cell based vaccines in rhesus monkeys challenges with SIV or a SIV-HIV hybrid virus (SHIV) (21, 36, 476). A phase I clinical trial Merck's replication defective adenovirus-based (Ad5) DNA vaccine containing clade B gag protein showed successful generation of anti-gag CTL responses (87). A year later the STEP trial was launched with an Ad5-based trivalent vaccine formulation containing the highly conserved gag, nef and tat HIV sequences. To the great disappointment of the whole field the vaccine failed to prevent infection or decrease viral load in the ones infected, and the trial was stopped prematurely (202, 493). In depth analysis demonstrated that the STEP vaccine induced a very narrow CTL response targeting an average of 2-4 epitopes, mostly in less conserved

regions (217, 290). Yet, comparisons of the vaccine strain with breakthrough virus infections in placebo recipients and vaccines found evidence for selection pressure induced by the Ad5 vaccine (433). A disconcerting finding was the increased HIV incidence in vaccinated men with pre-existing Ad5 immunity, however vaccine-induced immune responses were not responsible for the apparent increased risk (71, 329), nor were pre-existing Ad5-specific CD4+ T cell responses or Ad5 antibodies (245, 379). Ultimately confounding factors were found in circumcision and HSV status (71).

CD4+ T cell help

Not much attention has been paid to the role of CD4+ T lymphocytes in the control of HIV infection, even though antigen-specific CD4+ T cell responses are exceedingly important for the clearance of viral infections in general as they provide immune “help” to both B cells and CD8+ T cells. During primary HIV infection, HIV-1-specific responses expand, but they contract after the first few months. Even though numerous studies have demonstrated that HIV replication can be predominantly controlled by CTLs, a strong CD4+ T cell response has also been associated with viral control (437). Furthermore, CD4+ T cells have been shown to also induce escape mutations in CD4-targeted epitopes, especially early in infection (249, 440).

Even though HIV-mediated CD4+ T cell depletion is well documented, not much is understood about how remaining CD4 T cell subsets modulate HIV infection. T helper 17 (Th17) cells reside in the gut-associated lymphoid tissue (GALT), are involved in the immune response to pathogens at mucosal sites, and have been implicated in the immune activation (119). The loss of Th17 cells in the GALT and periphery has been documented for HIV and SIV infection (311, 410, 415). While nonpathogenic SIV-infected sooty mangabeys retain normal Th17 counts in the GALT and periphery (65, 66), an imbalance of Th17 cells has been proposed to contribute to the persistent immune activation in SIV-infected macaques (89, 161). These studies provide a potential mechanism between depletion of mucosal Th17 cells and disease progression, which warrants further investigation, and demonstrate the effect of HIV on mucosal immune dysfunction (483).

Follicular helper T (Tfh) cells are a subset of CD4+ lymphocytes that have been recently identified (99, 378). Tfh cells express PD-1 and CXCR5, and reside in the B cell follicles of germinal centers (GCs) providing stimulatory signals for activated antigen-specific B cells to undergo somatic hypermutation and thereby produce high-affinity antibodies (420). Due to their

expression of CD4 and their location in lymph nodes, these cells are highly susceptible to HIV and SIV, and are potential viral reservoirs (222, 243). A study of SIV-infected macaques followed the dynamics of Tfh cells and B cells *in situ*, and showed that, as infection progressed, increasing numbers of CD4+ Tfh cells were harbored in the GCs, while seemingly excluding CD8+ T cells (235). This provides a mechanism for sequestering a viral reservoir. In addition, increasing numbers of activated memory B cells accumulated in germinal centers, which correlated with increasing anti-SIV Ab levels. In fact, the role of Tfh cells in generating high-affinity antibodies makes them an important area of HIV research, as these are the type of immune response most likely to be protective of HIV infection. Due to the focus of this study, a detailed review of the antibody response to HIV will be covered in detail below.

THE ANTIBODY RESPONSE TO HIV-1

The antibody response is considered a key component of an effective HIV vaccine (321, 328, 490). In the past 20 years a lot has been learned about the neutralizing antibody response to HIV infection in terms of emergence, epitope specificity, potency, breadth, selection pressure on the virus and escape. Neutralization of free virions by antibodies results in the inability of the virus to infect target cells. Most neutralizing antibodies **(NAbs)** mediate this by preventing receptor binding or fusion (262), although NAbs to some viruses can prevent downstream budding or release from infected cells (143, 432, 472). The existence of anti-HIV NAbs in infected individuals was first recorded in the late 80's (427, 438, 543) and has sparked great interest in the field in the hopes of harnessing this immune response in HIV vaccines and therapeutics. Although it has proven extremely challenging to elicit NAbs by immunization that would be effective against the wide range of diverse circulating isolates, great strides have been made in our understanding of the interaction between neutralizing antibodies and HIV. Further details on this topic are provided later on, but first, a closer look at other antibody effector functions and their role in HIV infection.

Antibody effector functions

While neutralization is considered the most efficient humoral anti-viral defense, studies of other pathogens have demonstrated that non-neutralizing antibodies can have substantial anti-viral effects through effector functions **(Figure 1.7)** (212, 268, 401, 418, 481). The majority of antibodies elicited during HIV-1 infection are non-neutralizing – while they bind the gp120 or

gp41 subunit, they fail to recognize the functional trimeric spike or block viral entry (172, 225, 389). Non-neutralizing antibodies can capture HIV virions presumably by binding to the nonfunctional forms of Env present on the viral envelope, such as monomeric gp120/gp41 monomers, and gp41 stumps (354, 407, 568). Gp120 shed from virions upon CD4 receptor binding can also elicit non-neutralizing antibodies (350). Thus, while their biological role has not been fully elucidated, it is thought that these antibodies could activate effector functions against HIV and so contribute to viral clearance through phagocytosis of opsonized viruses, lysis of HIV-infected cells via NK cells or complement, and lysis of virions via complement.

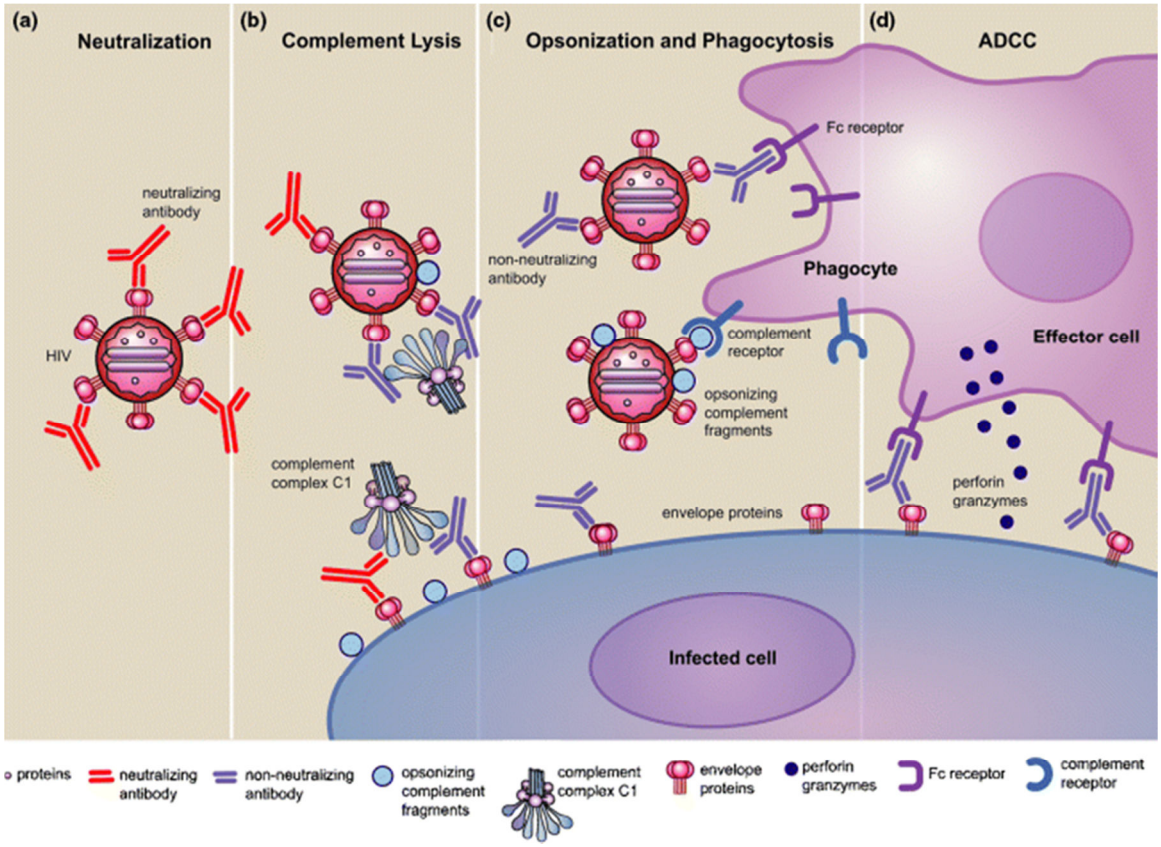

Figure 1.7 Humoral immunity to HIV - neutralization and antibody effector functions. a) Neutralization of virions by antibodies. **b)** Complement-mediated lysis triggered by antibodies. **c)** Phagocytosis of opsonized virions and infected cells. **d)** Antibody-dependent cellular cytotoxicity (ADCC) against infected cells. Adapted from Huber & Trkola, 2007 (242).

While antibodies recognize and bind to antigen through their variable Fab fragment, a lot of functional activity is mediated through the constant Fc segment – the one that interacts with Fc receptors on a variety of immune cells involved in the response to HIV. Receptors for the Fc of IgG antibodies (FcγRs) are found on NK cells, monocytes, macrophages, DCs, and neutrophils.

Antibody-dependent cellular cytotoxicity (ADCC) is a process in which antibodies binds to antigens on the surface of infected cells and through Fc-FcγR interaction recruit effector cells, most importantly NK cells, which can lyse the target cell. In terms of HIV infection, a number of studies have documented the presence of ADCC-inducing antibodies, predominantly directed to Env, in infected individuals (169, 274, 304, 309, 436, 497). More recently, ADCC activity against HIV has been associated with better disease outcome (6, 38, 169, 281). The importance of ADCC in reducing viral load and potentially in the prevention of infection was illustrated in a study of passive transfer of the anti-CD4BS monoclonal antibody **(MAb)** b12 in macaques that were later challenged with a SIV HIV chimeric virus (SHIV) (226).

Animals treated with b12 defective in complement and ADCC functions were significantly less protected from infection than the ones treated with wild type b12 or b12 lacking only complement activity. In addition, ADCC responses exercise selection pressure on HIV, which makes them a desirable part of a future HIV vaccine (101). As HIV infection progresses, ADCC, along with all other branches of the immune system, lose their effector function and become impaired, which has been associated with decreased FcγR expression on NK cells (147). Complement is an important component of the innate immune system. It can be activated by antibody-bound virions, and can play an important role in HIV infection through virus or target cell lysis, opsonization, induction of phagocytosis, and immune activation. *In vivo* complement-mediated lysis of HIV virions can be detected during acute infection, and is maintained throughout the course of infection (6, 241, 484, 494). Importantly, complement lysis of HIV can impact viral replication in acute infection (6, 241). Finally, the coating of virions by antibody and complement renders them susceptible to phagocytosis by macrophages and monocytes through their Fc receptors. Although the role of phagocytosis in HIV infection is not well understood, defects in both complement and Fc-mediated phagocytosis have been documented during disease progression (24, 254).

Escape strategies from neutralizing antibodies

As the only virally-encoded surface protein, and consequently, the only target of the humoral immune response, the HIV Env is also the most variable HIV gene. HIV-1 has a highly error-prone reverse transcriptase, capable of generating tremendous viral variation within each infected individual, and Env can tolerate many mutations without losing function or fitness. Additionally, Env has evolved multiple strategies to escape the action of neutralizing antibodies, which are briefly summarized below (76, 247, 556).

The variable regions of HIV Env are highly immunogenic (352), however, these loops can tolerate multitude of escape mutations. Consequently, neutralizing antibodies generated to the variable loops, are typically type-specific and readily escaped by the virus. In addition, the structure of unliganded gp120 has demonstrated that the V1V2 likely partially occludes the CD4BS, while V3 might interfere with recognition of the bridging sheet located between the inner and outer domains (94). Furthermore, as previously mentioned, the HIV Env is extensively glycosylated and the virus uses glycans to occlude underlying antibody epitopes (43). Studies have shown that glycans can also shift location in vivo (96, 117, 542), which has led to the proposition that this dynamic glycan shield contributes to viral escape from type-specific neutralizing antibody responses. Another antibody escape strategy by HIV is conformational masking of conserved Env regions, particularly the receptor binding site (278). Entropic barriers are imposed upon antibodies that contact the CD4BS on gp120, whereby, typically, large entropic changes are observed upon binding by non-neutralizing antibodies and smaller changes with more broadly neutralizing Abs (278, 366). Consequently, it has been proposed that the extensive conformational changes induced by non-neutralizing Abs are not compatible with the gp120 structure in the context of the native trimer, making such Abs incapable of binding their epitope and neutralizing the virus. To complicate matters further, the Env spike structure imposes steric hindrance on Abs that recognize conserved epitopes – the narrow recessed pocket of the CD4BS is further made inaccessible by neighboring protomer and bulky carbohydrates (464), the MPER on gp41 is in close proximity to the viral membrane, and the CD4i epitope is only transiently exposed and sterically restricted (280).

More recently, a new escape mechanism has been proposed stemming from the fact that Env spikes are present at unusually low density on HIV virions. Biochemical studies and cryo-electron tomography reconstructions estimate approximately 14 HIV spikes per viral particle (303, 581, 582). In contrast, the similarly sized influenza A virus, on average, has 450 spikes per viral particle (562). The sparse density of Env on the surface of the virus has raised the question whether this is one of the factors that make it difficult to raise a broadly neutralizing response to HIV (252, 266). Studies have shown that bivalent engagement of IgG improves HIV and SIV neutralization (88, 261, 359, 539, 569) and low Env density would make bivalent binding by neutralizing antibodies very difficult. The result is antibody-antigen interaction with diminished avidity, which is needed to achieve high-affinity binding and potent neutralization. A publication by Mouquet et al, has shed light on how the immune system might circumvent the

problem of the few and far between Env spikes by sequencing a large number of anti-gp140 Abs from HIV-infected individuals (358). The authors reported that 75% of the 134 anti-gp140 MAbs isolated from memory B cells were polyreactive, compared to 20% in uninfected controls. The most polyreactive and lipid-reactive were anti-gp41 Abs at 85-90%, which has been corroborated by other reports since then (127, 298, 356, 357). Yet another study found a statistically significant correlation between anti-cardiolipin activity with neutralization breadth, and anti-MPER titers (200), further supporting the idea that polyreactivity is a common feature of HIV-specific Abs. Mouquet et al proposed that the majority of anti-HIV antibodies were polyreactive to increase affinity through heterogeneous ligand binding, meaning that one scFv arm bound with high affinity to the Env spike, and the other with low affinity to another epitope on the HIV surface (358). One possibility is that the phospholipid bilayer of the viral envelope provides the secondary epitope, although this remains to be definitively shown. Another report from the same group characterized a bispecific MAb, which was engineered to bind to gp120 with one arm and to gp41 with the other arm (359). The biMAb demonstrated enhanced affinity and neutralization over the parental MAbs, providing more concrete proof that heteroligation is functionally relevant.

Epitope specificities of human monoclonal antibodies

Despite the multitude ways that HIV can escape the NAb response, a number of potent broadly neutralizing antibodies have been characterized that have circumvented the difficulties imposed by the structure of Env. For decades, only a handful of broadly neutralizing monoclonal antibodies to HIV had been isolated from chronically infected subjects. However, in the last few years, with the advent and improvement of technologies for their isolation, there has been a boom in the generation of novel broadly neutralizing MAbs (211, 357, 460). The basic concept is that, by understanding the mechanisms of neutralization by these antibodies, researchers will gain a better understanding of sites of vulnerability on Env, and will be able to design immunogens that would elicit similar specificities in vaccinees. Although a good understanding of the immunological factors associated with their development and a successful immunogen are still out of reach, the fact that broadly neutralizing MAbs exist is a proof of concept that the humoral response is at least capable of producing potent cross-neutralizing antibodies against HIV. These monoclonal antibodies have also been extensively used to design mapping strategies aimed at identifying similar specificities in cross-neutralizing polyclonal sera. To guide vaccine design and to measure breadth of neutralization in a standardized way, panels of isolates have been established (291, 292, 318, 466). Tier 1 isolates are relatively easy to

neutralize, while Tier 2 and 3 viruses are more representative of circulating strains and more difficult to neutralize. The MAbs characterized so far target distinct epitopes on the HIV envelope – CD4BS, CD4i, glycans, and the MPER of gp41, which are described in detail below **(Figure 1.8)**.

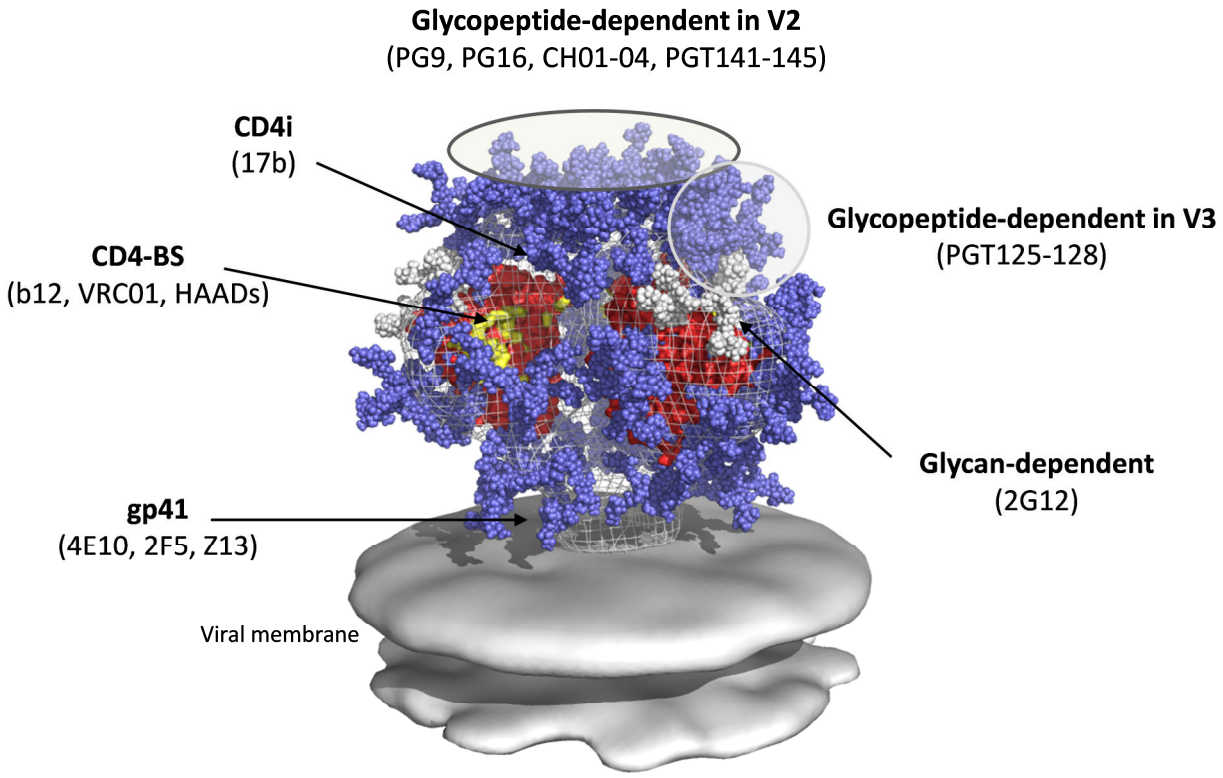

Figure 1.8 Epitope specificities of known anti-HIV broadly neutralizing MAbs on the native Env spike. The model of the Env trimer is as described in Figure 1.5. Indicated are approximate positions of the CD4BS (b12 epitope in yellow), CD4i epitope, 2G12 epitope (white glycans), MPER (gp41) epitope, PGT127-128 epitope and the quaternary epitope of PG9/PG16. Adapted from Schief et al, 2009 (464).

CD4BS (b12, VRC01, HAADs)

An important site of vulnerability for the virus is the highly conserved CD4 binding site, however extensive structural constraints from the narrow CD4 docking site and conformational masking by neighboring glycans and variable loops have made it exceedingly difficult to elicit broadly neutralizing antibodies to that region. Yet, the number of characterized broad and potent MAbs that map to that region provides proof that it is possible for the immune system to produce such responses. The recombinant monoclonal antibody b12 was isolated as a Fab fragment from a combinatorial phage display library from the bone marrow of a chronically clade B-infected asymptomatic subject, and neutralizes ~ 40% of circulating isolates (31, 47, 77, 79). To fit in the

narrow pocket of the CD4BS, b12 uses heavy chain only recognition, while generally most epitopes are bound by the juncture between the heavy and light chains on Fab (580). The ability to penetrate the recessed CD4BS pocket is further made possible by a long third complementarity determining region of heavy chain (CDRH3), which in its unliganded state extends 15Å above the antigen binding site (448). Upon passive transfer into macaques or humanized mice, mAb b12 can also provide sterilizing immunity after virus challenge (185, 227, 390, 526). Interestingly, mutations in the Fc portion of b12 that eliminate its ADCC function, also make the mAb much less protective, underlying the importance of this effector function in humoral anti-HIV activity (226). The non-neutralizing antibody b13 was also isolated from the individual that the potently neutralizing b12 was obtained. This mAb also binds to the CD4BS on gp120 with a similar approach angle as b12, however, only a 17° difference in recognition, moves the b13 epitope towards the bridging sheet and results in substantial conformational changes in the bound gp120 (95). Such changes are thought to be incompatible with the native viral spike, the deflection of the epitope thus constraining binding and neutralization by the b13 antibody.

MAb VRC01 was isolated from a chronically infected subject by fishing out individual B cell clones with a resurfaced stabilized core gp120 protein with antigenic specificity for the initial site of CD4 binding (552). VRC01 has remarkable breadth and potency, neutralizing over 90% of diverse HIV-1 isolates. There is substantial structural mimicry between VRC01 and CD4 interaction with the gp120 core, with a slight shift in orientation which allows the antibody to overcome the structural constraints of the variable loops and the surrounding glycans on the CD4BS (579). Computer modeling and alanine scanning mutagenesis suggest that the interaction of VRC01 with the native Env spike does not cause major structural changes in the configuration of the trimer (294) but mimics interactions with CD4 by exposing the co-receptor binding site. VRC01 is also characterized by the extensive affinity maturation of its IgG V-genes, with 41 amino acid alterations from the germline (genomic) variable heavy (V_H) gene. This phenomenon has been reported for HIV-specific antibodies in general, and especially for broadly neutralizing ones (239, 358, 459, 545, 553).

The pronounced levels of somatic hypermutation seen in MAbs like VRC01 prompted researchers to develop new assays for isolating MAbs that would also detect the highly divergent clones. After combining this strategy with a slightly modified resurfaced stabilized gp120 core or a gp140 trimer, a set of MAbs similar in binding to CD4 were isolated from four

unrelated HIV infected individuals, one of which the VRC01 donor (461). Together, these highly active agonistic anti-CD4BS (HAAD) antibodies neutralized 96% of the 118 viruses tested. Interestingly, despite extensive somatic hypermutation and origin in different individuals, the MAbs shared a consensus sequence of 68 residues in the IgG V_H gene. On the other hand, a different group also looking for VRC01-like anti-CD4BS antibodies demonstrated that three MAbs with low sequence similarity had, remarkably, converged to very similar structures in the mode of recognition of gp120 (553). MAb NIH45-46 is a more potent clonal variant of VRC01, and includes an insertion of four amino acids in the CDRH3 acquired through somatic hypermutation. Crystal structures demonstrate that the insertion creates additional contacts with gp120 that allow NIH45-46 to mimic even more closely binding to CD4 than VRC01 does, and most likely contribute to its increased potency (134). Overall, the extensive affinity maturation of anti-CD4BS antibodies – a result of prolonged antigenic stimulation – has raised concerns and debates about how this can be accomplished by vaccination.

Another concerning finding, reported for a number of broadly neutralizing MAbs, is that the predicted unmutated ancestral (or germline) versions of these antibodies did not bind the recombinant Env proteins used to isolate the mature Abs (461, 558, 559). This is largely problematic if such responses are to be elicited by recombinant Env immunogens, and offers an explanation why cross-reactive NAbs have not been elicited by any immunogen so far (488). In that case, current vaccine design methods need to be re-evaluated and any new immunogens should to be tested for binding to germline versions of broadly neutralizing MAbs.

CD4i (17b)

The CD4 induced (CD4i) epitope is so named because it is exposed or induced upon CD4 receptor binding. This region overlaps the chemokine co-receptor binding site, which is highly conserved among HIV strains and highly immunogenic. CD4i antibodies bind to their target epitope preferentially, or only, after CD4 has been engaged, and can have very broad reactivity, neutralizing as unrelated isolates as HIV-2 (123, 453, 500). Co-crystal structures of the broadly neutralizing CD4i MAb 17b in complex with a deglycosylated gp120 core and CD4, revealed that the epitope overlaps with the CCR5 co-receptor binding site and includes a substantial part of the bridging sheet on gp120 (279, 555). A long acidic CDR3 region of 17b, which is thought to mimic the tyrosine-rich acidic N-terminus of CCR5, largely facilitated interaction with the basic surface of the bridging sheet. Electron tomographic analysis of the trimeric Env spike in a ternary complex with CD4 and 17b demonstrated that CD4 binding caused reorganization of the

trimer with an outward displacement and rotation of each gp120 monomer, and a rearrangement of gp41 (303), thus exposing the 17b epitope and the co-receptor binding site. Consequently, despite high degree of conservation and immunogenicity, CD4i epitopes are only transiently exposed. One attempt to elicit such antibodies by vaccination has been to derive Env immunogens from viruses serially passaged in CD4-negative CCR5-positive or CXCR4-positive cell lines, generating CD4 independent strains that are bound by CD4i Abs without the need for CD4 (153, 233, 419). However, antibodies elicited by such an immunogen have to be able to bind to and neutralize the transmitted CD4-dependent isolates where steric constraints occlude the co-receptor binding site (280).

Glycan-specific (2G12, PG9/PG16, PGTs)

The HIV Env spike is very heavily glycosylated with host-cell derived sugar moieties, which shield the underlying protein epitopes from the host immune system (421). Carbohydrates are poorly immunogenic, and the dense cluster of glycans on gp120 is referred to as the silent face (555). And yet, despite the low immunogenicity and the fluidity of the glycan shield, with frequent mutations able to remove or add N-linked sugars, a number of broadly neutralizing MAbs have been found to map to conserved glycosylation sites. The broadly neutralizing MAb 2G12 recognizes a conserved cluster of oligomannose residues on gp120 (445, 456, 511, 512). The 2G12 epitope involves two primary glycan binding sites – N295 and N332, identified by site-directed mutagenesis and carbohydrate analysis (456). It is thought that the unique 'domain-swapped' structure of 2G12, whereby the variable heavy chains are exchanged on adjacent Fab arms, is what allows the antibody to recognize the clustered sugars as non-self (83, 277). The unusual architecture converts the antigen-binding regions of the MAb from a 20x30 $Å^2$ bivalent binding surface to a 20x60 $Å^2$ continuous multivalent surface able to span the heavily-glycosylated epitope (the dense clustering being a non-self, distinguishing feature of HIV). It is what allows 2G12 to not only bind to more than one N-linked glycan, but also to bind with nanomolar affinity. Multiple unsuccessful attempts have been made to elicit 2G12-like antibodies by vaccination. The question arises of how difficult it is for the immune system to evolve such unusual Ab architecture. Interestingly, a non-domain exchanged form of 2G12 was generated with only a single mutation, and could no longer bind the HIV Env glycan shield (137). This finding suggests that very few substitutions are required for domain exchange, which makes generation of Ab responses with similar specificities by vaccination plausible.

Recent efforts to identify new targets for HIV neutralization and to isolate novel, more potent neutralizing antibodies, led to the characterization of a class of broadly and potently neutralizing MAbs with conformational epitopes (276). These MAbs demonstrate higher affinity for the membrane-associated trimeric Env over soluble recombinant gp120 monomers, require an essential glycan at position 160, and a protein segment of the V1V2 region of gp120, and neutralize 70-80% of circulating isolates from diverse clades and recombinant forms (535). The class includes the prototypic MAb PG9, the somatically related PG16, and CH01-04, and PGT141-145 from two more donors (54, 535, 538). The crystal structure of PG9 in complex with scaffolded V1V2 forms has been solved and has demonstrated points of contact primarily with the glycan at position 160, and also at 156, and with one of the four β strands on V1V2 (332). X-ray crystallography of unliganded PG9, PG16, CH04 and PGT145 revealed a remarkable similarity among these MAbs in an unusually long and extended anionic CDRH3 region, which forms a stable 'hammerhead' subdomain, towering 20Å above the other combining loops (385, 392). Extensive levels of somatic hypermutation contribute to the breadth and potency of these MAbs. A distinctive feature of PG9-like antibodies is their sensitivity to a specific glycosylation inhibitor. Kifunensine is a mannose analogue that inhibits type-I alpha-glycosidases **(Figure 1.3)**, and viruses produced in kifunensine-treated cells become completely resistant to these MAbs (136, 535, 538). This tool has been used to map similar specificities in polyclonal sera of HIV-infected subjects (285, 536).

Another set of clonally-related glycan-specific broadly neutralizing MAbs are PGT125-128, which bind a complex epitope involving high mannose glycans at positions 301 and 332, and the amino acid backbone of the V3 loop (391, 538). PGT128 is the broadest of these antibodies, and neutralizes over 70% of circulating isolates from different clades; it is also one of the most potent anti-HIV MAbs known to date. Unlike PG9, this group of MAbs does not have 'quaternary' specificity and recognizes both monomeric gp120 and membrane-associated trimers. In addition, while PG9 binds simpler Man_5 sugars, PGT125-128 MAbs interact with the bulkier $Man_{8/9}$ carbohydrates. Structures of PGT128 in complex with a fully glycosylated gp120 outer domain demonstrated that a six-amino acid insertion in the CDRH2, and an extended CDRH3 greatly contribute to the high binding affinity to mannose by making contacts with multiple terminal mannose residues of the conserved glycans at positions 301 and 332 (391). The complex epitope also involves a short β strand segment of the V3 loop, with contacts primarily through the backbone amino acids that are tolerant of side-chain variations. Overall,

what accounts for PGT128's unusually high affinity for glycans is that it deeply penetrates the glycan shield on the HIV Envelope.

MPER (2F5, 4E10, Z13)

The membrane proximal external region (MPER) on gp41 is another highly conserved, functionally-relevant region of the HIV Env. It is also the target of three broadly neutralizing antibodies 2F5, 4E10 and Z13 (364, 588). Although the above features make the MPER an attractive vaccine target, neutralizing antibodies to the MPER are found rarely in HIV-infected individuals (42, 197, 295, 339, 449, 459, 474), and the proximity of this region to the membrane make it difficult for neutralizing antibodies to access. All three of these broad MAbs recognize highly conserved, linear epitopes, closely spaced on the 24-amino acid MPER. The minimal epitope for 2F5 is located in the N-terminus of MPER, the 4E10 epitope is proximal to the C-terminus, and Z13 binds in between, overlapping some of the 4E10 epitope (69, 70, 364, 412, 588). 2F5 is the most potent of the three MAbs, while 4E10 neutralizes the widest range of isolates; consequently the majority of studies have focused on the two antibodies.

Both 2F5 (11, 218, 381, 462) and 4E10 (11, 218, 462) are polyreactive with membrane phospholipids (i.e. cardiolipin) and several other autoantigens. It has been proposed that these antibodies mediate HIV neutralization through a two-component mechanism, which involves initial nonspecific interaction with the viral membrane that facilitates high-affinity recognition of the residues on the MPER (9, 175). This is a clever solution to the problem of steric hindrance on antibody access to the MPER caused by the adjacent lipid membrane. Crystal structures of 2F5 with gp41 peptides demonstrate that the MAb forms both hydrogen bonds and hydrophobic contacts with its epitope (382). As has been the trend for most broadly neutralizing anti-HIV MAbs, the CDRH3 of 2F5 is unusually long; its apex forms a hydrophobic surface that does not interacts with gp41 but likely makes direct contact with the viral membrane (382). Indeed, altering CDRH3 hydrophobicity had little effect on the binding of 2F5 to its peptide epitope, but caused profound decrease in viral neutralization (381). A mouse monoclonal antibody that shares the minimal tripeptide epitope of 2F5 but is non-neutralizing, lacks 2F5's long hydrophobic CDRH3, and only recognizes the postfusion formation of MPER (374). The stable postfusion six-helix structure is immunogenic, but antibodies that interact with it arrive too late to prevent membrane fusion and infection of the target cell. In contrast, 2F5 recognizes the prefusion or intermediate conformation of gp41 (122, 168, 454). In fact, experimental models of the neutralization mechanism of 2F5 and 4E10 suggest that their epitopes are likely not present

on the free virus, but most likely in the stages after CD4 receptor binding (44, 113). Two recent studies have questioned the extensive role of the 2F5 CDRH3 loop in lipid penetration because, although changes in CDRH3 hydrophobicity affected neutralization capacity, they had no effect against membrane components alone (250, 314).

The crystal structure of 4E10 in complex with an MPER peptide demonstrated that the core epitope WFXI(T/S), where the identity of X does not play a role in 4E10 binding, makes the most contacts with the antibody, with Tryptophan being the most critical (85, 443). This sequence is highly conserved among HIV-1 isolates, which explains the broad range of neutralization by this MAb. Similar to 2F5, 4E10 has a long hydrophobic CDRH3 loop, which also forms a flat hydrophobic surface at its tip, most likely contacting the neighboring viral membrane. Only the base of the CDRH3 makes contacts with the MPER peptide, and typically this region is crucial for antibody-antigen interaction (561). In addition, an unusually hydrophobic CDRH2 contributes to the hydrophobicity of the 4E10-combining site. Interestingly, when bound the 4E10 epitope adopts an unusual helical structure (85), when β-turns are the norm for antibody-bound peptides (491). This configuration could explain the conformational requirement of 4E10 for full epitope expression, which, despite the apparent linear nature of its epitope, does not bind denatured recombinant gp41 (588).

Numerous vaccine strategies have been attempted to elicit broadly neutralizing antibody responses to the MPER but without much success. The 2F5 epitope has been presented to the immune system in different contexts (111, 152, 154, 232, 297, 299, 331, 363, 560), and a number of immunogens with membrane components are under evaluation (128, 325, 380, 540). More recently, efforts have also focused on designing better 4E10 epitope vaccine candidates (259, 286, 325, 506, 533), which have met with similar challenges. Because of their reactivity to self-antigens and to lipids, elicitation of 2F5- and 4E10-like antibodies could be affected by immunologic tolerance mechanisms, specifically, that these polyreactive MAbs arose from autoreactive precursor B cells due to loss of tolerance checkpoints (218, 219, 367, 558). Importantly, studies in IgH knock-in mice bearing the 2F5 variable region transgenic for the V_H of 2F5 showed developmental arrest of 2F5-expressing B cells (529, 530). This theory has been called into question by data demonstrating that 2F5 and 4E10 have fundamentally different self-antigen reactivity than pathogenic autoantibodies (463, 479), and that despite their anti-cardiolipin activity, sustained passive infusion of these MAbs in humans is well tolerated (510, 523).

Autologous neutralizing antibody response and viral escape

As mentioned previously, 80% of Clade B and C infections are established by a single transmitted / founder variant, (7, 255, 442). Ten days later, p24 protein and viral RNA can be detected in the plasma, which marks the end of the eclipse phase (102, 116, 178, 302). Tomaras et al conducted a detailed study of the timing and specificity of the first HIV-specific Abs (503). The first detectable B cell response to HIV was found in the form of immune complexes 8 days after detectable plasma RNA, while the first free plasma antibodies were anti-gp41 IgM, detected approximately 13 days after initial viremia **(Figure 1.9)** (503). This is followed by class switching to IgA and IgG, making gp41 the first Env protein recognized by antibodies. Two weeks after anti-g41 Abs, is when binding antibodies to gp120 could be detected, all with specificity to the V3 loop. Over the next few weeks the specificity of anti-HIV Abs expands to include those to the CD4BS and the MPER (10, 503). However, neutralizing antibodies (NAbs) were not detectable in any of the subjects during the 40 days of follow-up. Indeed, autologous NAbs on average appear 3 months post-transmission (199, 424, 542), which is considerably delayed compared to other infections, and too late to prevent the establishment of latency. A recent study by Bar et al used increasingly sensitive DNA sequencing methods to isolate autologous Env variants early in infection, and proposed that low-titer NAbs appear earlier than previously recognized due to the limit of detection of laboratory neutralization assays (29). At the time that autologous NAbs were detected *in vitro*, in each individual the transmitted / founder virus had already been replaced by NAb escape mutants, indicating a pre-existing NAb response. Still, it is not known why the initial Ab responses are not neutralizing; one possibility is the immunodominant nature of denatured or non-functional Env forms (112, 501). It is also not completely understood why the NAb response is so substantially delayed. A role for HIV-induced immune dysfunction of B cells, destruction of germinal centers, and lysis of follicular B-cells has been implicated (360, 577).

In recent years, the autologous NAb response has been of increased interest in the HIV field because of the need to gain a better understanding of how it evolves, what kind of selection pressure it exerts on the virus, and potentially, how it can be elicited by vaccination. A number of longitudinal studies have sequenced viral Env's from the plasma of infected patients and have characterized the temporal NAb response to the virus. Studies of clade B and C infection unanimously show that, although most individuals mount an autologous NAb response of high titer, this response is largely type-specific, with little to no cross-reactivity (5, 22, 73, 199, 289, 400, 424, 542).

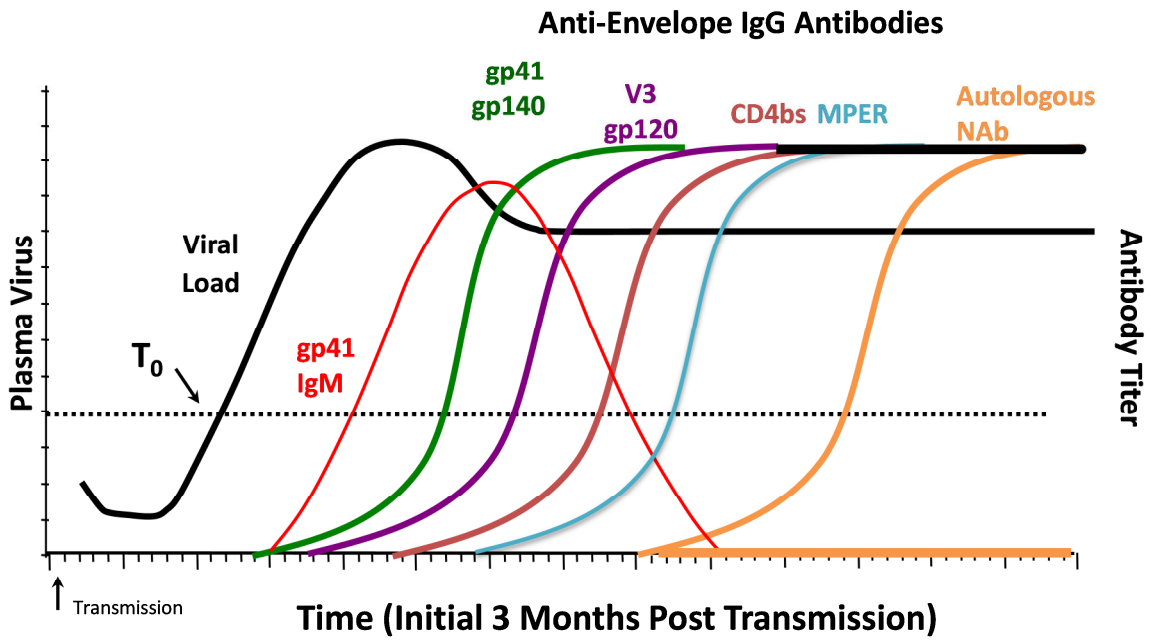

Figure 1.9 Timeline of anti-Env antibodies elicited during acute/early HIV-1 infection. After infection and the initial peak in viral load (black line), anti-gp41 IgM (red) are detected first. Days to weeks later, binding anti-Env IgG Abs are elicited sequentially – to gp41 (green), the V3 loop (purple), CD4BS (dark red), and MPER (light blue). Autologous NAbs (orange) develop within 3 months post infection. The dotted line indicates when plasma viremia or anti-HIV Abs are detectable in plasma. T0 is the time when plasma viremia reaches 100 copies/ml (the limit of detection of standard assays), and also marks the end of the eclipse phase. Adapted from Tomaras & Haynes, 2009 (502).

Frequent targets of autologous NAbs are the Env variable regions, but the virus promptly escapes their action with a complete replacement of the neutralization-sensitive virus with a population of resistant variants (29, 346, 351, 424, 434, 542). This evokes the development of new NAbs, leading to a cycle of antibody selection pressure and viral escape. Large insertions and deletions in the variable loops, particularly in V1V2, and changes in the number of potential N-linked glycosylation sites have been associated with viral escape (90, 144, 199, 353, 402, 434, 435, 441, 518, 520). Consequently, the autologous NAb response may play a pivotal role in driving Env diversity (177), although there seems to be limits to the ability of HIV to escape their action (125, 144). Interestingly, decades of global circulation of HIV and continuous rounds of NAb escape have resulted in an adaptation of Env to the humoral response at a population level. A study by Bunnik et al reported increased resistance of recently-transmitted HIV variants to broadly neutralizing MAbs over the course of 20 years (72). The observed change in resistance was particularly significant for anti-CD4BS antibodies and coincided with longer V1 loops and increased number of potential N-linked glycosylation sites (72, 155, 520).

Heterologous broadly neutralizing antibody response

The neutralizing antibody response is crucial for vaccine-mediated protection from a number of viral diseases (20, 78, 406), and it is also considered an important component of an effective HIV vaccine (321, 330, 490). However, bearing in mind the incredible viral diversity of circulating clades and recombinant forms, such Ab responses will have to be effective against an unprecedented variety of viral isolates. The broad and potent MAbs characterized to date provide a basis for studying epitope specificities to conserved regions on Env, while studies of broadly neutralizing antibodies **(bNAbs)** in human sera improve our understanding of the characteristics of such responses elicited during natural HIV infection, and the factors associated with their development.

Evidence for the protective role of neutralizing antibodies

Even though autologous NAb responses appear weeks after anti-HIV cytotoxic CD8+ T cells and after establishment of latency, their effect on viral control is greater than previously thought. The fact that NAbs exert selection pressure on the virus, and that HIV needs to escape their action has now been well documented (125, 177, 424, 542). One study reported an association between the presence of autologous NAb responses in chronic infection and the control of HIV replication in the absence of antiretroviral therapy (125). Huang et al demonstrated a direct link between long-term NAb control and HIV replication in a study of a chronically HIV-infected patient who underwent B-cell depletion as part of lymphoma treatment (240). Removal of B cells resulted in a decline of autologous NAb responses and a 1.7 log increase in plasma viral load. Reconstitution of B cells was followed by a decline in viral load, diversification of Env sequences and selection of escape mutants. This study provided direct evidence that NAb responses can play an important role in the control of HIV replication in chronic infection, a role that has likely been underestimated until recently.

Perhaps the most compelling evidence for pursuing a NAb-based vaccine comes from studies in non-human primate models where passive transfer or post-exposure prophylaxis with broadly neutralizing MAbs provide complete protection from infection. In the last 10 years numerous trials with either single or a combination of a few broad MAbs have demonstrated sterilizing protection in experimentally infected rhesus macaques. These include the anti-CD4BS MAb b12 (80, 185, 226, 227, 390, 526), the anti-glycan 2G12 (25, 165, 166, 228, 319, 320, 323), and anti-MPER MAbs 2F5 and 4E10 (25, 165, 166, 229, 319, 320, 323), which have been effective against intravenous and mucosal SHIV challenges. Importantly, while broadly neutralizing MAbs

confer protection, non-neutralizing MAbs do not provide sterilizing immunity in the non-human primate model (80). Purified IgG from HIV-infected chimpanzees has also been used successfully in passive transfer studies to provide sterilizing protection (375, 475). In addition, broadly neutralizing MAbs have been shown to delay viral rebound after cessation of antiretroviral therapy in HIV-infected humans (335, 510). Initially, the more qualitative of the non-human primate studies demonstrated that sterilizing immunity requires at least two orders of magnitude greater serum IgG concentrations than *in vitro* neutralizing concentrations (375, 390). The observation led many researchers to question the plausibility of inducing and maintaining such high titers through vaccination, suggesting instead to rely on vaccine-induced cellular immunity to blunt the infection and decrease viral load. However, two recent studies by Hessell et al argued that a major limitation of previous macaque studies has been the high dose of viral inoculums to ensure that control animals were infected by a single challenge, which are not a realistic representation of the low (0.1%-0.01%) probability of heterosexual transmission in humans. The authors demonstrated that much lower titers of MAbs b12 and 2G12 than previously thought were enough to completely protect macaques upon repeated low-dose mucosal challenge (227, 228). In terms of how many molecules of Ab are needed to prevent HIV infection on the molecular level, recent studies disfavor the previous theory of one Ab per virion (133), suggesting instead that all it takes is one Env spike to mediate viral entry so all need to be bound to prevent it (262, 564). Altogether, data from passive transfer studies provide proof-of-concept that a low-titer pre-existing antibody responses of sufficient breath can provide sterilizing immunity against HIV infection.

Nevertheless, most of the studies mentioned above represent an ideal setting of controlled infection with a single virus that is effectively neutralized by the transferred IgG. Mother-to-child transmission (MTCT) provides a unique natural setting to study the role of maternal NAbs in prevention of infection. MTCT can occur in utero (during gestation), intrapartum (during labor and delivery) or postpartum (through breast milk). In the absence of treatment, transmission occurs in 20-30% of cases, the majority of which happen postpartum (110, 370), and this has implicated a role for the immune system in the protection of the uninfected cases. Indeed, maternal neutralizing antibodies are transmitted across the placenta and into the developing infant (25, 269) and an inverse correlation has been observed between maternal bNAb titers and the risk of in utero and intrapartum transmission (33, 444). Anti-viral innate immune factors in the breast milk, such as lactoferrin, mucins, and alpha-defensins have been associated with reduced risk of HIV transmission (61, 275). Breast milk also contains innate and adaptive

immune cells, and is a source of IgA and IgG antibodies (81, 305). Although bNab titers in the breast milk are much lower than in plasma, antibodies in the milk have been shown to offer protection through their ADCC function (170, 310). Important evidence for the role of maternal NAbs in transmission are the reports that in most cases a genetic bottleneck occurs where a single virus is transmitted to the infant that is generally resistant to maternal plasma, and is phylogenetically more closely related to the mother's escape variants (132, 282, 439, 550, 572). These results argue for NAb selection pressure on the virus, although conflicting data have also been reported (528). A number of studies have demonstrated that transmitting mothers had lower autologous NAb titers than non-transmitting mothers (132, 267, 284, 457), with some studies not finding such an association (206, 244, 439). It is unclear if these discrepancies are due to experimental differences or differences in immunologic factors associated with the populations where MTCT was studied.

Superinfection occurs when an individual with established HIV infection and an ongoing immune response becomes infected with a second viral strain, suggesting gaps in the immune system. It is another natural setting in which to study the role of NAbs in transmission, and what the effect of antigenic diversity from two unrelated variants is on the development of NAbs. The majority of documented superinfections have occurred in the first year of infection when the immune response is not fully mature, however, cases in chronic infection are also observed when the immune response should be effective at protecting from another infection (395). Initially, it was suggested that superinfection occurred because of a weak NAb response that was unable to neutralize the second viral strain (482). However, this theory was challenged by a larger study that found no difference in the NAb breadth or potency between superinfected individuals and matched singly-infected controls (50). Two recent studies have provided evidence for the broadening of the NAb response after superinfection occurred (355, 408), although caveats such as potential confounding by viral load, CD4 counts, and unknown dates of seroconversion have to be considered. A more controlled nested cohort study by Cortez et al also demonstrated an increase in the breadth of the NAb response against heterologous isolates upon superinfection (108). A recent report aimed to address the role of the enhanced bNAb responses in neutralization of the early autologous virus, and in this case, the authors found no difference in the ability of late plasma to neutralize the first strain between superinfected and singly infected individuals (326). Although the role of NAbs in superinfection is not well understood, this is a useful model in to study the development of bNAbs in a setting of

increased antigenic stimulation. Unexplored areas of research are changes in NAb epitope specificities upon superinfection.

Although not neutralizing, antibody responses were for the first time associated with protection from HIV infection in the human vaccine trial known as RV144. The large-scale clinical efficacy trial took part in Thailand where volunteers were immunized with the live replicating canarypox vector ALVAC containing HIV env, gag, protease (vCP1521), and a boost of bivalent gp120 protein (AIDSVAX B/E). The modified intention-to-treat analysis, which excluded participants infected at the time of randomization, demonstrated a modest, yet statistically significant, 31.2% efficacy in the first year of follow-up (187, 422). Detailed studies of the NAb response in vaccinees found increased levels of ADCC activity, and weak neutralizing activities against easy-to-neutralize Tier 1 isolates mostly targeting the V3 loop, which waned rapidly (345). This could potentially mean that weakly neutralizing Abs were responsible for the modest protection in this low risk heterosexual population, or that other immune factors attributed to the acquisition protection. Robust analyses looking for immune correlates of protection identified a strong inverse correlation between infection risk and the concentration of plasma IgG specific for the V1V2 region, and a direct correlation between anti-Env plasma IgA levels and acquisition of infection (220). The report generated the hypothesis that high anti-V1V2 IgG levels are involved in protection against acquisition of HIV-1, and that high levels of plasma IgA mitigate the protective effect of Abs (220). Ongoing studies are investigating whether there is a direct mechanistic link between these immune parameters and the risk of infection or whether they are markers for other factors (333).

Studies of bNAbs in humans

For many years only a handful of broadly neutralizing MAbs had been isolated from HIV-infected individuals. It is also known that the autologous NAb responses that develop during natural infection are largely type-specific and easily escaped by the virus. This begs the question how likely is a vaccine to elicit cross-reactive neutralizing antibody responses effective against a wide range of diverse isolates if such responses are only rarely found during natural infection. A number of recent studies have addressed this question by studying the neutralizing activities in the plasma of large cohorts of infected subjects and systematically measuring the breadth and magnitude of their NAb responses against panels of unrelated (heterologous) isolates from multiple clades. Reports indicate that 10%-30% of chronically HIV-infected individuals have bNAb responses in their plasmas that can neutralize not only intraclade but

also interclade heterologous isolates (138, 157, 201, 295, 449, 477, 490), and that it takes years for these responses to develop (201, 519). Attempts to understand why only a proportion of subjects have cross-reactive NAb responses and what factors are associated with their development has resulted in multiple reports of statistically significant association between bNAbs and time since infection, plasma viral load, early Env diversity, and antibody avidity (125, 138, 201, 396, 449, 519). In particular, the apparent requirement for moderately high viral load suggests that antigenic stimulation is needed for the development of cross-reactive NAb responses.

Important information missing from the above-mentioned studies is data on the interplay of the autologous NAb response with the ever-evolving virus, and how it affects the mechanism of bNAb development. A recently published study by Wu et al characterized HIV Env variants from the donor of the broadly neutralizing MAb VRC01 in an attempt to identify virologic factors that might have elicited this specificity (551). All the sequenced plasma Env variants were resistant to VRC01 neutralization, while three other MAbs from the same donor could neutralize some VRC01 escape variants. These data indicated strong antibody-based selection pressure and a NAb response that continued to evolve in response to viral escape, similar to the pattern described for strain-specific autologous NAbs. Only a small number of archival Env variants from proviral DNA were sensitive to VRC01, but did not provide any detailed information on how this MAb was originally elicited. Euler et al studied the kinetics of the development of bNAb responses by looking longitudinally at the evolution of the autologous and heterologous neutralizing activities, and at the adaptation of the virus to the humoral response (156). Individuals with cross-reactive neutralizing activity had higher peak autologous IC50 titers than those without, and their infecting viruses escaped from NAb responses more rapidly, potentially indicating less immune pressure in the subjects with type-specific activity. The authors also reported an increase in autologous NAb titers that coincided with an increase in heterologous neutralizing activity, and found the highest Env sequence diversity in the individual with the most broad and potent NAb responses. Overall, the results suggest that an increase in the potency of autologous NAb responses may have driven viral diversity, which in turn could have driven the affinity maturation of these antibodies, contributing to their increased potency and cross-reactivity. This theory is in agreement with the moderate-to-high viral load associated with the development of bNAbs. In the same vein, another study reported that anti-gp140 MAbs from individuals with high titers of bNAb responses were exceptionally highly mutated, indicating that prolonged antigenic stimulation is driving continued somatic hypermutation and selection for

high-affinity Abs (459). Still, more information on the underlying factors that determine the mechanism of the development of bNAb responses is needed to improve our understanding of how such responses can be elicited by a vaccine.

The targets of bNAbs are of great interest in the HIV field, both in terms of understanding how they evolve and how they can be elicited by vaccination. Numerous studies have investigated the epitope specificities of bNAb responses in chronic infection using many different strategies, including competitions and depletions of defined specificities with Env peptides and proteins (monomers or trimers that could be WT, denatured, or mutated), neutralization of chimeric, point mutant or treated isolates, competitions with non-neutralizing MAbs, and binding assays (113, 120, 131, 198, 293, 295, 536, 570). Yet, despite thorough efforts to map specificities of cross-reactive NAb responses, substantial portion of target epitopes remain unidentified – a reflection of the inadequacy of the available laboratory reagents to faithfully mimic the native spike, and of the difficulty in working with polyclonal sera.

Mapping analyses have focused primarily on known conserved regions of HIV Env, which have been defined through the use of well-characterized broadly neutralizing MAbs, as discussed in the section ‘Epitope specificities of human monoclonal antibodies’. Although in some cases specificity could be mapped to a single epitope, for the most part, a limited number of specificities is responsible for the plasma bNAb activity in any one individual (131, 200, 285, 293, 449, 504, 536). In one chronically HIV-infected subject broadly neutralizing activity could be recapitulated by two clonally unrelated monoclonal antibodies, one targeting the CD4-BS and the other targeting a conformational V1V2 epitope (55), while in another case two distinct specificities were also identified – to the CD4BS and to an epitope exposed after CD4 binding (265). In fact, the CD4BS is highly immunogenic, and antibodies with such specificity are commonly elicited in HIV-infected and vaccinated individuals, although for the most part are weakly neutralizing with limited breadth. Still, broadly neutralizing activities targeting the CD4BS have come up multiple times in epitope mapping studies, in rare cases contributing exclusively to the sera bNAb responses (43, 293, 295, 368, 449). In the above studies fractionation using gp120 immobilized on beads demonstrated that most of the broadly neutralizing activity was due to anti-gp120 Abs. However, using a gp120 variant unable to bind the CD4 receptor (D368R mutant) could not deplete bNAb activity, suggesting that the epitope of the bNAb response in these individuals overlaps the CD4BS. This fractionation technique has been used extensively since it was first described in 2007. CD4i antibodies are found in more than 90% of

infected individuals (123, 200, 295, 430, 459). Although some have broadly neutralizing activity, the majority does not as they are restricted by steric constraints from the close proximity to the co-receptor binding site to the cell membrane after CD4 engagement (280). Gp41, and MPER specifically, is another Env conserved region of interest. Strategies to map such epitope specificities include depletions and competitions with gp41 peptides, and neutralization of chimeric viruses that present MPER peptide epitopes on the background of SIV or HIV-2 isolates. While anti-gp41 binding Abs are commonly found in HIV-infected subjects, little to no anti-MPER NAbs have been reported in most studies, with only a few cases where potent bNAb responses to this region have been identified (42, 197, 198, 295, 356, 449, 459, 474, 586).

Recently a number of broadly neutralizing MAbs have been isolated that recognize glycan-dependent epitopes, and studies have tried to identify how frequently such specificities are found in HIV infected subjects. Techniques used in their identification include neutralization assays of glycan point mutant isolates, and treating virus producer cells with glycosylation inhibitors. A 2010 study by Walker at all analyzed the epitope specificity of individuals with highly potent bNAb responses and found PG9/PG16-like specificity in 20% of subject in which specificity could not be absorbed by monomeric gp120 but was dependent on a glycan at position 160 and was sensitive to kifunensine treatment (536). A similar proportion of bNAb sera were sensitive to the presence of a glycan at position N332, perhaps indicative of PGT127-128 – like activity. The study also identified one subject with 2G12-like NAbs, which are typically not reported by other mapping studies [with one exception (64)], indicating the paucity of this specificity among HIV infected individuals. Another report identified a V2- and also N160-dependent quaternary specificity in a Clade C-infected subject, where potency was also modified by the length of the neighboring V1 loop (355). Interestingly, the fine specificity of the bNAb response varied a little over time, and the dependence on some of the residues identified as part of the neutralization motif changed over time.

Clinical relevance of bNAb responses

A frequent question in the HIV field is whether the presence of bNAb responses, which often target conserved Env regions and neutralize a broad range of isolates, provides any clinical benefit for the infected individual. Studies have addressed this question indirectly by looking for associations between plasma broadly neutralizing activity and higher CD4 count or slower disease progression (often measured as time to start therapy) but have found no significant correlation (157, 396). Others have researched the setting of natural control of HIV – individuals

called long-term non-progressors (LTNP) that have low or undetectable plasma viremia. A number of studies have found increased autologous or broadly neutralizing Ab levels in LTNP compared to progressors (84, 248, 300, 575). Others, however, have not found such an association, reporting instead in some cases strong CTL responses (28, 138, 214, 519). Even though bNAb responses may play a role in the control of infection in some LTNP, there is no direct mechanistic link between the presence of such responses and slower disease progression. The underlying theory is that bNAbs would be protective if present at the time of transmission when a small inoculum of a single or small number of variants initiates the infection, and when viral replication is much slower and not yet systemic.

GOALS OF THESIS PROJECT

As previously mentioned, a number of studies have reported that cross-reactive NAb responses are present in 10%-30% of HIV-infected subjects. However, these studies were cross-sectional and took part in chronic infection, and so missing the development of the earliest such responses. In addition, not much is known about the epitope specificities of these initial bNAb responses, how they evolve over time and how they compare with what has been reported during chronic infection. A better understanding of the factors associated with their development will likely aid the design of a successful HIV vaccine. The overall goal of this project was to characterize the earliest cross-reactive NAb responses. Specific aims were to define how much time after infection it takes for them to become detectable, what underlying factors are associated with their development, and to characterize the evolution of their epitope specificities. To this end, we took advantage of the availability of well-characterized cohorts of acute/early HIV infection with longitudinal collection of plasma samples, known date of infection or seroconversion, and absence of antiretroviral therapy. Chapter II of this work contains detailed characterization of the cross-reactive neutralizing activities in two cohorts, reporting that it takes 2 to 3 years for these activities to be detected, and that they are present in 27% of the participants in this study. Our findings led to the hypothesis that there is a window of opportunity for bNAbs to develop during natural HIV infection. The earliest cross-reactive NAb responses target a limited number of conserved epitopes, while type-specific Ab responses seem to target more variable regions on gp120. In chapter III is described a case study of an HIV infected individual who developed cross-reactive NAb responses that target two distinct regions of Env and are complimentary in their anti-HIV activities. The results indicate that the two epitope

specificities target the CD4BS and the apex of the Env spike. The anti-CD4BS specificity developed around 3 years post infection, while the anti-‘apex’ specificity became detectable after 4 years of infection. The above findings suggest that to prevent infection by diverse HIV isolates globally, a vaccine would only have to elicit antibodies against one or two well-defined, conserved Env regions, rather than antibodies to a greater number of epitopes.

CHAPTER II

Characteristics of the Earliest Cross-Neutralizing Antibody Response to HIV-1

ABSTRACT

As mentioned in Chapter I, a number of cross-sectional analyses of HIV-1+ plasmas have indicated that broadly cross-reactive neutralizing antibody responses are developed by 10% - 30% of HIV-1+ subjects. The timing of the initial development of such anti-viral responses is unknown. It is also unknown whether the emergence of these responses coincides with the appearance of antibody specificities to a single or multiple regions of the viral envelope glycoprotein (Env). Here we analyzed the cross-neutralizing antibody responses in longitudinal plasmas collected soon after and up to seven years after HIV-1 infection. We find that anti-HIV-1 cross-neutralizing antibody responses first become evident on average at 2.5 years and, in rare cases, as early as 1 year following infection. If cross-neutralizing antibody responses do not develop during the first 2-3 years of infection, they most likely will not do so subsequently. Our results indicate a potential link between the development of cross-neutralizing antibody responses and specific activation markers on T cells, and with plasma viremia levels. The earliest cross-neutralizing antibody response targets a limited number of Env regions; primarily the CD4-binding site and epitopes that are not present on monomeric Env but the virion-associated trimeric Env form. In contrast, the neutralizing activities of plasmas from subjects that did not develop cross-neutralizing antibody responses, target epitopes on monomeric gp120, other than the CD4-BS. Our study provides information that is not only relevant to better understand the interaction of the human immune system with HIV but may guide the development of effective immunization protocols. Since antibodies to complex epitopes that are present on the virion-associated envelope spike appear to be key components of earliest cross-neutralizing activities of HIV-1+ plasmas, then emphasis should be made to elicit similar antibodies by vaccination.

INTRODUCTION

The initial antibody response to the HIV-1 viral envelope glycoprotein (Env) manifests itself within the first 2 weeks of infection and is non-neutralizing (348, 503). Autologous neutralizing antibodies develop during the first months after infection (289, 351, 424) and recent studies indicated that approximately 10% -30% of chronically-infected HIV-1 subjects develop cross-reactive neutralizing antibody responses of significant breadth (138, 449, 477). These latter responses are the ones an effective vaccine should elicit (490). Several studies indicated that the breadth of plasma cross-neutralizing antibody responses is positively associated with plasma viral load (125, 138, 396, 449, 519), but very little is known about the time course of these responses. A recent study by van Gils et al, using samples collected at 2 and 4 years following infection, indicated that a greater number of infected subjects displayed cross-neutralizing activities at 4 than at 2 years (519). However, the earliest timing of the development of such responses was not determined. Defining the timing of emergence of cross- neutralizing antibody responses following HIV-1 infection and identifying factors associated with their development, will advance our understanding of the complex interaction of HIV-1 with the immune system, will improve our understanding on how HIV-1 infection leads to immune dysfunction, and will also be useful to the development of immunization protocols that hopefully would elicit similar antibody responses.

The epitope specificities of the anti-HIV-1 cross-reactive neutralizing antibody responses in HIV-1+ plasmas collected during chronic infection are complex, with many specificities remaining undefined. Although there is general consensus that these neutralizing activities rarely target the transmembrane subunit gp41, but mostly the extracellular gp120 subunit (42, 295, 368, 449, 459, 474), there remains quite an uncertainty whether the overall cross-neutralizing activities of HIV-1+ plasmas are due to a single, a limited number of, or many different epitope specificities (42, 131, 293, 295, 368, 398, 449, 459, 536, 537). The above studies were conducted with samples from chronically-infected subjects and very little, if anything, is known about the epitope specificities of the earliest cross-neutralizing antibody responses in HIV-1+ plasmas. Defining these epitope specificities would be informative for future immunogen design efforts.

Here we analyzed the cross-neutralizing antibody responses in longitudinal plasmas collected soon after and up to seven years after HIV-1 infection. We found that the subset of HIV-1-infected subjects that develop cross-neutralizing antibody responses do so on average within

the first 2.5 years of infection, although in rare cases such responses became detectable as early as 1 year after infection. Epitope-mapping analyses indicated that the earliest cross-neutralizing antibody responses target primarily epitopes within and around the CD4-BS of gp120, or epitopes that are present on the virion-associated trimeric Env, but not on the corresponding monomeric gp120 or gp41 Env subunits. In contrast, the neutralizing activities of plasmas from subjects that did not develop cross-neutralizing antibody responses, target epitopes on monomeric gp120, other than the CD4-BS. These observations are indicative of the presence and long-term survival of B cells that recognize complex but conserved epitopes on the viral Env in those HIV-infected subjects that develop cross-neutralizing antibody responses.

MATERIALS AND METHODS

Cohorts

Patients from the Vanderbilt University and the Ragon Institute of Massachusetts General Hospital 'acute / early' HIV infection cohorts (also referred to 'primary' cohorts) were used in this study. The subjects selected for the present study were infected with clade B HIV-1, had no AIDS-defining illnesses, and were not on antiretroviral therapy at the time of sample collection. In the MGH Acute HIV Infection Cohort, 'primary infection' was defined by detectable HIV RNA in the presence of either (i) a negative p24 ELISA, or (ii) a positive ELISA but evolving WB, or (iii) documented negative HIV ELISA within past 6 months. The plasma samples from the 'Vanderbilt Cohort' were collected mostly during the first year of seroconversion. All early infection subjects in this cohort had a documented negative HIV antibody test within one year of their first positive western blot result. In the case of the 'MGH Acute HIV Infection Cohort' the date of infection was known and samples were collected longitudinally from a few months post infection to up to 7 years post infection. In total, 53 plasma samples (collected longitudinally up to 2.5 years post-infection) from 21 HIV+ subjects from the 'Vanderbilt Cohort' and 69 plasma samples from 17 HIV+ subjects from the 'MGH Cohort' were evaluated.

Ethics statement

The Ragon Institute's and Vanderbilt University's Institutional review boards approved the study. Written informed consent was provided by all study participants and/or their legal guardians. The data were analyzed anonymously.

Plasma antibody adsorptions to monomeric gp120 or gp41

Plasma anti-HIV Env antibodies were adsorbed on beads coated with either recombinant SF162 gp120 or HxB2 gp41 (amino acids 541-682, Viral Therapeutics, Inc Ithaca NY) as previously described (293, 449). The proteins were coupled to MyOne Dynabeads Tosylactivated (Invitrogen) following the manufacturer's instructions. Briefly, 40 mg of magnetic beads were reacted with 1 mg protein ligand overnight at 37^{o}C with gentle rotation. After collecting the beads on a magnet, the supernatant was removed and the beads were incubated overnight at 37ºC in PBS, 0.5% BSA, 0.05% Tween 20. The magnetic beads were washed twice with PBS, 0.1% BSA, 0.05% Tween 20, and stored at 4ºC in the same buffer, with the addition of 0.02% Sodium Azide. Bead-coupled Env proteins were tested for antigenic integrity by flow cytometry using known MAbs b12, 447-52D, 2G12, IgG-CD4, and 4E10, followed by detection with goat-

anti-human-IgG-FITC secondary antibody (data not shown). Mock adsorption/elution experiments using several anti-HIV Env MAbs at a concentration of 10 µg/ml in naïve plasma were performed as a positive control (data not shown). 500µl of plasma, diluted 1:5 in DMEM/10%FBS, were incubated with 200µl Env protein-coupled beads at room temperature for 120 min with gentle rotation. The samples were placed on a magnet and the beads were isolated.

The antibodies bound to the bead-coupled Env proteins were eluted in a series of increasingly acidic solutions as previously described (293). The beads from each serial adsorption were combined and incubated in 0.1M Glycine-HCl, pH 2.7 for 30 seconds with vortexing. The beads were collected by brief centrifugation and held in place by a magnet. The supernatant was removed and adjusted to pH 7.5 with 1M Tris (pH 9.0). The process was repeated with the beads in 0.1M Glycine-HCl, pH 2.3 and then again in pH 1.7. The final supernatants were buffer-exchanged in PBS and washed over a 30kD Amicon Ultra centrifugation concentrator (Millipore). Concentration of immunoglobulin was determined by absorbance at 280 nm (NanoDrop Spectrophotometer ND-1000, Thermo). The depleted plasmas and the antibodies that were eluted from gp120-coated beads were tested by ELISA for reactivity to gp120, and for neutralizing activity.

Neutralization assays

The neutralizing activities of plasmas were determined using the Tzm-bl-based neutralization assay (130). Briefly, plasma dilutions (starting at 1:20) were pre-incubated with single-round competent virions (pseudovirus) for 60 minutes at 37ºC. The plasma / pseudovirus mixture was added to TZM-bl cells (3000 cells per well in a 96-well plate) for 72 hrs at 37ºC. The supernatant was removed and 100µl of Steady-Glo Luciferase Assay Substrate (Promega) was added to each well. Plates were incubated for 15 minutes at room temperature and 75µl of the lysate was transferred to micro titer plates. The cell-associated luciferase activity for each well was determined on a Fluoroscan Luminometer (Thermo). Percent neutralization was calculated at each plasma dilution as the percent inhibition of viral entry by the plasma sample compared to the absence of plasma. For each plasma/virus combination tested, a neutralization curve (percent neutralization versus plasma dilution) was generated using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego California, USA), and the plasma dilution at which 50% neutralization was recorded (IC50) was determined by transforming the data to a log10 scale with fitted sigmoidal dose-response curves.

Neutralization breadth of a plasma sample is defined as the percent (0% - 100%) of the 20 isolates neutralized by that sample.

All plasmas were tested against single round competent virions expressing Envs from 10 Clade B, 6 Clade C and 4 Clade A primary viruses. The clade B SF162.LS (EU123924), JRFL (U63632) and YU2 (M93258) viruses were isolated during chronic HIV-1 infection and the remaining isolates were isolated during acute infection, with published accession numbers (51, 291, 292, 306). All plasma samples were also screened for non-HIV-specific neutralization using the murine leukemia virus (MLV) pseudotyped into the HIV backbone. Neutralization activity was not detected against MLV at 1:20 by any of the plasma samples (data not shown).
In certain cases, competition neutralization experiments were performed in the presence of the D368R gp120 or an MPER-derived peptide. Serially diluted MAbs or HIV+ plasmas were pre-incubated with D368R (25 µg/ml) or the MPER peptide (10 µg/ml) for 1 hour at 37^0C and then the mixture was incubated with virus for another hour at 37ºC, and subsequently with cells as described above. The fold decrease in log10 IC50 neutralization titers of each plasma tested against each virus in the presence of D368R or the MPER peptide was determined.

Statistical analysis

Logarithmic transformation was used for viral load, and nonparametric regression with two-tailed p-value analysis was used to determine correlations between the breadth of cross-neutralizing antibody responses in HIV-1+ plasmas and plasma viremia levels. Mann-Whitney Test and Pierson correlation and linear regression analysis were used to determine correlations between immune activation and breadth of neutralizing activities.

RESULTS

Detection of cross-reactive neutralizing responses as early as one year following HIV-1 infection

To define the earliest period following HIV-1 infection when cross-neutralizing antibody responses appear in plasma we determined the neutralizing activities of plasmas collected within a few months and up to several years post HIV-1 infection from anti-retroviral naïve subjects infected with clade B viruses, against 20 heterologous clade A, B and C primary isolates **(Figures 2.1 and 2.2)**. Plasma samples from two independent cohorts were examined. The samples from the Vanderbilt cohort (VC) were, for the most part, collected within the first year of infection **(Figure 2.1)**. The breadth of cross-neutralizing activity (i.e., the percentage of viruses neutralized by any given plasma out of the total number of viruses the plasma was tested against) was minimal (less than 50%), in agreement with previous observations (424, 503). In most cases, these 'early' plasmas efficiently neutralized the 'easy-to-neutralize' primary SF162.LS virus, but not other primary viruses examined here. In the few cases where neutralizing activity against viruses other than SF162 was observed, the potency of neutralization was for the most part very weak and the neutralizing activities targeted clade B viruses. In two cases (subjects VC20017 and VC20027) the 'early' plasmas also neutralized a few non-clade B viruses. Plasma VC20027 collected within the first year of infection neutralized 6/9 clade B, 3/6 clade C and 1/4 clade A viruses. Plasma sample collected from subject VC20017 during the first year of infection neutralized 4/9 clade B viruses, the clade A virus Q259d2.17, and the clade C viruses ZM214M and Du422.1. These observations indicate that cross-neutralizing antibody responses begin to emerge during the first year of HIV-1 infection, but that such responses are weak in potency and narrow in breadth; rarely targeting viruses from clades other than the one the patient is infected with.

		Clade B									Clade C						Clade A				
PID	YPI	SF162.LS	JRFL	YU-2	REJO4541.67	TRO.11	SC422661.8	QH0692.42	PVO.4	CAAN5342.A2	ZM249M.PL1	ZM214M.PL15	ZM109F.PB4	Du422.1	Du172.17	CAP45.2.00.G3	Q769h5	Q461e2	Q259d2.17	Q168a2	Breadth
VC20001	3 yr	582	24	295	27	44	42	33	222	37	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	47%
VC20003	< 1 yr	736	116	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	11%
	1.5 yr	1257	336	(-)	(-)	(-)	39	62	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	21%
VC20005	< 1 yr	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	0%
	1 yr	274	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	1.5 yr	310	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
VC20007	1 yr	20	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	1.5 yr	435	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	2 yr	1009	(-)	(-)	(-)	45	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	11%
	3 yr	455	(-)	(-)	(-)	23	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	11%
	4 yr	718	(-)	(-)	21	20	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	16%
VC20008	1 yr	73	20	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	11%
	~1.5 yr	316	68	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	11%
VC20009	< 1 yr	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	0%
	< 1 yr	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	0%
	< 1 yr	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	0%
VC20014	< 1 yr	1700	32	(-)	41	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	16%
	1 yr	974	20	(-)	(-)	20	(-)	27	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	21%
VC20015	< 1 yr	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	0%
	< 1 yr	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	0%
	1 yr	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	0%
	1 yr	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	0%
VC20016	<1 yr	92	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	1 yr	1589	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	1 yr	>2560	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	2 yr	2068	29	(-)	65	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	16%
	2.5 yr	1019	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	2.5 yr	593	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	11%
VC20017	<1 yr	903	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	113	(-)	11%
	<1 yr	1455	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	398	(-)	11%
	1 yr	2207	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	123	(-)	11%
	1 yr	1921	20	(-)	26	20	(-)	(-)	(-)	(-)	(-)	(-)	(-)	71	(-)	(-)	(-)	(-)	162	(-)	32%
	1.5 yr	>2560	21	(-)	60	88	(-)	22	(-)	(-)	(-)	21	(-)	143	(-)	(-)	(-)	(-)	(-)	(-)	37%
VC20019	< 1 yr	144	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
VC20020	< 1 yr	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	0%
	< 1 yr	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	0%
	1 yr	131	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	1.5 yr	230	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
VC20021	< 1 yr	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	0%
	< 1 yr	263	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	1 yr	924	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
VC20022	< 1 yr	439	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	< 1 yr	354	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	< 1 yr	860	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
VC20023	< 1 yr	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	0%
	< 1 yr	23	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	1 yr	234	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
VC20024	< 1 yr	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	0%
VC20025	< 1 yr	72	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
VC20026	< 1 yr	216	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
VC20027	< 1 yr	754	25	(-)	20	119	20	(-)	(-)	(-)	(-)	20	(-)	83	(-)	134	(-)	(-)	(-)	20	47%
VC20028	<1 yr	1070	21	87	28	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	21%
VC20029	< 1 yr	194	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%

Figure 2.1. Cross-neutralizing activities in plasmas from the Vanderbilt Cohort.
The cross-neutralizing activities of plasmas from the indicated subjects (PID) were evaluated against the indicated clade B, C and A viruses. The values are the plasma titers at which 50% neutralization (IC50) was recorded. For clarity this information is color-coded: (blue) IC50<1:100; (orange) 1:100≤IC50≥1:250; (red) IC50>1:250. With the exception of SF162.LS (tier 1 virus), all other viruses are tier 2 (51, 291, 292). (-): less than 50% neutralization was recorded. YPI: years post-infection. 'breadth': the percent of isolates neutralized by a plasma sample, out of the total number of isolates tested, irrespective of the potency of neutralization (449). Each experiment was performed at least two independent times.

In the case of the MGH Acute HIV Infection Cohort (AC), plasma samples were collected longitudinally within a few months after infection and up to approximately 7 years post infection, with an average follow-up of 3.31 years. Here, too, samples collected during the first year of infection did not display broad cross-neutralizing activities **(Figure 2.2)**. In only one case (subject AC128), a plasma sample collected approximately 1.4 years after infection neutralized 65% of the heterologous viruses tested (7/10 clade B, 5/6 clade C and 1/4 clade A). A plasma sample collected a year later from the same subject neutralized 90% of the viruses tested with greater potency: an indication of a continuous evolution and increase in the breadth of the cross-neutralizing antibody responses during the first 2 years of infection in this subject. Overall, plasma samples from 7/17 subjects (41%) (AC049, AC053, AC071, AC089, AC128, AC131, and AC180) displayed cross-neutralizing activities against 50% of the isolates tested against at some point during the period of observation. Samples from 5/17 subjects (29%) (AC049, AC053, AC128, AC131, and AC180) displayed broad cross-neutralizing activities against at least 75% of the viruses tested at some point during the period of observation. This percentage is in agreement with numerous previous reports on the frequencies of broad cross-neutralizing activities in sera collected during chronic HIV-1 infection (138, 449, 477). In these 5 cases, a gradual increase in the breadth of cross-neutralizing activities was recorded over time, even though plasmas collected longitudinally from individual subjects did not always neutralize the same isolates, nor with the same potency. Such changes in potency by samples collected over time from individual subjects could be due to changes in the number of epitopes recognized by the circulating antibodies (i.e., the relative proportions of NAbs with diverse epitope specificities change over time), and/or due to changes in the plasma concentrations of antibodies with epitope specificities that do not change over time. Collectively, the above results indicate that the mean time it took for the breadth of cross-neutralizing activities to reach 50% was 2.13 years and the mean time to reach 75% was 3.08 years. Although the percentage of subjects developing cross-neutralizing antibody responses increased during the first 3 years of infection, that percentage did not further increase in year 4 **(Figure 2.3)**. Clearly, additional longitudinal analysis is required to determine whether cross-neutralizing antibody responses increase past that time.

		Clade B										Clade C						Clade A				
PID	YPI	SF162.LS	JRFL	YU-2	REJO4541.67	TRO.11	SC422661.8	QH0692.42	CAAN5342.A2	PVO.4	TRJO4551.58	ZM214M.PL15	ZM249M.PL1	ZM109F.PB4	ZM53M.PB12	Du422.1	CAP45.2.00.G3	Q769h5	Q461e2	Q259d2.17	Q168a2	Breadth
AC049	0.62	1642	(-)	(-)	(-)	(-)	125	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	10%
	1.09	3201	(-)	(-)	20	(-)	184	(-)	(-)	(-)	(-)	(-)	(-)	20	(-)	(-)	21	(-)	(-)	(-)	(-)	25%
	2.62	6247	75	21	80	153	259	(-)	(-)	(-)	(-)	97	(-)	30	(-)	84	70	(-)	(-)	(-)	45	55%
	3.64	4503	120	46	93	266	516	334	56	49	22	168	(-)	61	104	45	235	(-)	21	(-)	199	85%
	4.39	6114	102	35	89	356	442	592	27	60	20	291	(-)	57	199	40	160	(-)	(-)	(-)	130	80%
AC053	0.82	389	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	1.75	769	(-)	(-)	20	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	36	(-)	(-)	64	(-)	(-)	(-)	(-)	20%
	2.29	1180	23	(-)	41	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	221	(-)	(-)	(-)	(-)	20%
	3.29	6713	1467	612	46	114	82	(-)	(-)	393	(-)	(-)	(-)	(-)	(-)	153	117	(-)	(-)	(-)	(-)	45%
	4.30	6134	1117	543	78	151	100	409	2723	426	(-)	109	(-)	44	(-)	232	231	(-)	(-)	(-)	(-)	65%
	5.31	2821	1268	951	72	374	251	409	2809	271	44	86	29	46	(-)	271	150	(-)	(-)	21	(-)	80%
	5.88	3428	1403	594	63	328	440	230	1539	211	29	(-)	(-)	(-)	(-)	147	107	(-)	(-)	(-)	(-)	60%
	6.85	1590	623	330	30	288	37	172	766	131	(-)	(-)	(-)	(-)	(-)	106	44	(-)	(-)	29	(-)	60%
AC071	0.39	23	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	1.30	88	21	(-)	30	29	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	42	(-)	(-)	(-)	(-)	(-)	25%
	2.28	178	52	(-)	(-)	81	181	(-)	(-)	(-)	(-)	(-)	(-)	55	(-)	32	40	47	(-)	(-)	(-)	35%
	3.34	583	276	21	(-)	50	133	188	(-)	(-)	(-)	(-)	(-)	(-)	(-)	20	(-)	215	(-)	51	52	50%
	3.80	322	311	(-)	(-)	69	131	164	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	376	(-)	177	89	40%
AC083	0.88	1070	(-)	(-)	71	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	10%
AC089	0.00	1024	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	0.42	831	(-)	(-)	20	36	(-)	(-)	(-)	(-)	(-)	(-)	(-)	24	(-)	(-)	(-)	(-)	(-)	(-)	(-)	20%
	1.94	1035	70	(-)	30	224	81	535	(-)	(-)	(-)	54	(-)	56	(-)	(-)	540	(-)	30	(-)	(-)	50%
AC093	0.12	24	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	0.90	324	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	1.88	3845	(-)	(-)	(-)	21	(-)	(-)	(-)	(-)	(-)	(-)	(-)	23	(-)	(-)	(-)	(-)	(-)	(-)	(-)	15%
	2.91	3934	(-)	(-)	(-)	29	(-)	(-)	(-)	(-)	(-)	(-)	(-)	31	(-)	(-)	(-)	(-)	(-)	(-)	(-)	15%
	3.99	6130	36	(-)	94	39	(-)	250	(-)	(-)	(-)	(-)	(-)	22	(-)	(-)	47	(-)	(-)	(-)	(-)	35%
	4.97	7274	31	(-)	151	96	77	491	(-)	(-)	(-)	(-)	(-)	38	(-)	(-)	(-)	(-)	(-)	(-)	(-)	35%
	5.74	5578	34	(-)	149	54	(-)	407	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	25%
AC098	0.37	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	0%
	1.17	97	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	2.21	950	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	3.02	1750	(-)	(-)	(-)	(-)	30	91	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	15%
	4.31	2580	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
AC110	0.41	82	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	1.03	340	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	2.69	384	(-)	(-)	45	38	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	15%
AC115	0.65	1347	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	1.58	1530	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
AC128	0.43	5740	(-)	(-)	45	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	41	(-)	(-)	(-)	(-)	(-)	(-)	(-)	15%
	1.41	5183	103	(-)	354	25	91	(-)	25	(-)	45	46	71	83	(-)	33	40	(-)	(-)	21	(-)	65%
	2.47	6504	245	66	306	95	28	385	62	109	274	252	237	177	(-)	83	219	33	(-)	112	35	90%
AC131	0.63	48	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	1.52	846	75	(-)	142	485	34	685	(-)	(-)	(-)	265	92	(-)	(-)	(-)	25	(-)	35	(-)	(-)	50%
	2.38	361	71	(-)	82	546	65	766	(-)	(-)	(-)	(-)	37	20	(-)	(-)	(-)	(-)	24	(-)	(-)	45%
	3.19	439	107	30	106	376	25	952	35	(-)	(-)	238	20	51	(-)	57	112	(-)	23	(-)	241	75%
AC160	0.20	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	0%
	1.04	732	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	2.13	2603	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	3.23	5007	94	(-)	427	50	(-)	453	(-)	(-)	(-)	(-)	(-)	206	1015	(-)	(-)	(-)	(-)	(-)	(-)	35%
AC167	0.68	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	0%
	1.72	178	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	2.25	375	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	4.51	521	(-)	(-)	40	51	(-)	29	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	20%
AC180	0.06	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	0%
	1.21	769	475	(-)	71	24	(-)	(-)	(-)	(-)	(-)	20	(-)	(-)	(-)	75	51	(-)	(-)	(-)	(-)	35%
	2.19	2388	2585	106	255	502	64	209	179	53	33	21	(-)	(-)	(-)	113	114	58	(-)	57	(-)	75%
AC183	0.34	34	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	1.54	2500	(-)	(-)	(-)	(-)	52	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	10%
	2.53	7169	(-)	(-)	73	46	165	241	(-)	(-)	(-)	(-)	(-)	56	(-)	(-)	(-)	(-)	(-)	(-)	(-)	30%
	3.54	7604	(-)	(-)	55	(-)	(-)	366	(-)	(-)	(-)	(-)	(-)	35	(-)	(-)	(-)	(-)	(-)	(-)	(-)	20%
AC194	0.23	44	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	1.17	1908	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	2.13	972	(-)	(-)	(-)	(-)	34	380	(-)	(-)	(-)	(-)	(-)	50	(-)	(-)	(-)	(-)	(-)	(-)	(-)	20%
	2.37	2050	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
AC212	0.07	21	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	1.03	462	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	2.04	168	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%
	2.56	162	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	5%

Figure 2.2. Cross-neutralizing activities in plasmas from the MGH Acute HIV infection Cohort. The cross-neutralizing activities of plasmas from the indicated subjects (PID) were evaluated against the indicated clade B, C and A viruses. The values are the plasma titers at which 50% neutralization (IC50) was recorded. For clarity this information is color-coded as in Figure 2.1. With the exception of SF162.LS (tier 1 virus), all other viruses are tier 2 (51, 291, 292). Each experiment was performed at least two independent times.

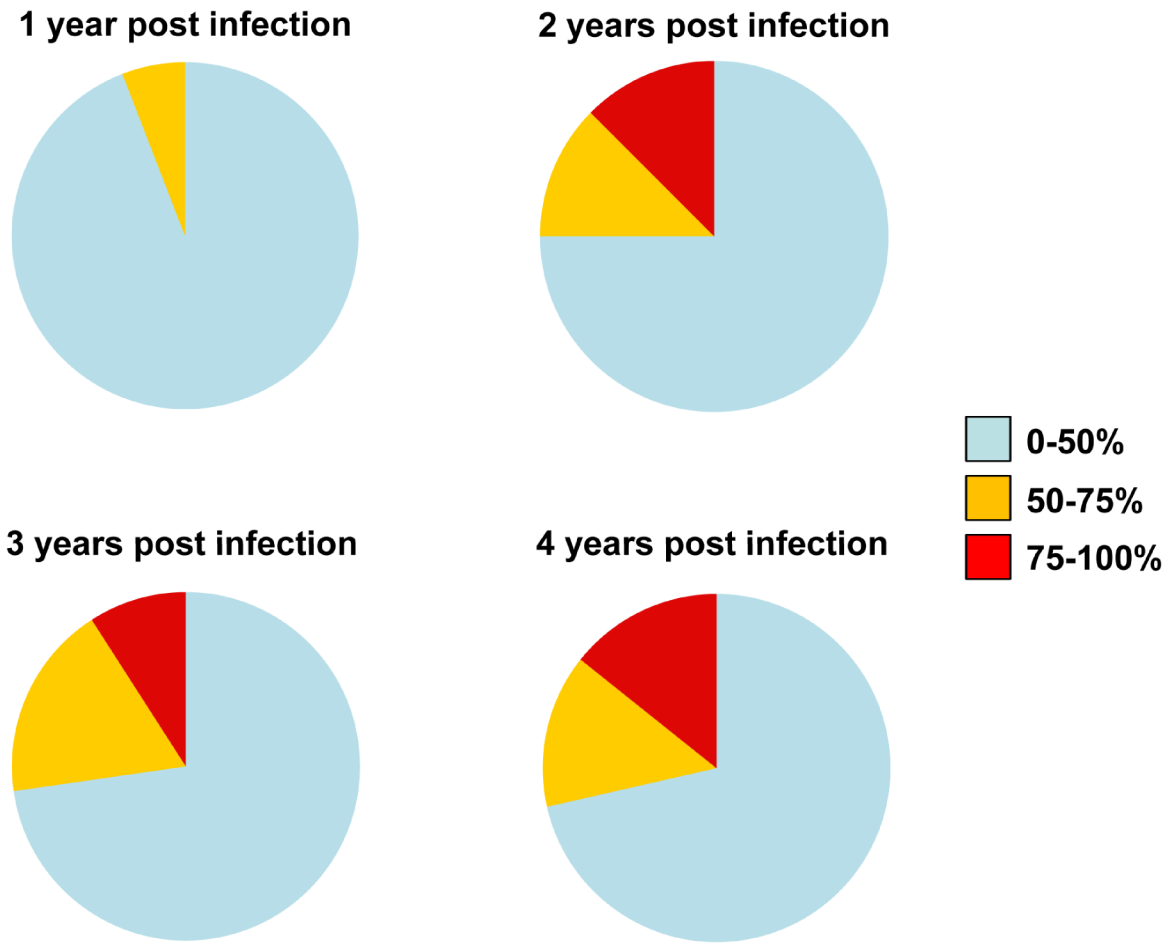

Figure 2.3. Evolution of the potency and breadth of cross-neutralizing antibody responses. The pie charts represent the evolution of breadth and potency of serum neutralizing activities in the MGH Acute HIV Infection Cohort. Subjects who developed cross-neutralizing activities within the first 2 years of infection, but who were not followed longitudinally post that period, were not included in our calculations in the subsequent years. Samples from 17 subjects were available during the 1st year of infection, samples from 16 during the 2nd year, 11 during the 3rd, and 7 during the 4th year.

Factors associated with the emergence of cross-neutralizing antibody responses

In chronic HIV-1 infection the breadth of serum cross-neutralizing antibody activities positively correlates with the levels of plasma viremia (138, 396, 449, 519). Here, we recorded a positive correlation (p=0.0026; R=0.3615) between the breadth of the earliest cross-neutralizing antibody responses in HIV+ plasmas and the levels of plasma viremia. Because of the association between plasma viremia and immune activation during early HIV infection (124), we examined potential associations between the development of serum cross-neutralizing activities and markers of immune activation and exhaustion **(Figure 2.4)**.

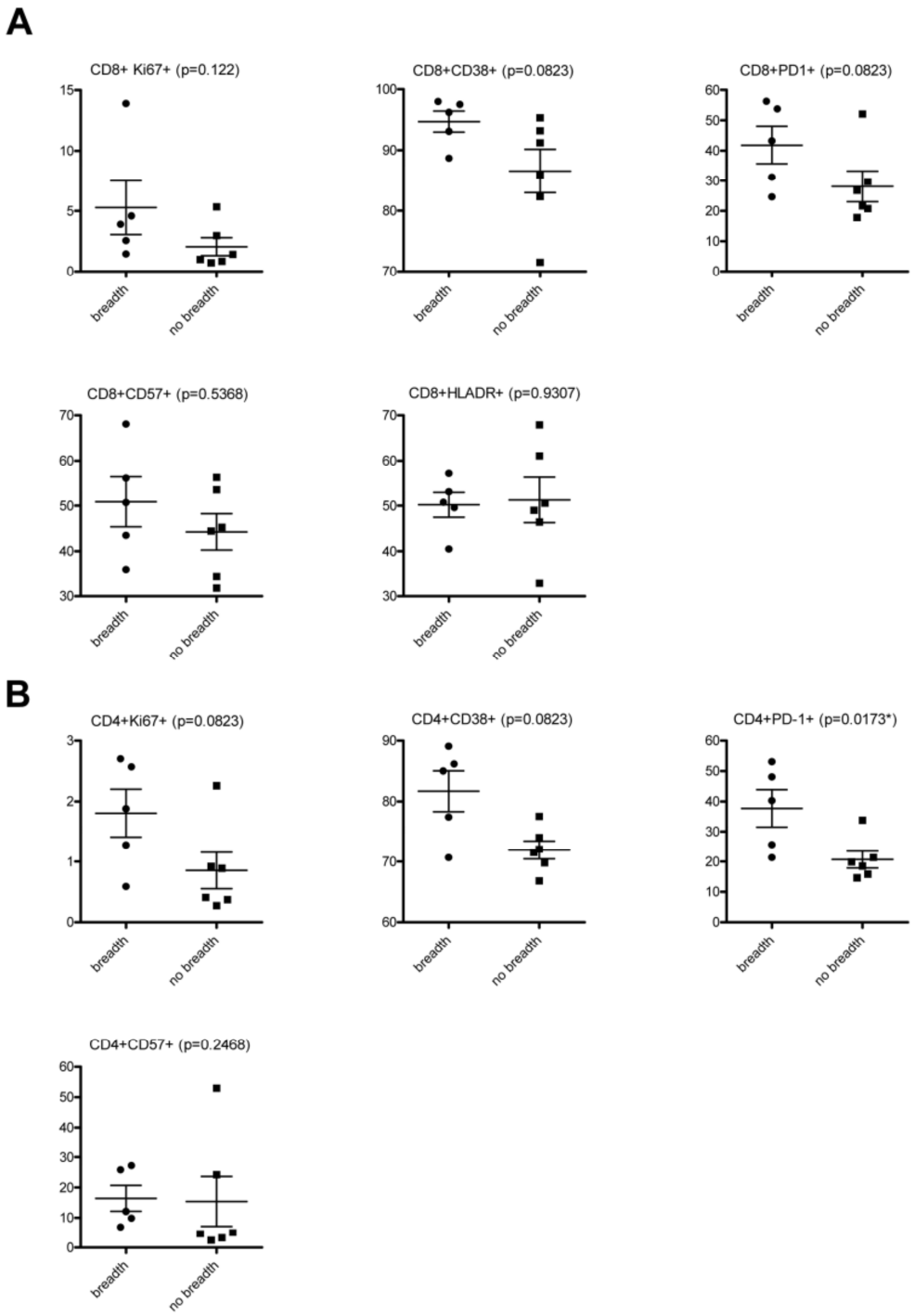

Figure 2.4. Immune activation markers and cross-neutralizing antibody responses. The frequencies of **(A)** CD8+, **(B)** and CD4+ T cells expressing the indicated markers in subjects who developed cross-neutralizing antibodies (at least 75% breadth) (AC049, AC053, AC128, AC131 and AC180) and those who did not (no breadth) (AC093, AC110, AC167, AC183, AC194, AC212) are shown. These frequencies were determined at the earliest time point when cross-neutralizing antibody responses were evident: for AC049 at 2.62 year post-infection (ypi), for AC053 at 3.29 ypi, for AC128 at 1.41 ypi, for AC131 at 1.52 ypi, and for AC180 at 2.19 ypi. Similar time points of infection were used for those subjects who did not develop cross-neutralizing antibody responses.

Specifically, we compared the percent of CD4+ and CD8+ T cells expressing Ki67, CD57, CD38, PD1, and HLADR in subjects that developed cross-neutralizing antibody responses and those who did not. The immune activation status of subjects who developed broad cross-neutralizing antibody responses (at least 75% breadth at some point during the period of observation) was determined at the earliest time point when cross-neutralizing antibody responses were evident: for AC049 at 2.62 year post-infection (ypi), for AC053 at 3.29 ypi, for AC128 at 1.41 ypi, for AC131 at 1.52 ypi, and for AC180 at 2.19 ypi. Similar time points of infection were used for those subjects who did not develop cross-neutralizing antibody responses (AC093, AC110, AC167, AC183, AC194, and AC212).

A trend towards higher percentages of CD8+ T cells expressing Ki67 (p:0.122), CD38 (p:0.0823), and PD1 (p: 0.0823) was recorded in subjects with breadth. It is likely that, because the number of subjects who developed cross-neutralizing antibody responses is small, these differences did not reach statistical significance. A similar trend towards higher expression of Ki67 (p:0.0823) and CD38 (p:0.0823) was recorded in the case of CD4+ T lymphocytes. A statistically significant difference (P:0.0173) was, however, recorded in the percent of CD4+ PD1+ T cells between those subjects that developed cross-neutralizing antibody responses and those who did not. In addition, we performed correlation analysis between the degree of breadth and the frequencies of T cells expressing the various activation markers. A statistically significant positive association was observed between breadth and the frequency of CD4+ PD1+ T cells (p: 0.0174, Pearson r: 0.6961), and CD4+ CD38+ T cells (p: 0.0306, Pearson r: 0.6494). Overall, these results link for the first time the state of immune activation (within approximately 2 years of infection) to the development of cross-reactive neutralizing antibody responses.

Epitope specificities of the earliest cross-neutralizing antibody response

Taking advantage of the availability of longitudinal samples from the MGH Acute HIV Infection Cohort, we performed epitope-mapping studies to determine: (a) whether the initial cross-neutralizing antibody responses developed by subjects infected with different viruses were due to the emergence of antibodies that target one or multiple epitopes on heterologous Env, and (b) whether the initial epitope specificities of cross-reactive neutralizing antibody responses in HIV-1+ plasmas evolve over time.

Cross-neutralizing activities targeting the transmembrane gp41 Env subunit

The extracellular part of the transmembrane subunit gp41 is immunogenic (45, 394) and the target of the initial anti-HIV-1 antibody responses generated following infection (503). However, the vast majority of human anti-gp41 MAbs are non-neutralizing and only a handful of anti-gp41 neutralizing MAbs have been isolated from HIV-1-infected subjects (109, 230, 588, 590). Two of these anti-gp41 MAbs, 2F5 and 4E10, display broad cross-neutralizing activities and they recognize two distinct epitopes within MPER (588, 590). In between the 2F5 and 4E10 epitopes lies the epitope recognized by a third anti-HIV-1 antibody, Z13, whose breadth of neutralization is much narrower than those of 2F5 and 4E10 (372, 588).

We first performed peptide competition neutralization experiments, during which the plasmas were pre-incubated with a MPER-derived peptide and then incubated with viruses (JRFL and TRO.11) **(Table 2.1)**. MPER-derived peptides block the neutralizing activities of MAbs 4E10 and 2F5, and have been used to define the contribution of anti-MPER neutralizing activities in HIV+ sera (42, 197, 295, 449, 450). Control experiments confirmed that the MPER peptide specifically competes the neutralizing activities of the anti-MPER MAbs 2F5 and 4E10, but not those of neutralizing antibodies to other regions of Env (such as the anti-gp120 MAbs b12, 2G12, P3C8 and P3E1) (data not shown). In only one subject (AC131) the MPER-derived peptide consistently reduced (by a modest 0.3-0.6 Log10) the plasma's overall neutralizing activity against both HIV-1 viruses tested, indicating that the anti-MPER antibodies in this plasma moderately contribute to its overall anti-HIV-1 cross-neutralizing potential. Interestingly, the relative contribution of anti-MPER antibodies to the anti-JRFL and -TRO.11 neutralizing activity decreased during the period of observation in that subject, although the breadth of cross-neutralizing activity increased from 45% to 75% during that period.

To better define the epitope(s) within the MPER region targeted by these antibodies we utilized viruses expressing chimeric HIV-2/HIV-1 Envs (42, 197, 199). These chimeric Envs are based on the HIV-2 Env 7312A, on which the entire MPER region, or portions of it, have been replaced by those of the HIV-1 clade B YU2 Env. Since plasmas isolated from HIV-1-infected subjects rarely neutralizing HIV-2 isolates, these chimeras are useful tools to detect the presence of anti-HIV-1 MPER neutralizing antibody responses (42, 197, 199). The MPER sequences of the chimeras used here are shown in Table 1. As expected, plasma from AC131 was very effective in neutralizing the C1 chimera, which expresses the entire HIV-1 MPER region **(Table 2.1)**. This plasma also potently neutralized the C8 chimera, which expresses the domain of MPER

encompassing the Z13 and 4E10 epitopes, while it did not efficiently neutralize the C3 or C4 chimeras, which express the 2F5 and 4E10 epitopes, respectively, of the HIV-1 MPER. Most likely therefore, this plasma contains neutralizing antibodies whose epitope(s) overlaps the Z13 and 4E10 epitopes. Overall, these results indicate that anti-MPER neutralizing antibodies rarely (and only modestly) contribute to the earliest cross-neutralizing antibody responses following HIV-1 infection. They are in agreement with numerous recent studies indicating that anti-MPER targeted antibodies rarely contribute to the breadth of cross-neutralizing activities of HIV-1+ sera from chronic infection (42, 197, 295, 368, 449, 450, 459, 474).

Table 2.1. Contribution of anti-MPER antibodies to the plasma's cross-neutralizing activities.

			Log decrease in presence of MPER (d)		IC50 of HIV2/HIV1 MPER chimeras (g)			
PID (a)	**YPI (b)**	**Breadth (c)**	**JRFL**	**TRO.11**	**C1 (MPER)**	**C3 (2F5)**	**C4 (4E10)**	**C8 (Z13, 4E10)**
AC131	**2.38**	**45%**	**0.48**	**0.68**	**>2560**	**43**	**119**	**>2560**
	3.19	**75%**	**0.39**	**0.35**	**>2560**	**24**	**157**	**>2560**
AC049	3.64	85%	(--) (e)	(--)				
	4.39	80%	(--)	nd (f)				
AC053	5.31	80%	(--)	(--)				
	5.88	60%	(--)	(--)				
AC071	3.34	50%	(--)	(--)				
	3.8	40%	(--)	nd				
AC089	1.94	50%	(--)	(--)				
AC093	4.97	35%	(--)	(--)				
AC128	2.47	90%	(--)	(--)				

(a) Patient ID

(b) Years post infection

(c) Breadth: percentage of HIV-1 isolates neutralized by each plasma out of the total number of isolates the plasma was tested against

(d) The neutralizing activities of plasmas collected at the indicated time points following infection were evaluated against the HIV-1 viruses JRFL and TRO.11 in the absence or presence of the MPER peptide (NEQELLELDKWASLWNWFDITNWLWYIRKKK). The Log10 decrease in IC50 neutralization titers in the presence of the MPER peptide is indicated.

(e) No affect in the neutralizing activities of plasmas was recorded in the presence of the MPER peptide

(f) experiment not performed

(g) IC50 neutralizing titers of plasma samples collected from AC131 against four HIV2/HIV-1 MPER chimeric viruses are indicated.

We did not investigate whether anti-gp41 antibodies that target regions other than the MPER were present and contributed to the early cross-neutralizing activities of the above plasmas. Although several monoclonal antibodies against the HR1 domain of gp41 have been shown to display anti-HIV neutralizing potentials, such antibodies are generally not broadly neutralizing (109, 340, 373).

Cross-neutralizing activities targeting the gp120 Env subunit

Potentially, the above results suggested that the earliest cross-neutralizing activities in HIV-1+ plasmas are primarily targeting the extracellular gp120 subunit. To address this point, we depleted the anti-gp120 antibodies from six plasmas (AC049, AC053, AC071, AC128, AC131, AC180) displaying cross-neutralizing activities, and three plasmas (AC098, AC115, AC212), which only neutralized SF162 **(Figure 2.5)**. Although during these depletion experiments we used gp120 from one clade B virus (SF162), we verified that this treatment eliminated anti-gp120 antibodies against other gp120s, from both clade B and clade C viruses **(Figure 2.5)**.

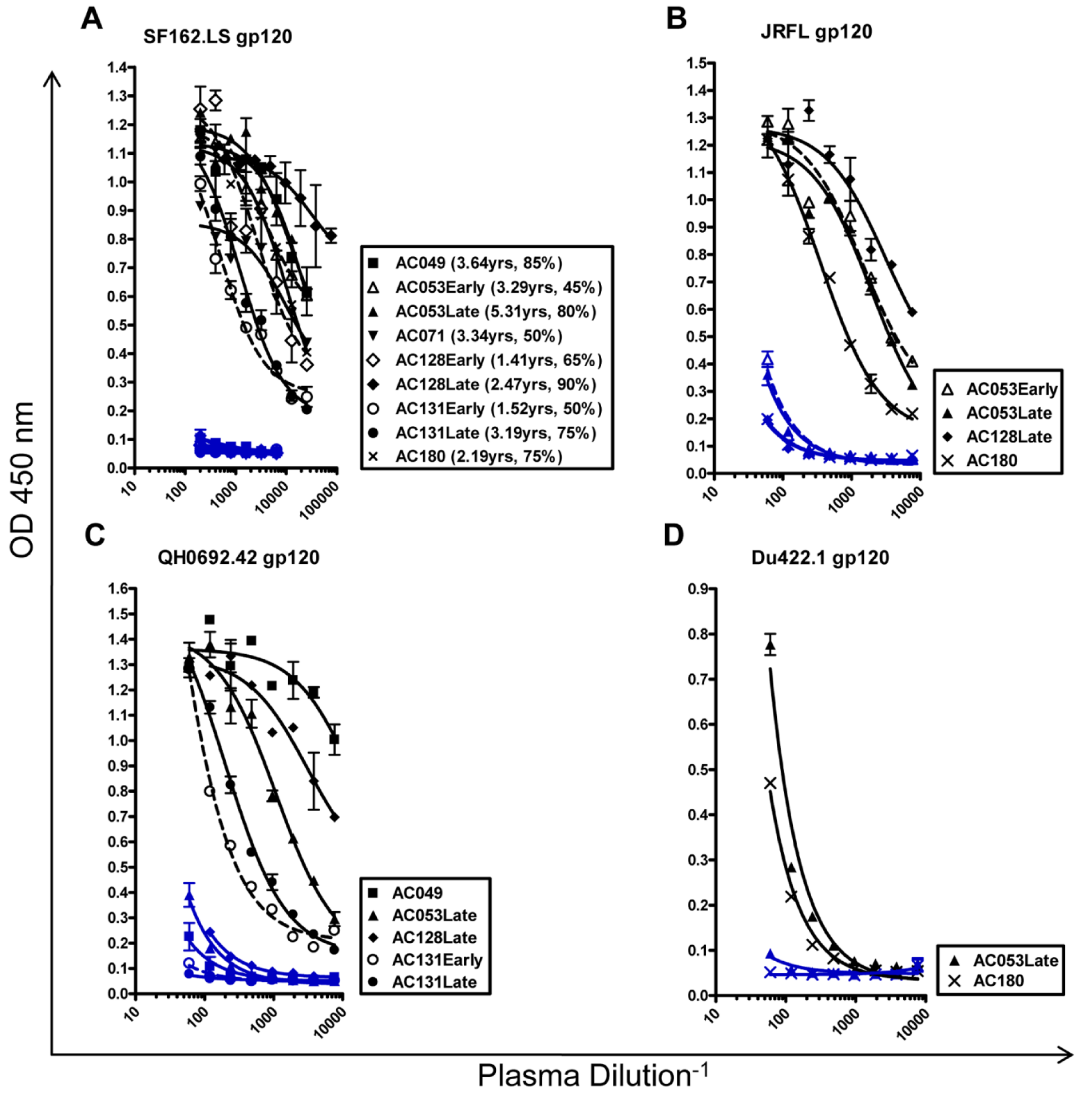

Figure 2.5. Depletion of anti-gp120 antibodies. Anti-gp120 reactivities, prior to and following depletion of indicated plasmas, were evaluated against 4 gp120s: **(A)** SF162.LS, **(B)** JRFL, **(C)** QH069.42, and **(D)** Du422.1. Black lines: undepleted plasmas; blue lines: gp120-depleted plasmas. Years post infection and breadth are indicated in parenthesis in **(A)** next to each subjects ID.

Afterwards, the neutralizing activities of non-depleted and of the corresponding gp120-antibody-depleted plasmas were compared against several clade B and C viruses (the data are summarized in **Figure 2.6A** and representative examples are shown in **Figure 2.7**).

A

			Clade B							Clade C	
PID	YPI	Breadth	SF162.LS	JRFL	YU2	TRO.11	REJO4541.67	QH0692.42	CAAN5342.A2	Du422.1	ZM214M.PL15
AC049	3.64	85%	>1.00	0.76	nd	0.28	nd	nd	nd	nd	0.02
AC053	3.29	45%	>1.00	0.88	>1.00	0.21	nd	--	--	>1.00	--
	5.31	80%	0.74	0.68	>1.00	0.24	>1.00	0.75	0.47	0.81	0.01
AC071	3.34	50%	nd	0.00	--	0.00	--	nd	--	--	--
AC128	1.41	65%	nd	>1.00	--	0.57	0.47	--	--	--	nd
	2.47	90%	nd	0.51	nd	0.14	0.24	0.86	0.03	0.38	0.00
AC131	1.52	50%	>1.00	0.00	--	0.00	0.37	0.92	--	nd	0.00
	3.19	75%	0.80	0.25	0.32	0.23	0.33	0.74	0.16	0.23	0.00
AC180	2.19	75%	>1.00	>1.00	0.49	0.43	0.74	0.92	0.17	0.28	--
AC098	4.31	5%	>1.00								
AC115	1.58	5%	>1.00								
AC212	2.04	5%	>1.00								

B

			Clade B							Clade C	
PID	YPI	Breadth	SF162.LS	JRFL	YU2	TRO.11	REJO4541.67	QH0692.42	CAAN5342.A2	Du422.1	ZM214M.PL15
AC049	3.64	85%	nd	0.33	0.20	0.00	0.10	0.35	0.00	0.30	0.00
AC053	3.29	45%	nd	0.19	0.48	0.11	0.24	--	--	0.15	--
	5.31	80%	0.22	0.26	>1.00	0.20	0.00	0.29	0.46	0.48	0.00
AC071	3.34	50%	nd	0.10	--	0.00	--	0.00	--	--	--
AC128	1.41	65%	nd	0.46	--	0.56	0.46	--	--	--	0.36
	2.47	90%	>1.00	0.48	0.50	0.08	0.26	0.70	0.00	0.00	0.00
AC131	1.52	50%	nd	0.10	--	0.08	0.00	0.00	--	0.00	0.00
	3.19	75%	0.51	0.36	0.00	0.11	0.00	0.32	0.00	0.00	0.45
AC180	2.19	75%	0.49	0.00	0.00	0.17	0.00	0.29	0.00	0.00	--
AC098	4.31	5%	>1.00								
AC115	1.58	5%	>1.00								
AC212	2.04	5%	0.88								

Figure 2.6. Contribution of anti-gp120 antibodies to the overall neutralizing activity of HIV+ plasmas. (A) Log10 decrease in neutralizing activity caused by the elimination of anti-gp120 antibodies, and **(B)** Log10 decrease in neutralizing activity of plasmas in the presence of D368R. The values are the average from 2-3 independent experiments in most cases. Light blue: no effect or less than 0.5 Log10 decrease; Yellow: decrease between 0.5 and 0.9 Log10; Red: over 0.9 Log10 decrease. >1.00: depletion resulted in complete loss of the neutralizing activity. (--) plasma did not neutralize that particular virus. nd: experiment was not performed. YPI: years post infection.

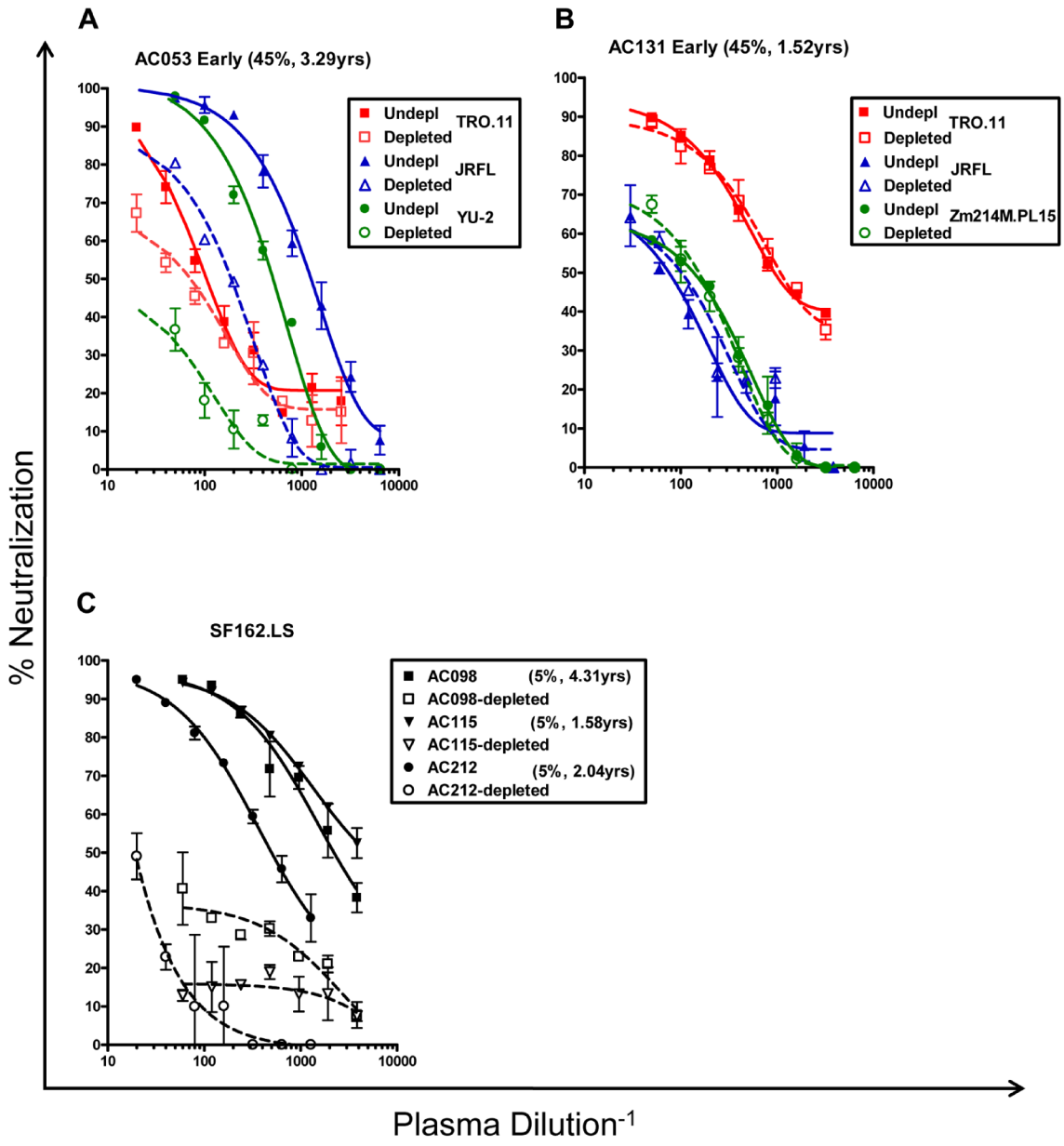

Figure 2.7. Neutralizing activities of anti-gp120 antibody-depleted plasmas. (A and B) Neutralizing activities of 2 plasmas against the indicated primary isolates prior to and following the removal of anti-gp120 antibodies. **(A)** Plasma AC053 against the TRO.11, JRFL, and YU2 viruses. **(B)** Plasma AC131 against TRO.11, JRFL, and Zm214M viruses. TRO.11 (red squares), JRFL (blue triangles), YU2 and ZM214M (green circles). Patient ID, breadth and years post infection are shown. Undepl: Undepleted plasma. Depleted: plasma depleted from anti-gp120 antibodies. Filled symbols and solid lines – undepleted plasmas; clear symbols and dashed lines – gp120-depleted plasmas. **(C)** Plasmas from subjects AC098 (squares), AC115 (inverted triangles), and AC212 (circles) did not display significant breadth and neutralized only SF162.LS (Figure 2). Their anti-SF162.LS neutralizing activities were determined before and following depletion of the anti-gp120 antibodies. Filled symbols and solid lines: prior to depletion of anti-gp120 antibodies. Clear symbols and dashed lines: following the depletion of anti-gp120 antibodies. Each experiment was performed at least three independent times.

Removal of the anti-gp120 antibodies from plasmas with narrow breadth resulted in complete loss in neutralizing activity. In contrast, removal of the anti-gp120 antibodies from plasmas with breadth had a diverse effect on the neutralizing activities of plasmas, depending on the plasma / targeted virus pairing. In most cases examined, either no changes in IC50 titers were recorded or changes smaller than 0.5Log10 in IC50 were recorded. However, in specific cases the neutralizing activity of given plasma against a given virus was completely lost when the anti-gp120 antibodies were removed. That was the case of plasma AC131 and the QH0692 and SF162 viruses (but not other viruses tested); or the case of plasma AC053 and the YU2, REJO and Du422 viruses (but that was not the case for this plasma's anti-TRO.11, -CAAN or -ZM214 neutralizing activities).

It was recently reported by Walker and colleagues that a significant fraction of cross-neutralizing activities in plasmas collected during chronic HIV-1-infection are recognizing complex epitopes on the trimeric Env spike that are not efficiency presented on (or are absent from) monomeric gp120 (536). Antibodies with epitope specificities overlapping those of MAbs PG9 and/or PG16 were defined as being partially responsible for the overall cross-neutralizing activity of some sera tested in that study. We were interested in determining whether similar PG9- and PG16-like antibody specificities are contributing to the earliest cross-neutralizing anti-HIV-1 activities.

To address this point we generated mutants of HIV-1, on which the asparagine at position 160 (within the V2 loop) was substituted by a lysine. Position 160 is critical for the binding and neutralization of MAbs PG9 and PG16 (535, 536), and recently it was suggested that the sugar molecules present on N160 may also directly participate in the binding of these two MAbs (136). In agreement with these previous studies, we observed that when asparagine 160 was mutated into a lysine (N160K) or to an alanine (N160A), MAbs PG9 and PG16 no longer neutralized HIV-1 (TRO.11) **(Figure 2.8)**. Next, the susceptibilities of the WT and mutant TRO.11 virus against plasmas were determined, once gp120-antibodies were eliminated from these plasmas **(Table 2.2)**. We selected plasmas from subjects AC053, AC131 and AC181, since removal of anti-gp120 antibodies from those samples had a minimal effect on their anti-TRO.11 neutralizing activities **(Figure 2.6A)**. In all cases a decrease of that plasmas' neutralizing activity was recorded in the case of the N160K mutations. However, the N160A mutation had no effect on the neutralizing activity of the same plasmas. Combined, the results indicate that the sugars at position 160 are not part of the epitope recognized by these antibodies. Potentially, they suggest that the nature of the amino acid at position 160 is very relevant to the neutralizing

activity of the earliest cross-neutralizing antibody response in HIV-1+ plasmas. In summary, our results suggest that the earliest cross-neutralizing antibody response to HIV-1 includes antibodies whose epitopes on the virion-associated Env spikes are overlapping, but are not identical to those of MAbs PG9 and PG16.

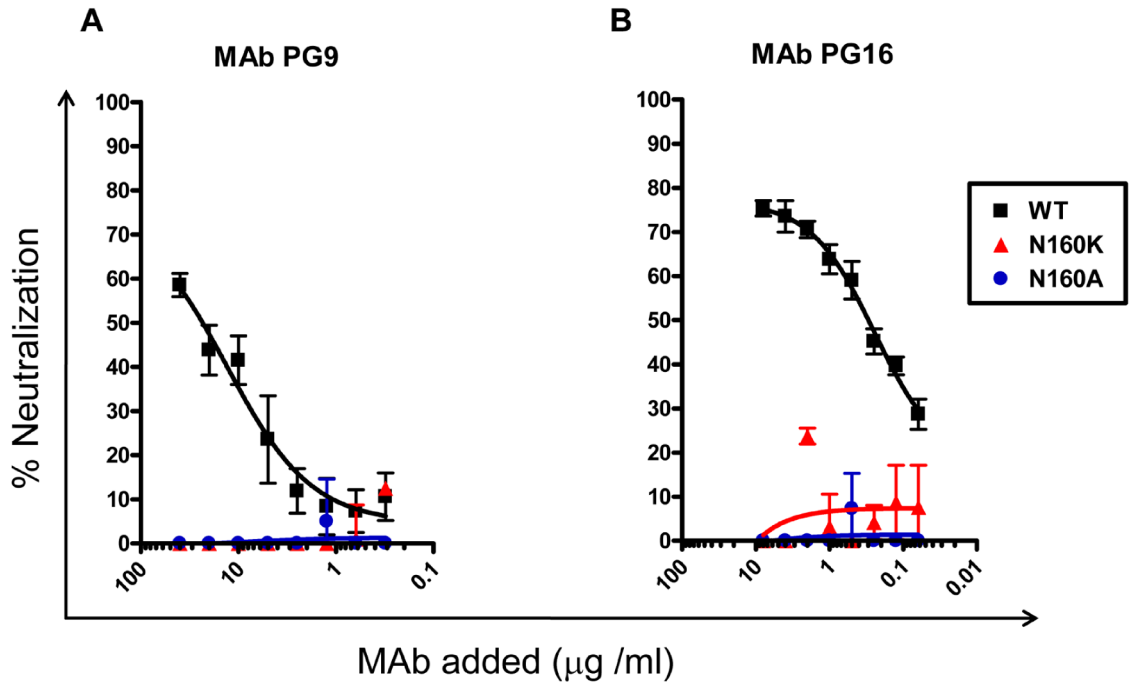

Figure 2.8. Effect of N160 mutation on the neutralizing activities of MAbs PG9 and PG16. The neutralizing activities of MAbs (A) PG9 and (B) PG16 against TRO.11 are shown. WT: wild type TRO.11; N160K: TRO.11 with the asparagine at position 160 mutated to a lysine; N160A: TRO.11 with the asparagine at position 160 mutated to an alanine.

Table 2.2. Percent reduction in neutralizing activity of gp120-depleted plasmas due to amino acid substitutions at position N160.

Patient ID	YPI (a)	Breadth	TRO.11 N160K	TRO.11 N160A
AC053	5.31	80%	**36% (b)**	(--) (c)
AC131	3.19	75%	**53%**	(--)
AC180	2.19	75%	**25%**	(--)

Gp120-depleted plasmas were tested for neutralization of TRO.11 N160 mutants.
(a) Years post infection
(b) % neutralizing activity is the % reduction in IC50 of mutant compared to wild type.
(c) no difference between neutralization of wild type and mutant TRO.11.

Epitope-specificities on monomeric gp120

We performed epitope-mapping analysis to define the regions within monomeric gp120 that were targeted by cross-reactive NAb responses in certain plasmas. Usually, such epitope-mapping analysis studies are performed by incubating HIV-1+ plasmas with soluble peptides derived from the variable regions of the extracellular HIV-1 Env subunit gp120 (42, 295, 449). However, soluble peptides do not accurately represent the conformations of the variable regions of HIV-1 Env and thus do not accurately report on the contribution of antibodies that recognize conformational epitopes within the variable regions. To deal with this limitation we developed a competition neutralization assay based on a variant of monomeric gp120, termed D368R.

The D to R mutation at the conserved position 368 within the CD4-BS abrogates the binding of CD4 and of most known anti-CD4-BS MAbs to gp120 (with a few exceptions; see below), while it does not affect the binding of antibodies that target epitopes outside the CD4-BS (293, 368, 449). Because D368R does not bind cellular CD4, it does not interfere with the ability of the virus to enter CD4+ target cells during *in vitro* neutralization assays. We confirmed that D368R competes the neutralizing activities of known MAbs that recognize epitopes outside the CD4-BS, such as P3C8 (anti-V1), P3E1 and 447-52D (anti-V3) (129, 192) and 2G12, which recognizes a conformational e(445, 456, 512)pitope made of mannose residues **(Figure 2.9A)**. D368R had no effect on the neutralizing activities of the anti-CD4-BS MAb b12 or of CD4-IgG2 **(Figure 2.9B)**.

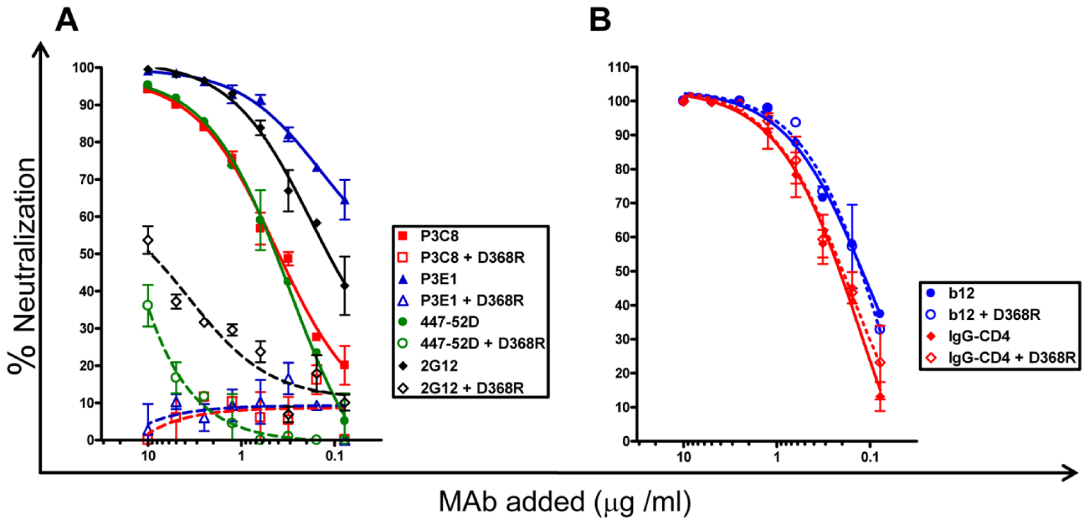

Figure 2.9. Competing the neutralizing activities of known MAbs by D368R. The neutralizing activities of known anti-HIV neutralizing MAbs were determined in the presence and absence of the competing D368R gp120 protein. **(A)** The anti-V1 MAb P3C8, anti-V3 MAbs P3E1 and 447D, and MAb 2G12 (recognizes a complex glycan epitope on gp120). **(B)** Anti-CD4-BS MAb b12 and of IgGCD4. Solid lines and filled symbols: absence of D368R. Dashed lines and clear symbols: presence of D368R.

We next examined if the neutralizing activities of the above-described plasmas were affected (and to what extent) by the presence of D368R (the data are summarized in **Figure 2.6B** and representative examples are shown in **Figure 2.10**). The neutralizing activities of the three plasmas (AC098, AC115, and AC212) with narrow breadth were significantly reduced in the presence of D368R; an indication that the anti-SF162 neutralizing activities of these plasmas are due to antibodies that primarily target epitopes outside the CD4-BS. In contrast, the cross-neutralizing activities of plasmas with breadth were either not affected or only modestly reduced by D368R. The exception was plasma AC053, whose anti-YU2 neutralizing activity was significantly reduced by D368R. This result suggests that the anti-YU2 neutralizing activity of this plasma (but not its neutralizing activity against any other viruses tested) is due to antibodies that recognize epitopes on gp120 located outside the CD4-BS.

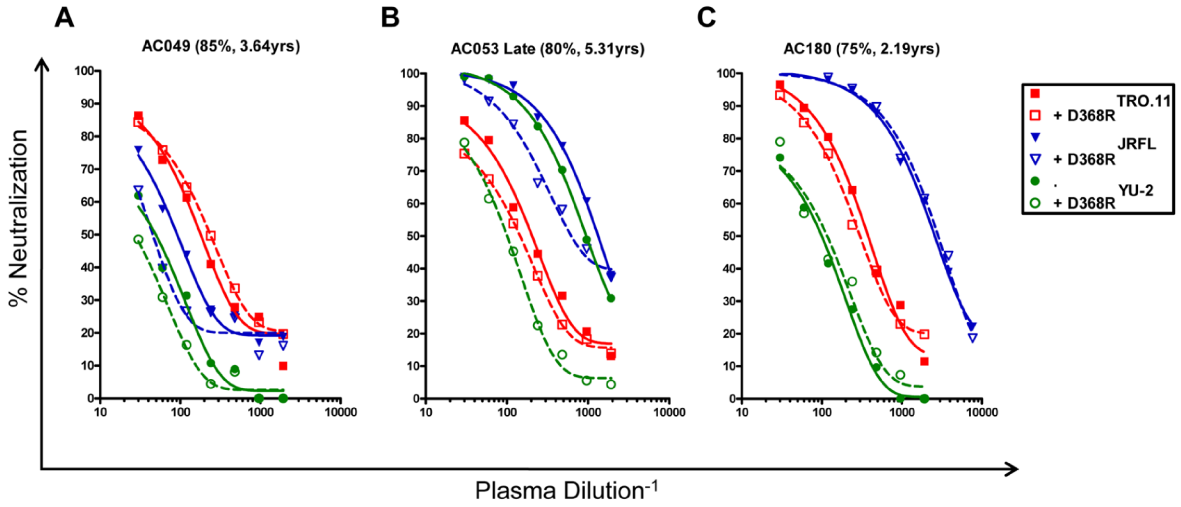

Figure 2.10. Neutralizing activities of plasmas competed with gp120 D368R. The neutralizing activities of plasmas **(A)** AC049, **(B)** AC053, and **(C)** AC180 against TRO.11 (red squares), JRFL (blue triangles) and YU2 (green circles) were determined in the absence (solid lines and filled symbols) and presence (dashed lines and clear symbols) of D368R gp120. Patient ID, breadth, and years post infection are shown.

As expected, in the cases where depletion of the anti-gp120 antibodies had no or only minimal effect on the neutralizing activity of a plasma sample against a set of viruses, the D368R protein had a similar effect. In cases, however, where the neutralizing activity of plasma against a particular virus was significantly affected by depletion of the anti-gp120 antibodies, our results indicate that the major fraction of anti-gp120 antibodies responsible for that neutralizing activity

was due to anti-CD4-BS antibodies. This is the case for example of plasmas AC049, AC053, AC128, and AC180 against JRFL, of plasma AC053 (3.29 ypi) against YU2, or plasmas AC053 (3.29 ypi), AC131 (3.19 ypi), and AC180 against the QH0692 virus.

Here we need to clarify that although the D368R mutation abrogates the binding of most known anti-CD4-BS antibodies, it does not abrogate the binding of all such antibodies. Corti et al (109) recently discussed the binding and neutralizing properties of such a MAb, HJ16. In addition, Scheid et al (459) identified a group of anti-HIV Env antibodies generated during infection, termed anti-gp120 core, whose epitopes overlap part of the CD4-BS and whose binding is not affected by the D368R mutation. Thus, anti-core antibody specificities in HIV-1+ plasmas will be 'removed' if the plasmas are incubated with D368R. Potentially, the fraction of the plasma neutralizing activities that was 'eliminated' in the presence of D368R could be due to anti-core antibodies.

Longitudinal evolution of anti-D368R neutralizing activities

With the above caveats in mind, we examined whether the cross-neutralizing activities of plasmas that target epitopes on the D368R protein evolve over periods of time during which the breadth of cross-neutralization increases. We thus performed D368R competition neutralization experiments with plasmas collected longitudinally from subjects who gradually developed breadth **(Figure 2.11)**. In the vast majority of cases, the neutralizing activities of plasmas remained minimally affected by D368R over several years of observation, as the breadth of cross-neutralization increased. The exception was plasma AC053 and virus YU2. Here, the presence of D368R had a modest (less than a 0.5 Log10 decrease in IC50) on the anti-YU2 neutralizing activity at 3.29 years following infection when breadth was less than 50%, but in the subsequent years, when breadth varied between 60% and 80%, the anti-YU2 neutralizing activity was significantly reduced (~1Log10 or more decrease in IC50). In this particular case, therefore, the anti-YU2 neutralizing activity of this plasma gradually focused on epitopes present on D368R. This 'focusing' was however only observed for YU2 and not for other viruses tested, such as JRFL, TRO.11, or REJO.

			JRFL		YU2		TRO.11		REJO4541.67	
PID	YPI	Breadth	IC50	Decrease	IC50	Decrease	IC50	Decrease	IC50	Decrease
AC049	2.62	55%	75	0.53	21	--	153	0.08	80	0.05
	3.64	85%	120	0.33	46	0.20	266	0.00	93	0.10
	4.39	80%	102	0.25	35	0.10	356	0.05	89	0.15
AC053	3.29	45%	1467	0.19	612	0.48	114	0.11	46	0.24
	4.30	65%	1117	0.41	543	0.97	151	0.30	78	0.15
	5.31	80%	1268	0.26	951	>1.00	374	0.20	72	0.00
	5.88	60%	1403	0.30	594	1.00	328	0.29	63	0.18
	6.85	60%	623	0.16	330	0.90	288	0.28	30	0.00
AC071	2.28	45%	52	0.01			81	0.15		
	3.34	50%	276	0.10			50	0.00		
	3.80	35%	311	0.00			69	0.28		
AC128	1.41	65%	103	0.46	--	--	25	0.56	354	0.46
	2.47	90%	245	0.48	66	0.50	95	0.08	306	0.26
AC131	1.52	50%	75	0.10	--	--	485	0.08	142	0.00
	2.38	45%	71	0.03	--	--	546	0.14	82	0.00
	3.19	75%	107	0.36	30	0.00	376	0.11	106	0.00
AC180	1.21	30%	475	0.00	--	--	24	0.22	71	0.00
	2.19	75%	2585	0.00	106	0.00	502	0.17	255	0.00

Figure 2.11. Contribution of anti-CD4-BS antibodies in the overall neutralizing activities of plasmas collected longitudinally. The values indicate the Log10 decrease in neutralizing activity in the presence of the D368R construct. The values are the average from 2-3 independent experiments in most cases. The color-coding is the same as in Figure 5. (--) the experiment was not performed because that particular plasma did not neutralize that particular virus. YPI: years post infection. Cross-neutralizing antibodies that bind the CD4-BS do not recognize the D368R mutant (293), thus, the majority of the neutralizing activities that remain in the plasmas that have been incubated with D368R most likely are due to cross-neutralizing antibodies that bind the CD4-BS. There are some exceptions, which are discussed in the Results section.

DISCUSSION

It is now recognized that 10-30% of HIV-1 chronically infected subjects develop cross-neutralizing antibody responses of significant breadth (138, 449, 477, 519). We and others have previously discussed that the duration of HIV infection is positively associated with the breadth of cross-neutralizing antibody responses (138, 396, 449, 519). Here we show that such anti-viral responses become detectable in the blood of these subjects, on average, at 2.5 years after infection. In rare cases, cross-neutralizing antibodies appear as early as 1 year post-infection. A recent study indicated that the development of cross-neutralizing antibody responses does not delay the onset of AIDS (157), however, it is currently unknown whether an unusually early emergence of such responses will offer a long-term clinical benefit to the patient or not. The observation that the development of cross-neutralizing antibody responses is associated with higher levels of plasma viremia potentially indicates a more efficient viral escape from autologous anti-viral responses (neutralizing antibody responses and/or cellular-mediated anti-viral responses) in those subjects that eventually develop cross-neutralizing antibodies. Our data indicate that those subjects who develop cross-neutralizing antibodies have higher frequencies of CD4+ T expressing PD1. This observation is intriguing and potentially of high importance. A fraction of CD4+ T cells that express high levels of PD1 (termed follicular T helper cells, T_{FH}) have a distinct gene expression profile from other effector T cells and develop independently of the classic TH1 or TH2 lineages (99, 378) They are not 'exhausted', they secrete IL-4 and IL-21 for extended periods of time, and they are crucial for the formation of germinal centers and the proliferation and survival of circulating plasma cells (189). One possible reason for the development of broad neutralizing antibody responses in only a subset of HIV-1-infected subjects is that optimal interactions between the T_{FH} and B cells are taking place in those subjects who develop such antibody responses, while the T_{FH}-B cell interactions are limited by the smaller number of T_{FH} cells in those subjects who do not develop such responses. Clearly, follow up studies are required to determine whether the CD4+PD1+ T cells found in the periphery actually behave like T_{FH} cells and whether or not the early development of cross-neutralizing antibody activities impacts the rate of disease progression.

The subjects examined here were infected with clade B HIV-1 viruses and it is not known whether the timeline for the emergence of cross-neutralizing antibody responses in non-clade B HIV-1 infections is similar, or whether infection with a particular HIV-1 subtype elicits an earlier or a delayed development of cross-neutralizing antibody responses. In the 'early' cases

examined here, 29% of subjects developed cross-neutralizing antibody responses. This percentage is in agreement with several previous studies conducted with sera collected during chronic infection (138, 449, 477). Potentially our data, in combination with data on the frequency of broad cross-neutralizing antibody responses in sera collected during chronic HIV-1 infection (139, 449, 477), suggest that if cross-neutralizing antibody responses are not generated during the first 2-3 years of infection, they may not emerge later. However, further follow up of these subjects is required to address this important point. It is currently unknown whether the emergence of cross-neutralizing antibody responses, at that particular time period of HIV-1 infection, in only approximately a third of those infected, is the result of a stochastic event or due to genetic predisposition, and whether it is related to particular evolutionary pathways the virus follows in response to other types of anti-viral immune responses.

In agreement with publications with sera from chronic HIV-1 infection (42, 536), we found that the earliest cross-neutralizing antibody response targets only a few regions of Env. It appears, therefore, that a few Env regions are targeted early and late during HIV-1 infection by cross-neutralizing antibodies. The fine specificities of such antibodies within these Env regions may evolve over time.

A significant portion of, or the entire, 'early' cross-neutralizing antibody response was due to antibodies that target virion-associated Env, rather than epitopes present on monomeric gp120 or gp41. The fact that the antibodies that bind such complex epitopes were elicited in response to infection by viruses unrelated to the heterologous viruses used to assess the cross-neutralizing potentials of HIV-1+ plasmas is strongly suggestive that these epitopes are common and most likely present on the viruses circulating even in those subjects who do not develop such antibody responses.

Since such epitopes are conserved among diverse viruses, we assume that they are also present on the transmitted viruses. Then why is the appearance of antibodies that target these epitopes delayed by 2-3 years? Potentially, antibodies that recognize epitopes which are exclusively present on the virus but not the monomeric form of HIV-1 Env could very well be generated earlier following infection, but may specifically target the autologous virus. In fact, MAbs with such complex epitope specificities that display only autologous virus neutralizing activities, or activities only against SF162 and related viruses, have been isolated from SHIV-infected macaques and from a chronically HIV-1-infected human (191, 429). As infection

progresses and in response to a continuous viral evolution, the B cell response to such complex epitopes may also evolve, and this evolution may eventually lead to the generation of antibodies with broader cross-neutralizing activities (535, 536).

The fact that anti-CD4-BS antibodies contribute to the initial cross-neutralizing activities of diverse HIV-1+ plasmas is not surprising since the CD4-BS is one of the most conserved regions of the HIV-1 Env. Numerous studies have already reported the contribution of such antibodies in defining the cross-neutralizing activities of plasmas collected during chronic HIV-1 infection (42, 131, 293, 295, 368, 449). However, anti-CD4-BS antibodies are present in plasmas with and without 'breadth', and several anti-CD4-BS MAbs displaying very narrow cross-neutralizing activities have been isolated from HIV-1-infected individuals (47, 225). Presently, it is not known why only a subset of HIV-1-infected subjects generates anti-CD4-BS antibodies that are cross-neutralizing, while the majority of subjects generate anti-CD4-BS antibodies of narrow neutralizing breadth. The angle of recognition of the CD4-BS by anti-CD4-BS antibodies with narrow and broad neutralizing activities is different (95), which implies that the CD4-BS is recognized differently by the B cell receptors (BCRs) of subjects who develop cross-neutralizing anti-CD4-BS antibodies and the BCRs of subjects who develop anti-CD4-BS antibodies of narrow neutralizing activities.

It is important to note that a fraction of the cross-neutralizing activities in some subjects could be adsorbed on both gp120 and the D368R mutant. Such specificities may be similar to those reported by Scheid et al (459) and more recently by Pietzsch et al (397) that target the 'core' part of gp120. Overall therefore, our data indicate that the 'earliest' cross-neutralizing antibody response to HIV is primarily comprised of antibodies that target the CD4-BS, the core of gp120, and epitopes present on the trimeric Env. The positive association, however, between plasma viremia levels and the breadth of the earliest cross-neutralizing antibody responses suggests that HIV is able to escape the action of the antibodies that recognize conserved regions of Env. Viral escape from antibodies that preferentially bind the Env trimeric spike may involve changes in the V1V2 region of Env, since the epitopes of this type of antibodies include elements of the V1V2 Env region (535, 536). In fact, the V1V2 region of Env undergoes extensive alterations (including increases in length and in glycosylation) early following infection (215, 434, 441). These changes are associated with early escape from autologous neutralizing antibody responses. Our data suggest that such changes may also be involved in the escape from the early cross-neutralizing antibody responses.

Our results provide information that may guide the development of effective immunization protocols. Since antibodies to complex epitopes that are present on the virion-associated envelope spike appear to be key components of the earliest cross-neutralizing activities of HIV-1+ plasmas, then emphasis should be made to elicit similar antibodies by vaccination. As a first step, HIV envelope glycoproteins that readily display such complex epitopes must be identified and tested as immunogens. However, if the development of such cross-neutralizing antibodies is somehow linked to genetic factors, then the outcome of immunizations with such immunogens will largely depend on the population the immunogens are evaluated, since only those vaccinees with the appropriate genetic makeup will respond appropriately.

CHAPTER III

Characterization of the emergence and evolution of cross-neutralizing antibody specificities to the CD4-BS and the carbohydrate cloak of the HIV Env glycoprotein in an HIV-1-infected subject

ABSTRACT

I the previous chapters I have addressed the issue that broadly neutralizing antibodies are considered an important part of a successful HIV vaccine. A better understanding of the factors underlying their development in HIV-infected subjects and the epitopes they target is needed to be able to rapidly elicit such responses by vaccination. We and others have reported that, on average, it takes 2 to 3 years for cross-reactive neutralizing antibodies to become detectable in the sera of HIV-infected subjects, and that they target a limited number of epitope specificities on the HIV Envelope. Here we investigated the development of the earliest cross-reactive neutralizing antibody specificities in one HIV-infected individual, AC053. We defined two distinct epitopes on Env that are targeted by the broadly neutralizing antibody responses developed by AC053. The first specificity became evident at 3 years post infection and targeted the CD4-binding site of Env. Antibodies responsible for that specificity neutralized most, but not all, viruses susceptible to neutralization by the plasma of AC053. The second specificity became apparent approximately a year later. It was due to antibodies to PG9-like antibodies, which were able to neutralize those viruses not susceptible to the anti-CD4-BS antibodies in AC053. These finding support the concept that an effective neutralizing antibody-based HIV vaccine should elicit multiple specificities.

INTRODUCTION

A neutralizing antibody (NAb) response of sufficient duration and magnitude is considered an important part of a successful HIV vaccine (321, 330, 490). Numerous studies have demonstrated sterilizing protection by NAbs against challenge with simian-human immunodeficiency virus (SHIV) in nonhuman primate models (227, 228, 323, 390), and the pressure that NAbs exert on the virus during natural infection in humans (240, 353, 542, 551). These observations overwhelmingly suggest that the presence of similar types of NAbs elicited by a vaccine would be beneficial to the vaccinee. The only target for neutralizing antibodies on HIV is the virally encoded envelope glycoprotein (Env) spike. The functional unit of Env, as expressed on the surface of infectious virions, is a trimer of non-covalently-associated extracellular subunit (gp120) and transmembrane subunit (gp41). Due to the tremendous genetic diversity of the HIV Env, the antibodies elicited by a successful vaccine will have to neutralize a wide range of circulating HIV-1 isolates (321). Such antibodies are referred to as broadly neutralizing antibodies (bNAbs). Although eliciting such responses by vaccination has not yet been achieved, numerous studies have investigated the development and characteristics of broadly neutralizing antibodies produced during natural HIV-1 infection in humans. Such studies provided novel information on the epitopes targeted by these cross-clade neutralizing activities and the factors associated with their development. Numerous studies of infected subjects in early and chronic HIV-1 infection have demonstrated broadly neutralizing antibody responses develop in approximately 15% of infected individuals (138, 157, 201, 295, 339, 449, 477, 490), and become detectable within 2 to 3 years post infection (201, 339, 519). In contrast, autologous NAb responses develop weeks to months after infection in virtually all infected subjects, but although potent, are largely strain-specific and rapidly escaped by the virus (5, 29, 199, 424, 542).

Systematic analyses of the epitope specificities of the serum broadly neutralizing antibody responses have demonstrated that a limited number of specificities are responsible for the serum cross-neutralizing activity in any given individual (42, 131, 200, 285, 293, 295, 449, 536). Monoclonal antibodies (MAbs) with broad neutralizing activities have been isolated from chronically-infected HIV+ subjects and were shown to target structurally-conserved epitopes of Env: the CD4 binding site (CD4-BS) (77, 461, 552, 553, 579), conserved elements of the V2 loop and associated carbohydrates (332, 535), and conserved elements of the V3 loop and

associated carbohydrates (391, 538) on gp120. In addition, a few broadly neutralizing MAbs target the membrane proximal external region of gp41 (364, 588).

In a previous study we sought to determine the timing of the development of the broadly neutralizing antibody response to HIV-1 clade B in a cohort of anti-retroviral naïve subjects that have been monitored longitudinally from a few months to up to 7 years post infection (339). Our findings indicated that cross-neutralizing antibody responses emerged gradually and became detectable at approximately 2.5 years post infection. Subsequently, these responses increased both in potency and breadth. Others have also reported on a similar time-dependent development of cross-neutralizing antibody responses during HIV-1 infection (156, 201, 519). Epitope mapping studies of the polyclonal IgG responses in plasmas from the cohort we examined indicated that earliest cross-neutralizing antibody responses targeted either the CD4-BS on gp120 or epitopes not present on monomeric gp120 (339). Since neutralizing activities against the gp41 subunit of Env were not detectable in the plasmas, we assumed that these later neutralizing activities target epitopes present on the oligomeric Env, but not present on monomeric gp120. We also reported that in certain plasmas a small number of epitope specificities contributed to the overall cross-neutralizing activity of a plasma sample. For example, anti-CD4-BS antibodies were responsible for neutralizing a certain number of viruses, and antibodies that could not be mapped to gp120 were responsible for neutralizing different viruses against which the plasma was tested. Recently, it was demonstrated that indeed the overall cross-neutralizing activity in a chronically-infected HIV-infected subject could be recapitulated by two monoclonal antibodies isolated from that subject, one targeting the CD4-BS and the other targeting a conformational V1V2 epitope (55, 265). However, those studies did not examine whether both specificities emerged at the same time or not and how they evolved over time. In the present study we aimed to better understand the relative emergence and evolution of dual epitope specificities in a well-characterized case control from the above-described cohort.

MATERIALS AND METHODS

Human plasma samples

Samples from subject AC053 from the Ragon Institute of Massachusetts General Hospital (MGH) 'acute / early' HIV infection cohort (also referred to 'primary' cohorts) were used in this study. The subject was infected with clade B HIV-1, had no AIDS-defining illnesses, and was not on antiretroviral therapy at the time of sample collection. In the MGH Acute HIV infection Cohort 'primary infection' was defined by detectable HIV RNA in the presence of either (i) a negative p24 ELISA or (ii) a positive ELISA but evolving WB, or (iii) documented negative HIV ELISA within past 6 months. The date of infection for AC053 was known and 9 samples were collected longitudinally starting at a 0.82 years post infection to 6.85 years post infection, after which CD4 T cell count fell below 200 and the subject was placed on antiretroviral therapy. Samples were heat-inactivated at 56°C for 1 hour before use in neutralization assays. The neutralizing activity of AC053 plasma has been reported by our group previously (339).

Plasma antibody adsorptions to monomeric gp120

Recombinant monomeric SF162 gp120 WT or SF162K160N gp120 proteins were coupled to MyOne Dynabeads Tosylactivated (Invitrogen) as previously described (293, 449). Briefly, 50 mg of magnetic beads were reacted with 1 mg protein ligand overnight at 37°C with rotation. After collecting the beads on a magnet, the supernatant was removed and the beads were incubated overnight at 37°C in PBS, 0.5%BSA, 0.05% Tween 20. The magnetic beads were washed twice with PBS, 0.1%BSA, 0.05% Tween 20, and stored at 4°C in the same buffer, with the addition of 0.02% Sodium Azide. Bead-coupled Env proteins were tested for antigenic integrity by flow cytometry using known MAbs b12, 447-52D, 2G12, PG9, and 4E10, followed by detection with goat-anti-human-IgG-FITC secondary antibody (data not shown). Mock adsorption/elution experiments using several anti-HIV Env MAbs at a concentration of 10 µg/ml in naïve plasma were performed as a positive control. 250µl or 500µl of plasma diluted 1:5 in DMEM/10%FBS were incubated with 100µ or 200µl Env protein-coupled beads, respectively, at 37°C for 120 min with gentle rotation. The samples were placed on a magnet and the beads were isolated. Four rounds of bead adsorptions were performed per sample.

The anti-gp120 plasma antibodies bound to the bead-coupled Env proteins were eluted in a series of increasingly acidic solutions as previously described (293). The beads from each serial adsorption were combined and incubated in 0.1M Glycine-HCl, pH 2.7 for 30 seconds with

vortexing. The beads were collected by brief centrifugation and held in place by a magnet. The supernatant was removed and adjusted to pH 7.5 with 1M Tris (pH 9.0). The process was repeated with the beads in 0.1M Glycine-HCl, pH 2.3 and then again in pH 1.7. The final supernatants were buffer exchanged in PBS and washed over a 30kD Amicon Ultra centrifugation concentrator (Millipore). Concentration of immunoglobulin was determined by absorbance at 280 nm (NanoDrop Spectrophotometer ND-1000, Thermo). The anti-gp120-antibody depleted plasmas and the anti-gp120 antibodies eluted from gp120-coated beads were tested by ELISA for binding activity and for neutralizing activity (data not shown).

Neutralization assays

Single round-competent viruses expressing Envs from Clades A, B, and C primary isolates were used in this study. The clade B SF162, JRFL and YU2 isolates were isolated during chronic HIV-1 infection and the remaining isolates were isolated during acute infection (289, 291, 306). Single-round competent viruses were produced in 293 cells as previously described (327) with the modification that GeneJuice (EMD Millipore) was used as the transfection reagent. In the cases when kifunenisne- or swainsonine-treated pseudoviruses were used, the 293T producer cells were treated with 50μM of the glycosidase inhibitor after transfection and the pseudovirus was collected after 72 hour incubation. All treated pseudoviruses were tested for neutralization resistance to PG9 or PG16.

The neutralizing activities of plasmas were determined using the Tzm-bl-based neutralization assay (130). Briefly, plasma dilutions were pre-incubated with single-round competent virions (pseudovirus) for 90 minutes at 37°C. The plasma / pseudovirus mixture was added to TZM-bl cells (3000 cells per well in a 96-well plate) for 72 hrs at 37°C. The supernatant was removed and 100μl of Steady-Glo Luciferase Assay Substrate (Promega) was added to each well. Plates were incubated for 15 minutes at room temperature away from light and 75μl of the lysate was transferred to micro titer plates. The cell-associated luciferase activity for each well was determined on a Fluoroscan Luminometer (Thermo). Percent neutralization was calculated at each plasma dilution as the percent inhibition of viral entry by the plasma sample compared to the absence of plasma. For each plasma/virus combination tested, a neutralization curve (percent neutralization versus plasma dilution) was generated using GraphPad Prism 5 (GraphPad Software, San Diego California, USA) and the plasma dilution at which 50% neutralization was recorded (IC50) was determined by transforming the data to a log10 scale with fitted sigmoidal dose-response curves. Neutralization breadth of a plasma sample is

defined as the percent (0% - 100%) of the 20 isolates neutralized by that sample (339). All plasma samples were screened for non-HIV-specific neutralization using the murine leukemia virus (MLV) pseudotyped into the HIV backbone.

RESULTS

Plasma neutralizing activities of subject AC053

To determine how the epitope specificities of the cross-reactive neutralizing antibody response evolved over time we focused our analysis on a single subject in the MGH cohort - AC053. Yearly plasma samples were available for AC053 starting at 0.82 years to 6.85 years post infection, after which the subject initiated antiretroviral therapy. Previously, we characterized the development of the cross-neutralizing antibody response in this subject and demonstrated that at 3.29 years this subject's plasma could neutralize 45% of the 20 cross-clade isolates tested, and at 5.31 years – 80% (339). The cumulative IC50 titers at all time-points are shown in **Figure 3.1**.

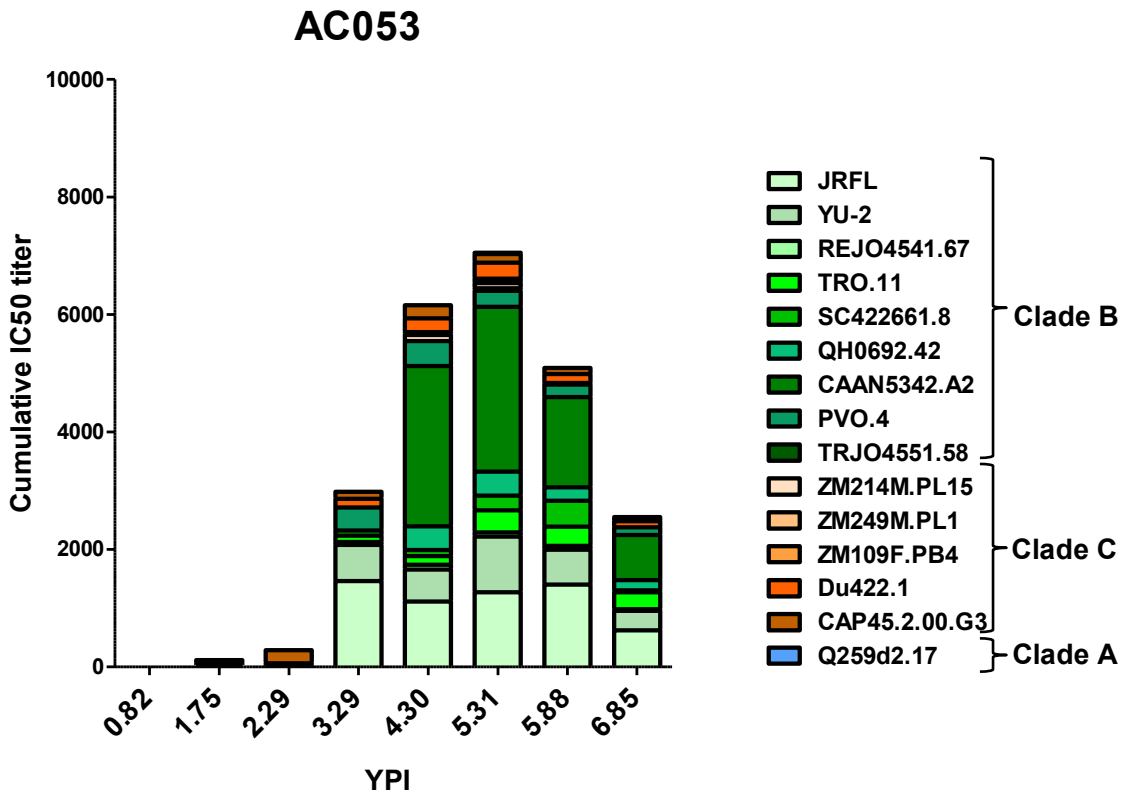

Figure 3.1. Cumulative IC50 titers of AC053 longitudinal plasma samples as previously reported (339). Plasma neutralizing activities were previously titrated against 20 heterologous Clade A (blue), B (green), and C (orange) isolates, 15 of which were included in the graph. The neutralizing antibody response gradually increased in breadth and potency, and at the highest recorded breadth (5.31 ypi), AC053 neutralized 16 of these isolates amounting to 80% breadth (Titers to SF162 are not depicted). This clade B-infected subject had highest titers against Clade B isolates.

Initial epitope-mapping studies demonstrated that the broadly neutralizing antibody response of AC053 at 5.31 years was primarily focused on the CD4-BS of monomeric gp120 (339). However, for some isolates tested (for example TRO.11, CAAN, and Zm214M), neutralizing activity of the plasma was unaffected by the depletion of anti-gp120 or anti-MPER antibodies. This led us to hypothesize that at least one additional antibody specificity was present in that subject's plasma; a specificity that targeted an epitope on the virion-associated Env, outside the CD4-BS.

Kifunensine-treated viruses are no longer neutralized by AC053 plasma

MAbs PG9, PG16 and CH01-04 represent a class of cross-neutralizing specificities that primarily target the virion-associated Env over corresponding soluble recombinant Env forms, and map outside the CD4-BS (54, 535). These types of antibodies recognize a complex epitope within the V2 loop that is formed both by amino acids and glycan molecules (332). MAbs PGT125-128 represent a second class of cross-neutralizing specificities that also recognize two conserved glycans on gp120 but target a complex epitope that includes the amino acid backbone of the V3 loop (391, 538). A known distinctive feature of the PG9/PG16 epitope-like specificities (as compared to the PGT-like epitope specificities) is the loss of neutralizing activity against kifunensine-treated viruses (136, 535). Kifunensine is a mannose analogue that inhibits type-I alpha-glycosidases, and HIV virions produced by kifunensine-treated cells are resistant to neutralization by PG9 and PG16 (136). However, this treatment does not affect substantially the neutralization of other broad mAbs, such as VRC01 or 2G12 **(Figure 3.2)** and others (285). In addition, the neutralizing activity of the PGT125-128 MAbs (which recognize a conserved epitope in the V3 loop and associated carbohydrates) is not affected by such treatment (538). As a negative control we included treatment with swainsonine, an enzyme that inhibits mannosidase-II, which does not affect neutralization by PG9 and PG16 (136). Interestingly, swainsonine-treated HIV was more susceptible to PG9, PG16 and 2G12 than the untreated virus **(Figure 3.2)**.

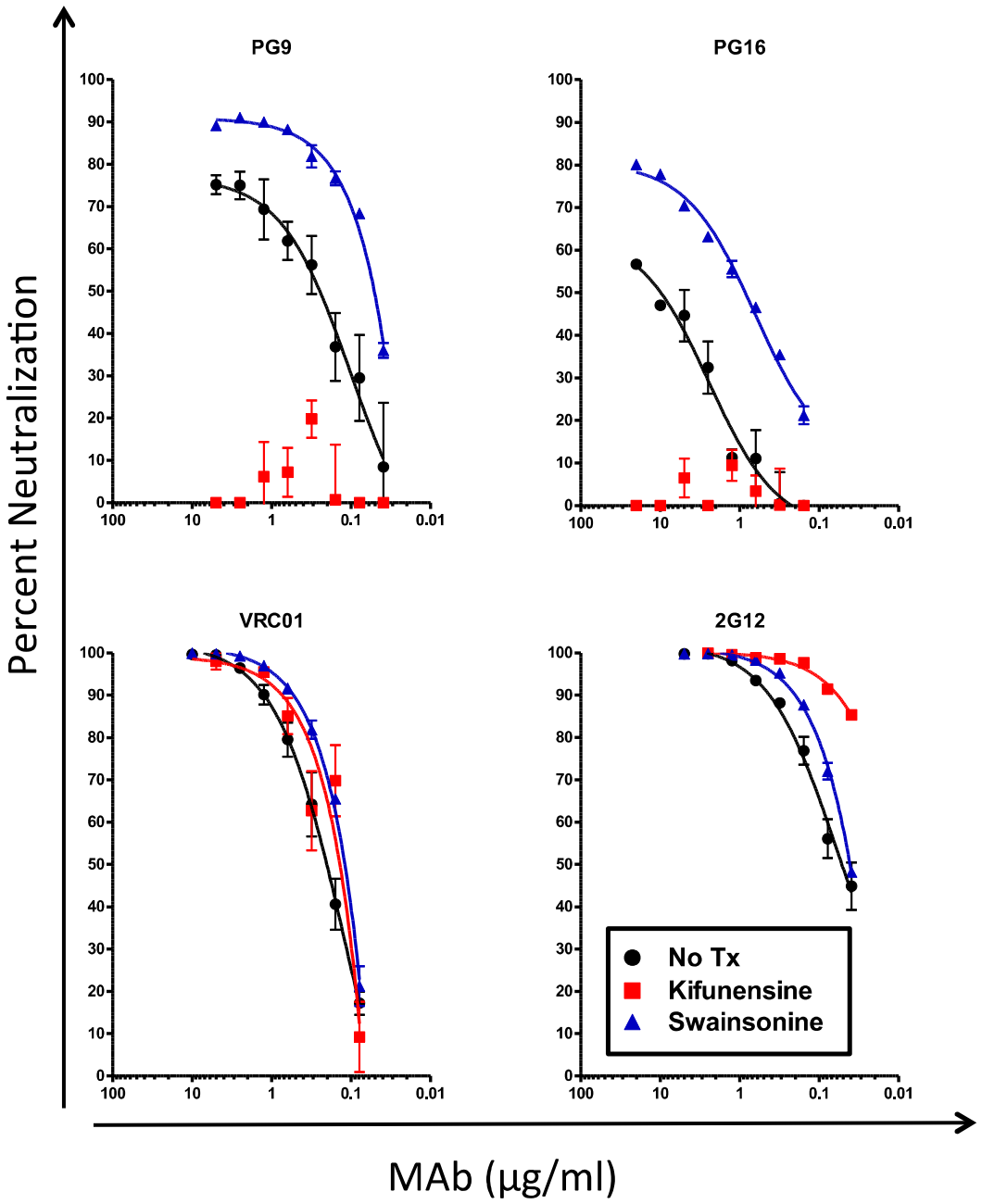

Figure 3.2. Neutralization of kifunensine- and swainsonie-treated pseudoviruses by monoclonal antibodies. Neutralization curves were plotted for MAbs (µg/ml) PG9, PG16, VRC01 and 2G12 with untreated (black circles), kifunensine-treated (red squares), and swainsonine-treated viruses (blue triangles). Percent neutralization was calculated as the percent of inhibition of viral entry by each MAb compared to a 'No MAb' control.

Initially, we examined whether PG9/PG16-like neutralizing activities were present in AC053 plasma, by comparing the neutralizing activity of this plasma using viruses produced in the absence or presence of kifunensine. PG9/16-like antibodies do not recognize the SF162 gp120

because it lacks glycans at position 160, which are necessary for PG9/16-Env binding (535, 549). This would therefore explain why the anti- TRO.11, CAAN, or Zm214M neutralizing activity of AC053 plasma could not be eliminated by SF162gp120-based antibody adsorptions (339). The introduction of an asparagine at that position (SF162K160N) renders the virus highly susceptible to PG9/16 (429). Therefore, we tested AC053 plasma at 5.31 yrs PI against SC422661, PVO.4 and SF162 K160N viruses grown in the presence or absence of kifunensine or swainsonine **(Figure 3.3)**. Neutralizing activity against all kifunensine-treated viruses was either completely absent (SC422661 and PVO.4) or markedly decreased (SF162K160N) compared to untreated or swainsonine-treated viruses. This result suggested that, potentially, the AC053 plasma contained PG9/16-like antibodies.

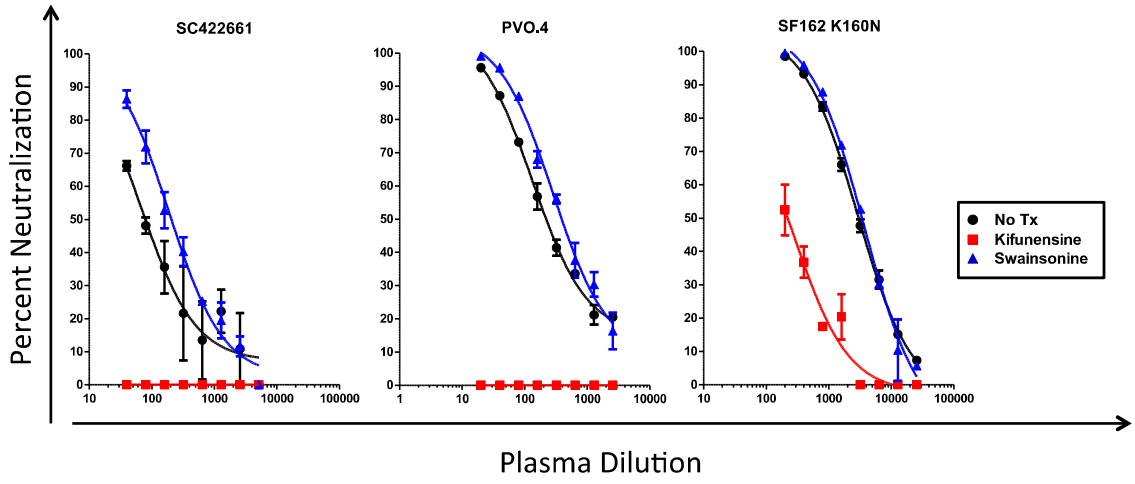

Figure 3.3. Neutralization of kifunensine- and swainsonine-treated pseudoviruses by AC053 at 5.31 yrs post infection. Percent neutralization of viruses produced in 293T cells treated with 20 μM kifunensine or swainsonine glycosidase inhibitors (as described in the Materials and Methods) was calculated. Black circles – no treatment, red squares – kifunensine treatment, blue triangles – swainsonine treatment. Percent neutralization was calculated as the percent of inhibition of viral entry by the plasma compared to a 'No plasma' control.

The above analysis of AC053 was performed with plasma collected at 5 years post infection, at a time when the broadly neutralizing activities in this subject were well established. To determine how early this specificity emerged in the plasma of AC053 and whether it coincided with the emergence of the overall cross-neutralizing activity in this subject, we performed similar studies with plasmas collected longitudinally. The earliest samples, however, do not display cross-neutralizing activities and do not neutralize SC422661 or PVO.4 (339) and therefore we could not use those viruses for this experiment. All samples, however, do neutralize the SF162K160N virus. The neutralizing activities of longitudinal plasmas from AC053 were

evaluated against SF162K160N that was grown in the presence or absence of kifunensine **(Figure 3.4)**. The earliest plasma (collected at 0.82 yrs after infection) could not neutralize either the kifunensine or swainsonine-treated viruses. In contrast, plasma collected at 1.75 yrs post-infection could only neutralize the untreated virus and the swainsonine-treated virus, but not the kifunensine-treated virus. These results suggest that, potentially, PG9/16-like neutralizing activities began emerging in this subject within the first two years of infection, sometime between 0.82 and 1.75 yrs post-infection (at the same time as the overall cross-neutralizing activity of AC053 plasma began to be detectable (339)). This relatively early development of PG9/16-like antibodies during HIV infection was recently reported in other HIV+ subjects (201, 536). Plasmas collected after that point also neutralized the untreated virus and the swainsonine-treated virus, but not the kifunensine-treated virus. Therefore, the PG9/16-like specificities were maintained for the duration of the observation (over 6 years of infection).

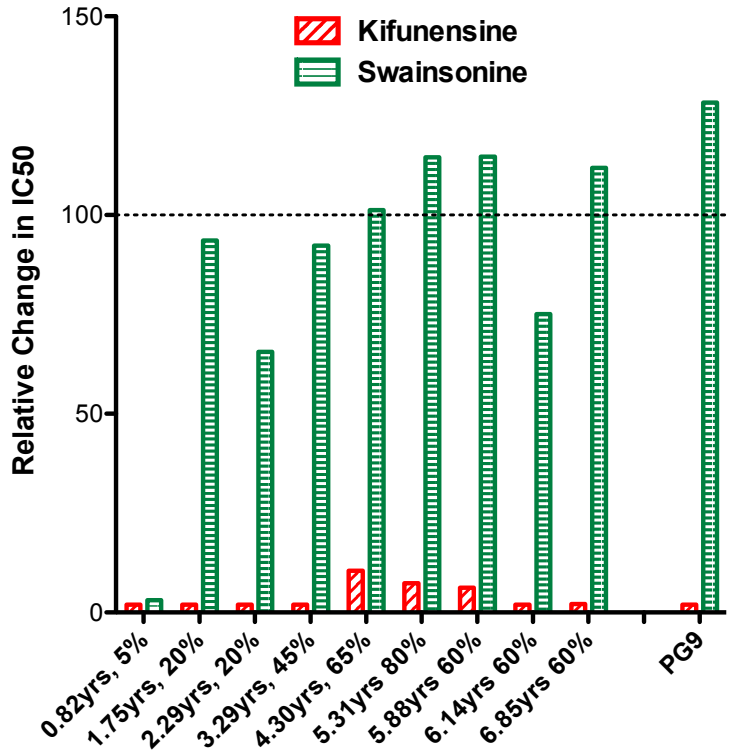

Figure 3.4. Evolution of glycan-dependent specificity in AC053. Percent change in the IC50 titers of kifunensine-treated (red) and swainsonine-treated (green) SF162 K160N by plasma samples was calculated relative to the untreated virus. Years post infection and percent isolates neutralized are indicated for each sample. PG9 is included as a positive control.

In light of these results, we wanted to investigate how common the kifunensine-dependent neutralizing specificity was in the MGH cohort. The effect of kifunensine treatment was tested on plasmas from 3 other subjects from that cohort that developed cross-neutralizing activities and on plasmas from 4 subjects from that cohort that did not develop such responses **(Figure 3.5)**. In contrast to the results obtained with the AC053 plasma, kifunensine treatment had no effect on the neutralizing activities of these plasmas. Our findings suggested, therefore, that the PG9/16-like pattern of sensitivity to kifunensine but not swainsonine was a unique feature in the AC053 cross-reactive NAb response in this cohort. Of note, two studies of distinct cohorts of chronically HIV-1-infected subjects reported higher frequencies of PG9/16-like neutralizing antibody specificities (285, 536).

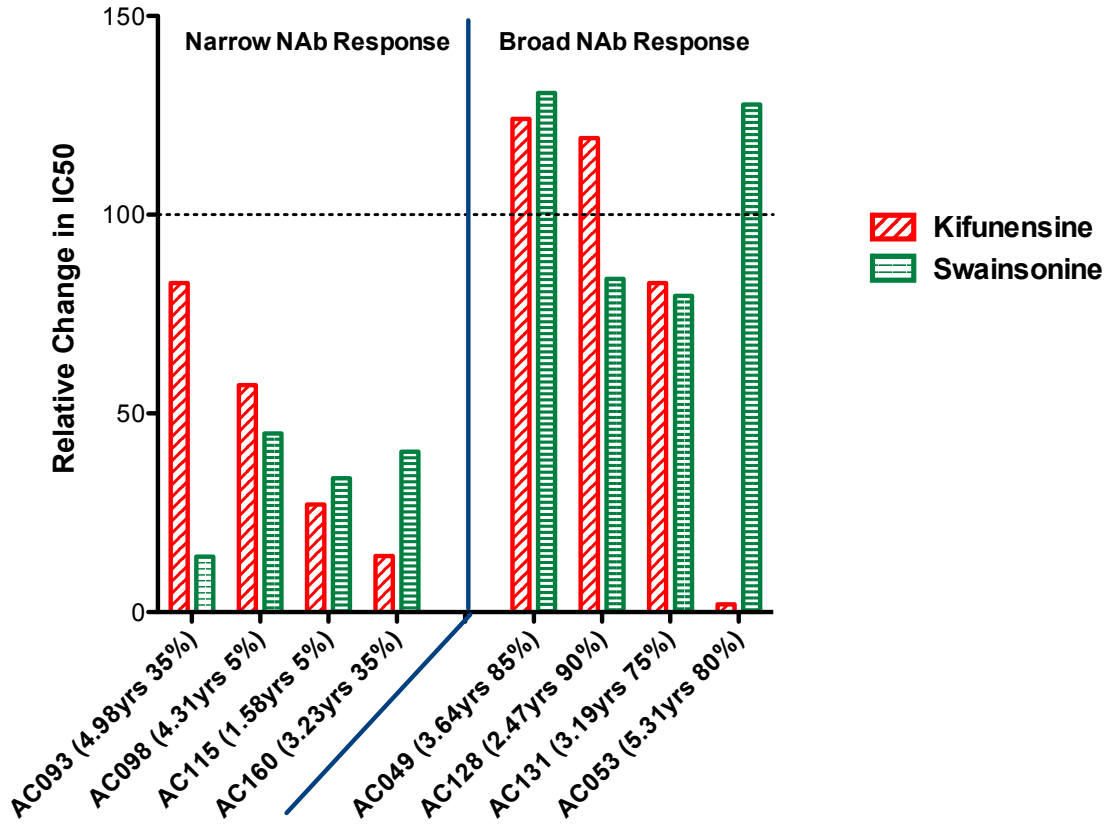

Figure 3.5. The PG9/16-like resistance of kifunensine-treated but not swainsonine-treated viruses is a unique feature of AC053 in the MGH cohort. Plasma samples from the MGH cohort, characterized in detail in a previous report (339), were tested for neutralization of kifunensine-treated (red) and swainsonine-treated (green) SF162K160N. Four plasmas with narrow breadth (≤ 35% breadth) and four plasmas with cross-reactive NAb responses (≥ 75% breadth) were selected. AC053 was the only plasma that demonstrated the PG9/16-like resistance of kifunensine-, but not swainsonine-treated pseudoviruses. Years post infection and percent isolates neutralized are indicated for each sample.

Depletions of PG9-like NAb activities from AC053 plasma

We next tested the hypothesis that the glycan-specific, PG9-like cross-neutralizing activity in AC053 plasma would be 'depleted' if appropriate reagents are used. To this end, we performed the following set of experiments. Plasmas collected at four time-points after the development of cross-neutralizing activities were depleted from their anti-gp120 antibodies with either WTSF162gp120 (which does not deplete PG9-like antibodies) or SF162K160Ngp120 (which depletes PG9-like antibodies) **(Figure 3.6)**. Both Envelope proteins deplete anti-CD4-BS antibodies (data not shown).

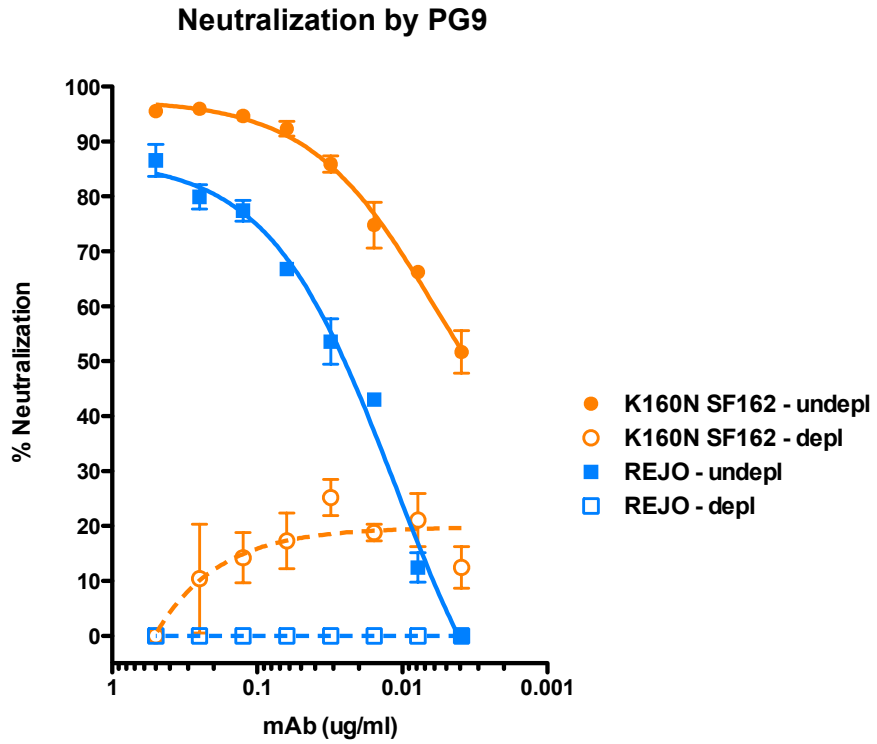

Figure 3.6. Depletion of PG9 neutralizing activity with SF162 K160N gp120. PG9 in naïve human sera was depleted with 4 consecutive incubations with SF162K160N gp120-coupled beads. Neutralization by undepleted (filled symbols and solid lines) and depleted (clear symbols and dashed lines) PG9 was tested against SF162 K160N (orange circles) and REJO (blue squares) viruses, demonstrating the substantially diminished neutralization activity.

The neutralizing activities of the undepleted and depleted plasmas were evaluated and compared, using CAAN-derived viruses as targets **(Figure 3.7)**. CAAN is one of the viruses that, although susceptible to the neutralizing activity of AC053 plasma, is not susceptible to the anti-CD4-BS antibodies present in AC053 **(Figure 3.7A)** and ref (339). Aside from the WT CAAN, two variants of CAAN were also included in these experiments. One (CAAN N301Q)

lacks the N-linked glycosylation site at the N-terminus of the V3 loop at position 301, and the other (CAAN 332) lacks the N-linked glycosylation site at the C-terminus of the V3 loop. These two glycans are part of the epitopes of the PGT-like antibodies (391, 538). The high-mannose glycan at position 332 is also part of the 2G12 epitope – another glycan-dependent MAb (456). The neutralizing activities of the AC053 plasmas were not affected by the WTSF162gp120 depletion treatment **(Figure 3.7B)**. However, the SF162 K160Ngp120 depletion resulted in incremental loss of the AC053 plasmas' neutralizing activity. The IC50 neutralizing titers of AC053 plasma increased (i.e., less potent neutralization) by more than half a Log 10, initially against CAAN N301Q, (4.3 ypi), then against CAAN N301Q and CAAN N332Q (5.31 ypi) and finally against all three viruses tested (CAAN N301Q, CAAN N332Q and CAAN) (5.88 ypi).

A.

	3.29 yrs		5.31 yrs	
Viral Isolate	WT-depl	D368R comp	WT-depl	D368R mut
CAAN WT	not neutralized		0.47	0.46

B.

	4.30 yrs		5.31 yrs		5.88 yrs		6.14 yrs		6.85 yrs	
Viral Isolate	WT-depl	K160N-depl	WT-depl	K160N-depl	WT-depl	K160N-depl	WT-depl	K160N-depl	WT-depl	K160N-depl
CAAN WT	0.43	0.49	0.12	0.47	0.26	0.98	0.37	0.79	0.18	1.22
CAAN N301Q	0.39	0.61	0.03	0.65	0.13	0.76	0.17	0.53	0.00	0.93
CAAN N332Q	0.21	0.43	0.02	0.51	0.13	0.94	0.20	0.75	0.00	0.91

Figure 3.7. Contribution of different anti-gp120 specificities for the cross-reactive neutralizing activity of AC053. A. Log10 decrease in IC50 titers by elimination of anti-WT SF162 gp120 or competition of anti-D368R SF162 gp120 antibodies, as reported in (339), which demonstrates the CAAN is not susceptible to the anti-CD4BS cross-reactive NAb responses of AC053. **B.** Log10 decrease in IC50 titers by elimination of anti-WT SF162 gp120 or anti-K160N SF162 antibodies, indicative of a glycan-specific cross-neutralizing response. Light blue: no effect or less than 0.5 Log10 decrease; Orange: decrease between 0.5 and 0.9 Log10; Red: over 0.9 Log10 decrease.

Relative emergence of cross-reactive neutralizing antibody responses to the CD4-BS and to the Env carbohydrates in AC053

Our results indicate that the glycan-dependent neutralizing activity in the plasma of AC053 emerged sometime between 0.82 and 1.75 ypi **(Figure 3.8)**. At the time this activity became evident (at 1.75 ypi), the overall breadth of neutralizing activity in the blood was very narrow with ~20% of heterologous clade B and C isolates tested being susceptible to neutralization by AC053 plasma (339). At approximately 3 ypi, anti-CD4-BS antibodies with cross-neutralizing

potential became detectable **(Figure 3.8)**, and ref (339). As mentioned above, and in our previous study (339), these antibodies were capable of neutralizing a wide range of isolates, but not every isolate tested against (for example, TRO or CAAN). The neutralizing activity that was dependent on the presence of glycans at position 160 became evident much later, sometime after 4.30 ypi. We believe that PG9-like antibodies, rather than PGT-like antibodies or 2G12-like antibodies, emerged at that time in AC053 because: (a) this neutralizing activity is affected by the kifunensine treatment of the target virus, (b) it is dependent on the presence of carbohydrates at position 160, and (c) is independent on the presence of carbohydrates at positions 301 or 332.

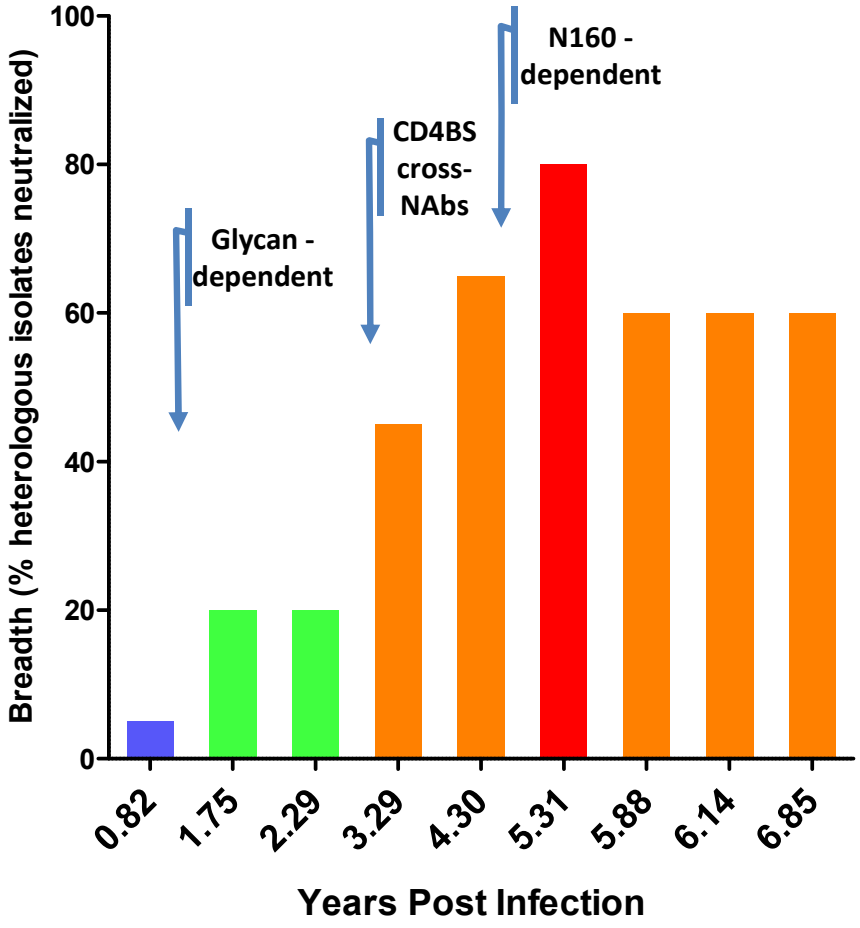

Figure 3.8. Timeline of the epitope evolution of cross-reactive NAb responses in AC053. The breadth of NAb responses, previously reported as percent of n=20 heterologous isolates neutralized (339), was plotted for all available time-points for subject AC053. Breadth is color-coded as follows: blue < 20%; green, 20%-39%, orange, 40-74%, red ≥ 75%. The arrows on the timeline correspond to approximate years post infection when particular NAb specificities became evident.

DISCUSSION

In the past few years several groups reported that approximately 15% of those infected with HIV-1 develop broadly neutralizing antibody responses (138, 157, 201, 295, 339, 449, 477). MAbs with potent and broad anti-HIV neutralizing activities have been isolated from such subjects (54, 364, 461, 535, 538, 552, 588). We and others have shown that broadly neutralizing antibody responses become detectable on average at 2 to 3 years post infection, but very rarely before that (201, 339, 519). Our initial epitope-mapping analysis indicated that, similar to what has been observed in studies of chronic HIV infection (42, 536), a relatively small number of Env regions are targeted by the earliest cross-neutralizing antibody responses (339).

The characterization of such MAbs has provided new information on the structure of broadly neutralizing antibodies, the location and structures of their epitopes, and has expanded our knowledge on the mechanisms by which such antibodies prevent HIV-infection (95, 134, 332, 461, 545, 553, 579, 580). Whether the overall broadly neutralizing activity of an individual's plasma is due to a single or multiple antibody epitope specificities remains a topic of intensive investigation. Recent studies, conducted with samples collected from a very small number of HIV-1-infected subjects, indicate that one or two distinct epitope specificities can recapitulate the majority of the cross-neutralizing activity of the corresponding plasma (55, 160, 265). In one report, the anti-CD4-BS cross-neutralizing activity was complemented by a PG9/16-like neutralizing activity (55), while in the other report the anti-CD4-BS cross-neutralizing activity was complemented by an activity targeting a not well-defined epitope that is preferentially exposed once Env engages CD4 (265). This information is very relevant not only to future HIV vaccine-design efforts, but also to our understanding of the way the human immune system (specifically its B cell arm) responds to HIV infection. The above observations were, however, made in the context of chronic HIV infection and it remains unknown whether dual specificity broadly neutralizing antibody responses emerge at the same time of infection, or sequentially, in a given subject.

To address the topic of the relative timing of emergence of broadly neutralizing antibody responses that target two (or more) distinct Env regions within a given subject, we focused our attention on patient AC053. The clade B-infected AC053 was part of a cohort of HIV-1-infected subjects that were monitored longitudinally from the time of their HIV-1 infection (339). We previously reported that the cross-neutralizing activity of this subject's plasma was due to at

least two specificities: one that targets the CD4-BS on gp120 and was effective against many, but not all, isolates the plasma was tested against, and another specificity that was not targeting the CD4-BS or the gp41 Env subunit (339). The second specificity was effective against viruses that were naturally resistant to this subject's anti-CD4-BS NAbs. The availability of nine plasma samples from AC053 collected between 0.82 to 6.85 years post infection, and the knowledge of when plasma broadly neutralizing antibody responses became detectable in this individual, made it a perfect case for studying the evolution of dual epitope specificities of broadly neutralizing antibody responses during HIV-1 infection.

Sometime between 0.82 and 1.75 years of infection a glycan-dependent neutralizing activity became apparent in the blood of AC053. The timing of the emergence of this activity coincides with the initial appearance of cross-neutralizing antibodies in this subject. At 0.82 ypi the plasma only neutralized SF162 (out of 20 isolates tested), while at 1.75 ypi the plasma neutralized four out of 20 isolated tested (339). Two of these four isolates were clade C and two were clade B. At that point, however, the potency of neutralization was weak and the breadth of neutralization was narrow. In addition, several isolates that are susceptible to PG9 were resistant to neutralization by this plasma. Overall, these observations suggested to us that at its earliest development the glycan-dependent neutralizing activity in AC053 plasma was not due to PG9-like antibodies. Of course, one could also argue that PG9-like antibodies begun emerging at that point of infection, but that their VH and VL antibody domains had not yet incurred somatic mutations that are required for the broad neutralizing ability of PG9. In the absence of longitudinally isolated MAbs from AC053 it is not possible to address this point directly. Broader cross-neutralizing antibody responses capable of neutralizing at least 50% of isolates tested (from clades A, B and C) became first apparent at approximately 3 ypi and were due to anti-CD4-BS neutralizing antibodies **(Figure 3.8)**, and ref (339). As we extensively discussed previously, these anti-CD4-BS cross-neutralizing activities were not effective against all isolates that were susceptible to neutralization by the AC053 plasma (339). For example, they were not effective against the CAAN or TRO.11 viruses. Even the anti-CD4-BS neutralizing activities of plasmas isolated later in infection, which displayed broader and more potent neutralizing activities, were ineffective against these and other viruses. At 3 ypi, cross-neutralizing specificities that are dependent on the presence of a glycan at position 160 were not evident in AC053. This second cross-neutralizing specificity became apparent sometime around 4.30 ypi. Because of its dependency on the 160 glycan, but not on glycans positioned in regions of Env targeted by the PGT-like antibodies or 2G12-like antibodies, we believe that this second cross-

neutralizing specificity is due to PG9-like antibodies. We do not believe it is due to PG16-like antibodies, because the neutralizing activity of PG16 cannot be blocked by SF162K160N gp120, while that of PG9 and of the AC053 plasma antibodies are efficiently blocked by that recombinant protein.

Our results are in agreement, and extend, those recently reported by Bonsignori et al (55) that two distinct broadly neutralizing antibody specificities, one against the CD4-BS and the other against the V1V2 loop (PG9-like), can be simultaneously present in an HIV+ subject with broad serum neutralizing activities. The timing of relative emergence of these two distinct specificities was unknown in that study. Here we independently show similar dual specificities in a different subject, but we also report that the anti-CD4-BS specificity emerged first while the V1V2-directed specificity emerged approximately 1-2 years later. It remains unknown whether these two types of specificities can be generated simultaneously in an HIV-infected subject and whether they can be elicited simultaneously by vaccination. We do not currently have viral sequence information on AC053 to define how the virus evolved in that subject before and after the development of the anti-CD4-BS neutralizing antibodies. It is possible that in its effort to escape their action, the virus altered the amino acid composition of the V2 loop, and such escape viral Env clones were the ones that stimulated B cells to produce PG9-like antibodies. We believe that the earliest development of glycan-dependent neutralizing specificities aided in the development of PG9-like antibodies later in infection.

In summary, our studies support efforts to elicit multiple specificities of broadly neutralizing anti-HIV antibodies by vaccination. Most likely this will be achieved by prime-boost immunization protocols, during which sequential immunizations with distinct Env proteins will stimulate the development of broadly NAbs of distinct epitope specificities.

CHAPTER IV

CURRENT STATUS OF THESIS PROJECT

The results presented in Chapter II have been published as the following: Mikell I, Sather DN, Kalams SA, Altfeld M, Alter G, Stamatatos L. 2011. Characteristics of the Earliest Cross-Neutralizing Antibody Response to HIV-1. PLoS Pathog 7(1): e1001251. doi:10.1371/journal.ppat.1001251.

The results presented in Chapter III have been submitted for review as: Mikell I, and Stamatatos L. 2012. Characterization of the emergence and evolution of cross-neutralizing antibody specificities to the CD4-BS and the carbohydrate cloak of the HIV Env glycoprotein during HIV infection.

I am also a secondary author on the following manuscripts, submitted for review.

Alter et al[1] investigated the contribution of antibody Fc-mediated effector functions such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-mediated cellular viral inhibition (ADCVI) in protection during acute HIV infection, and their kinetics and evolution in relation to the neutralizing antibody responses. The authors studied subjects in the MGH cohort and reported that ADCC/ADCVI responses were generated soon after HIV infection, and peaked at 6 months post infection. However, while the cross-neutralizing antibody responses in some of these subjects broadened over time, the Fc-mediated antibody activities declined in the setting of increasing immune activation, suggesting that these two distinct antibody activities are modulated by different inflammatory signals.

[1] Manuscript submitted for review as follows: Stamatatos L, Dugast AS, Suscovich TJ, Tonelli A, Licht A, **Mikell I**, Ackerman ME, Streeck H, Klasse PJ, Moore J, Alter G. 2012. Independent evolution of Fc- and Fv-mediated HIV-specific antiviral antibody activity starting in acute HIV infection.

Sather et al[2] studied the effects of extraordinarily broad and potent cross-neutralizing antibody activity on the replication of, and escape by the autologous virus in a chronically infected individual. The broadly neutralizing antibody response targeted the conserved CD4 binding site (CD4BS) on the HIV envelope. Resistance to these responses involved mutations in the CD4BS that restricted access to that region by the autologous antibodies, yet still allowed the virus to enter target cells through the use of the CD4 receptor. Escape through these mutations, however, amounted to a substantial fitness cost to the virus in terms of slower replication kinetics and reduced target cell entry.

[2] Manuscript submitted for review as follows: Sather DN, Carbonetti S, Kehayia J, Kraft Z, **Mikell I**, Scheid J, Klein F, Stamatatos L. 2012. Broadly neutralizing antibodies developed by an HIV+ elite neutralizer exact replication fitness cost to the contemporaneous virus.

DISCUSSION AND IMPLICATIONS

For over 200 years vaccines have played an essential role in protection from multiple infectious diseases with spectacular success against a number of viral pathogens, as demonstrated by the eradication of smallpox, the impending eradication of poliomyelitis, the drastic decrease of measles, mumps, and rubella cases (405), and the recent successful introduction of rotavirus vaccines (114, 313, 423). Most vaccines are thought to protect against viral pathogens through the induction of neutralizing antibodies (NAbs) in sera or in the mucosa, which block infection at the site of entry or prevent virions from invading the bloodstream (20, 406, 426). Finding correlates of protection, be they absolute or relative, is an important part of vaccine research because it provides an empirical measurement of a specific response level, above which protection is provided, and also an objective criterion for assessing the expected level of protection in vaccinees.

After almost 30 years of HIV research, identifying correlates of protection from HIV infection has become an intensive, challenging, and hotly debated area of study. Immune correlates are needed to guide vaccine design and to provide better clinical trial endpoint measurements, but those are difficult to obtain for a pathogen that is never effectively cleared by the immune system, and in the absence of an efficacious vaccine. A number of lines of evidence point to neutralizing antibodies as an important part of a successful HIV vaccine as discussed in Chapter I. Particularly encouraging is the sterilizing protection provided by passive infusion of anti-HIV IgG or monoclonal antibodies (MAbs) in the experimental challenge of rhesus macaques and chimpanzees, and in the protection of juvenile and neonatal macaques (25, 166, 226-229, 319, 323, 375, 376, 390, 475). However, the MAbs used in these studies are potent and cross-reactive in their neutralization of diverse Env isolates, and are administered before or soon after experimental challenge. Most HIV infected individuals develop autologous NAbs months after infection, after latency has been established (424, 542). This response is typically of narrow specificity and is quickly escaped by the virus, leading to a cycle of immune selection pressure and viral escape (177, 425, 542). The so-far-insurmountable challenge is that an effective HIV vaccine has to elicit neutralizing antibodies of sufficient potency, magnitude and duration that will protect against the wide diversity of circulating variants and recombinant isolates a vaccinated person can encounter (76, 321, 488). Yet, no single immunogen has generated antibodies that can neutralize diverse isolates. An effective way to understand how such cross-reactive NAb responses are elicited is through studies of natural HIV infection.

Numerous cross-sectional studies of chronic infection have demonstrated that broadly neutralizing antibody (bNAb) responses are detectable in 10%-30% of HIV-infected subjects, and that they target a limited number of conserved epitopes on the HIV Envelope [reviewed in (490)]. This is encouraging news for HIV vaccine development – the fraction reported is higher than previously thought. However, not much was known about the timing of the development of the earliest cross-reactive NAb responses, and about their specificities compared to chronic infection. Chapter II contains results from a longitudinal study of two acute/early HIV infection cohorts, in which we assessed the breadth of neutralization at consecutive time-points against a standardized panel of isolates from different clades. We reported that 29% of the subjects in the cohort developed bNAb responses, similar to what has been observed for chronic infection. In terms of factors associated with the development of the earliest bNAb responses, there was statistically significant positive correlation between the breadth of plasma neutralizing activities and plasma viral load, and time since infection, again similar to what has been reported for chronic infection (138, 396, 449). A word of caution here; despite this association, high viral load is not an absolute predictor of neutralization breadth, as some highly viremic individuals do not develop cross-reactive NAb responses even after 5 years of follow-up (157). Some groups have suggested that set-point viral load, rather than contemporaneous plasma viremia, is a better predictor of the development of neutralization breadth (201, 396).

Our longitudinal analysis demonstrated that cross-reactive NAb responses become detectable *in vitro* at an average of 2 to 3 years post infection, but not after 3-4 years of infection. This finding has been supported by other groups as well (201, 519), and suggests that potentially there is a window of opportunity during which cross-reactive NAb responses develop, but not afterwards. If development of broadly neutralizing antibodies was simply a stochastic event, then it would be expected that such antibodies would be generated at any time during infection. Potentially, the development of broadly neutralizing antibody responses around 2-3 years of infection, but not after that point, is most likely due to a gradual immune dysregulation that takes place during HIV infection. For example, one study demonstrated that autologous NAb responses waned after 3 to 4 years post infection with the apparent loss of their ability to neutralize the relentlessly escaping viral isolate (518). This finding could explain why de novo cross-reactive NAb responses are likely not generated after 4 years of infection. However, the above report included a small number of HIV-infected subjects, and their cross-reactive responses did not diminish as autologous NAbs did, so further studies are needed to test this hypothesis.

Our studies established for the first time an association of cross-reactive NAb responses with markers of immune activation. A trend towards higher percentages of CD8+ T cells expressing Ki67, CD38, and PD1, and CD4+ T cells, expressing Ki67 and CD38 was observed, with statistically significant association with PD1 expression on CD4+ T cells. The medium-to-high viral load of subjects with bNAb responses compared to the lower viral load of those with narrow responses could be related to an increased immune activation status. A positive association between plasma viremia and immune activation has been reported starting very early on after infection (124). CD38 is a marker for cell activation, and Ki67 is strictly associated with proliferating cells. Upregulation of PD1 is considered a marker of exhaustion of CD8+ T cells during persistent viral infections, most likely the result of prolonged antigenic stimulation (32, 148, 393, 516). In HIV infection, it is associated with higher viral load and impaired CD8+ T lymphocyte function (121). Interestingly, PD1 is also normally expressed at high levels on the surface of follicular helper T (Tfh) cells. Tfh are a recently characterized subset of CD4+ T lymphocytes (99, 378), and by engaging PD1 ligands on B cells, regulate the survival of germinal center B cells, and the formation of long-lived plasma cells during humoral immune responses (189). These cells are essential for the generation of high affinity, effective antibody responses (531). One potential implication of the association between higher percentage of PD1+ CD4+ T cells and broad NAb responses is that increased frequency or activation of Tfh cells in subjects with cross-reactive NAbs could potentially accelerate B cell activation and maturation in the germinal centers. This could promote higher levels of somatic hypermutation of the VH and VL antibody domains, thereby selecting for high-affinity, cross-neutralizing Abs. In fact, broadly neutralizing monoclonal Abs (including b12, VRC01, HAADs, PG9 and PG16) isolated from chronically HIV-1-infected subjects have VH regions with exceptionally high number of somatic mutations and long complementarity-determining region of the heavy chain (CDRH3) (277, 385, 461, 535, 579, 589).

The above hypothesis could also explain the delay of 2-3 years in the acquisition of cross-reactive NAb responses. A requirement for antigen-driven selection and increased somatic hypermutation, beyond what is seen with NAb responses to other viruses (409), combined with an increasingly dysfunctional immune system, could set the stage for years of multiple rounds of affinity maturation by B cells to finally achieve cross-neutralizing activity.

Why 10%-30% of the population generate cross-reactive NAb responses, and what is different about their infection compared to the rest of HIV-infected subjects is currently not known. As

mentioned above, a simply random event is likely not the whole story. The observed association of bNAb responses with antigenic stimulation and Ab avidity (449), and the likely need for extensive somatic mutation can provide researchers with some clues as to how cross-reactive NAb responses are generated. One hypothesis is that efficient viral escape from the autologous antiviral response (NAb and cell-mediated) leads to higher viral load which increases the chances of a circulating isolate with the "right" epitope that would elicit cross-reactive Abs **(Figure 4.1)**. However, this epitope could also be present on Envs in subjects who develop only isolate-specific Ab responses. This is something that can be tested by looking at Envelope diversity and escape from the neutralizing Ab response over time. What seems to be different in subjects who develop cross-reactive NAb responses is the higher level of "early" immune activation, particularly higher frequencies of CD4+ T cells expressing PD1. If this population is Tfh cells, their increased frequency and / or activation could accelerate B-cell maturation and increase levels of somatic hypermutation, selecting for high-affinity cross-reactive Abs, prior to the onset of extensive immune dysregulation.

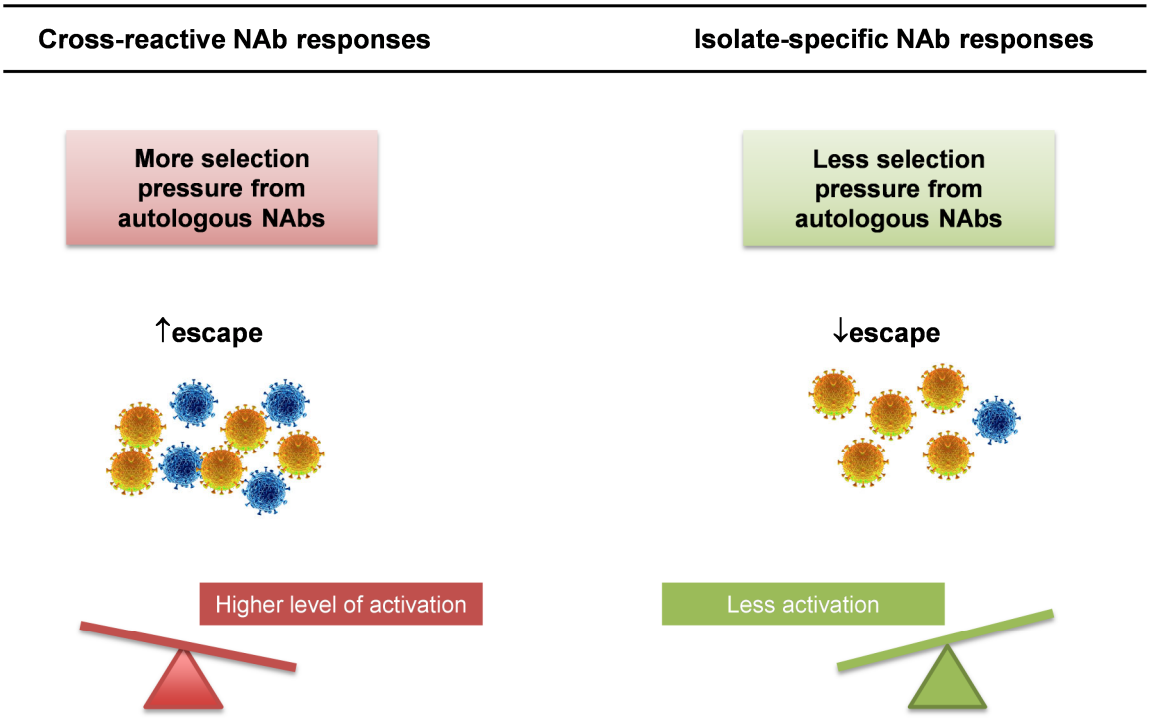

Figure 4.1. Hypothesis for the generation of cross-reactive NAb responses. Autologous NAb responses in subjects that generate cross-reactive NAb responses are hypothesized to exert more selection pressure on the virus (orange virions), resulting in a faster emergence of escape viral variants (blue virions), and higher Env diversity. This could increase the chances of eliciting NAbs to more conserved regions of Env. The higher viral load could also result in increased levels of immune activation, accelerated B cell affinity maturation and increased somatic hypermutation of IgG genes in subjects that develop cross-reactive NAb responses.

In Chapter II, I reported that, in agreement with what has been described for sera from chronic infection (42, 131, 200, 536), the earliest cross-neutralizing antibody responses target only a few specificities on the HIV Env. Chapter III contains our findings on how these specificities evolve over time from acute/early into chronic infection in one infected individual. Availability of yearly plasma samples for subject AC053 from a few months to almost 7 years post infection made such a study possible. Cross-neutralizing responses in the sera targeting the CD4BS became detectable at approximately 3 years post infection, and a second specificity to a glycan at position 160 in the V2 region appeared after 4 years of infection. This finding is in agreement with two recent studies of bNAb epitope specificities in two distinct HIV-infected subjects, in which cross-reactive NAb responses during chronic infection targeted the CD4BS, and either a glycan in the V2 region or a new epitope exposed after engagement of the CD4 receptor (55, 265). In addition, a combination of the anti-CD4BS MAb VRC01 and the V1V2 MAb PG9 displayed no cross-competition, and was found to have additive effect on the breadth of neutralization in vitro, covering 97% of the 208 diverse isolates tested (140). These observations support the direction of vaccine efforts towards a rather limited set of target epitope specificities. Overall this is a finding that is very relevant to HIV immunogen-design efforts: instead of having to elicit neutralizing antibodies with many specificities, these results indicate that 1-2 specificities will be sufficient to block infection of almost 100% of diverse circulating viruses globally.

FUTURE DIRECTIONS

The results of the two studies described in Chapter II and III, and data on the cross-reactive NAb response to HIV from other groups provide a few important directions for future research.

Development of bNAb responses

A better understanding of the virologic and immunologic factors underlying the development of cross-reactive NAb responses is needed if they are to be elicited by vaccination. The availability of a well-characterized MGH Cohort with longitudinal plasma samples provides an ideal setting to study the generation of cross-reactive NAb responses and the reason that they are elicited in only a fraction of those infected with HIV-1. On the virus side, sequencing longitudinal Envelope variants from both, subjects with broad and narrow responses, is an essential first step in understanding whether specific viral variants are associated with the development of broadly

neutralizing antibody responses. Importantly, Env sequences can be obtained from isolates circulating very early on in infection, before cross-reactive NAb responses can be detected, and generation of Env diversity can be followed phylogenetically over the years as breadth begins to emerge or not. Functional comparison can be made between the interplay of virus with autologous NAbs in subjects with cross-reactive responses and those with isolate-specific Ab responses. A recently published study has demonstrated that individuals with cross-reactive NAb responses have higher peak autologous neutralizing antibody titers and quicker viral escape than individuals with type-specific neutralizing antibody responses (156). Higher levels of selection pressure from autologous NAb responses could play a pivotal role in increasing Env diversity (through escape) (177), and finding a similar increase in the MGH cohort in the setting of cross-reactive NAb responses would provide important information about designing an immunogen that would elicit such responses in vaccinees.

Aside from the infecting virus, immunological factors are likely to also play a role in determining whether broadly neutralizing antibodies develop or not. Focusing again on the MGH cohort, for which there is longer follow-up, comparisons can be made between multiple immune parameters in subjects with broad and narrow neutralizing antibody responses. The statistically significant levels of PD1 on CD4+ T cells led to an ongoing study in the lab investigating the role of Tfh cell in the development of cross-reactive NAb responses. A detailed look at Tfh and B cell phenotypes over time and noting any differences in levels of expression of surface markers of activation can provide clues as to why only a subset of individuals develop cross-reactive NAb responses. An additional marker of immune activation is plasma levels of LPS (67). Another relevant comparison is of levels of expression of select cytokines associated with immune activation and immunomodulation, in terms of both protein levels in blood and mRNA expression by PBMCs.

High-affinity, somatically hypermutated broad NAb responses

Another interesting area of future research is to test the hypothesis that cross-neutralizing antibodies are of higher affinity and more highly mutated from the germline than type-specific NAbs. A way to test for accelerated B-cell maturation is to stain B cell populations for the upregulation of the enzyme AID, and the transcription factors Bcl-6 and Blimp-1, comparing B cells from subjects who do or do not develop cross-reactive NAb responses. These proteins are known regulators of germinal center reactions that ultimately result in the production of high-affinity, somatically mutated Abs. In addition, qPCR of AID mRNA levels will provide a more

quantitative analysis of AID expression in B cells in the setting of broad vs narrow NAb responses. B cells from chronically HIV-infected subjects have been shown to express higher levels of AID compared to healthy controls (82). Given the hypothesis that subjects with broad NAb responses might produce more somatically hypermutated Abs, I expect to observe higher AID expression in this group compared to those with Ab responses of narrow specificity.
A way to directly examine the rate of somatic hypermutation of the variable regions of Abs, is to isolate HIV Env-specific B cells from subjects with broad and narrow NAb responses, and amplify and sequence the light and heavy variable antibody regions. Comparing the number of mutation from the germline, in Env-specific and non-Env specific sequences from subjects with broad and narrow cross-reactive NAb responses would demonstrate an overall increase in the mutation rate of antibodies from subjects with broad cross-reactive NAb responses, although somatic hypermutation might not be targeted only to Env-specific antibodies.

Evolution of cross-neutralizing Ab responses

The report in Chapter III detailed the development of two distinct epitope specificities of the cross-reactive NAb response in the HIV-infected subject AC053. One, targeting the CD4BS, became detectable at approximately 3 years post infection, and the other, specific to a glycan at position 160, appeared some time after 4 years of infection. Almost 7 years of follow-up and a well-characterized epitope specificity make this individual a basis for studying the specifics of the evolution of the cross-reactive NAb response and how it relates to the autologous NAb response. An ideal study would involve sequencing viral Env variants from all time-points, including from the earliest available plasma draw post infection, to acquire the closest version of the transmitted/founder virus. Another priority is to obtain HIV-specific autologous monoclonal Abs for sequence analysis to determine levels of somatic hypermutation and CDRH3 length, and for susceptibility of AC053 viral isolates to autologous NAbs. Most informative will be comparison not only between sequences from AC053 HIV-specific and HIV-nonspecific MAbs, but also with HIV-specific and non-specific MAbs from a matched control with only type-specific Ab responses. The availability of monoclonal antibodies from AC053 would help verify the epitope specificities proposed in our previous study, will allow fine-mapping of changes in the target epitopes over time, and will provide a way to test if the two specificities recapitulate the neutralizing breadth of the sera.

CONCLUDING REMARKS

The search for an effective HIV vaccine immunogen continues but great strides have been made in our knowledge of the interaction of HIV with the immune system. HIV-infected subjects develop cross-reactive NAb responses, the kinds of responses that an effective vaccine should elicit. The results included in this thesis contribute important information on the development of cross-reactive NAb responses and may guide future immunogen design and vaccine regimens that elicit such responses.

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