SPECIFICITY AND POLYREACTIVITY OF THE ANTIBODY RESPONSE DURING NATURAL HIV-1 INFECTION

by

Xin Wang
M.D., Shanxi Medical University, 1998
M.Sc., The Fourth Military Medical University, 2001

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

In the
Department
of
Molecular Biology and Biochemistry

SIMON FRASER UNIVERSITY

Fall 2006

APPROVAL

Name: Xin Wang

Degree: Master of Science

Title of Thesis: Specificity and Polyreactivity of the Antibody Response During Natural HIV-1 Infection

Examining Committee:

Chair: **Dr. Mark Paetzel**
Assistant Professor, Department of Molecular Biology and Biochemistry

---

Dr. Jamie Scott
Senior Supervisor
Professor, Department of Molecular Biology and Biochemistry

Dr. Bruce Brandhorst
Committee Member
Professor, Department of Molecular Biology and Biochemistry

Dr. Nicholas Harden
Committee Member
Professor, Department of Molecular Biology and Biochemistry

Dr. Frederic Pio
Internal Examiner
Assistant Professor, Department of Molecular Biology and Biochemistry

Date Defended/Approved: Dec. 1, 2006

ABSTRACT

The specificity and polyreactivity of the antibody response in natural HIV-1 infection were studied. First, to investigate the overall antibody response, overlapping linear peptides were used to screen sera taken from HIV-1-infected individuals. The polyclonal antibody response was relatively stable during long-term infection, compared with acute infection, and mostly directed against immunodominant regions. Low level, transient antibody responses were detected against membrane proximal external region of gp41. To test if these Abs are neutralizing, an affinity purification method was developed to isolate these serum antibodies.

Second, in terms of polyreactivity of antibody response, we found that two broadly-neutralizing monoclonal antibodies bound only weakly to self antigen, cardiolipin. Moreover, sera were screened against cardiolipin; no significant reactivity was observed.

We conclude that the antibody response in HIV-1 natural infection is relatively stable over time; the MPER is weakly immunogenic *in vivo,* and that broadly-neutralizing antibodies do not seem to be autoreactive.

DEDICATION

To my parents

and

my husband, Honggang,

for their unconditional love

ACKNOWLEDGEMENTS

Many people have contributed to this thesis. First, I would like to thank my supervisor Dr. Jamie Scott for accepting me into her lab when I came to Canada as a visitor, and giving me support when I decided to leave the lab because of family reason. I want to thank her for teaching me to think critically and independently, from which I will benefit in my future research. I would also like to thank my committee members, Drs. Nicholas Harden and Bruce Brandhorst for their support and helpful input. Also thanks to the other members of my examining committee, Drs. Frederic Pio and Mark Paetzel, for their time and effort in evaluating my research.

I would like to thank my colleagues and friends in Scott lab. Special thanks to the technician, Sampson Wu. This thesis would not be possible without the huge amount of experiments he did. Thanks to two excellent undergraduate students worked with me and contributed to the data of this thesis, Chao Wang and Amy Lubik. I want to thank Alfredo Menendez and Marinieve Montero for their advices and helpful discussions. Thanks to Martinal Mai for always being a good listener, and supporter for me. Thanks to Nienke van Houten for always being a great help in my writing, and cooking. Thanks to Melita Irving, Tannika Grant, Joanna Chodowska, Sandy Henderson and Matthew Parsons, for together making the lab a happy place for working everyday.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

GLOSSARY

aa	Amino acid
Ab	Antibody
ABTS	2,2;-azino-bis(3-ethylbenzthiazoline-6) sulfonic acid
AIDS	Acquired immune deficiency syndrome
aMLV	amphotropic murine leukemia virus
AP	Alkaline phosphotase
Bio-	Biotinylated
bNt/Nt	Broadly neutralizing/neutralizing
BSA	Bovine serum albumin
CDR	Complementarity determining regions
CHR	C- terminal heptad repeat on gp41
CL	cardiolipin
CTL	Cytotoxic T lymphocytes
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
H3	Hypervariable loops 3 (CDR3) of the heavy chain
HIV-1	Human immunodeficiency virus-type 1
HRP	Horse radish peroxidase
IC_{50}	Inhibitory concentration required to reduce ELISA signals to 50%
SC	Seroconverter
SP	Long-term, slow progressor
MAb	Monoclonal antibody
MHC	Major histocompatibility complex
MPER	Membrane proximal external region on gp41
NFDM/DM	Non fat dry milk
NHR	N- terminal heptad repeat on gp41
NP	Normal progressor
O.D.	Optical density
OVA	ovalbumin
PBS	Phosphate-buffered saline
p-NPP	p-nitrophenyl phosphate substrate
RP	Rapid progressor
RT	Room temperature
SA	Streptavidin
TBS	Tris-buffered saline
TCLA	T-cell laboratory-adapted

CHAPTER 1: THESIS INTRODUCTION

1.1 The AIDS epidemic

The acquired immune deficiency Syndrome (AIDS) pandemic has been one of the most destructive epidemics in history and has killed more than 25 million people since the report of its first case. In spite of broader access to highly active anti-retroviral therapy (HAART) around the world, the AIDS epidemic claimed an estimated 2.8 million lives in 2005 of which more than a half-million were children. Globally, ~46 million people are currently living with HIV and Sub-Saharan Africa remains the worst affected region (UNAID, 2006, http://www.unaids.org/en/HIV%5Fdata/).

The first indication of AIDS epidemic was reported in 1981, with the outbreak of several rare pneumonia cases in homosexual men in Los Angeles, U.S.A.(CDC, 1981, http://www.cdc.gov/mmwr/preview/mmwrhtml/june_5.htm). In 1983, Montagnier *et al.* at the Pasteur Institute in France first discovered the pathogen causing AIDS, Human Immunodeficiency Virus (HIV) (Barre-Sinoussi *et al.* 1983). Based on sequence analysis and serology, HIV was divided into two types. Type 1 HIV (HIV-1) is more virulent, more easily transmitted and is the cause of the majority of HIV infections globally, whereas type 2 HIV (HIV-2) is less easily transmitted, has a different clinical picture, and is largely confined to West Africa (Reeves *et al.* 2002). HIV-1 has three subtypes / groups: M (majority), O (outliers), and N (non-M/non-O). Group M is further classified into 10 different sub-subtypes / clades, designated clades A through J, and a number of recombinant forms (RFs; e.g., the BC RF). Clade B is the most common viral isolate in

the United States and Western Europe; clades A, C, D, and E are most common in the developing world. The focus of work presented in this thesis is on HIV-1.

1.2 HIV-1 structure and life cycle

HIV-1 belongs to the *Lentivirus* genus, and *Retroviridae* family (Popovic *et al.* 1984). Lentiviruses have RNA genomes contained within a protein capsid surrounded by a lipid envelope. HIV-1 is ~120 nm in diameter (60 times smaller than a red blood cell). It is composed of two copies of a single-stranded RNA genome enclosed within a conical capsid, which is in turn surrounded by a lipid bilayer membrane (envelope) derived from the host cell. The p24 protein forms the capsid that encloses the two genomic RNA strands and viral enzymes, such as integrase, p32 and reverse transcriptase, p64. The matrix protein, p17, is anchored to the internal face of the envelope.

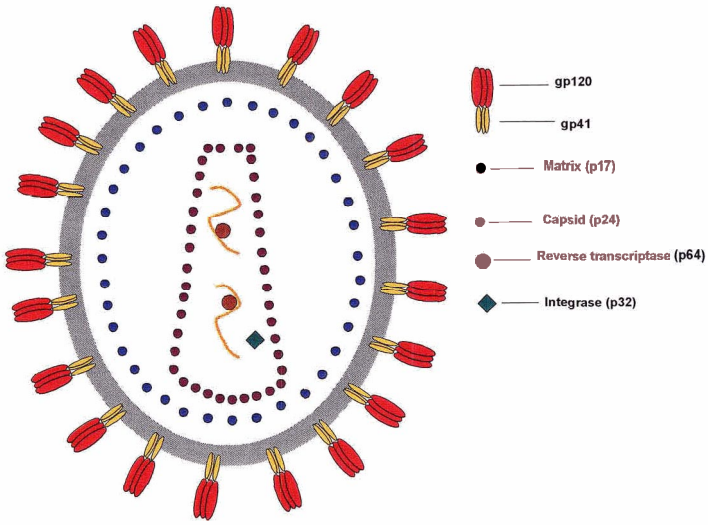


Figure 1-1: Basic organization of HIV-1. See text for details.

HIV-1 targets T lymphocytes, macrophages, and dendritic cells, which all have important functions in the human immune system, via a primary receptor, the CD4

surface molecule, and a secondary chemokine receptor: either CXCR4 (X4) or CCR5 (R5) (Alkhatib *et al.* 1996; Deng *et al.* 1996; Doranz *et al.* 1996; Dragic *et al.* 1996; Feng *et al.* 1996). The cellular preference (tropism) of an HIV-1 varant is largely determined by its ability to use X4 and/or R5. The X4 coreceptor is mainly present on $CD4^+$ T cells, whereas the R5 coreceptor is presented on macrophage/ dendritic cell lineages.

The life cycle of HIV-1 in $CD4^+$ T cells begins with an effective infection, which is mediated by binding of envelope protein, gp120, to the CD4 receptor and X4 coreceptor on the cell surface. This initiates the process of viral entry, which is described in detail below. After successful entry into the target cell, the viral reverse-transcriptase enzyme transcribes the single-stranded viral RNA into double-stranded DNA that is integrated as a proviral genome into the genome of the host cell. In this state, the virus is latent. When $CD4^+$ T cell is activated by antigen, it is stimulated to translate the integrated proviral DNA into proteins needed to build new viral particles. After the viral components assemble inside the cell,

When the infected cell synthesizes new protein, integrated viral DNA is also translated into the proteins needed to build new viral particles, such as Gag, Env and Pol proteins. The Env protein (gp160) is cleaved into the mature gp120 and gp41 subunits by cellular enzymes within the Golgi apparatus. After these proteins assemble at the cytoplasmic side of the cell plasma membrane, the viral core buds through the membrane and obtains an envelope composed of cell membrane components spiked with viral envelope proteins (Freed *et al.* 1995).

1.3 HIV-1 envelope proteins

The HIV-1 envelope spike is a trimeric structure formed by three copies of the membrane-spanning protein, gp41, and three copies of the extracellular surface protein, gp120, non-covalently bound to gp41 (Lu *et al.* 1995). The envelope spike is encoded by the *env* gene, and is translated first as a single polypeptide precursor during viral replication. The *env* gene product subsequently enters the Golgi apparatus and undergoes oligomerization and extensive glycosylation to become gp160, which is then cleaved by cellular proteases into gp120 and gp41 during transfer via the Golgi network to the cell surface. The envelope proteins remain associated through weak, noncovalent (mainly hydrophobic) interactions after cleavage, and gp120-gp41 heterotrimers are incorporated into the envelope of the virion (Earl *et al.* 1991).

1.3.1 The structure of gp120

The outer component of the trimeric spike, gp120, is a heavily glycosylated protein, with carbohydrate accounting for 40% to 50% of its molecular weight (Leonard *et al.* 1990). Glycosylation masks the protein surface of Env, and is important for viral evasion from host immune response (see section **1.6**). Based on comparative sequence analyses, gp120 sequence has been divided into five alternating conserved and variable regions (C1–C5 and V1–V5) (Leonard *et al.* 1990; Starcich *et al.* 1986). Conserved regions of gp120 form a central core, whereas variable regions V1-V4, form loops that are anchored to the surface of the protein by disulfide bridged cysteine residues at their bases.

The V3 loop is a 35-residue-long, glycosylated, disulfide-bonded structure that has been extensively studied, since it is involved in viral infection and dominates the anti-

gp120 antibody (Ab) response (immunodominant region, see **Chapter 2**). The central segment of V3 has been represented by synthetic peptides (KRKRIHIGPGRAFYTT) (HIV-1 MN) (Hartley *et al.* 2005). In the literature, the "tip" or "crown" of the V3 loop has frequently been used to describe the beta-turn, GPGR/GPGQ, of the V3 loop and an unspecified number of residues flanking it. Although highly variable, V3 possesses some relatively conserved features, such as positively-charged patches and a predicted β-turn at the tip. The β-turn motifs, GPGR and GPGQ, are the most common motifs found among all HIV-1 strains, with the former being in subtype B viruses and the latter in viruses from other subtypes (Hartley *et al.* 2005).

It has long been recognized that the V3 loop has a major influence on HIV-1 tropism, which, in turn, substantially affects pathogenesis (Moore *et al.* 2004; Douek 2003). An early example of this influence dates from before the identification of the viral coreceptors. Huang *et al.* observed that some T-cell laboratory-adapted (TCLA) strains of HIV-1 replicated poorly in macrophages, but substitution of 20 residues from V3 of a "macrophage-tropic" viral strain conferred tropism to macrophages (Huang *et al.* 1991). Once different coreceptors (see **1.3.3**) were identified for macrophage/ lymphocyte tropic viruses, it was soon shown that if the V3 region of an X4 virus was genetically replaced by its counterpart from an R5 strain, the chimeric virus had the R5 phenotype, and vice versa (Moore *et al.* 2004; Choe *et al.* 1996; Cocchi *et al.* 1996).

The crystal structure of the gp120 core (comprising gp120 lacking the V1/V2 and V3 loops, and portion of the N and C termini) has been resolved in complex with the receptor CD4 domain and the Fab fragment of the chemokine receptor mimic, monoclonal (M) Ab, 17b (Kwong *et al.* 1998). **Figure 1-2** shows that gp120 is

structurally organized into inner and outer domains that are linked by a bridging-sheet minidomain. The inner domain faces the axis of the trimeric complex, and contributes most of the contacts made with gp41. The outer domain of gp120 is heavily glycosylated, and is thought to face away from the trimer axis. The bridging sheet is formed by four antiparallel β-sheets. Among different clades of HIV-1, the inner domain is more conserved than the outer domain. The crystal structure also reveals that all three domains of the gp120 core contribute to receptor and coreceptor binding (Kwong *et al.* 1998). Packing of the envelope proteins into the trimeric spike structure, and the heavy glycosylation of gp120, lead to very little of the protein surface being exposed on infectious envelope spikes.

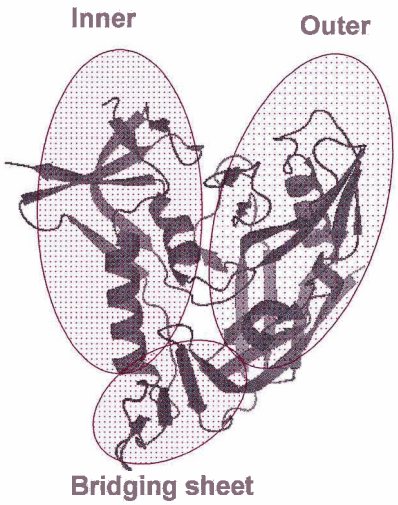


Figure 1-2 HIV-1 gp120 structure.
Crystal structure of gp120 core composed of inner domain, outer domain and bridging sheet.

1.3.2 The structure of gp41

The gp160 precursor is cleaved to form gp41. At the amino-acid sequence level, gp41 shares many features with the transmembrane proteins of other retroviruses. As shown in **Figure 1-3**, gp41 includes following regions: **(a)** a hydrophobic N-terminus forming the fusion peptide which comprises a characteristic stretch of about 15 hydrophobic amino acids (Gallaher 1987). The fusion peptide is believed to insert into and destabilize the target cell membrane during fusion process (see section **1.3.3**). **(b)** C-terminal to the fusion peptide are two regions containing hydrophobic heptad repeats(HRs), the N-terminal (N) HR and C-terminal (C) HR, respectively (Gallaher *et al.* 1989). Both NMR and crystallographic studies have shown that three NHRs fold in parallel to form a trimeric coiled-coil structure surrounded by three antiparallel CHR helices. Each NHR-CHR pair is connected by an extended, disulfide-bridged loop, the clusterI region (Chan *et al.* 1997; Caffrey *et al.* 1998; Skehel *et al.* 1998). Drawing from the extensive structural and functional studies done on the corresponding transmembrane proteins of human Influenza virus (Wilson *et al.* 1981; Carr *et al.* 1993; Bullough *et al.* 1994), it is currently believed that the six-helix bundle structure of gp41 represents post-fusion conformation (Melikyan *et al.* 2000; Chan *et al.* 1997; Tan *et al.* 1997; Weissenhorn *et al.* 1997). The "native" or "pre-fusion" conformation remains unknown. **(c)** The region C-terminal to C-NHR, named membrane-proximal external region (MPER), is highly conserved. (Zwick 2005). The MPER has been an important target for HIV-1 vaccine design because it binds to three broadly (b) neutralizing (Nt) MAbs and is one focus of this thesis. **(d)** Immediately C-terminal to the MPER is the transmembrane domain (TM) of gp41. **(e)** The C-terminus of gp41, cytoplasmic domain (CT), comprises a long, intercellular tail of about 150 amino acids.

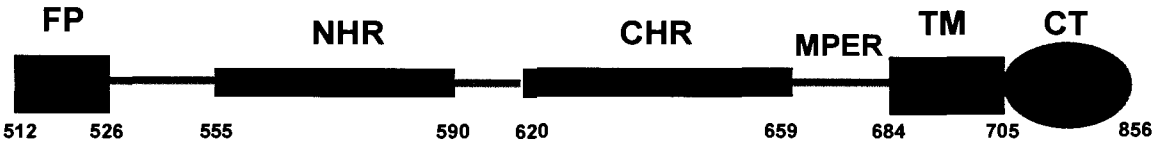


Figure 1-3: Schematic diagram of HIV-1 gp41 protein.

The important functional regions of HIV-1 gp41 including the hydrophobic fusion peptide (FP, aa512~526), N-terminal and C-terminal heptad repeat regions (NHR and CHR) with loop regions in between, MPER (defined here as residues 660-683 of gp160), the transmembrane (TM) domain, and the internal cytoplasmic domain (CT) (Chan *et al.* 1997).

1.3.3 Envelope proteins mediate HIV-1 entry

HIV-1 entry is a sequential multi-step process mediated by envelope protein gp120 and gp41. Surface protein gp120 is responsible for viral binding to receptors on target cells. The binding of gp120 to receptor CD4 molecule induces conformational changes in gp120, and leads to the exposure of the coreceptor binding site, also called the CD4-inducible (CD4i) site (Rizzuto *et al.* 1998). The binding of gp120 to the coreceptor (R5/X4) results in further conformational changes that lead to gp41 activation, fusion of the viral and cellular membrane, and entry of the nucleocapsid into the cell. These latter steps are mediated by gp41.

On native virus, gp41 exists in a metastable (pre-fusion) conformation and is largely buried by gp120 on its top. Following receptor and coreceptor binding, gp120 is shed, freeing the N-terminal fusion peptide of gp41 that is otherwise buried by gp120. The fusion peptide (**Figure 1-3**) then inserts into the target cell membrane. This extended intermediate form of gp41 is not stable, and is followed by the formation of a gp41 coiled-coil structure that acts as a spring-loaded mechanism to bring the viral and cell membranes into close proximity (**Figure 1-4**). The details involving membrane fusion, pore formation and entry of the viral capsid into the cell are poorly understood.

For example, it is not known how many gp41 trimers are required to form a fusion pore. After the pore has been formed, the viral capsid, including RNA genomes and various enzymes, is injected into the target cell though the pore.

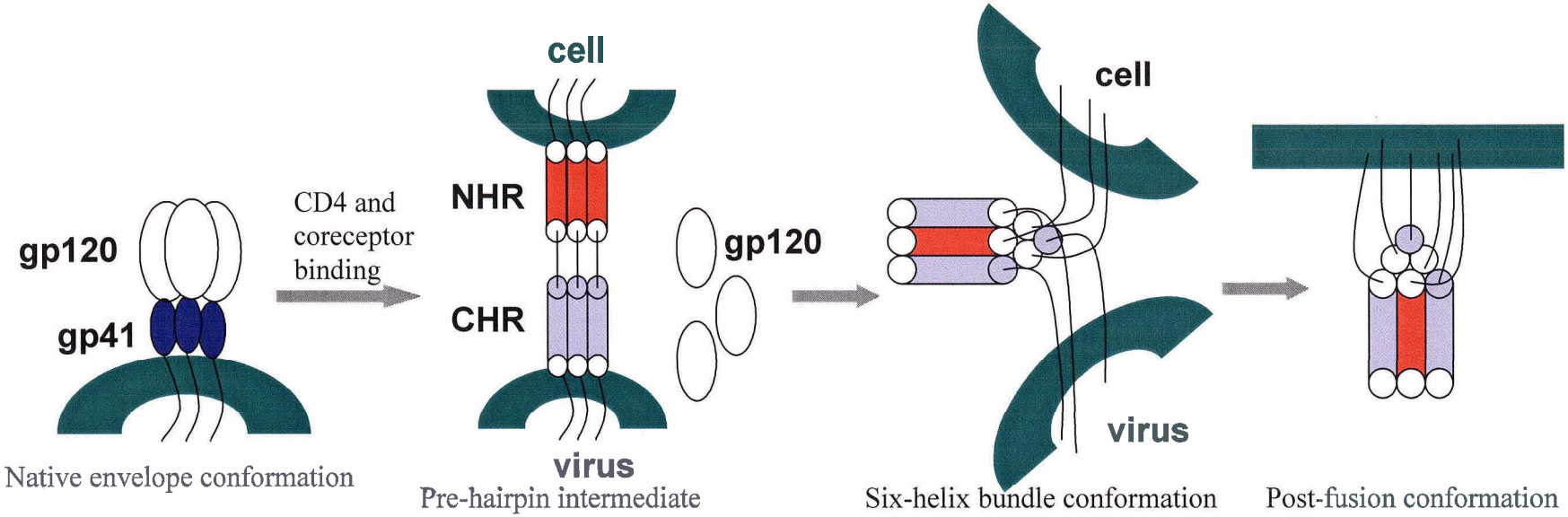


Figure 1-4: A schematic model for HIV-1 entry.

After gp120 binds to receptor CD4 and coreceptors on the target cell membrane, gp41 forms an extended conformation and the hydrophobic fusion peptide inserts into the target membrane (pre-hairpin intermediate). The next step is the formation of six-helix bundle conformation and the energy thereby released causes the membranes to bend towards each other. After two membranes fuse together, the viral capsid will then be injected into the target cell.

1.4 The structure of antibodies and their epitopes

Abs are globular proteins made by B lymphocytes. Recognition of an antigen by receptor on the B cell (surface antibody) triggers proliferation and differentiation into memory B cells and plasma cells that secret soluble Abs with identical specificity to the parent B cell surface receptor. Ab molecules have a common structure of four polypeptide chains (**Figure 1-5**), comprising two identical light (L) chains, and two identical heavy (H) chains. Each L chain comprises two homologous, ~110 amino acid (aa) domains, V_L and C_L, each of which folds into a two-layer sandwich of antiparallel β-strands arranged in two β-sheets, named immunoglobulin (Ig) domain. Depending on the isotype of the H chain, it comprises four to five Ig domains (VH, CH_1, CH_2, CH_3 and sometimes CH_4); CH_1 and CH_2 are linked by a non-Ig domain, hinge region of varying length. Each light chain is bound to a heavy chain by a disulfide bond, and by noncovalent interactions between Ig domains that form H-L chain heterodimers. Similar noncovalent interactions and disulfide bridges link the two identical H-L chain combinations to each other to form the basic four-chain $(H\text{-}L)_2$ Ab structure (Edelman 1991; Janeway, 2005).

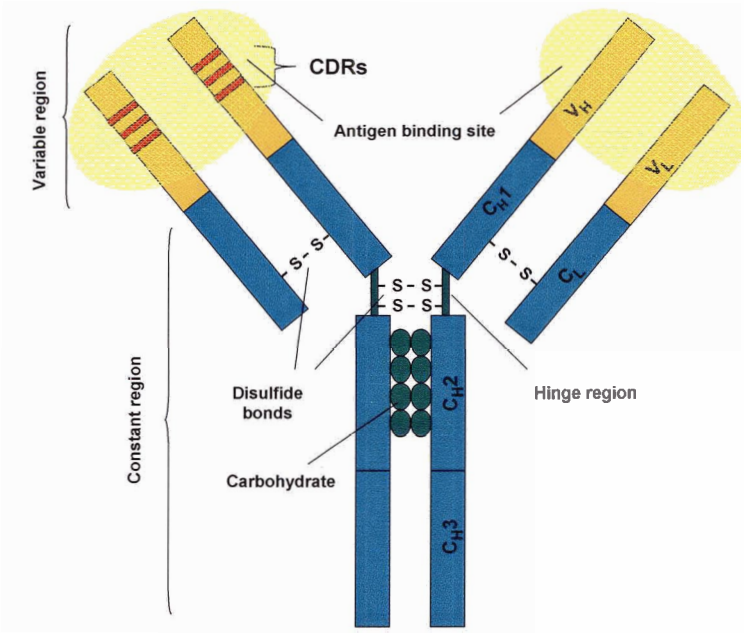


Figure 1-5 Schematic diagram of an intact immunoglobulin antibody.

The structure includes the variable (V) and constant (C) regions of heavy (H) and light (L) chains. The CDRs are illustrated in the variable-region domains. Also shown are the intradomain disulfide bonds and associated N-linked carbohydrates.

Sequence comparison revealed that there are variable regions and constant regions in each H and L chain (Wu *et al.* 1970). The amino-terminal variable (V) Ig domains of the H and L chains (V_H and V_L) together make up the V region of the Ab and confer the ability to bind specific antigen, whereas the constant (C) Ig domains of the H and L chains (C_H and C_L) make up the C-region (Janeway 2005). When an Ab binds to an antigen, only a very small part, representing the six hypervariable loops at the tips of the V domains is actually involved in binding the antigen. This region of antigen contact is called the paratope of the Ab; the region on the antigen it contacts is called the epitope. Since these loops determine what shapes on the antigen the Ab will bind to, they are referred to as complementarity determining regions (CDRs) (Janeway 2005). There are

three CDRs on both H and L chains, namely CDR1, CDR2 and CDR3. In this thesis, the CDR3 loop on the H chain will be referred to as "H3".

An epitope is a site on an antigen recognized by an Ab (B cell epitope) or an antigen receptor (T cell epitope); epitopes are also called antigenic determinants (Laver *et al.* 1990). There are two types of protein epitopes, discontinuous and linear epitopes. A discontinuous epitope is composed of residues from different parts of the polypeptide chain that are brought together by the three-dimensional conformation of folded protein. Because denaturation of the protein normally changes such conformation, Abs that bind to discontinuous epitopes on a native protein typically do not bind to denatured protein. In contrast, a linear epitope is often composed of a single segment of polypeptide chain (also called linear epitope) and is present whether the protein is in its native or denatured state. However, linear epitopes may be conformational, requiring protein folding for Ab to bind. On the surface of a protein antigen (such as gp120 or gp41), a large number of potential epitopes are presented; the epitopes recognized by the immune system are called immunogenic. Furthermore, certain epitopes are said to be immunodominant, meaning they preferentially stimulate a stronger immune response than other epitopes within a particular antigen. The epitopes that are immunodominant for B cells tend to be on the surface because they are more available than internal epitopes on the antigen.

1.5 The antibody response during HIV-1 infection

In general, Abs provide protection from extracellular pathogens and cytotoxic T lymphocytes (CTLs) operate against intracellular pathogens. Both extracelluar (before entry) and intracellular stages exist in the HIV-1 life cycle; therefore, an immune response involving both Abs and CTLs is produced in HIV-1 infection. The host

immune response can temporarily control but not eliminate the virus due to the large pool of latent infected cells (mainly $CD4^+$ T lymphocytes) and the virus's ability to generate large numbers of mutants. There is a dynamic interplay between HIV-1 and the host immune system in which HIV-1 mutants arise and are suppressed by strain specific Ab and CTLs. However, eventually, the viral replication and evasion, and infected cell killing leads to decline of the CD4+ T cell population and to the clinical symptoms of AIDS.

HIV-1 CTLs have been shown to be well correlated with the initial decline of viremia (Pantaleo *et al.* 1994; Koup *et al.* 1994; Connick *et al.* 1996; D'Souza *et al.* 1996). In contrast, Nt Abs usually appear after viremia has begun to decline, suggesting that they are not responsible for the initial reduction in viremia (Ariyoshi *et al.* 1992; McKnight *et al.* 1992). However, studies have suggested that the NtAbs may be more pronounced in individuals with long-term, slowly-progressing infection (SP). NtAb responses in SP are improved in magnitude and breadth compared with other HIV-1 infected individuals (Montefiori *et al.* 1996). In addition, sera from SP have a greater probability of neutralizing heterologous primary isolates than sera from progressors (Cao *et al.* 1995; Montefiori *et al.* 1996). Note that whether the improved Ab response is a cause or effect of SP is still a question needs to be answered.

More direct evidence of the protective role of the Ab response comes from passive transfer studies. In chimpanzee models, it has been demonstrated that passive immunization with bNt MAbs followed by infection with a SIV-HIV chimeric virus (SHIV) can prevent chronic infections (Emini *et al.* 1992; Mascola 2003). In human, there is also direct evidence that passively transferred bNt MAbs have the capacity to

contain viremia in established HIV-1 infection (Trkola *et al.* 2005). Given such data, the general consensus is that an effective HIV-1 vaccine should produce bNtAbs in order to prevent new infections (i.e., that produces sterilizing immunity).

1.6 Mechanisms of HIV-1 evasion from Ab response

One reason that HIV-1 is not eliminated by the immune response during infection is that HIV-1 has evolved a capability to generate escape mutants. Many escape mechanisms from Ab-mediated neutralization have been described. First of all, the exceptionally high genetic variability of HIV-1 envelope proteins including the sites of glycosylations allows the virus to escape from Ab recognition by mutation of the epitopes on the envelope proteins (Moog *et al.* 1997; Wei *et al.* 2003; Richman *et al.* 2003). Second, both gp120 and gp41 are largely covered by carbohydrate glycans that are synthesized by the host and therefore considered "self" by the host's immune system at low density. As a result, the glycosylation renders the underlying "foreign" proteins gp120 and gp41 invisible to the immune surveillance (glycan-masking). The protective effect of the carbohydrate glycans has been demonstrated by Malenbaum *et al.*, who showed that a gp120 mutant with a deletion of a single glycosylation site rendered much higher sensitivity to neutralization (Malenbaum *et al.* 2000). Third, the complex organization of gp120 and gp41 as heterotrimers, has important implications for immune evasion since functionally important sites for viral neutralization on envelope proteins are conformationally protected from binding to Abs; this has been called "conformational masking" (Kwong *et al.* 2002). In contrast, it has been suggested that *in vivo*, non-functional envelope falls apart due to non-covalent interactions that tether gp120 to gp41, thus exposing new immunogenic sites that are not present on functional envelope. These

non-infectious envelope proteins act as the predominant immunogen, and divert the immune system to produce Abs to surfaces that are occluded on the functional spike. For example, the gp41/gp120 interfaces and areas on gp120 buried along the trimer axis are now presented to the immune system. Both gp120 and gp41 may also change conformation upon dissociation. As a result, most Abs elicited *in vivo* are raised against these decoy epitopes and do not bind the functional envelope glycoprotein complex, therefore can not block viral infection (Parren *et al.* 1997a; Parren *et al.* 1997b; Burton 1997). In supportive of this idea, it has been shown that there is a clear correlation between antibodies that are capable of neutralization and those that bind to mature oligomeric Env protein (Fouts *et al.* 1997; Roben *et al.* 1994; Sattentau *et al.* 1995).

1.7 HIV-1 primary isolates and T-cell laboratory-adapted strains

There was an enigma misleading HIV-1 research for years, which is the difference in neutralization resistance between HIV-1 primary isolates and TCLA strains. Primary isolates represent viruses isolated directly from patients such as the molecular clones SF162, BaL, ADA, 89.6, JR-CSF, JR-FL, and YU2. In contrast, TCLA strains represent HIV-1 that has been cultured and passaged in immortalized T-cell lines *in vitro* for a period in the laboratory, such as the molecular clones HXBc2, IIIB, and MN. In general, primary isolates are more resistant to Ab neutralization compared to TCLA. Most of the early studies on NtAbs have used TCLA strains whereas it is believed now that primary isolates are more representative of the original *in vivo* HIV-1. The difference in neutralization resistance may be attributed to Ab-mediated selection pressure on primary isolate viruses *in vivo*. In other words, viruses grown in tissue culture without Ab pressure become more neutralization sensitive (Daar *et al.* 1990;

Gorny *et al.* 1994; Mascola *et al.* 1994; Moore *et al.* 1992; Zhang *et al.* 1997; Fouts *et al.* 1997; Moore *et al.* 1995).

1.8 Broadly neutralizing antibodies and their epitopes on envelope proteins

During HIV-1 infection, strain-specific NtAbs are elicited early on. Once the Ab response reaches a certain level, a resistant virus emerges. Then, a neutralizing Ab response to this resistant virus develops and a new resistant virus emerges. In most cases, the virus always stays one step ahead of the evolving neutralizing Ab response and the autologous viruses are replaced quickly by populations of resistant viruses. To eliminate the established HIV-1 infection by natural immune response or to prevent a new infection by vaccines, the presence of Abs that can neutralize HIV-1 from a wide variety of clades and origins, the so-called bNt Abs, is important. Yet, up to now, only a small panel of bNt MAbs (b12, 2G12, 447-52D, 2F5, 4E10 and Z13) have been identified that can neutralize a broad range of HIV-1 primary isolates (Burton *et al.* 2005; Zolla-Pazner 2004). Therefore, the epitopes of these bNt MAbs have been attractive targets for vaccine design.

bNt MAb b12 binds to a conformational epitope that overlaps the CD4 receptor binding site on gp120. The CD4 binding site is a recessed pocket located at the interface of the outer and inner domains with the bridging sheet (Kwong *et al.* 1998). In the gp120-CD4 interaction, amino acid Phe43 on CD4 plays crucial role by protruding into the pocket on gp120 (Wyatt *et al.* 1998). The b12 crystal structure has been determined and at the same time, docked onto the gp120 structure with CD4-liganded (Saphire *et al.* 2001). In the crystal structure, a notable feature is that b12 has an ususually long

protruding H3 loop (18aa) compared with the average (13aa long)in anti-protein human Abs (Collis *et al.* 2003). It was suggested that this H3 loop inserts into the pocket of the CD4 binding site on gp120 and therefore blocks viral infection by preventing CD4 attachment.

bNt MAb 2G12 recognizes an epitope composed of oligomannose residues located on the outer domain of gp120 (Calarese *et al.* 2003). It has been hypothesized that the neutralizing activity of 2G12 is an indirect, steric effect manifested by a binding site that is physically close to the receptor-binding sites of gp120 (Scanlan *et al.* 2002). 2G12 is unique in terms of both specificity as well as structure. Crystallographic study has revealed that two Fabs of 2G12 assemble into an interlocked Vh domain-exchanged dimmer, forming an extended antigen binding site that comprises two V_H-V_L interfaces and one V_H-V_H interface (Calarese *et al.* 2003). Thus, two conventional light chains are flanking this swapped heavy-heavy chain. The 2G12 structure indicates that the humoral arm of the immune system has the capacity to overcome the glycan-masking strategy (see section **1.6**) that HIV uses to protect itself from Ab recognition.

The epitope of bNt MAb 2F5 was mapped to a linear epitope ELDKWA (aa662~667) on the MPER of gp41 (Muster *et al.* 1993). This epitope has been since confirmed and further expanded from the original 6-mer epitope to a longer 17-mer (655~671) linear epitope (Menendez *et al.* 2004; Conley *et al.* 1994; Parker *et al.* 2001; Tian *et al.* 2002; Barbato *et al.* 2003. Site-directed mutational study has demonstrated that the critical binding sites on the peptide are DKW (Zwick *et al.* 2005). Consistent with this, peptides bearing the DKW motif are repeatedly selected from phage-displayed peptide libraries (Menendez *et al.* 2004). In the crystal structure of 2F5 in complex with

the 17-mer peptide, a notable feature is that 2F5 has a 22 aa long CDR3 loop on heavy chain (Ofek *et al.* 2004). Curiously, only 10 out of 22 amino acids at the base of H3 show interactions with the peptide and the apex remains largely unbound. However, mutagenesis studies showed that mutation on a Trp at the tip of this CDR3 loop significantly decreases the binding affinity of 2F5 to both gp41 and its epitope peptide. This mutation also decreases neutralization activity, indicating that the long loop is not only required for the creation of the peptide binding site, but also that the tip of the loop may be involved in further interactions (Zwick *et al.* 2004).

Another bNt MAb 4E10 also binds to a linear epitope NWFDIT (aa 671-677) that is C-terminal to the 2F5 epitope within MPER (Zwick *et al.* 2001b). Mutagenesis has shown that W, F in the core epitope and another W at the C-terminal (7aa away) are important for 4E10 neutralization (Zwick *et al.* 2005). In the crystal structure of 4E10 in complex with a peptide (WNWFDITNW) (Cardoso *et al.* 2005), the peptide adopts an unusual helical conformation compared to the typical β-turn that is the predominant secondary structure of the Ab bound peptide (Stanfield *et al.* 1995). Furthermore, the binding site of 4E10 is remarkably hydrophobic. The hydrophobicity of the combining site is derived in part from the H3 loop as well as from an unusually hydrophobic H2 loop. This makes the 4E10 combining site considerably more hydrophobic than those of most antibodies. 4E10 H3 is also relatively long (18aa). Similar to 2F5, only two residues at its base contact the C-terminal region of the peptide epitope and the other part of the loop bends away. The tip of the H3 loop of 4E10 is composed of mainly nonpolar residues that form a hydrophobic, flat surface that has been suggested to interact with the adjacent viral membrane (Cardoso *et al.* 2005).

The existence of bNtAbs demonstrates the ability of the human immune response to recognize B cell epitopes on the HIV-1 envelope that are shared and that, when explexed with Abs, prevent virus infectivity. However, the fact that bNtAbs are produced in only few individuals, and all demonstrate quite extraordinary characteristics highlights the challenges, that targeting bNtAbs poses to vaccine design.

1.9 Summary of my contribution to Chapter 2, 3 and 4

The work presented in this thesis was performed by a team of people that included myself. For **Chapter 2,** Chao Wang, an undergraduate student in our lab had finished a great amount of preliminary work before I started my project. However, I repeated most experiments to duplicate the data. I also analyzed the screening data. Sampson Wu, a technician in our lab, has helped me complete and replicate the serum screening experiments. For **Chapter 3**, I developed the initial affinity purification method. Amy Lubik, an undergraduate student under my supervision, optimized the method and compared two different purification systems. For **Chapter 4**, I was involed in experimental design and paper writing based on our data. Sampson Wu executed most experiments, and greatly helped Dr. Scott and me in writing this section as a manuscript for publication. I have revised it for this thesis.

CHAPTER 2: ANTIBODY RESPONSE AGAINST HIV-1 ENVELOPE PROTEINS IN NATURAL INFECTION

2.1 Introduction

A bNt Ab response is likely to be necessary for a preventive HIV-1 vaccine. The ideal Abs should have potent neutralizing activity against a broad range of HIV-1 primary isolates, and target conserved epitopes on the envelope protein that are not able to evolve in response to immune response. However, vaccine efforts have not been successful to elicit such bNtAbs *in vivo* (Belshe *et al.* 1998; Graham 1994; Hanson 1994; Letvin 1998; Mascola *et al.* 1996; Matthews 1994). The reason for this failure partly lies in our incomplete understanding of the Ab response in HIV-1 natural infection.

As with most pathogens, HIV-1 induces a polyclonal Ab response against various epitopes on different viral proteins, especially gp120 and gp41. Despite the ability of HIV-1 to evade the Ab response, as mentioned in the introduction, serology studies have shown that NtAbs are made in most HIV-1 infected individuals. Most patients during natural HIV-1 infection produce autologous NtAbs that neutralize the HIV-1 primary isolate the patients were infected with (Mascola *et al.* 1996; Cao *et al.* 1995; Ho *et al.* 1987; Moog *et al.* 1997; Richman *et al.* 2003; Weiss *et al.* 1985). In addition, such NtAbs appear during the acute phase of HIV-1 infection, within three months of seroconversion (Richman *et al.* 2003). Unfortunately, autologous NtAbs can not clear infection, and is followed by the appearance of escape variants. The continuous cycle of autologous neutralization followed by the emergence of escape variants, as shown in many studies

(Bradney *et al.* 1999; McKnight *et al.* 1992; Quinnan *et al.* 1998; Zhang *et al.* 1999; Richman *et al.* 2003; Wei *et al.* 2003), is particularly impressive in HIV-1 infection because of the virus's extremely high mutation rate.

As the HIV-1 virus evolves, the Ab response some patients develop heterologous NtAbs that neutralize variants that are not present in the individual's viral population. Heterologous neutralization always arises after autologous neutralization (Bradney *et al.* 1999; McKnight *et al.* 1992; Quinnan *et al.* 1998; Zhang *et al.* 1999; Wei *et al.* 2003; Moog *et al.* 1997; Richman *et al.* 2003). A few heterologous Nt sera have been found capable of Nt a very broad spectrum of primary HIV-1 isolates. Some examples of these bNt sera include FDA-2 (Vujcic *et al.* 1995), HNS2 (Polonis *et al.* 2001; Quinnan *et al.* 1999; Vujcic *et al.* 1995), and serum from a South African sex worker DU179 (Bures *et al.* 2002). Slow progressors of HIV-1 infection have been characterized, and some found to have high titre of NtAbs (Cao *et al.* 1995; Binley *et al.* 1998; Loomis-Price *et al.* 1998). A few potent bNt MAbs have been discovered, namely: b12, 2G12, 447-52D, 2F5, 4E10 and Z13 (Zolla-pazner 2004; Burton 2005). One important strategy for HIV-1 vaccine design therefore involves the design and optimization of immunogens that mimic these epitopes and thus possibly elicit bNtAbs *in vivo*.

Although it is known that neutralization activity becomes broader over time, it is still not known whether bNt activity is correlated with the breadth of the anti-HIV Ab response. Most previous studies that characterized the breadth of the Ab response against Env proteins were focused on peptide reactivity in natural infection. The immunodominant region has been shown to be the V3 region on gp120 (Garrity *et al.* 1997; Devash *et al.* 1990; Zwart *et al.* 1991; Zwart *et al.* 1992), and cluster1 on gp41

(Horal *et al.* 1991; Chiodi *et al.* 1987; Norrby *et al.* 1987; Smith *et al.* 1987; Wang *et al.* 1986). Other regions on Env proteins have also been reported to be immunogenic, such as MPER (Geffin *et al.* 1998; Calarota *et al.* 1996) and CT (Buratti *et al.* 1998) on gp41. In terms of the correlation of Ab response against certain peptides or proteins with different clinical outcomes (protective epitopes), the results are contradictory. Peptide reactivity, especially those regions NtAbs bind to (*e.g.,* V3 and MPER), was related to better clinical outcomes in some studies (Loomis-price *et al.* 1998; Geffin *et al.* 1998; Srisurapanon *et al.* 2005; Ugen *et al.* 1997), but not others (Zwart *et al.* 1994; Moore *et al.* 1994).

The questions addressed in the study presented in this chapter were: First, what is the overall Ab response (in both strength and breadth) against Env proteins in different stages of HIV-1 infection? Second, is there reactivity against sites where bNt MAbs bind? And is there reactivity with peptide markers we have developed that are specific for the bNt MAbs? Third, is there a correlation between reactivity at sites bound by Nt MAbs, particularly the MPER, and stages of infection, clinical outcome, or neutralization activity in a serum?

We characterized the Ab response during natural HIV-1 infection by screening a large panel of serum samples (~150) with the envelope proteins, linear peptides derived from various regions on gp160 (MN strain), and peptides that are specific for the bNt MAbs. We used serum samples that were taken from 20 HIV-1 infected individuals. Some of these samples were grouped by clinical course as slow (SP), rapid (RP), and normal progressors (NP). Others were taken within the first 1-4 years of infection, starting at seroconversion (SC). Between four and eight samples were taken over time from each of these individuals, allowing the stability and breadth of each person's Ab

response to be followed. We also used a set of single serum samples taken from 13 individuals; these sera had been characterized as bNt. The peptides used in our study include, (1) linear peptides representing immunogenic regions in literature and our previous screening of a panel of HIV+ IgGs to assess the breadth of reactivity. These peptides have been shown to be immunodominant or have moderate/low reactivity. (2) Peptides covering the regions where NtAbs possibly bind. (3) Peptides that are specific for the bNtMAbs (b12, 2G12, 2F5 and 4E10) developed in our lab.

In summary, we found that the Ab response broadens over time during the first 1-2 years after seroconversion, and for the most part, becomes stable. Surprisingly, the reactivity profiles of the bNt sera were less broad than RP sera, indicating that broad neutralization is likely due to high titer oligoclonal, or monoclonal, Nt Abs, in these sera. Strong Ab responses against sites where Nt MAbs are reported to bind (V3, cluster1 and NHR) were detected, but most of these Abs are probably not Nt, as they were produced in all or most HIV-1 infected people. The MPER was an exception to this; as expected, low and mainly transient reactivity was found against the MPER through all stages of infection, as well as in some of the bNt sera. No envelope specific reactivity was detected for any of the peptides that were specific for the bNt MAbs. Given that we observed reactivity in all of the sites of broad neutralization, including the MPER, this indicates that: (i) Reactivities against these sites are not bNt; or (ii) these epitopes are different from those of the bNt MAbs; (iii) these peptides binding to the bNt MAbs are too specific to cross-react with other bNtAbs against the same epitope.

2.2 Materials and methods

2.2.1 Serum samples

HIV-1 positive human sera from rapid progressor (RP), normal pregressor (NP), and slow progressor (SP) groups were provided by Drs. L. Kingsley and C. Rinaldo (University of Pittsburgh) through the (Multicenter AIDS Cohort Study) MACS program. Samples from seroconverter group (SC) and the 11 bNt sera were provided by Drs. D. Richman and S. Little (UCSD). FDA-2 (#13 bNt serum), AD18 and AD28 sera were given by Drs. P. Parren and D. Burton (The Scripps Research Institute).

2.2.2 Reagents

High-binding microtiter plates (Corning Inc., Corning, NY) were used for all ELISA. Protein G (to determine serum IgG content), human IgG (as a standard), Bovine Serum Albumin (BSA) and 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich (St.Louis, MO). Horseradish peroxidase (HRP) conjugated protein A/G was purchased from Pierce (Rockford, IL). Tween-20 was from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ).

The reagents for the serum-screening ELISA are the same as above if not mentioned below. 15-mer synthetic peptides derived from HIV-1 envelope protein MN isolate (>80% HPLC purity) were provided by NIH AIDS Research and Reference Reagent Program; peptide sequences are listed in **Table2-1**. Ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO), and non-fat dried milk (DM) (Bio-Rad, Hercules, CA) were used as control antigens. Recombinant gp41 (MN strain) and gp120 (MN strain) envelope proteins were used as HIV-1 specific (ImmunoDiagnostics, Woburn, MA). Alkaline phosphatase (AP)-conjugated rabbit-anti-human IgG (Fc specific), (Pierce,

Rockford, IL), and the substrate, p-nitrophenyl phosphate (p-NPP) (Sigma-Aldrich, St.Louis, MO) were used to detect bound IgG.

2.2.3 Determination of serum IgG content

To IgG content was determined for each serum sample, using immobilized protein G (which binds to the Fc portion of IgG); this was used to capture IgG from the serum. One ug protein G was diluted in 35ul Tris-buffered saline (TBS) and used to coat microtiter wells. By overnight incubation at 4°C, wells were blocked with 200 ul TBS containing 2% BSA (w/v) (TBS/BSA) at 37 °C for 1 hour, then washed three times with TBS containing 0.01% (v/v) Tween-20 (TBST). Starting at a 1:300 dilution, 1:3 serial dilutions of serum samples were prepared in Ab diluent (TBST containing 1% (w/v) BSA). Second, 1:3 dilutions of 500 nM human IgG was used as standard with 35ul of each titration. The plates were incubated at room temperature for 2 hours. After six times wash with TBST, HRP conjugated proteinA/G, diluted 1 in 500 in Ab diluent was added with 35ul/well to the wells as secondary Ab. The plates were then incubated at room temperature for 45 minutes. All incubations were performed on a rocker.

After six washes with TBST, plates were developed with 0.03% (v / v) H_2O_2 and 400 ug/ml of ABTS in citrate/phosphate buffer. ELISA plate readings were done with a VersaMax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA). The optical densities (O.D.) at 405 nm and 490 nm were measured and reported as ($OD_{405\text{-}490}$) X 1000. Readings were typically taken every 10 minutes up to 50 minutes, and unless otherwise indicated, are presented at 30 minutes.

Calculation of serum IgG content was done by comparing the $OD_{405-490}$ of serum samples to a standard curve obtained from serial dilutions of human IgG with known concentrations. Three dilutions were used to obtain an average IgG content. Each serum sample was adjusted with TBST containing 1% (w/v) BSA to a final IgG concentration of 200 nM and used for all subsequent ELISA.

2.2.4 Direct enzyme-linked immunosorbent assay (ELISA) for epitope mapping

Microtiter wells were coated with 400 ng synthetic peptide in TBS, then incubated at 37°C for 6 hours to dry. Other antigens, 50 ng nvelope proteins (gp120 and gp41), protein G (used as a quality control since its signal presents the amount of serum IgG), 2%OVA, 2% DM and 2%BSA in TBS, were then coated. Following the incubateion at 4°C overnight, the plates were blocked with TBS/BSA at 37°C for 1 hour, then washed 3 times with TBST. Serum diluted to an IgG concentration of 200 nM was added, and rocked at room temperature for 2 hours; plates were then washed 6 times with TBST, and AP-conjugated rabbit anti-human IgG (Fc) (1:1000 dilution in Ab diluent) was added, and incubated for 1 hour at RT. Wells were developed by p-NPP, and was read in microplate reader at A405nm.

2.2.5 Phage-displayed peptides ELISA

Phage ELISA were performed as described in Menendez *et al.* 2004. Briefly, wells were coated with phage clones (10^{10} phage particles in TBS) overnight at 4°C, followed by blocking with TBS/BSA for one hour at 37°C. After washing 3 times with TBST, sera diluted to an IgG concentration of 200 nM were then added to the wells, and incubated for 2 hours at RT. After washing 3 times, HRP-conjugated goat-anti-human-

IgG was added (1: 1000 dilution), and incubated for 1 hour at RT. The plates were developed with 0.03% (v / v) H_2O_2 and 400 ug/ml of ABTS in citrate/phosphate buffer. The data was reported at $OD_{405\text{-}490}$ as in **2.2.3**.

2.3 Results

2.3.1 HIV-1 positive study population

Ab responses were studied over time among four groups of HIV-1 infected patients, namely the rapid progressor (RP), normal pregressor (NP), slow progressor (SP) and seroconverter (SC) groups. No patients from these groups have been treated with anti-retroviral therapy. The SP, NP, and RP groups have unknown seroconversion date, and were taken beween 1984 to 1999. The RP samples span between one to five years, with three to five samples taken from each of six individuals. The NP samples span between nine years, with four to seven samples taken from each of five individuals. The last sample from both RP and NP groups was drawn shortly before patient death. The SP samples span fifteen years, with eight samples taken from each of four individuals. Patients from the SP group never developed symptoms of AIDS by the time the last samples were taken. Contrast to the SP, NP and RP groups, the serum samples in SC group were taken between 1998 to 2003 in a different cohort. Most samples in SC group were drawn within a year after seroconversion (six to eight samples for each individual) except two SC patients (SC1 and SC2, **Figure 2-2**), which have more samples taken up to four years (ten and eleven samples in these two individuals, separately).

A set of 13 serum samples, taken after two to five years' infection with known broadly neutralizing reactivity was also screened. Note that there is only one sample

available within each individual; thus, the Ab response over time within this group could not be studied. These 13 samples are named broadly neutralizing group (bNt). Serum #1~12 were tested to be bNt as described in Richman *et al.* 2003. Serum #13 (FDA-2) was also tested to be a bNt serum (Vujcic *et al.* 1995). The purpose of using these sera was to investigate the strength and breadth of Ab response in these samples, and to identify the difference between sera from bNt group and other groups.

2.3.2 General Ab response in natural HIV-1 infection – characterized by gp160 linear peptides in serum samples

2.3.2.1 Gp160 linear peptides

To investigate the breadth and stability of the overall Ab response against the HIV-1 envelope proteins, twenty nine 15-mer linear peptides representing distinct regions on gp120 and gp41 were chosen for study from the gp160 (MN strain). The sequences of these peptides are listed in **Table 2-1**. Some of the peptides were chosen by our previous screening of a panel of HIV+ IgGs to assess the breadth of reactivity (data not shown). These peptides have been shown to be immunodominant or have moderate/low reactivity. Other peptides were chosen since they had been reported to be immunogenic in HIV-1 natural infection or immunization studies (Klasse *et al.* 1988; Goudsmit *et al.* 1990; Neurath *et al.* 1990; Horal *et al.* 1991; Xu *et al.* 1991; Broliden *et al.* 1992; Ugen *et al.* 1992; Vanini *et al.* 1993; Calarota et al. 1996; Cleveland *et al.* 2003; Chanh *et al.* 1986; Loomis-Price *et al.* 1998). These linear peptides reflect specific Ab responses against linear (continuous) epitopes on envelope proteins. The non-specific control antigens, 2% non-fat dried milk (DM), 2% OVA were also used in the study, represented non-specific Ab response in HIV-1 natural infection.

Table 2-1: Table of HIV-1 MN Env peptides obtained from the NIH AIDS Research & Reference Reagent Program.

Amino Acid	HIV-1 MN Env 15-mer peptide #	Corresponding region on gp160	Peptide sequence
97-111	6233	C1	NNMVEQMHEDIISLW
301-315	6284	V3	CTRPNYNKRKRIHIG
305-319	6285	V3	NYNKRKRIHIGPGRA
357-371	6298	C3	FKNKTIVFNQSSGGD
417-431	6313	C4	QCKIKQIINMWQEVG
501-520	2015*	C5	TKAKRRVVQREKRAAIGALF
533-547	6342	Polar region	AASVTLTVQARLLLS
561-575	6349	NHR	EAQQHMLQLTVWGIK
565-579	6350	NHR	HMLQLTVWGIKQLQA
569-583	6351	NHR	LTVWGIKQLQARVLA
573-587	6352	NHR	GIKQLQARVLAVERY
577-591	6353	NHR	LQARVLAVERYLKDQ
593-607	6357	Cluster 1	LLGFWGCSGKLICTT
597-611	6358	Cluster 1	WGCSGKLICTTTVPW
657-671	6373	CHR/MPER (2F5)	NEQELLELDKWASLW
661-675	6374	MPER	LLELDKWASLWNWFD
665-679	6375	MPER	DKWASLWNWFDITNW
669-683	6376	MPER (4E10)	SLWNWFDITNWLWYI
673-687	6377	MPER/TM	WFDITNWLWYIKIFI
725-739	6390	CT	PRGPDRPEGIEEEGG
729-743	6391	CT	DRPEGIEEEGGERDR
733-747	6392	CT	GIEEEGGERDRDTSG
769-783	6401	CT	YHHRDLLLIAARIVE
773-787	6402	CT	DLLLIAARIVELLGR
777-791	6403	CT	IAARIVELLGRRGWE
825-839	6415	CT	GTDRVIEVLQRAGRA
829-843	6416	CT	VIEVLQRAGRAILHI
833-847	6417	CT	LQRAGRAILHIPTRI
837-851	6418	CT	GRAILHIPTRIRQGL

*All the peptides are 15-mer except C5 (20-mer) taken from another set.

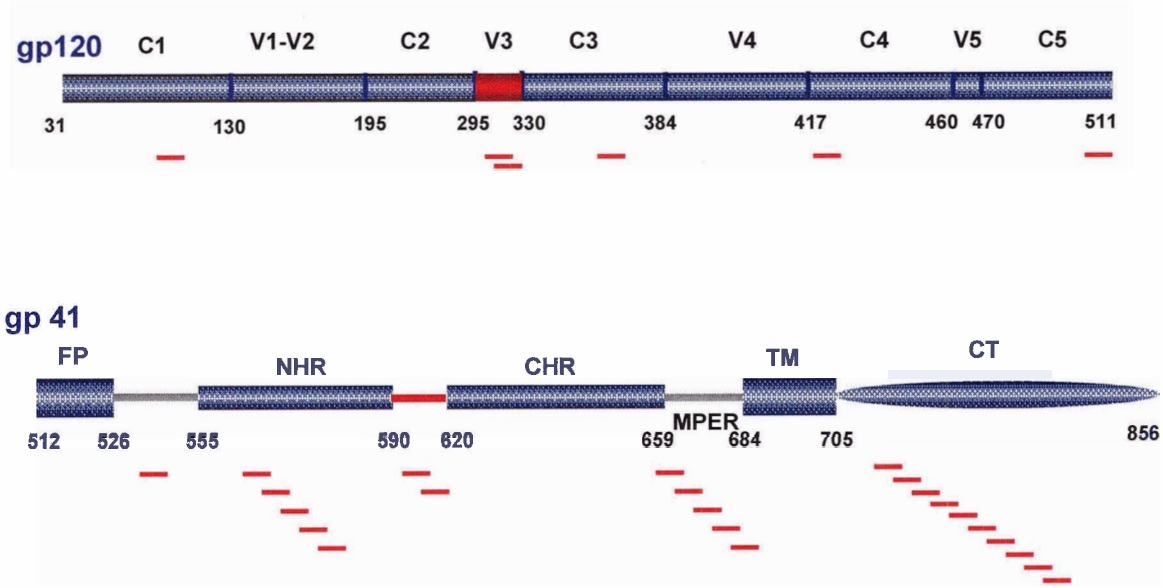
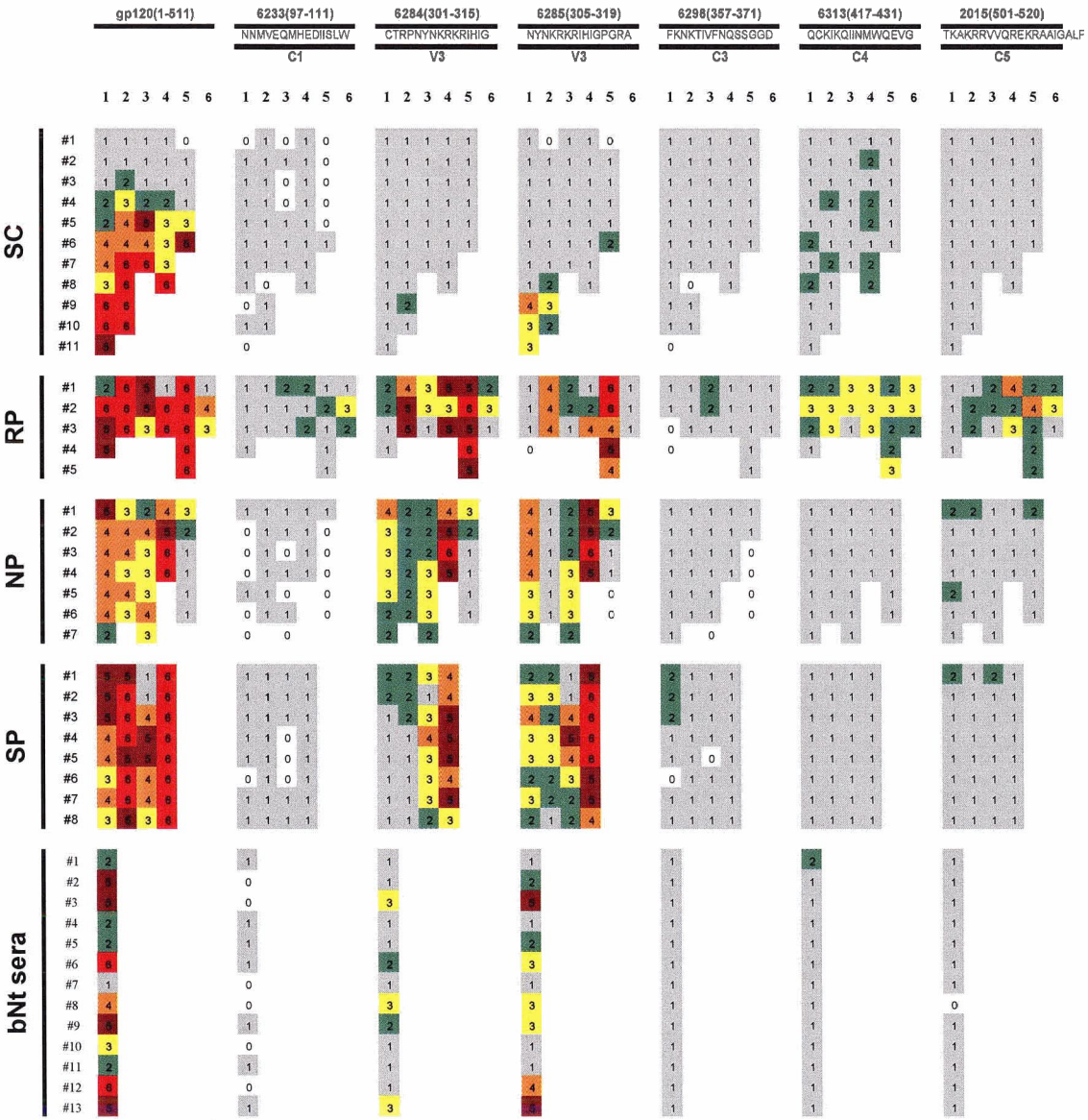
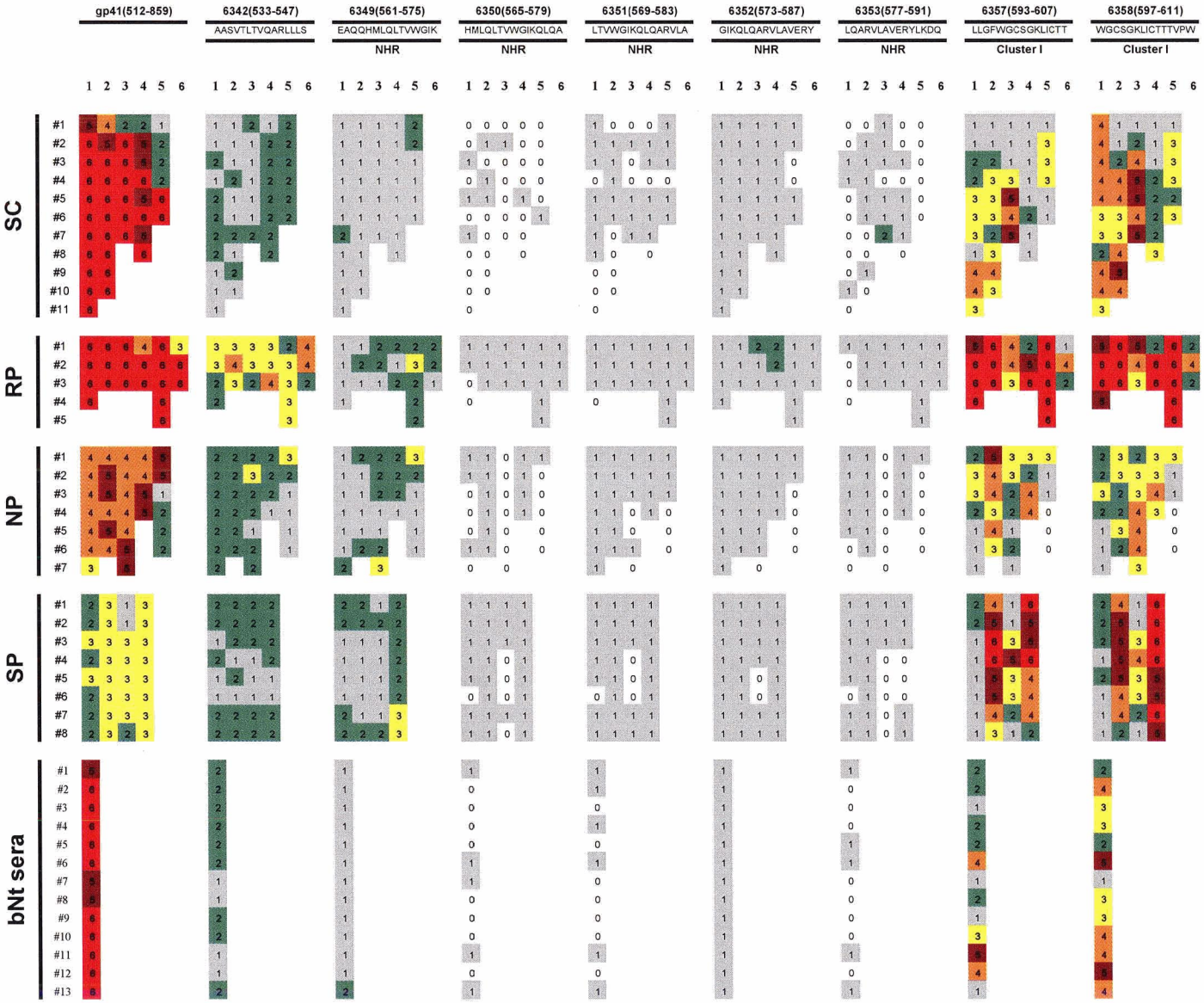
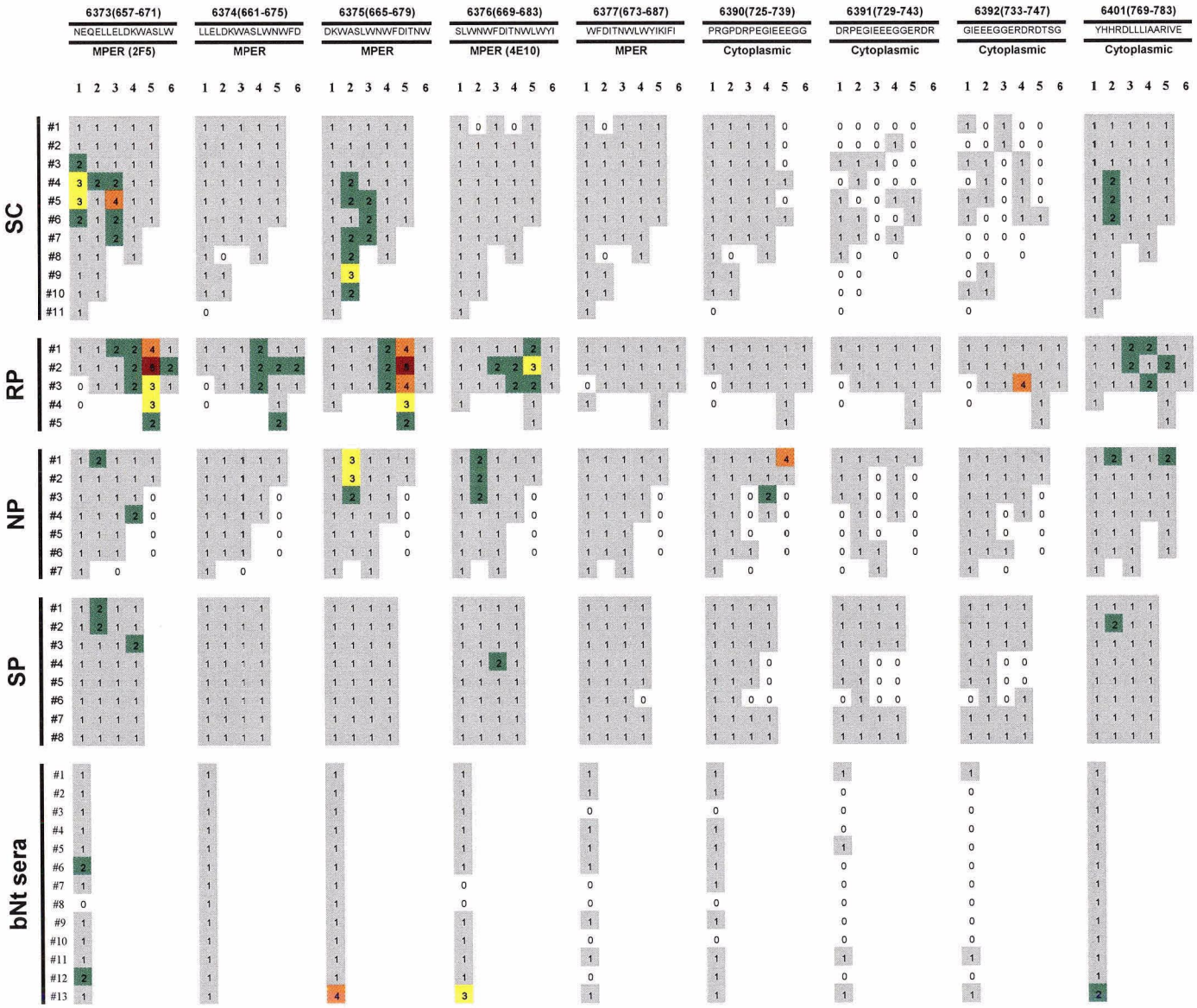


Figure 2-1: Summary of peptides used in the serum screening.

Each red line represents one peptide. Different regions the peptides covered on gp120 and gp41 were shown in blue boxes. See Table 2-1 for the sequences of the peptides.


gp120(1-511)
6233(97-111)
NNMVEQMHEDIISLW
C1
6284(301-315)
CTRPNYNKRKRIHIG
V3
6285(305-319)
NYNKRKRIHIGPGRA
V3
6298(357-371)
FKNKTIVFNQSSGGD
C3
6313(417-431)
QCKIKQIINMWQEVG
C4
2015(501-520)
TKAKRRVVQREKRAAIGALF
C5
1 2 3 4 5 6
SC
RP
NP
SP
bNt sera
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
#11
#12
#13


gp41(512-859)
6342(533-547)
AASVTLTVQARLLLS
6349(561-575)
EAQQHMLQLTVWGIK
NHR
6350(565-579)
HMLQLTVWGIKQLQA
NHR
6351(569-583)
LTVWGIKQLQARVLA
NHR
6352(573-587)
GIKQLQARVLAVERY
NHR
6353(577-591)
LQARVLAVERYLKDQ
NHR
6357(593-607)
LLGFWGCSGKLICTT
Cluster I
6358(597-611)
WGCSGKLICTTTVPW
Cluster I
1 2 3 4 5 6
SC
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
#11
RP
#1
#2
#3
#4
#5
NP
#1
#2
#3
#4
#5
#6
#7
SP
#1
#2
#3
#4
#5
#6
#7
#8
bNt sera
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
#11
#12
#13


6373(657-671)
NEQELLELDKWASLW
MPER (2F5)
6374(661-675)
LLELDKWASLWNWFD
MPER
6375(665-679)
DKWASLWNWFDITNW
MPER
6376(669-683)
SLWNWFDITNWLWYI
MPER (4E10)
6377(673-687)
WFDITNWLWYIKIFI
MPER
6390(725-739)
PRGPDRPEGIEEEGG
Cytoplasmic
6391(729-743)
DRPEGIEEEGGERDR
Cytoplasmic
6392(733-747)
GIEEEGGERDRDTSG
Cytoplasmic
6401(769-783)
YHHRDLLLIAARIVE
Cytoplasmic
1 2 3 4 5 6
SC
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
#11
RP
#1
#2
#3
#4
#5
NP
#1
#2
#3
#4
#5
#6
#7
SP
#1
#2
#3
#4
#5
#6
#7
#8
bNt sera
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
#11
#12
#13

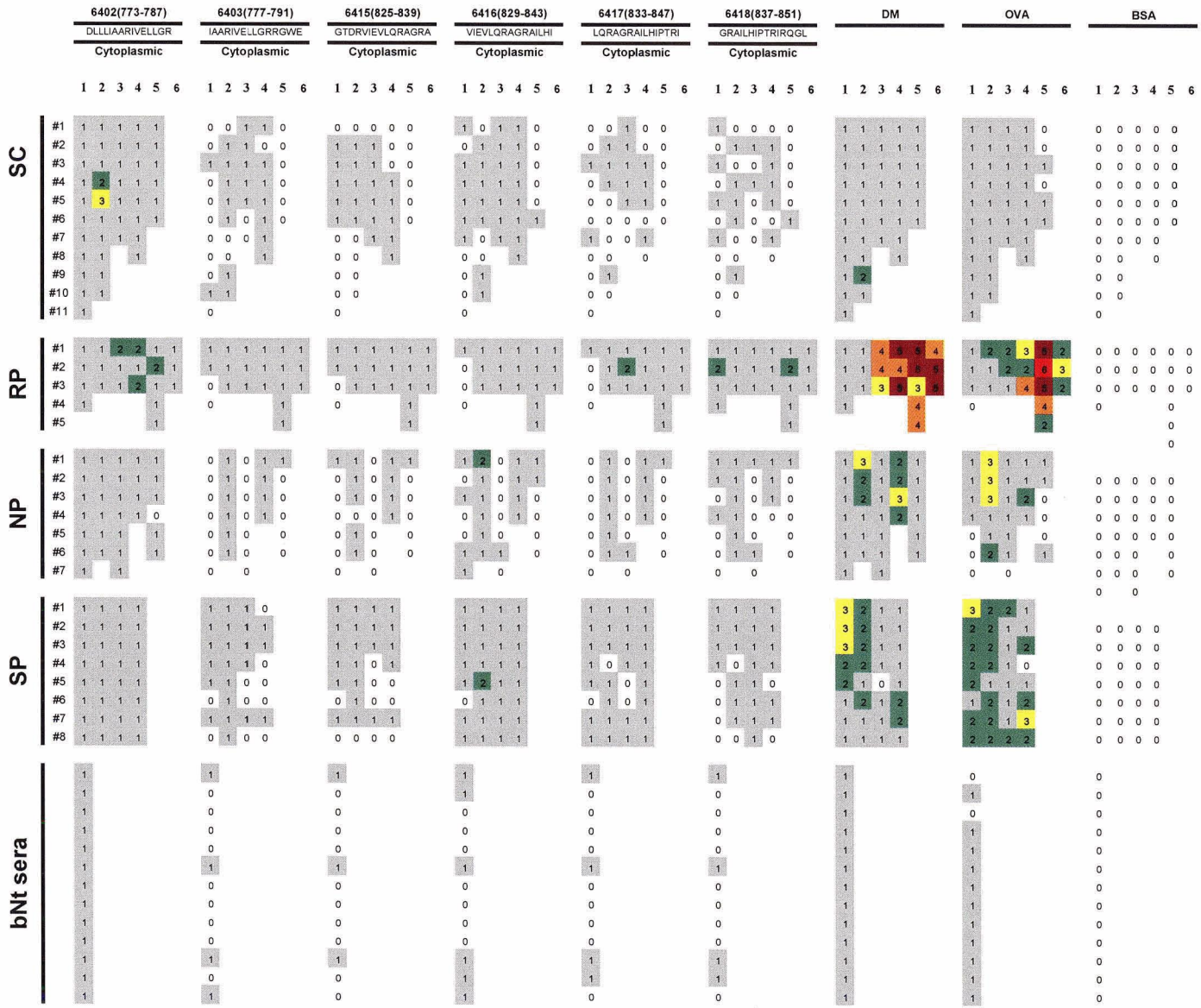

6402(773-787)
DLLLIAARIVELLGR
Cytoplasmic
6403(777-791)
IAARIVELLGRRGWE
Cytoplasmic
6415(825-839)
GTDRVIEVLQRAGRA
Cytoplasmic
6416(829-843)
VIEVLQRAGRAILHI
Cytoplasmic
6417(833-847)
LQRAGRAILHIPTRI
Cytoplasmic
6418(837-851)
GRAILHIPTRIRQGL
Cytoplasmic
DM
OVA
BSA
1 2 3 4 5 6
SC
#1
#2
#3
#4
#5
#6
#7
#8
#9
#10
#11
RP
#1
#2
#3
#4
#5
NP
#1
#2
#3
#4
#5
#6
#7
SP
#1
#2
#3
#4
#5
#6
#7
#8
bNt sera

Figure 2-2: Summary of antibody response against envelope proteins in serum samples.

(1). The strength of serum Ab response in ELISA experiment is shown by different numbers with colours. "0" (white) means O.D.≤0.1; "1" (grey) means 0.1<O.D.≤ 0.5; "2" (green) means 0.5<O.D.≤1; "3" (yellow) means 1<O.D.≤ 1.5; "4" (orange) means 1.5<O.D.≤ 2; "5" (brown) means 2<O.D.≤ 2.5; "6" (red) means O.D. above 2.5 (2). The numbers in a column (#1, #2...etc) in SC, SP, NP, RP represents samples taken at different time points from a same patient. In bNt sera group, #1~13, represent 13 bNt serum samples taken from 13 different individuals.

2.3.2.2 The antibody response against gp120

The early timepoints within SC group represent acute HIV-1 infection; over time, Ab cross-reactivity with gp120 (MN strain) appeared at least 6 weeks after seroconversion and peaked at the end of the first year (**Figure 2-2**). The trend of increasing reactivity was obvious for every individual. Note that cross-reactivity is observed for all these studies since the TCLA MN strain sequence (may be different from primary viral sequence) was tested; this was thus testing "heterologous" reactivity. For the peptide response, Ab against one V3 region (6285) peptide appeared at the end of the first year and showed increasing trend afterwards. Note that the last three serum samples from number 1 and 2 individuals were taken at the 2^{nd}, 3^{rd} and 4^{th} year after seroconversion. There was also some response against C4 region (6313 peptide); however this was low and not consistent over time. There was no reactivity against any other peptide on gp120, indicating a restricted Ab response against gp120 during early HIV-1 infection.

The RP, NP and SP groups were screened to represent the Ab response over the course of HIV-1 infection after diagnosis. Overall, the Ab response against both the gp120 protein and the peptides (if any) was stable over time regardless of clinical outcome. One exception was individual number 5 in the NP group, who had a strong Ab

response at the beginning, but which disappeared over time. Ab response against the V3 region (6284 and 6285 peptides) was the strongest and most cross-reactive (i.e., most individuals developed a V3-specific Ab response) in all three groups. The RP, but not the NP or SP groups, showed reactivity against the C1 (6233 peptide), C4 (6313 peptide) and C5 (2015 peptide) regions. Individual #1 in the RP group and #2 in the NP group developed a strong Ab response against gp120 protein, but showed no Ab response to any of the peptides. As discussed below, peptide mapping represents only a small fraction of the entire Ab response, as it detects only Abs that cross-react with linear peptide sequences. In comparison, the bNt serum group produced an overall strong Ab response against gp120 (except bNt serum #7, who did not show any reactivity against gp120 or its peptides). The Ab response against V3 region (6284 and 6285 peptides) was also the strongest among all the peptides. Note that only one sample available from each individual in this group.

Compared to the SC group, other groups showed a more stable Ab response. This is mainly indicated by reactivities against whole gp120 and the V3 region (6284 and 6285 peptides). In all five groups, the Ab response against V3 region was immunodominant for gp120, consistent with many previous reports (see **2.4,** discussion). While the stability of the Ab response seemed to be independent of clinical course after initial seroconversion, the breadth of the Ab response varied among RP, NP, SP and bNt groups. The RP group had the broadest Ab response, which is shown by a higher frequency of reactivity against the C1 (6233 peptide), C4 (6313 peptide) and C5 (2015 peptide) regions, but not the SP or NP groups. Interestingly, the breadth of the Ab response in bNt serum group did not seem to be greater than other groups, though there

was only one sample available from each individual. The breadth of peptide reactivity in bNt sera is more comparable to NP and SP groups than the RP group, indicating the breadth of neutralization activity in the sera does not correlate with the breadth of Ab response or more specifically, Ab response that cross-reacts with overlapping peptides studied here.

2.3.2.3 Antibody response against gp41

Reactivity against gp41 (MN strain) in all serum samples in the SC group appeared earlier than that against gp120. While gp41 reactivity in the SC group was strong throughout time, it appeared weaker in the NP and SP groups (especially SP). In all five groups, the Ab response against the cluster I region (6357 and 6358 peptides) was immunodominant; however in the SC group, this reactivity appeared to increase over time. In addition, reactivity against the region N-terminal to the NHR (6342 peptide), and within the NHR (6349 peptide) was very crossreactive, with this reactivity being the weakest in the SC group, and strongest in the RP group. Interestingly, there was also a difference in the extent of cross-reactivity against some MPER peptides (6373-6376) and cytoplasmic regions (6401 and 6402 peptides), with the RP group showing the strongest and broadest reactivity. Thus, compared to other groups, the RP group showed stronger and broader Ab responses against peptides in the gp41 region.

As mentioned in the introduction, the MPER is of particular interest because this region includes the epitopes for three bNt MAbs, 2F5, 4E10 and Z13. It is also the most conserved region on gp41; therefore, if other NtAbs are discovered against this region, it is possible the they will neutralize a broad range of HIV-1 isolates. For these reasons, we affinity purified specific Abs using the 2F5 and 4E10 epitope peptides (6373 and 6376,

repectively). The purpose was to see if the purified Abs that bind to 2F5 and 4E10 linear epitopes will have 2F5 or 4E10-like neutralization activity or, will neutralize HIV-1at all (see **Chapter 3**).

2.3.2.4 Non-specific reactivity against OVA, DM and BSA

The reactivity against dried milk (DM) and ovalbumin (OVA) in this study indicates the presence of non-specific or polyspecific Abs in serum samples. In acute infection, represented by SC group, a relatively clean response was shown by minimal non-specific reactivity with these antigens. Over the longer-term, the SP, NP and RP groups showed stronger non-specific reactivity, with the RP group showing the strongest. The bNt serum group also showed minimal non-specific response, similar to the SCs. Given the time period over which sera were collected for the bNt serum group (after 2~3 years of infection), its serum samples are most comparable to the NP group, yet the DM and OVA reactivity of the bNtserum group was most comparable to the SC group. This is consistent with the results for the gp120 and gp41 reactivity profiles, with the SC and bNt serum groups having the most restricted Ab response, and the RP group the broadest and strongest ones.

2.3.3 Serum reactivity with peptides specific for broadly neutralizing monoclonal antibodies

Our group has selected and optimized phage-displayed peptides that specifically bind to bNt MAbs b12, 2G12, 2F5 and 4E10 (Zwick *et al.* 2001a; Menendez *et al.* 2004; Menendez 2005; Bahr 2004). These phage-displayed peptides bind to the bNt MAbs at affinities similar to their cognate epitopes on the envelope proteins. In this thesis, these phage-displayed peptides were used to determine if those having similar reactivities to

the bNt MAbs were present in our panel of sera. These bNt MAb-specific peptides included one specific for the b12 MAb (B2.1); one specific for the 2G12 MAb (D10, ACPPSHYLDMKSGTCR); five for 2F5 MAb (AL4.22, ELDKWSFWPWGDDSPLLT; AA 4.22, AADKWSFWPWGDDSPLLT; EL1.1, ELDKWSHLSLTEFSPSHQ; EL10.5, ELDKWSDWPNIAQYTTTL; and AA10.5, AADKWSDWPNIAQYTTTL), and two for the 4E10 MAb (E6.8, RCRTIDVFRNCI; and 10A.3, AEPAETSWFYLTTFL).

In addition, as positive controls, three phage clones were used, with each displaying a peptide that commonly react with a significant fraction of sera from HIV-1 infected people. Briefly, the IgG from two sera, AD18 and AD28, were used to screen phage library, clones with high binding were chosen to screen sera. Three clones were selected and sequenced due to their common reactivity. These phage clones are, A4.3 (AQKQIKCGDACNFYF), A5.4 (KCMWTSPFQTCV) and A7.4 (TCSGKILCSLPIRSWEQ).

A serum panel, comprising sera from the SC, RP, NP and SP patient groups, was screened to determine whether they contain Abs that recognize peptides specific for the bNt MAbs. Phage displaying-peptides that were known to react with HIV positive sera, were used as a positive control. While the entire serum panel was screened, the results in **Figure 2-3** are a summry, and represent the highest ELISA signal produced from the serum samples of a given individual; thus, they are biased toward depicting the strongest reactivities in the serum panel. In all groups, the Ab response against bNt MAb-specific peptides was minimal (green and yellow colors) compared to the positive controls, which included gp120, gp41 and the A4.3, A5.4 and A7.4 phage; reactivity with control phage bearing no recombinant peptide was also minimal (data not shown). While some serum

samples showed reactivity with clones AA4.22 and 10A.3, it was not gp41 specific since the ELISA signal for these phage were unchanged in the presence of soluble gp41 (data not shown). In contrast, there was significant reactivity with phage clones A4.3, A5.4, and especially A7.4. Previous experiments had shown that serum binding to these clones is blocked by gp120 (A4.3 and A5.4) and gp41 (A7.4). Thus, while the sera reacted well with phage displaying peptide markers for reactivities commonly produced in HIV-1 positive sera, no envelope-specific reactivity was observed for any of the phage bearing peptides specific for the bNt MAbs. This is especially striking for the peptides specific for MPER-binding Nt MAbs, 2F5 and 4E10, since reactivity with these epitope peptides (6373 and 6376, respectively) was present in a number of sera (see **Figure 2-2**).

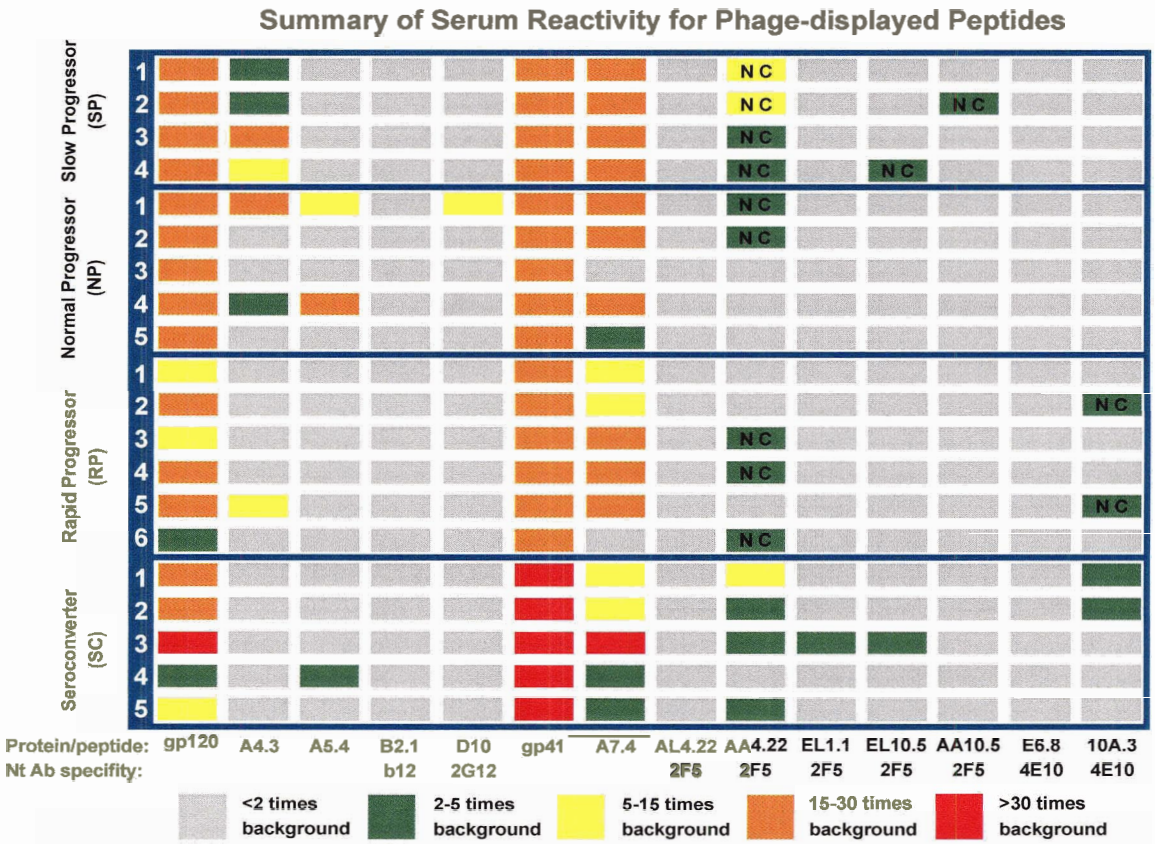


Figure 2-3: Summary of serum screening with bNt MAb peptide markers.

X-axis represents bNt MAb peptide markers and controls (see the text for details). Y-axis represents serum samples from SC, RP, NP and SP groups. As a summary of Each box represents the strongest reactivity in multiple serum samples taken from one person. ** NC: no competition, peptides do not compete against gp120 or gp41 for binding to serum Abs.

2.4 Discussion

2.4.1 The stability and breadth of peptide-specific antibody response

Figure 2-3 is the summary of the antibody strength over time in different groups.

In our mapping studies, we showed that, in comparison to the other patient groups, the

		SC(early)	SC(late)	RP	NP	SP	bNt
	gp120	-	+	+ +	+ +	+ +	+
C1	6233	-	-	+/-	-	-	-
V3	6284	-	-	+ +	+	+	+ / -
V3	6285	-	+	+ +	+ +	+ +	+ +
C3	6298	-	-	-	-	-	-
C4	6313	+ / -	+ / -	+	-	-	-
C5	2015	-	-	+	-	-	-
	gp41	+ +	+ + +	+ + +	+ +	+	+ + +
Polar region	6342	+ / -	+ / -	+	+ +	+	+
NHR	6349	-	-	-	-	-	-
NHR	6350	-	-	-	-	-	-
NHR	6351	-	-	-	-	-	-
NHR	6352	-	-	-	-	-	-
NHR	6353	-	-	-	-	-	-
Cluster I	6357	-	+	+ +	+	+	+
Cluster I	6358	-	+	+ +	+	+	+
MPER (2F5)	6373	+ / -	+ / -	-	-	-	-
MPER	6374	-	+ / -	-	-	-	-
MPER	6375	+ / -	+ / -	-	-	-	+ / -
MPER (4E10)	6376	-	+ / -	-	-	-	-
MPER	6377	-	-	-	-	-	-
CT	6390	-	-	-	-	-	-
CT	6391	-	-	-	-	-	-
CT	6392	-	-	-	-	-	-
CT	6401	-	+ / -	-	-	-	-
CT	6402	-	+ / -	-	-	-	-
CT	6403	-	-	-	-	-	-
CT	6415	-	-	-	-	-	-
CT	6416	-	-	-	-	-	-
CT	6417	-	-	-	-	-	-
CT	6418	-	-	-	-	-	-
DM		-	-	+ +	+	+	-
OVA		-	-	+ +	+	+	-
BSA		-	-	+ +	+	+	-

Figure 2-4: Summary of antibody response in serum screening with peptides.

The strength of antibody response from low to high is represented by "-", "+/-", "+", "++", and "+++".

c had the broadest and strongest Ab responses. The NP group had a broader reponse compared to the SP, though this trend was not significant. This is interesting considering a previous study showed that SPs are more likely to have broader NtAbs against primary isolates (Montefiori *et al.* 1996). In contrast, the response against the peptides studied increased and broadened over time in the SC group. We also found that the reactivity patterns of the 13 bNt sera were not broader than those of the other groups. Our results

indicate that after ~2 years of infection, viral load is positively associated with the breadth and strength of the Ab response. While broadening of the overall Ab response is more associated with viral load, broad neutralization is associated with more restricted (i.e., oligo- or monoclonal) Abs. Further study with a larger population should help to confirm these trends.

The Ab response was investigated over time in different HIV-1-infected populations using linear peptides derived from the gp160 sequence (epitope mapping). Although polyclonal sera were used in this study, our results are discussed in light of current knowledge of the conserved regions on the envelope proteins that are involved in Ab-mediated neutralization, as defined by Nt MAbs.

Overall, vigorous Ab responses against both gp120 and gp41 proteins were produced in most individuals. Some of the regions we analyzed are also recognized by MAbs reported to have Nt activity, or bNt activity; strong reactivity was observed against many of these regions (i.e., V3, cluster I and the NHR). Due to the strength of the Ab response against these sites, and the presumed lack of Nt activity in most of the sera, it is unlikely that most of the reactivity of these sites is Nt. We show here that, the MPER was an exception, as it was weakly immunogenic through all stages of HIV-1 infection. MPER reactivity was transient, and not associated with clinical outcome. As this region is recognized by three different bNt MAbs, it may be worthwhile to investigate whether the serum Abs that bind to this region are Nt, since the titer was probably too low for their sera to be Nt. Discussion of all these regions follows.

The V3 region (6284 and 6285 peptides) was shown to be the immunodominant region on gp120, and this is consistent with most of the early HIV-1 serology studies

which have shown that more than 90% of HIV-1 infected individuals produce Abs against the V3 region (Devash *et al.* 1990; Zwart *et al.* 1991; Zwart *et al.* 1992). Initial studies on HIV-1 suggested V3 as a main target for vaccine design because of its immunodominance and production early after infection or brief immunizations (Javaherian *et al.* 1989; Putney *et al.* 1986; Goudsmit *et al.* 1988; Javaherian *et al.* 1990; Mascola *et al.* 1996). However, as it is highly variable, many V3-specific Abs are isolate-specific and most V3-specific MAbs produced by infected individuals and selected using linear V3 peptides have little Nt activity for primary isolates despite broad immunochemical cross-reactivity (Hioe *et al.* 1997; Gorny *et al.* 1997; Gorny *et al.* 1993). Notably, broadly reactive V3 Abs with potent Nt activity do exist. MAb 447-52D has been found to be the most broadly Nt V3-specific MAb discovered so far (Gorny *et al.* 2002). This MAb interacts with the 14 residues at the crown of the V3 loop; its "core epitope" is defined by the sequence GPxR, a motif that is highly conserved among clade B viruses and which exists in a minority of other HIV subtypes (Gorny *et al.* 1992). The presence and recognition of the arginine (R) residue in the core epitope is required for MAb 447-52D to exercise its activity (Zolla-Pazner *et al.* 2004). This cross-reactivity reveals the presence of features within the V3 loop that are conserved despite the sequence variation in this region. Nevertheless, most reactivity against the V3 region, while strong, is not associated with bNtAbs.

Like the V3 region on gp120, cluster I (6357 and 6358 peptides) was shown to be the immunodomimant region in gp41. This finding is consistent with most of the previous studies to date (Horal *et al.* 1991; Chiodi *et al.* 1987; Gnann *et al.* 1987; Norrby *et al.* 1987; Smith *et al.* 1987; Wang *et al.* 1986). Cluster I induces high levels of Ab, most of

which are not Nt. MAb Clone 3 alone has Nt activity specific for this region. It binds to a linear epitope between aa 597 and 606 at the C-terminal end of cluster I (Cotropia *et al.* 1996). This epitope is quite conserved, a fact reflected by the Clone 3's ability to neutralize three diverse lab adapted T-cell line viruses from clade B and three primary isolates from group O (Cotropia *et al.* 1996; Ferrantelli *et al.* 2004). Beyond the activity of Clone 3 against these viruses, few details are known about its functional breadth. Thus again, the cluster I epitope, while able to elicit strong reactivity, typically does not elicit bNtAbs.

Other than immunodominant regions, our epitope mapping study revealed several other regions of interest, all of which are on gp41. The first regions are the NHR (6349 peptide) and an adjacent region N-terminal to NHR (6342 peptide). In our mapping study, most sera in all five groups showed medium reactivity against the 6342 peptide and half of the serum samples in the long-term infected samples (SP, NP and RP) showed reactivity against the 6349 peptide. This is the first report of the prevalence of Abs against these regions in natural infection. As mentioned in the introduction (**1.3.2**), gp41 undergoes major conformational changes during membrane fusion process. Therefore, intermediate states before six-helix-bundle formation may serve as targets for Nt Abs. Furthermore, Moreno *et al.* (2004) showed that the inner coil of the six-helix bundle, the NHR, may play a role in virus-host membrane fusion, as peptides derived from the NHR destabilize liposomes. Recently, a Nt MAb that targets the NHR was discovered (Miller *et al.* 2005). MAb D5 was derived from a normal human B-cell-derived scFv-expressing phage-display library by sequential selection with two synthetic mimics of the gp41 prehairpin intermediate. Its epitope overlaps a hydrophobic pocket on the NHR.

However, MAb D5 is not very potent (high concentrations are needed to neutralize the HIV-1), and has not been shown to have bNt activity. Thus the NHR, while a good target for therapeutic drug design, may not serve as a good target for vaccine design, given the strong response typically produced against this region (which is not Nt).

Another region included in our mapping is the long C-terminal cytoplasmic domain of gp41. Conventionally, this domain is considered entirely hidden within the virion. Recently it was suggested that ~40 residues within this region are looped out on the external surface of the virion. The evidence supporting this is that some Abs specific for this site neutralize HIV-1 infection (Buratti *et al.* 1998; Dalgleish *et al.* 1988; Cleveland *et al.* 2000). For example, the so-called "Kennedy" epitope (PRGPDRPEGIEEEGGERDRDRS) is located in this external tail, which is a hydrophilic region first identified 20 years ago (Kennedy *et al.* 1986). It was suggested that there are three antigenic regions within this epitope, centering on PDRPEG, IEEE and ERDRD (Cleveland *et al.* 2003). Since Ab (IgG) theoretically cannot pass through viral membrane, it is believed that the epitope must be available on the outside of the virion (Cleveland *et al.* 2003). However, in our mapping study, using three peptides spanning this region (6390, 6391 and 6392), we did not detect significant reactivity in most of the sera (**Figure 2-2**). The function of the cytoplasmic domain in the life cycle of the virus, and its possibility as a target in disease prevention or intervention remain to be determined.

Finally, we detected cross-reactivity against several peptides within the MPER of gp41 (peptides 6373-6376). This reactivity is weak and transient through all stages of infection; strong MPER reactivity was presented in only one bNt sera (#13). The MPER

has been reported to be immunogenic in several previous studies. Calarota *et al.* (1996) proposed that the C-terminus of the MPER is an immunodominant site on gp41. They screened 63 sera from HIV-1 infected patients using sequential overlapping peptides covering aa 647-684 (which includes the MPER, see **Figure 1-3**) and identified the C-terminus of the MPER as immunodominant, with residues WNWFDI (which overlaps with the epitope for bNt MAb 4E10) being most critical for Ab recognition. However, most other studies have shown that the MPER is not immunodominant (Horal *et al.* 1991; Srisurapanon *et al.* 2005; Geffin *et al.* 1998; Muhlbacher *et al.* 1999; Broliden *et al.* 1992; Braibant *et al.* 2006), especially when compared to cluster I. Although the frequencies of MPER Abs vary in different studies, our study agrees with most others, in showing that the MPER is weakly immunogenic, even within the first year after seroconversion. This is encouraging since it indicates that MPER reactivity can be produced early after infection.

Studies have been done to correlate Ab response against MPER linear peptides with serum neutralization activity or clinical outcomes. Most of these have focused on the 2F5 epitope (ELDKWA). In one study, a negative association was found between Ab reactivity to the ELDKWA epitope, and disease progression and viral load (Geffin *et al.* 1998). More recently, it was reported that Ab titers to the 2F5 epitope in sera from AIDS patients were significantly lower than those in sera from asymptomatic subjects which were collected in the same year (Srisurapanon *et al.* 2005). In our study, serum neutralization activity was not tested; therefore, we could not compare the peptide reactivity to neutralization.

2.4.2 Limitations of our epitope mapping study

Epitope mapping with linear peptides has been used early on in HIV-1 research as well as in our study to discover B cell epitopes on envelope proteins. However, there are inherent limitations of peptide-based assays (Moore *et al.* 1994). Most relevant is that linear peptide mapping can only reveal so called "linear" or "continuous" epitopes, yet most anti-protein Abs are against discontinuous epitopes. In addition, solid phase peptide (on ELISA plate) does not necessarily represent the conformation of the same region on the cognate protein. This is why in many cases, mapping by peptides derived from envelope proteins does not correlate with protective neutralizing Ab response. A well-studied example is V3 peptide: it was shown that the solid-phase peptide-binding assay could fail to or falsely predict reactivity of a MAb with a gp120 protein (Moore *et al.* 1994). In general, peptides in solution are considered more flexible and can adopt more conformations; however, peptides in solution have the drawbacks that they typically are of lower affinity than their immobilized counterparts, to some degree because of their close packing in ELISA plate wells, which allows bivalent Ab binding. Similar to our peptide mapping, a multiplexed Ab-binding and mapping assay was developed to characterize the specificity, breadth, and magnitude of the Ab response to gp41 (Opalka *et al.* 2004). This method is to immobilize the biotinylated peptides onto streptavidin (SA)-coated microspheres with different fluorescent colors. High-titer Ab responses were detected to the 2F5-4E10 regions by this assay using sera from HIV-1 positive individuals, but there was no direct correlation between Ab binding to these peptides and virus Nt activity.

Another limitation of our mapping study is the serum cohorts used in our study. The serum samples used from SC and most of bNt sera groups were taken from a separate cohort and in different decades compared to SP, NP and RP groups. Plus, the seroconversion times of the latter groups were not available. Although this will not impact the comparisons we did among SP, NP and RP groups, this inconsistency should be considered in the comparisons to SC and bNt groups.

CHAPTER 3: AFFINITY PURIFICATION OF PEPTIDE-SPECIFIC ANTIBODIES

3.1 Introduction

In **Chapter 2**, we investigated the breadth and strength of the Ab response during HIV-1 natural infection against sites where Nt MAbs bind (V3, cluster I, NHR and MPER). Strong Ab responses against V3, cluster I and the NHR were found in all or most sera, whereas weak, transient reactivity was found against the MPER through all stages of infection, as well as in some of the bNt sera. Many studies have been done to characterized Abs against some of these regions in natural infection, particularly against V3 and cluster I; most of these are not Nt or are strain specific, and have no or little Nt activity for primary isolates (Hioe et al. 1997; Gorny et al. 1997; Gorny et al. 1993). Immunization studies using the V3 region have also failed to elicit bNtAbs (Goudsmit et al. 1988; Mascola et al. 1996). Our data suggest that some of these regions (V3, cluster I and NHR) are immunodominant or strongly immunogenic. Based on the possibility that most sera are not bNt, it is not likely that the Ab response against V3 is bNt. In contrast, the MPER is weakly immunogenic, highly conserved, and is bound by three bNt MAbs (2F5, 4E10 and Z13). It may be worthwhile to investigate whether the serum Abs that bind to this region are Nt, since the low titer of these Abs in most sample is consistent with their lack of Nt activity; moreover, two of the 13 bNt sera (#6 and #13, described in **Chapter 2**) bound this region strongly.

The question addressed in this chapter is whether anti-MPER Abs from HIV-1 infected people, especially those Abs binding to 2F5 and 4E10 epitopes are Nt. In addition, we were particularly interested in the Abs produced early in the infection (i.e., by the SC group). The reason is that bNtAbs produced early might be easier to elicit *in vivo* by immunogens/vaccines, which typically expose the body to immunogens for a short period.

A common method to study NtAbs is to test the serum samples by an *in vitro* neutralization assay. Neutralizing antibody assays provide a measure of whether an antibody or serum sample blocks HIV entry into human T-cells. This is done by incubating virus with antibody for a set time, adding cells, incubating the mixture to allow for several rounds of viral replication and then measuring the amount of virus present, usually as a function of viral antigen expression. The traditional assay does this in peripheral blood mononuclear cells (PBMC) by collecting the cell culture supernatant and testing for p24 (viral core protein) secreted from the infected PBMCs. Other assays have also been developed. One of them is a straightforward variation of the traditional PBMC assay, using antiretroviral agents to stop viral replication after a single round. These cells are then enumerated by staining intracellular p24 and analyzed by using flow cytometry (Mascola, *et al.* 2002). Two other approaches both use "reporter" genes that luminesce when expressed, allowing quantification by a luminometer. One assay (Richman, *et al.* 2003), builds luciferase (a firefly enzyme) into an env-deleted HIV DNA construct. Target cells are co-transfected with this and an env-containing DNA construct, producing infectious virus particles that cannot produce new, functioning virus. Instead, replication stops after a single cycle, and infected cells, which luminesce, are counted.

The other assay is similar, in which the luciferase gene is incorporated into a target cell line (Wei, *et al.* 2003).

In all the in vitro neutralization assays, the possibility cannot be excluded that NtAbs at very low titer are not detectable in whole serum sample. In the presented study, we developed an affinity purification method to isolate peptide-specific Abs from the sera. Two different approaches to peptide-specific Ab purification were explored: one based on peptide conjugated on agarose support, and another based on biotin labelled peptide bound to streptavidin (SA)-coated magnetic beads. The goals in these methods were to isolate Abs with high yield, preserve peptide-binding activity, and produce low background in HIV-1 neutralization assay *in vitro*. Although the results from the neutralization assays for affinity-purified Abs were not conclusive due to very small amounts of purified Ab, the goals for this method were successfully accomplished.

3.2 Materials and methods

3.2.1 Reagents

Two different methods were explored in developing an Ab purification strategy. The SulfoLink® Coupling Gel Kit, which includes the reagents needed in the Trial 1 protocol (see below) was purchased from Pierce (Rockford, IL). The Dynabeads MyOne used in Trial 2 were from Dynal, Lake Success, NY. The magnetic apparatus (MagnaRack) was from Invitrogen (Carlsbad, CA). For both methods, biotinylated 2F5 and 4E10 peptides containing cysteine residues were purchased from NeoMPS (San Diego, CA), with more than 95% purity analyzed by HPLC, and mass spectrometry. 2F5

peptide sequence is NH_3^+-EQELLELDKWASLWSGK(Biotin)GC-$CONH_2$, and 4E10 peptide sequence is NH_3^+-SLWNWFDITNWLWYISGK(biotin)GC-$CONH_2$.

3.2.2 Antibody affinity purification - Trial 1

Affinity purification of peptide-specific Abs in the serum samples was conducted using the SulfoLink Coupling Gel Kit. The kit enables covalent immobilization of sulfhydryl-containing peptides (i.e., containing a cysteine residue) to an agarose gel support in affinity purification procedures. A 5 ml column was loaded with 2 ml gel slurry. Sulfhydryl-containing peptide (2 mg) was dissolved in coupling buffer and added to the column. After incubating for 20 minutes (end-over-end mixing) and 30 minutes (without mixing) at RT, the top and bottom column caps were sequentially removed and the unbound peptide solution was allowed to drain from the column into a clean tube. The column was then placed over a new collection tube and washed with 3ml coupling buffer. To block the remaining binding sites on the gel, a 1-ml solution of 50 mM L-Cysteine•HCl in coupling buffer was added to the column, and incubated first with mixing for 15 minutes, then without mixing for 30 minutes. The column was then washed with at least 6 ml of wash solution (1 M NaCl), followed by 2 ml of degassed storage buffer (phosphate buffered saline (PBS) containing 0.05% (w/v) sodium azide (NaN_3)). The column was then stored at 4°C.

For affinity purification of peptide-specific serum IgG, the peptide-coupled column was first equilibrated to room temperature, then washed with 6 ml PBS. Up to 500 ul of serum sample was applied to the column and allowed to run through the gel bed. (If a serum sample was > 500 ul, this step was repeated). After the serum had penetrated the gel, 200 ul PBS was added on top of the column, and the column was then

incubated at room temperature for 1 hour. The unbound serum (flow through) in the column was then washed out with 12 ml PBS and collected for later analysis. Bound Ab was eluted by flowing 8ml low pH glycine buffer (100 mM glycine, pH 2.5-3.0) through the column, then was collected into sequential 1ml fractions, and neutralized as soon as possible by the addition of 100 µl 1 M Tris, pH 7.5 (or 50 µl 1 M Tris, pH 9). Neutralized eluents were concentrated on an Amicon filter (30kD cutoff) to a volume of ~500ul.

The column was regenerated as soon as possible after chromatography to prevent damage to the immobilized peptide by the low pH elution buffer; 16 ml PBS was added to wash the column. The column was then equilibrated with 8 ml degassed storage buffer (PBS containing 0.05% NaN3) and stored upright at 4°C.

3.2.3 Antibody affinity purification - Trial 2

Biotin-containing peptides bind to SA with extremely high affinity (10^{-15} k_d), providing another way to immobilize the peptide for purification. The following protocol is for Ab purification for 200 ul serum. First, 500 ul SA coated magnetic beads were added to a 1.5 ul Eppendorf tube, and centrifuged for 5 min at 5000 rpm. The supernatant was aspirated with the aid of the magnet apparatus to immobilize the bead pellet. Beads were washed once in 500 ul PBS, then centrifuged for 5 min at 5000 rpm, placed in the magnet apparatus, and supernatant was aspirated. 500-ul of PBS solution containing 40 ug bio-peptide was added to beads and incubated on a ferris wheel for 1 hr at room temperature. Samples were centrifuged for 5 min at 5000 rpm, and placed in the magnet apparatus; supernatants were aspirated and kept for ELISA. Subsequently, 100 ul PBS containing 40 nM biotin was added to the beads and incubated for 15 min on the rocker at RT to block remaining free biotin binding sites. Samples were then centrifuged for 5 min

at 5000 rpm, and the supernatant was aspirated. After washing once in 500 ul PBS, samples were centrifuged for 5min at 5000 rpm and supernatant was aspirated. The beads were then resuspended in 500 ul PBS and stored upright at 4°C.

For affinity purification of peptide-specific serum IgG, the SA-coated magnetic beads were first equilibrated to room temperature, then centrifuged for 5 min at 5000 rpm. After the supernatant was aspirated, 200 ul serum was added to the beads, then incubated on the rocker for 1 hour at RT. The beads were then centrifuged for 5 min at 5000 rpm, and the supernatant (unbound serum, or flow through) was collected into a separate tube. The beads were then washed 2 times with PBS. Bound Ab was eluted by adding 500 ul low pH glycine buffer (100 mM glycine, pH 2.6) to the beads, and incubated for 20 min at RT. After centrifuging the beads for 5 min at 5000 rpm, the supernatant (purified Ab) was aspirated into a separate tube. After washing 3 times by PBS, the beads were stored upright at 4°C.

To estimate the amount of Ab that was purified, the purified Ab IgG content was tested using the same method as in **2.2.3**. To determine the amount of Ab removed from a serum, the binding of purified Ab and the remaining unbound serum (**3.2.2** and **3.2.3**) was tested for binding to specific peptide by direct ELISA, as in **3.2.4**.

3.2.4 Direct ELISA for characterizing purified IgG binding to specific peptide

Initially, 400 ng peptide were coated in the wells, and dried at 37°C for 6 hours. Next, TBS containing 2%BSA, used as a negative control, was coated. The plates were incubated at 4°C overnight. Next day, after blocking with 2% BSA in TBS followed by three washes, 1:2 serial dilutions of purified Ab IgGs and unbound serum were added to

wells, starting at 1:10 dilution. The plates were incubated for 2 hours at RT, and then washed six times with TBST. HRP conjugated goat-anti-human IgG (diluted 1 in 1000) was added to the plates, and incubated for 45 minutes at RT. After six washes, plates were developed using ABTS system, and read at 30 min, as in **2.2.3**.

3.2.5 Neutralization assay

Neuralization assay was performed by a recombinant virus assay as described in Richman *et al.* 2003. Briefly, virus particles containing envelope proteins plus an HIV genomic vector that contains a firefly luciferase indicator gene. Virus infectivity was determined by measuring the amount of luciferase activity expressed in infected cells compared with an antibody negative control after a single cycle of replication. NtAb titers were calculated as the reciprocal of the plasma dilution conferring 50% inhibition (IC50). HIV-1 MN strain was used in Trial 1 and 2, and an additional HIV-1 92HT594 strain was used in Trial 2. In both trials, amphotropic murine leukemia virus (aLMV), an unrelated retrovirus was used as negative (specificity) control.

3.3 Results

3.3.1 Ab affinity purification – Trial 1

In the epitope mapping study described in **Chapter 2**, MPER-specific Ab reactivity was detected in both early infection (SC group) and longer-term infection (RP group), as well as in two of the bNt sera. This was interesting because these Abs bind to the same region as bNt MAb 2F5 or 4E10. Do these Abs also neutralize HIV-1? We developed an Ab affinity purification method to answer this question. The initial purifications were performed using SC samples. The reason is that, although these MPER

signals were low, if there was any NtAb against this region in early infection, these Abs should be easier to elicit by vaccination compared to those Abs produced after long-term infection, thus an observation that MPER-specific NtAbs are in the SC sera would have greatest significance for vaccine design.

We chose the following SC samples for affinity purification based on their reactivity against the MPER: SC1.4, SC1.5, SC3.5 (specific for 2F5 epitope), SC2.8 and SC2.9 (specific for 4E10 epitope). The method used is described in section **3.2.2** (SulfoLink Coupling Gel Kit). In Ab purification, the harsh conditions used (low pH elution buffer) may have denatured Abs, which might then lose their binding property. Peptide ELISAs and IgG content ELISAs were used to determine the "specific activity" (i.e. peptide binding per unit IgG) of affinity-purified Ab preparation, and compared to the "specific activity" of the completely bNt MAb (2F5 or 4E10). Thus, samples of each affinity purified Ab, and the corresponding bNt MAb, were titrated on peptide and on protein G. This produced a rough idea of the quality of the affinity-purified Ab, in that low "specific activity" (compared to bNt MAb) indicated either denatured and/or low-affinity Ab.

Figure3-1 and **Figure 3-2** show the peptide binding (left panels) and IgG content (right panels) results from two representative purifications; sample SC1.5 purified on bio-2F5 peptide, and sample SC2.8 purified on bio-4E10 peptide. Both results showed that by using SulfoLink Coupling Gel Kit, most peptide specific Abs were removed from the serum, indicated by the low peptide signal in unbound serum. In addition, the purified Abs maintained the peptide binding reactivity in ELISA, as shown by the titration curves in the left panels. To produce the same O.D. (i.e., O.D.=2), ~2.1 nM purified SC1.5,

compared to ~0.3 nM 2F5 MAb, and ~3.4 nM purified SC2.8, compared to ~0.2 nM 4E10 MAb were required. Thus, larger amount of "2F5/4E10-like" Abs were required to produce the same ELISA signal as compared to 2F5/4E10 MAbs. Moreover, larger amount of "4E10-like" Abs may be required, as compared to "2F5-like Abs".

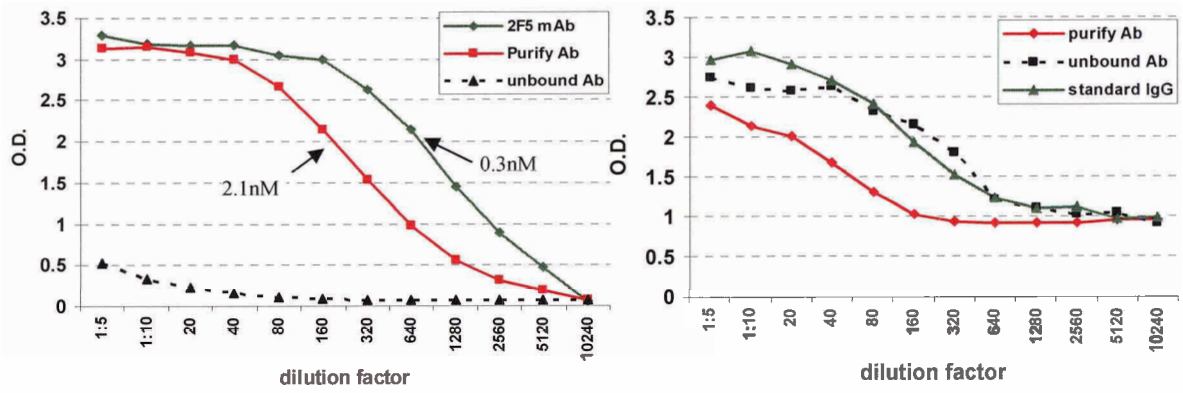


Figure 3-1: Serum SC1.5 affinity purification with 2F5 peptide.

Left panel is the purified Ab (red) reactivity against 2F5 peptide compared with 2F5 MAb (green) and unbound serum (black) side by side. Right panel is the IgG content of purified Ab, unbound serum Ab compared to standard IgG.

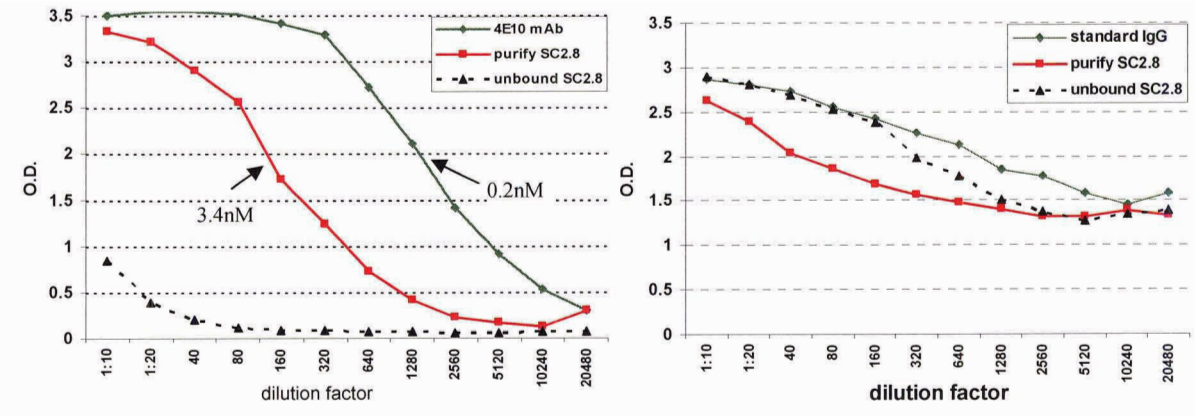


Figure 3-2: Serum SC2.8 affinity purification with 4E10 peptide.

Left panel is the purified Ab (red) reactivity against 4E10 peptide compared with 4E10 MAb (green) and unbound serum (black) side by side. Right panel is the IgG content of purified Ab, unbound serum Ab compared to standard IgG.

3.3.2 Neutralization assay – Trial 1

Following affinity purification study, the purified Abs were sent to Monogram Biosciences company for neutralization assay. The results for purified Ab specific for 2F5-peptide (SC1.4, SC1.5 and SC3.5) and 4E10 peptide (SC2.8 and SC2.9) are shown in **Figure 3-3** and **Figure3-4** separately. As a control for the neutralization assay, a high titer guinea pig antiserum, produced against the 2F5 peptide (Merck serum), was used. It has been shown no neutralization activity in this serum (McGaughey *et al.* 2003). Significant neutralization of the TCLA strain, MN pseudovirus was observed for all of the affinity purified Abs. However, in all cases, purified Abs showed different extents of neutralization against the negative-control aMLV, indicating that something in the purified Ab preparation was affecting the assay. Although the affinity purified Abs may have Nt activity, it is difficult to draw clear conclusions without eliminating this high background.

In our purification process, a large volume (8 ml) of elution buffer containing 1% BSA was used to elute Abs from the column. Considering the concentration of BSA in purified Abs will go up to 8% after concentration, this may be one possible reason causing high background problem. Besides this, the glycerol, used to store the Abs, and/or contents eluted from the agarose support, could also have affected the assay. This result led us to try another approach for Ab purification.

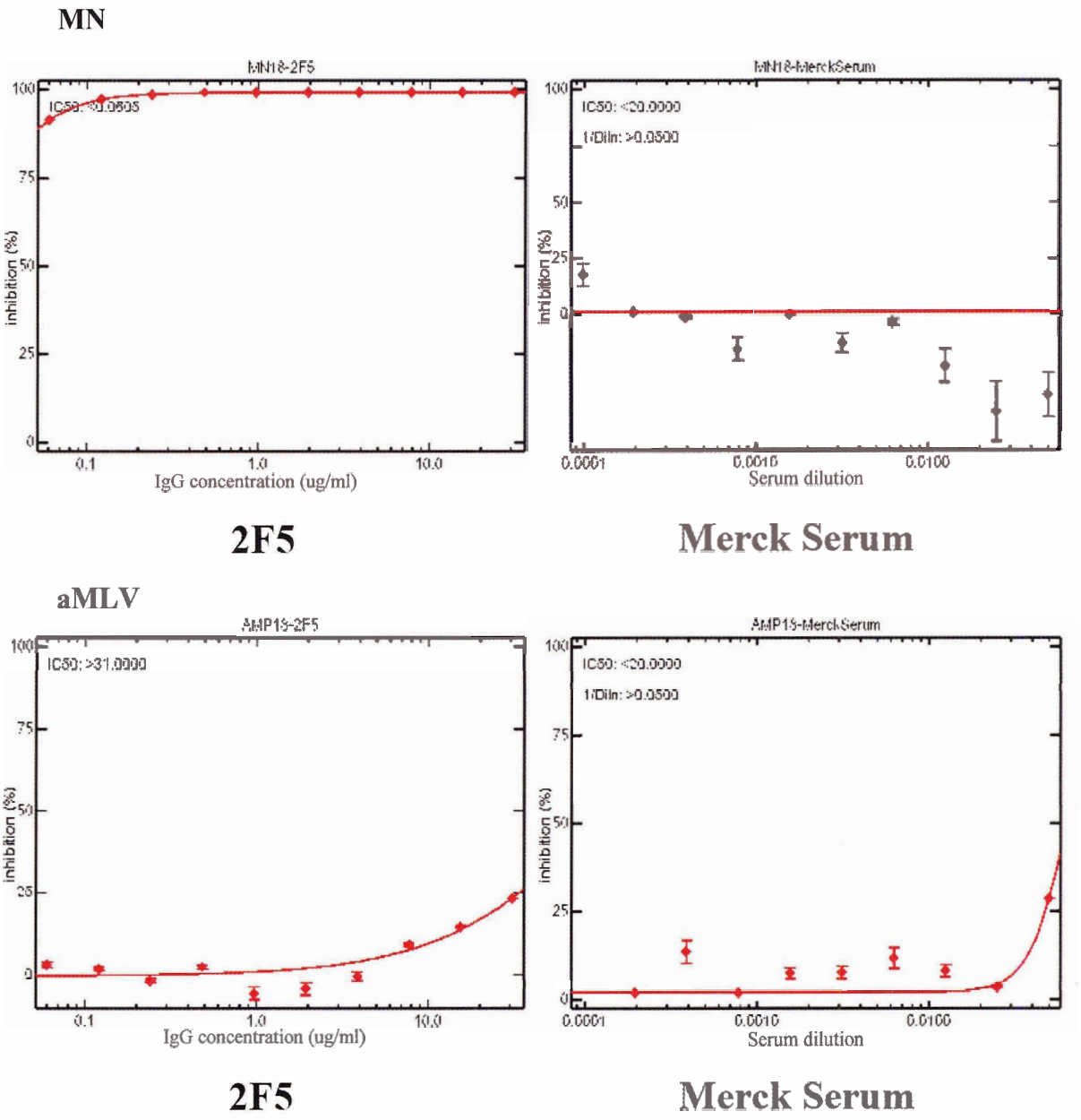
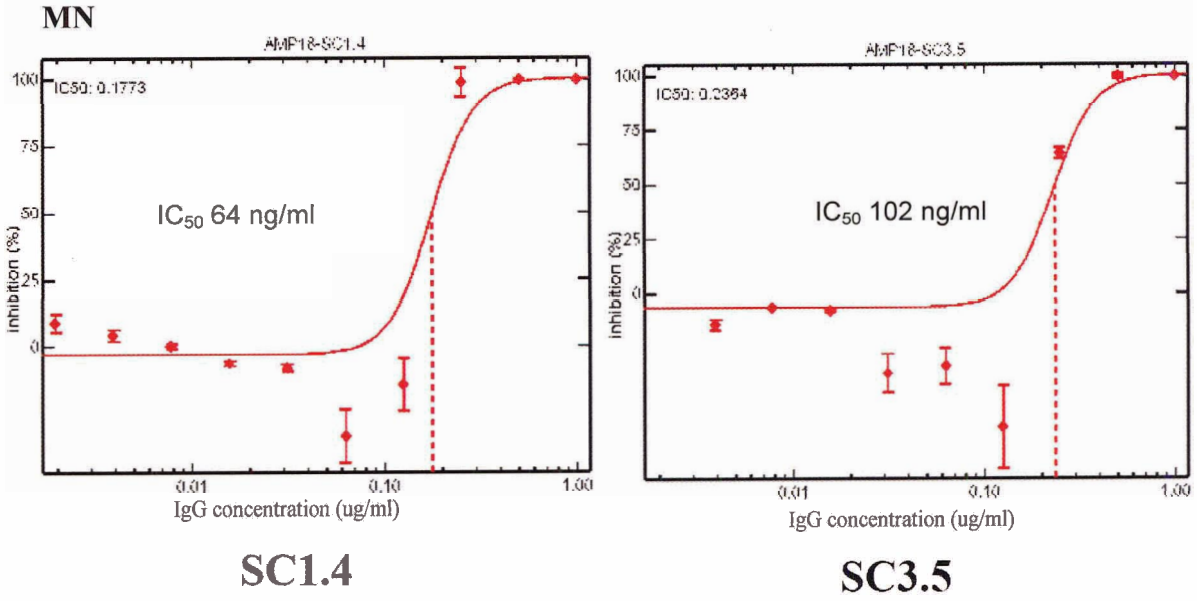


Figure 3-3: Neutralization data for an 18-hour neutralization assay using serum Abs affinity purified on the 2F5 peptide.

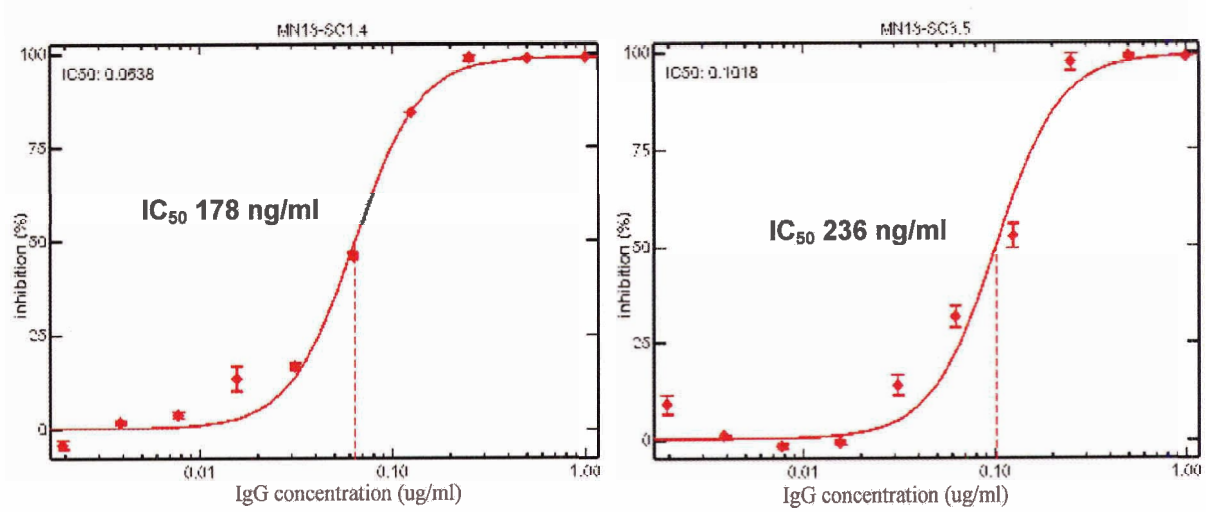
The Abs were purified on a 15-mer peptide bearing the 2F5 epitope sequence from gp41 MN sequence. The upper panel represents 2F5 MAb (positive control), Merck Serum (negative control) on this page, and purified Abs (from SC1.4, and SC3.5) neutralize HIV-1 MN strain. The bottom panel represents same Abs neutralize aMLV (negative control virus). X-axis represents serial dilutions of Abs. Y-axis represents percentage of neutralization (percentage of viral infection blocked). Data courtesy of T.Wrin, Monogram Biosciences.

MN



SC1.4 SC3.5

aMLV



SC1.4 SC3.5

Figure 3-3: continued.

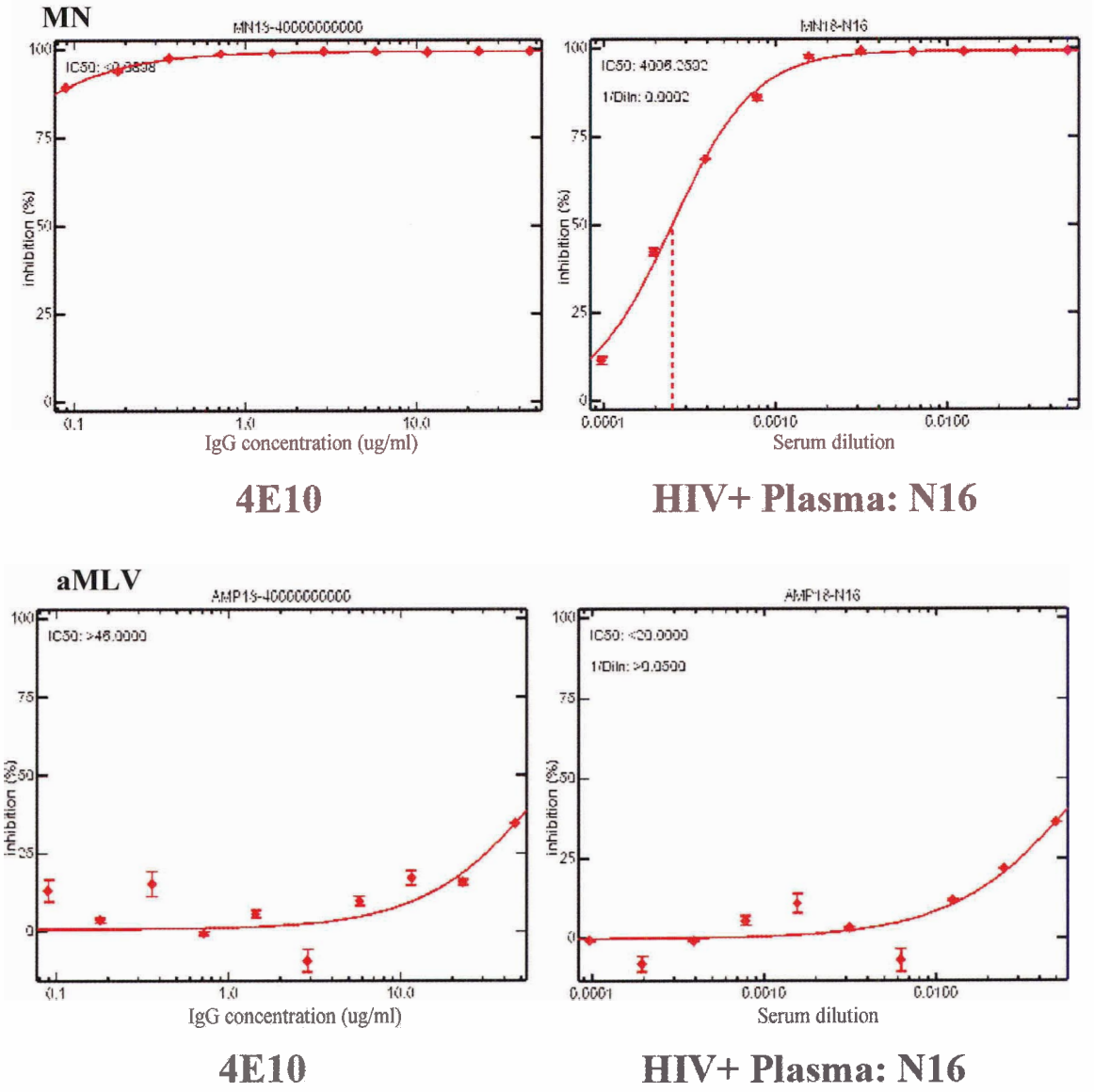


Figure 3-4: Neutralization data for an 18-hour neutralization assay using serum Abs affinity purified on 4E10 peptide.

The Abs were purified on a 15-mer peptide bearing the 4E10 epitope sequence from gp41 MN sequence. The upper panel represents 4E10 MAb (positive control), HIV^+ plasma (positive control) on this page, and SC2.8, SC 2.9 on the next page neutralize HIV-1 MN strain. The bottom panel represents same Abs neutralize aMLV (negative control virus). X-axis represents serial dilutions of Abs. Y-axis represents percentage of neutralization (percentage of viral infection blocked). Data courtesy of T.Wrin, Monogram Biosciences.

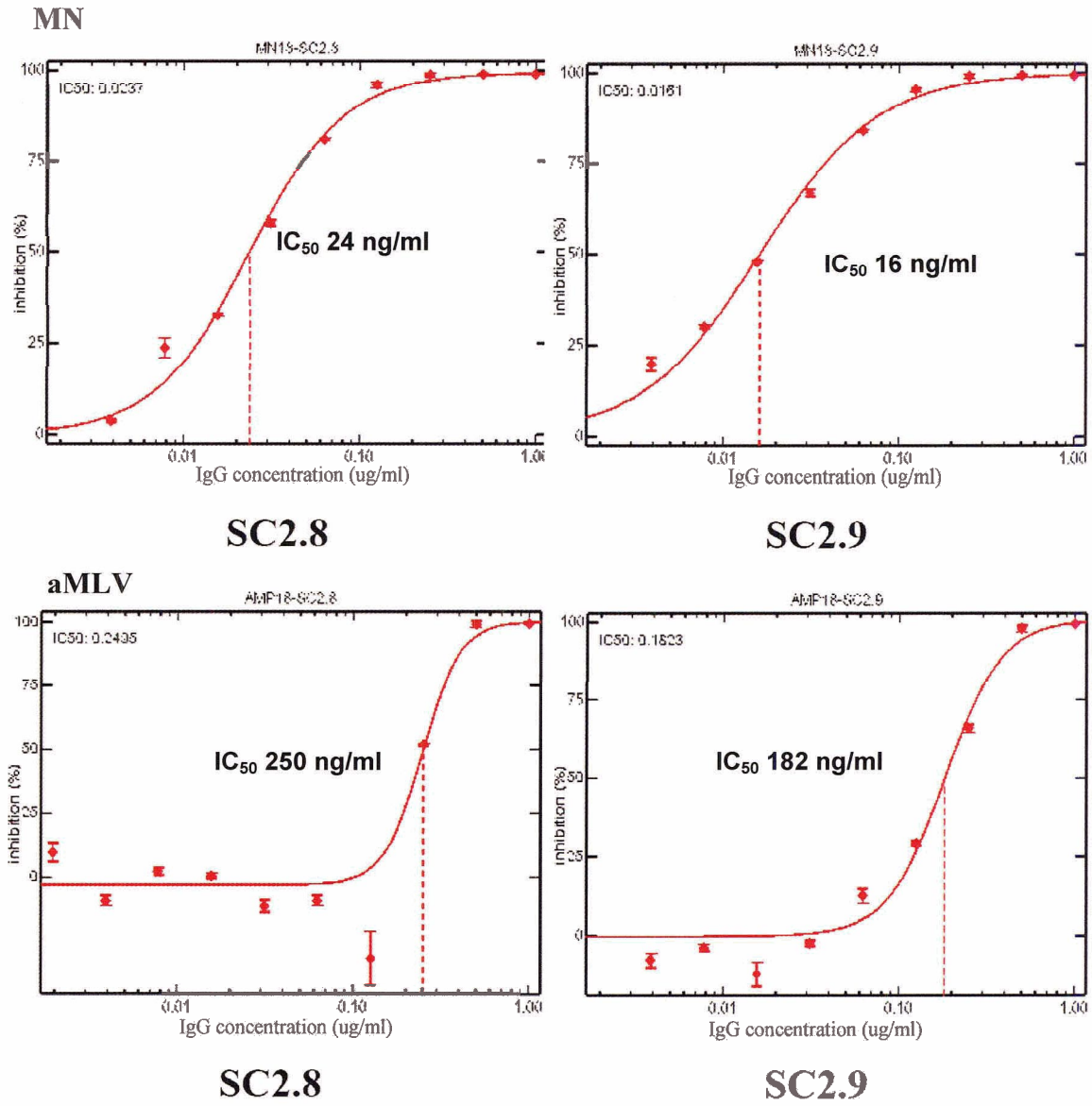


Figure 3-4: continued.

3.3.3 Ab affinity purification and neutralization assay– Trial 2

As shown in **Figure 2-2** in **Chapter 2**, there was strong 2F5-peptide reactivity in bNt serum #6. We thus purified this reactivity from #6 serum by Trial 2 method. As in Trial 1, Merck serum was used as negative control, and affinity purified by the Trial 2 method. To control the background problem observed with the previous SC samples, a high volume wash was performed using a 30-kD molecular-weight cut-off filter after elution of Ab. The molecular weight of IgG is ~150 kD. This may have helped remove small molecules and salt related to acid neutralization. Also, the magnetic beads are less likely to shed other contaminates as compared to the agarose-gel support used in the Trial 1 method.

Figure 3-5 and **Figure 3-6** show the peptide binding (left panels) and IgG content (right panels) results of affinity purified Abs from #6 and Merck serum on bio-2F5 peptide. The IgG content for Merck serum is 223 ug/ml, whereas for #6 serum is 6 ug/ml. To produce the same O.D. (i.e., O.D.=0.6 in **Figure 3-5**, or 0.8 in **Figure 3-6**), ~8 times more affinity purified Ab than 2F5 MAb was required, and ~5.6 times more purified Ab from Merck serum was required. **Figure 3-7** shows the titration curves in neutralization assay for Merck guinea pig serum (M1); purified antibody (M2, before concentrating, in 100mM glycine); purified antibody (M3, after concentrating, no glycine); #6 human serum (#6-1, Nt); #6 purified antibody (#6-2, before concentrating, in 100mM glycine); purified antibody (#6-3, after concentrating, no glycine); unbound #6 serum (#6-4). Purified Ab from Merck serum before removing acid (M2) showed neutralization activity in MN, 92HT594, and on the control virus aMLV; however after washing, there was no neutralization activity, as expected. This shows that small

molecules (either acid, glycine or Tris-HCl) in the purified Abs are causing high background, probably by compromising the cells in neutralization assay. Or, the concentrated #6 Ab after removing acid (#6-3) showed weak Nt activity, whereas the eluted Ab before washing produced high background, which obscured the Abs' Nt activity (**Table 3-1**). Thus, the Trial 2 approach, using affinity purification on peptide-loaded magnetic beads, in combination with extensive washing, appears to the best approach to serum Ab purification.

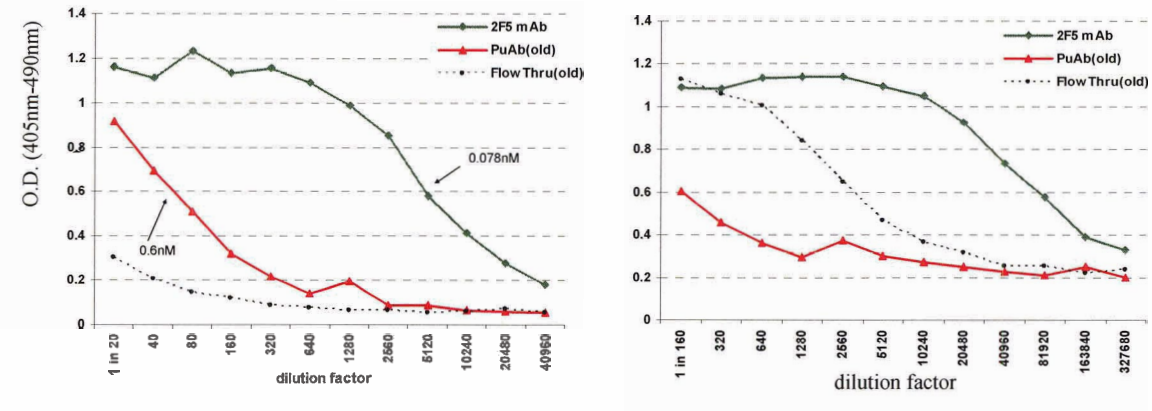


Figure 3-5: Serum #6 affinity purification with 2F5 peptide.

Left panel is the purified Ab (red) reactivity against 2F5 peptide compared with 2F5 MAb (green) and unbound serum (black) side by side. Right panel is the IgG content of purified Ab, unbound serum Ab compared to standard IgG.

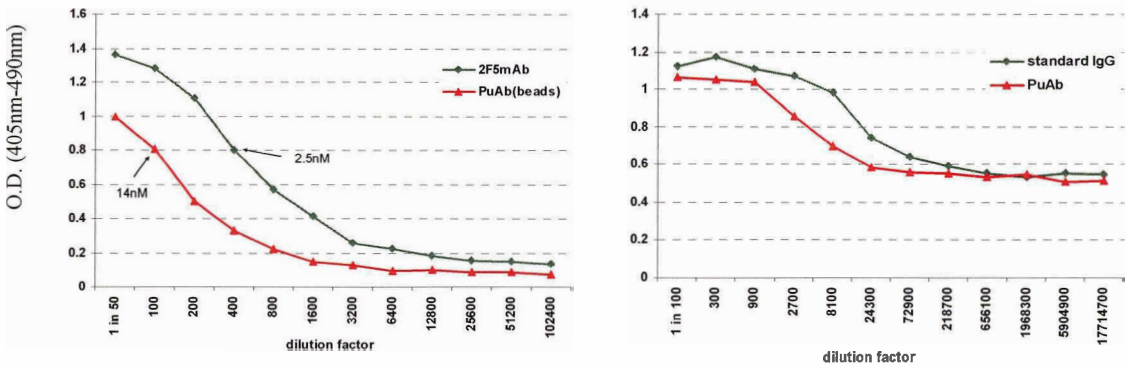
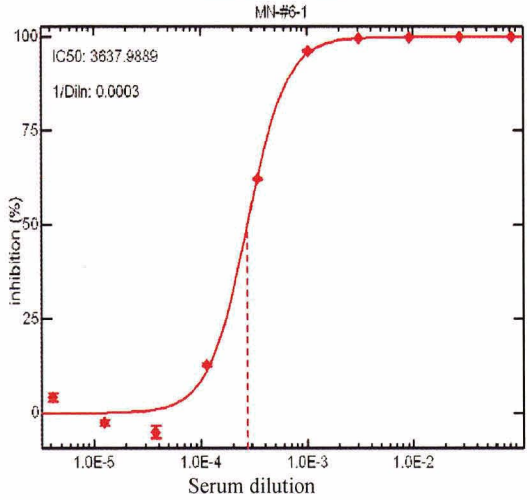


Figure 3-6: Merck serum affinity purification with 2F5 peptide.

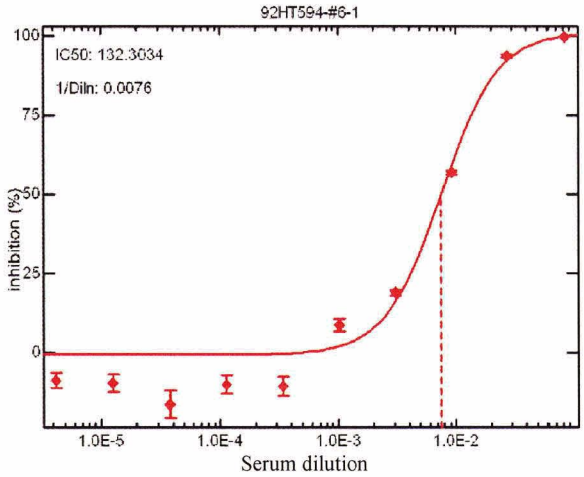
Left panel is the purified Ab (red) reactivity against 2F5 peptide compared with 2F5 MAb (green) side by side. Right panel is the IgG content of purified Ab, compared to standard IgG.

#6-1 (P)

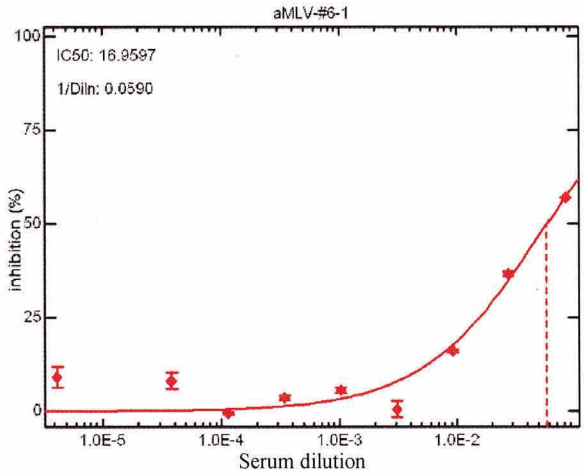
HIV-1 MN strain



HIV-1 92HT594 strain

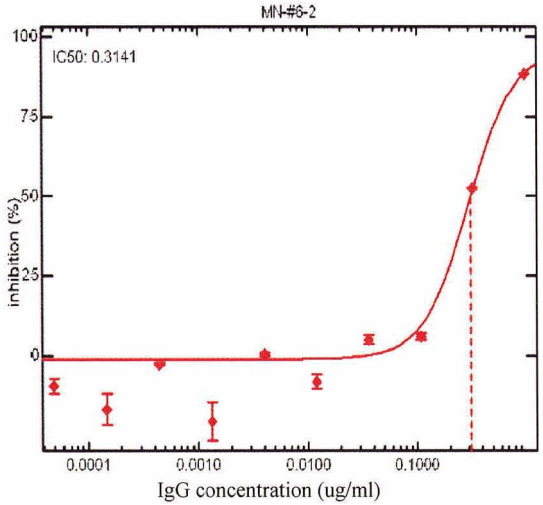


aMLV (negative control)

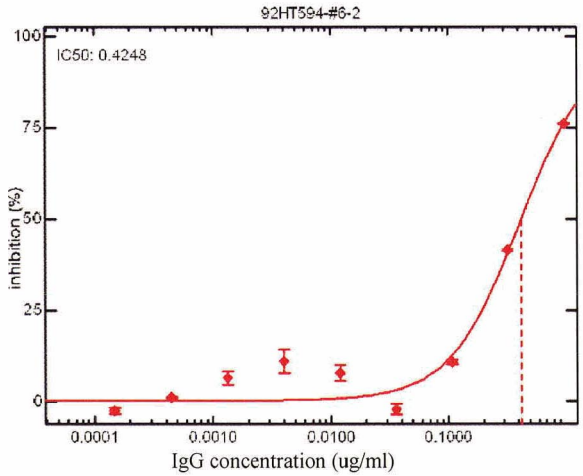


#6-2 (N)

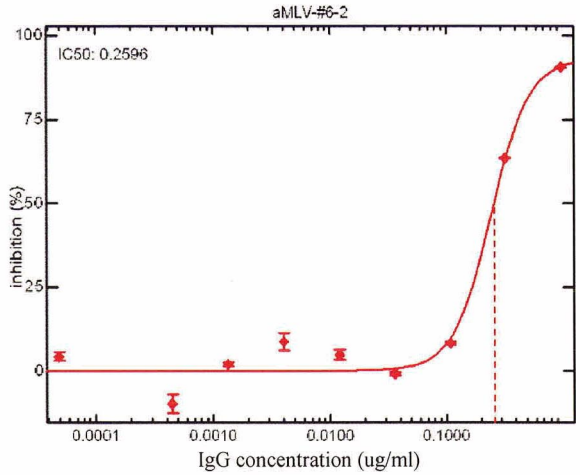
HIV-1 MN strain



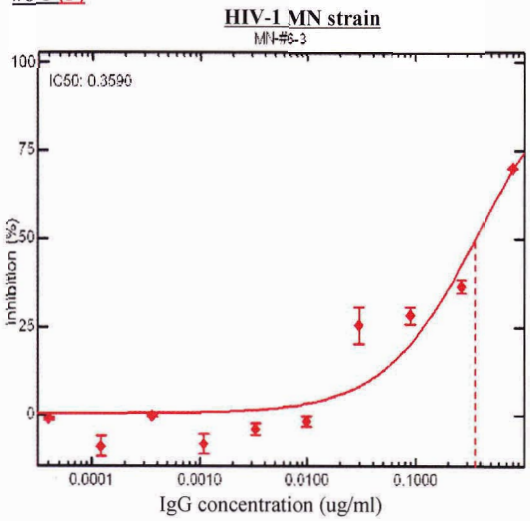
HIV-1 92HT594 strain

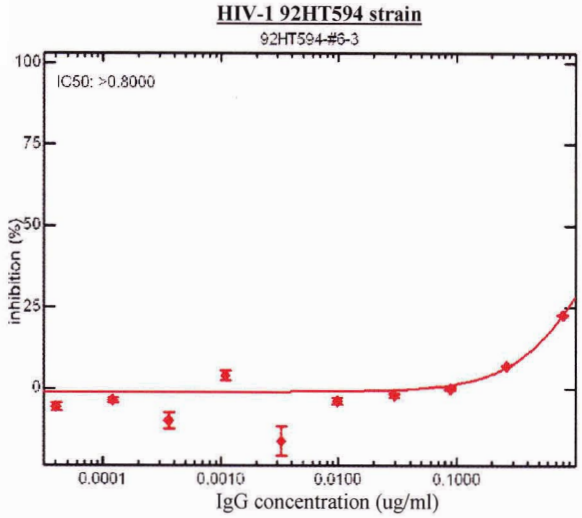


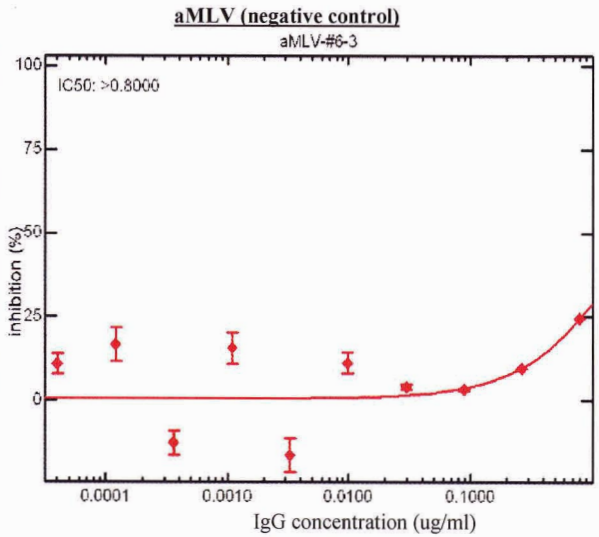
aMLV (negative control)



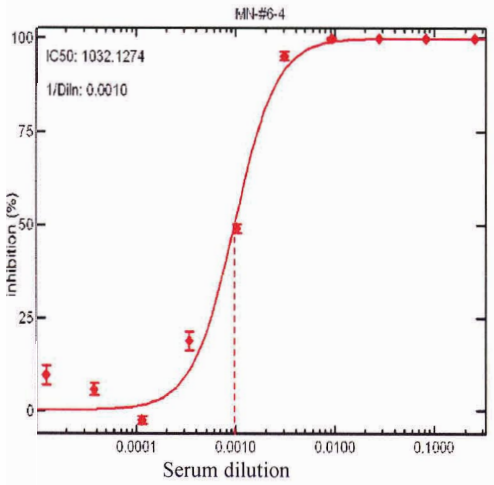
#6-3 (P)

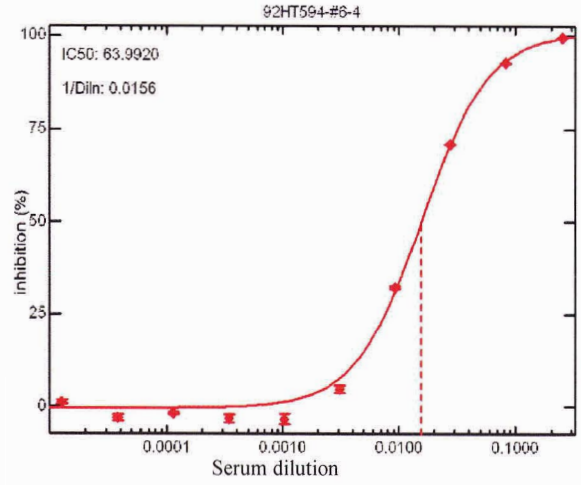

HIV-1 MN strain
MN-#6-3
IC50: 0.3590
inhibition (%)
IgG concentration (ug/ml)

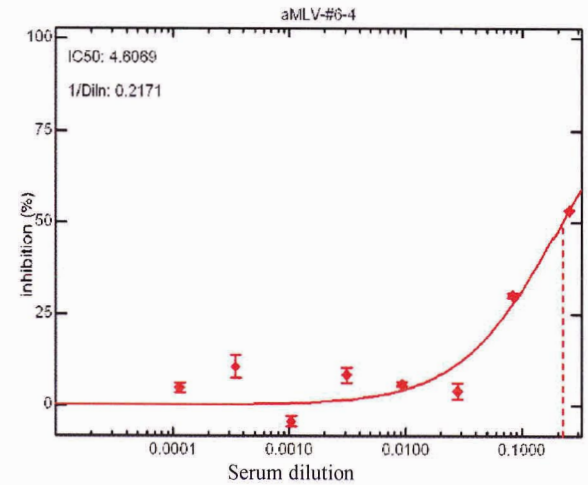

HIV-1 92HT594 strain
92HT594-#6-3
IC50: >0.8000
inhibition (%)
IgG concentration (ug/ml)


aMLV (negative control)
aMLV-#6-3
IC50: >0.8000
inhibition (%)
IgG concentration (ug/ml)

#6-4 (P)

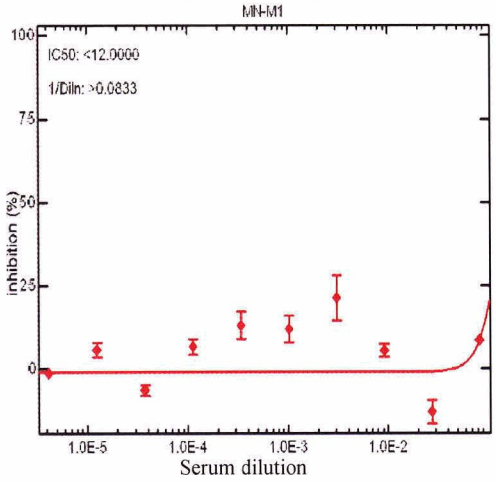

HIV-1 MN strain
MN-#6-4
IC50: 1032.1274
1/Diln: 0.0010
inhibition (%)
Serum dilution


HIV-1 92HT594 strain
92HT594-#6-4
IC50: 63.9920
1/Diln: 0.0156
inhibition (%)
Serum dilution

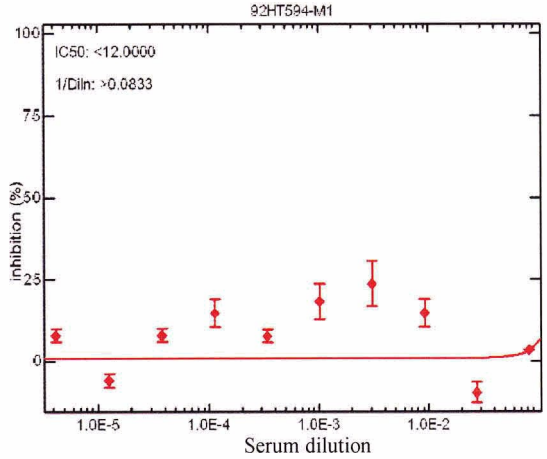

aMLV (negative control)
aMLV-#6-4
IC50: 4.6069
1/Diln: 0.2171
inhibition (%)
Serum dilution

M1

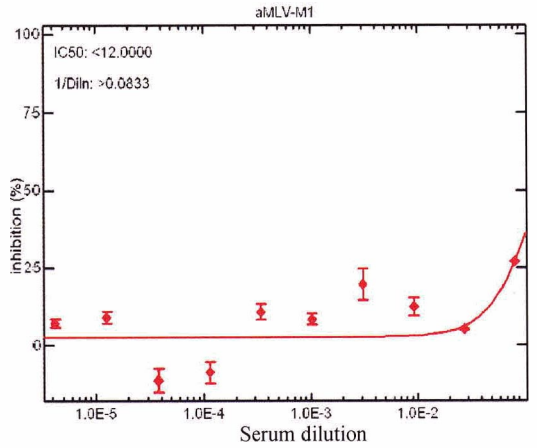
HIV-1 MN strain



HIV-1 92HT594 strain

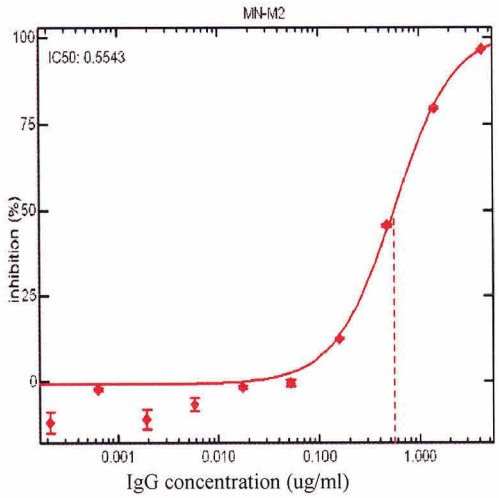


aMLV (negative control)

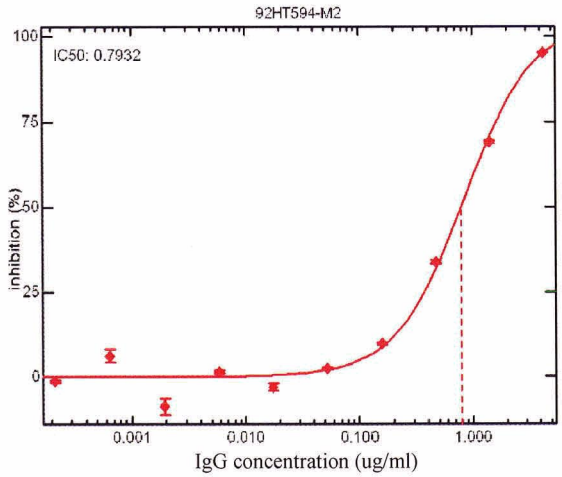


M2

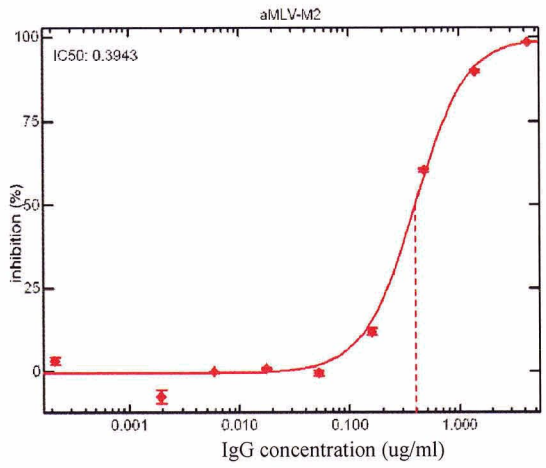
HIV-1 MN strain



HIV-1 92HT594 strain

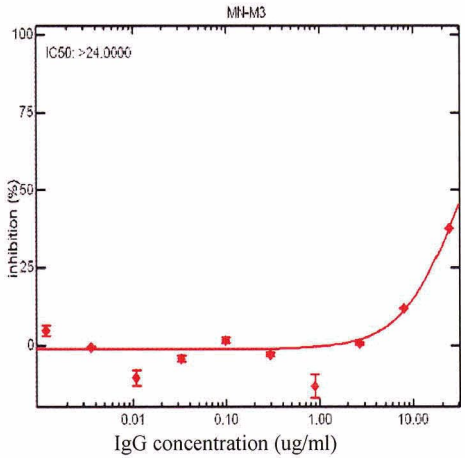


aMLV (negative control)

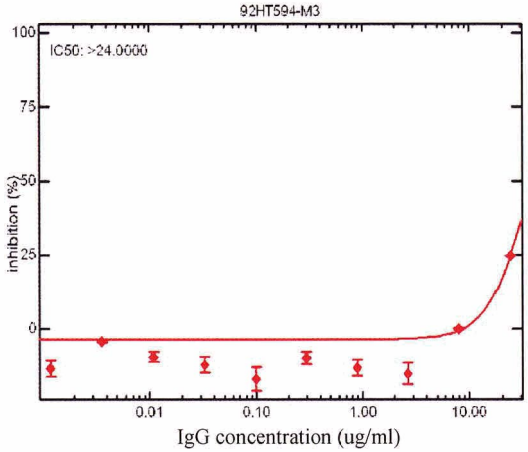


M3

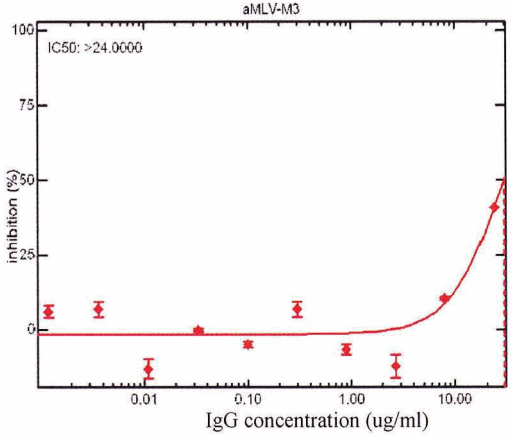
HIV-1 MN strain



HIV-1 92HT594 strain

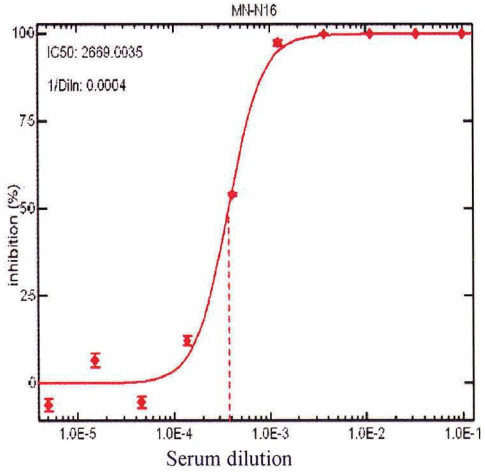


aMLV (negative control)

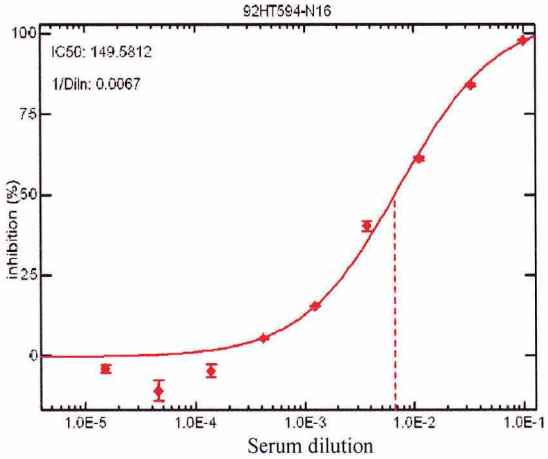


HIV+ Plasma N16

HIV-1 MN strain



HIV-1 92HT594 strain



aMLV (negative control)

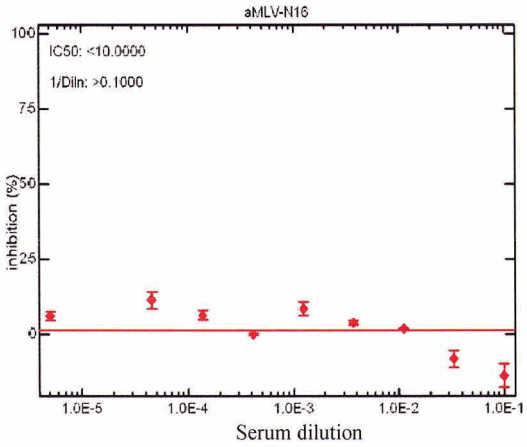


Figure 3-7: Summary of neutralization assay with bNt serum #6, Merck serum (negative control).

M(1): Merck Guinea pig serum, negative control, 50ul; M(2): purified antibody (before concentrating, in 100mM glycine and 1% FCS), 100ul, 14ug/ml; M(3): purified antibody (after concentrating, no glycine, ~16% FCS), 80ul, 223 ug/ml; #6 (1): #6 human serum, neutralizing, 50ul, 13mg/ml; #6 (2): #6 purified antibody (before concentrating, in 100mM glycine and 1% FCS), 100ul, 9 ug/ml; #6 (3): purified antibody (after concentrating, no glycine, ~16% FCS), 80ul, 6ug/ml; #6 (4): unbound #6 serum, should have no or very less 2F5 peptide specific antibody. The assay was done with pseudoviruses bearing the MN (TCLA), and 92HT594 (primary isolate) envelope proteins. aMLV was the negative control virus, and HIV+ plasma N16 was the positive serum sample. All Ab samples were tested in a 18-hour incubation with the viruses before the addition of cells. P/N represents positive and negative in neutralization assay. Data courtesy of T.Wrin, Monogram Biosciences

Table 3-1: Summary of IC50 in neutralization assay with bNt serum #6, Merck serum (negative control).

sample	Unit (1)	92HT594	MN	aMLV
#6-1	Diln(1/Diln)	132.3034	3637.9889	16.9597
#6-2	(ug/mL)	0.4248	0.3141	0.2596
#6-3	(ug/mL)	>0.8000	0.3590	>0.8000
#6-4	Diln(1/Diln)	63.9920	1032.1274	4.6069
M1	Diln(1/Diln)	<12.0000	<12.0000	<12.0000
M2	(ug/mL)	0.7932	0.5543	0.3943
M3	(ug/mL)	>24.0000	>24.0000	>24.0000

(1) For purified Abs, ug/ml concentrations were used; for sera, dilutions were used. (2) In order for a test sample to be called "positive" in the assay (the green cells) the IC50 must be at least 3X higher than the same sample when tested with aMLV. Data courtesy of T.Wrin, Monogram Biosciences.

3.4 Discussion

In this chapter, we compared methods for affinity purifying Abs that bind to the 2F5 or 4E10 epitope peptides, and their ability to produce Ab preparations that behave well in a HIV-1 pseudovirus neutralization assay. Two different purification systems were used: SulfoLink Coupling Gel Kit (Trial 1) and MyOne magnetic streptavidin beads (Trial 2). In the neutralization assay, purified Abs from SulfoLink Coupling Gel Kit showed high background (neutralized a control virus aMLV) (**Figure 3-3** and **Figure 3-4**). The possible reason might be the agarose gel contaminated the purified Ab, though we could not prove this speculation. In contrast, using the magnetic beads, we were able to purify Ab with clean background after removing other small molecules (**Figure 3-7**). Other advantages of the magnetic bead purification system are listed in **Table 3-2**. The comparison was done by using Streptavidin Beads to affinity purify MAb 2F5, compared with the serum purification data from SulfoLink Agorose Gel.

Table 3-2: Comparison of Two Affinity Purification Systems, Sulfolink Agrose Gel and SA Beads.

	SA Beads	SulfoLink Agarose Gel	Advantage
Ease of Protocol	Very easy	Moderate	Streptavidin Beads
Peptide Binding Efficity	100%	97%	Streptavidin Beads
Peptide capacity in column	0.056 mg/ml	1-10mg/ml	SulfoLink Agarose Gel
Ab capture capability	100%	70-80%	Streptavidin Beads
Peptide-Ab Molarity ratio	30	>3600	Streptavidin Beads
Optimum pH	2.5	could not be optimized	Streptavidin Beads
Elution Buffer Volume	1x Sample volume	20x Sample volume	Streptavidin Beads

From the comparison, it is obvious that SA beads are much easier to work with and more importantly, Abs purified by this method behave well in viral neutralization assays. One disadvantage of the SA bead system is that for same amount of beads and sulfolink gel, less peptide can be coupled to the beads, and that less Ab can be purified. The sacrifice of yield should be taken into account in future purification work since oftentimes we could not get enough purified Abs for the neutralization assay. We were encouraged by the fact that in Trial 2, 2F5-peptide specific Ab weakly neutralized a sensitive HIV-1 MN strain. Whether this Ab is indeed Nt needs confirmation. HIV-1 MN is a TCLA strain, and is relatively sensitive to Ab-mediated neutralization. In general, primary isolates are more representative of HIV-1 *in vivo* albeit more resistant to Ab neutralization. Thus, to purify more MPER Abs (both 2F5 and 4E10 peptide specific) and test them against more HIV-1 viral isolates is one of our future directions.

Several experimental approaches have been used to identify the epitope specificity of serum Abs of HIV-infected individuals (Berkower *et al.* 1991; Broliden *et al.* 1992; Kang *et al.* 1991; Matthews *et al.* 1986; Steimer *et al.* 1991; Vancott *et al.* 1995; Stamatos *et al.* 1998) (Braibant, *et al.* 2006). Similar to our approach (Ab

depletion), Stamatos *et al.* (1998) have successfully removed more than 95% of gp120 protein specific serum Abs by using Sepharose beads coupled with gp120 protein. It was shown that purified Abs neutralized HIV-1 and left over serum neutralization reactivity has mostly been removed (Stamatos *et al.* 1998). Therefore, although we have met technical difficulties, epitope specific Ab affinity purification is one way to circumvent the problem that NtAbs might not otherwise be detectable at extremely low titers in the serum assay.

CHAPTER 4: THE SPECIFICITY OF HUMAN MONOCLONAL AND POLYCLONAL ANTIBODIES AGAINST HIV-1

4.1 Introduction

As mentioned above, the production of bNtAbs is likely a requirement of an effective HIV-1 vaccine. Among the bNt MAbs identified so far, 2F5 and 4E10 both recognize conserved linear epitopes on the MPER of gp41(aa 660~683). Linear epitopes have advantages over conformational epitopes in that they are easier to design as immunogens. Therefore, intensive studies have been done to elicit Abs having similar bNt properties to 2F5. However, immunization with peptide bearing the 2F5 epitope sequence (ELDKWA) expressed in a number of contexts have failed to elicit Nt Abs (Muster *et al.* 1994; Liang *et al.* 1999; Eckhart *et al.* 1996). Although there are no data available in the literature for 4E10 immunization studies, the possibility of reproducing 4E10-like antibody *in vivo* by using simple peptide bearing its core epitope (NWFDIT) is small. This has led to the hypothesis that the recognition between 2F5 or 4E10 and their linear epitopes is critically dependent on the environment in which it is present on gp41 in the trimer, and/or that these linear epitopes are not the complete epitopes.

Crystal structures of bNt MAbs 2F5 and 4E10 revealed that both have long H3s, containing hydrophobic patches (see **Introduction, 1.8**). It has been suggested by Haynes *et al.* (2005) that such long hydrophobic H3s are generally typical of polyreactive autoantibodies (autoAbs). They tested all of the known bNt MAbs for their ability to bind a number of autoantigens, and it was found that b12 reacts with DNA, and 2F5 and 4E10

bind to cardiolipin (CL), a phospholipid found mainly in mitochondrial membranes. Serum reactivity in clinical, anti-CL assays is associated with anti-phospholipid syndrome (APS), an autoimmune, thrombogenic condition. This group concluded that 2F5 and 4E10 are polyspecific autoAbs, based on their CL reactivity, minimal extension of prothrombin time, and unusual cell-staining patterns. Furthermore, based on the hypothesis that these autoAbs originated from a pool of autoreactive B-cell precursors that are deleted during normal B-cell development, they questioned whether bNtAbs, particularly those specific for the membrane-proximal external region of gp41 (MPER), can be induced by current vaccination approaches (Haynes *et al.* 2005; Nabel 2005).

Our laboratory has developed specific peptide ligands for MAbs b12, 2G12, 2F5 and 4E10 (Zwick *et al.* 2001a; Menendez *et al.* 2004; Alfredo 2005; Sondra 2004). The affinity of the MAbs for these peptides is similar to that for their "cognate" antigens. Given the results of Haynes *et al.*, several questions arose for us: How would binding of 2F5 and 4E10 to CL compare if tested side-by-side with peptides bearing their gp41 epitopes? Are 2F5 and 4E10 polyreactive in general (i.e., with non-autoantigens)? And how does this reactivity compare to that of more classical autoimmune APS MAbs?

In this chapter, the reactivity of bNt MAbs 2F5, and 4E10 were compared to that of an APS MAb, CL15. The results showed that 2F5 and 4E10 prefer their epitope peptides to CL by orders of magnitude, while the binding of CL15 to its antigen CL is not distinguishable from non-specific proteins. We then compared the specificity of these MAbs by using a panel of peptides whose hydrophobicity and charge varied. The results showed that 2F5 and 4E10 specifically bind to their cognate-epitope peptides whereas

CL15 is clearly polyspecific. Taken together, our results support the idea that HIV-1 bNtAbs are not unusually polyreactive or autoreactive.

If 2F5 and 4E10 are autoAbs as suggested by Haynes *et al*, there should be a repertoire of autoreactive B cells induced by HIV-1 infection and such autoreactivity should theoretically increase over time. Therefore, we further tested a panel of sera (SP, NP, RP, SC and bNt sera, see **Chapter 2**) for CL reactivity. Our data show no significant autoreactivity or any increase trend following increased CL reactivity in the serum samples. Possible reasons are discussed in this chapter.

4.2 Materials and Methods

4.2.1 Reagents

The NIH AIDS Research and Reference Reagent Program provided 15-mer synthetic peptides derived from HIV-1 envelope protein MN isolate (>80% HPLC purity). The sequences of the peptides used for these studies are listed in Supplement Ib. The 2F5 MN peptide (NH2-EQELLELDKWASLWSGK(BIOTIN)GC-CONH2) was provided at >95% purity by NeoMPS (San Diego, CA). CL15 IgG was kindly provided by Dr. P. Chen (University of California, Los Angeles). HIV-1(MN) recombinant gp41 (MN) and gp120 (MN) envelope proteins were purchased from ImmunoDiagnostics (Woburn, MA). HIV-1 positive human sera were provided through the MACS program (University of Pittsburgh) and the Center for AIDS Research (UCSD). AP-conjugated goat anti-human IgG (Fab specific) were from Pierce (Rockford, IL). Cardiolipin (CL), bovine serum albumin (BSA), ovalbumin (OVA), p-nitrophenyl phosphate (pNPP) disodium salt tablets were from Sigma-Aldrich (St. Louis, MO). Non-fat dried milk

(DM) was from Bio-Rad (Hercules, CA). Tween-20 was from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ).

4.2.2 Direct ELISA of 2F5, 4E10 and CL15 IgGs

The procedures for coating wells with CL, blocking and washing microtiter plates were adapted from the optimized procedure used by Haynes *et al.*(2005) (pers. comm., B. Haynes, Duke University). The order of coating plates with antigen is described for complex ELISAs, using synthetic peptides, CL and proteins; for ELISAs using two of these antigen types, the order of coating was the same as described. Initially, the wells of 96-well microtiter wells (Corning Inc., Corning, NY) received 400 ng MN peptides from the NIH AIDS RRP (listed in **Table 4-1**) or the highly-purified 4E10 or 2F5 synthetic peptides in 50 ul PBS and dried at 55 °C for 4 hours. Only peptides from the NIH AIDS RRP were used for assays comparing different MN peptides (i.e., for **Figure 4-1B**). Next, wells received 2 µg CL in 50 ul methanol, and were dried at 55 °C for 45 minutes. Finally, wells were coated with proteins (50 ng gp41 in 50 µl PBS, or 50 µl PBS containing 2% (w/v) BSA, DM, or OVA) by overnight incubation at 4 °C. Next day, all wells were blocked at 37 °C for two hours with 100 µl blocking solution (0.05 M carbonate bicarbonate buffer, pH 9.6 (CBC buffer), containing 3% (w/v) BSA). The plates were washed three times with PBST (PBS, pH 7.4, containing 0.05% Tween-20). Starting at 600 nM, 1:4 serial dilutions of 4E10, 2F5 and CL15 IgGs were prepared in antibody diluent (PBST containing 3% (w/v) BSA and 2% (v/v) fetal calf serum (FCS)); 50 µl diluted IgG were added to each well and incubated at room temperature for one hour. The plates were washed four times with PBST, then 50 µl AP- conjugated goat anti-human IgG (Fab-specific), diluted 1:1,000 in antibody diluent, were added to wells

and incubated for one hour at room temperature. After four washes, wells were developed using 100 µl pNPP tablet, dissolved at 1 mg/ml in CBC buffer containing 2 mM $MgCl_2$. After 45 minutes' incubation, optical densities were measured at 405 nm using a VersaMax Tunable Microplate Reader (Molecular Devices, Sunnyvale, CA).

4.2.3 Neutralization assay

Recombinant HIV-1HxB2 virions, competent for a single round of infection, were generated using the luciferase reporter plasmid pNL4-3.Luc.R-E-, as described previously (Connor *et al.* 1995; Zwick *et al.* 2003), and the pseudotyped virus was assayed for neutralization using U87.CD4.CXCR4 cells as target cells (Bjorndal *et al.* 1997). Different concentrations of the mAbs were added (1:1 by volume) to HIV-1, and pre-incubated 1 h at 37°C. The mixture of mAb and and HIV-1 was then added (1:1 by volume) to the target cells, and the assay was developed using luciferase reagent (Promega) following a 72-h incubation at 37°C. The degree of virus neutralization was determined as a percentage reduction of viral infectivity against an antibody-free control. The experiment was performed in triplicate.

4.2.4 Serum ELISAs

Serum ELISAs were performed as described above. Briefly, synthetic peptides, gp41 and negative control proteins were adsorbed to microtiter wells as described. Next day, sera, which had been previously inactivated by heating at 56 °C for 30 minutes followed by the addition of Empigen detergent to 0.01% (v/v), were diluted 1:50 in antibody diluent. After the blocking and washing steps, 50 ul diluted sera were added to

each well, and incubated at room temperature for one hour. Plates were then washed, developed and read as described above.

4.3 Results

4.3.1 Comparison of the reactivity profiles of MAbs 2F5, 4E10 and CL15

We compared the reactivity profiles of MAbs 2F5 and 4E10 to that of the APS MAb, CL15 (Chukwuocha *et al.* 2002). The assay conditions of Haynes *et al.* were used to assess binding strength and antigen specificity in direct ELISAs (see section **4.2.2**). 2F5 and 4E10 were titrated on small amounts of specific antigen and on "blocking" amounts of CL and three non-specific antigens (OVA, DM and BSA) so as to gauge their level of generalized polyreactivity. **Figure 4-1A** shows that there is a >3-log difference between the concentration of 2F5 IgG producing a given ELISA signal on peptide bearing the 2F5 epitope (or on gp41) and that producing the same signal on CL; likewise, 4E10 produces a >2-log difference. Moreover, 4E10 reactivity for CL is similar to that for OVA. In contrast, reactivity of the APS MAb, CL15, is strong for CL, OVA and DM. Our side-by-side study shows that 2F5 and 4E10 prefer gp41 and their respective MPER peptides to CL by orders of magnitude, whereas a classic APS MAb is plainly polyreactive.

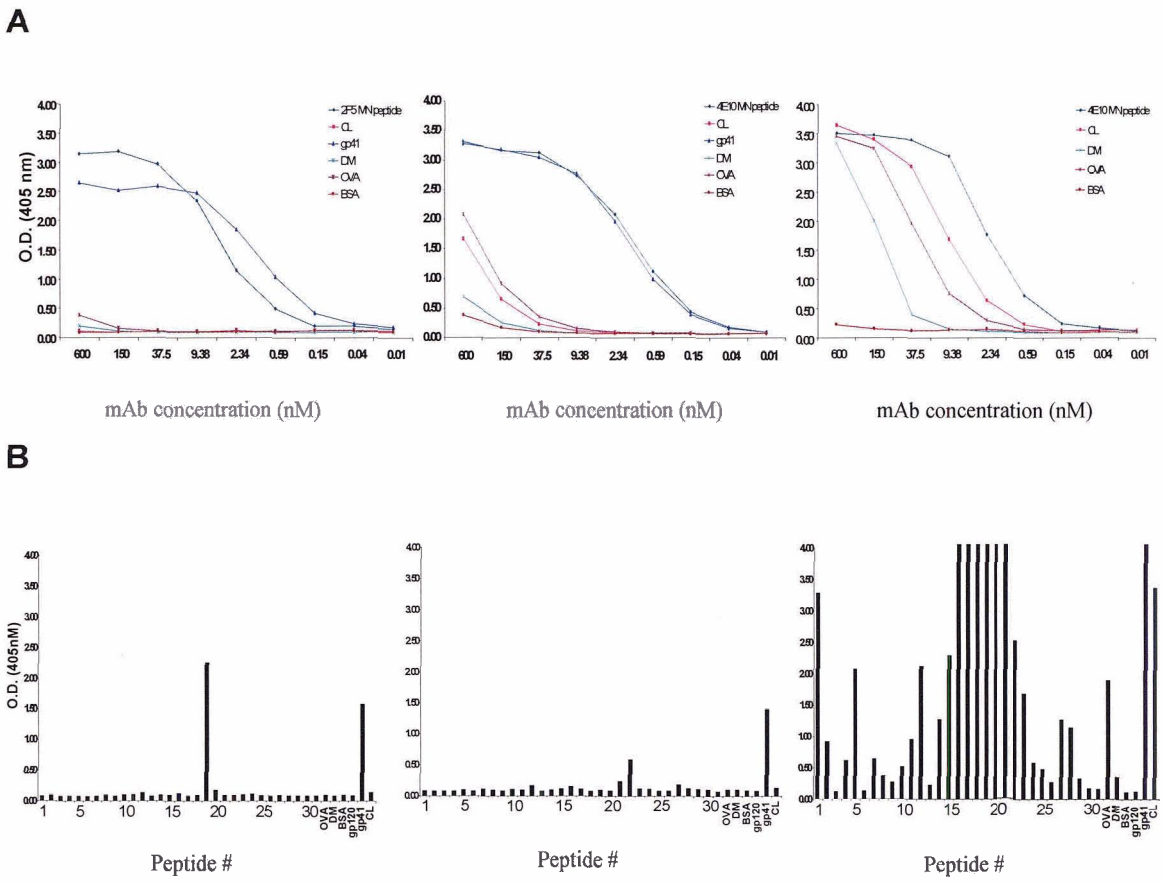


Figure 4-1: Comparison of MAbs 2F5, 4E10 and CL15.

(A) Titrations of MAb IgGs on antigens: 50 ng gp41; 400 ng 2F5 or 4E10 MN peptide; or "blocking" amounts of CL, non-fat dried milk (DM), ovalbumin (OVA) or bovine serum albumin (BSA) were used as antigens in direct ELISA following the washing and blocking procedures of Haynes et al.. O.D. values were recorded at 405 nm using an alkaline phosphatase/p-nitrophenyl phosphate developing system. (B) Antigen specificity of 2F5, 4E10 and CL15 IgG. ELISAs were performed as above, using 400 ng of each HIV-MN peptide and 10 nM MAb. The peptide sequences are shown in Table 4-1.

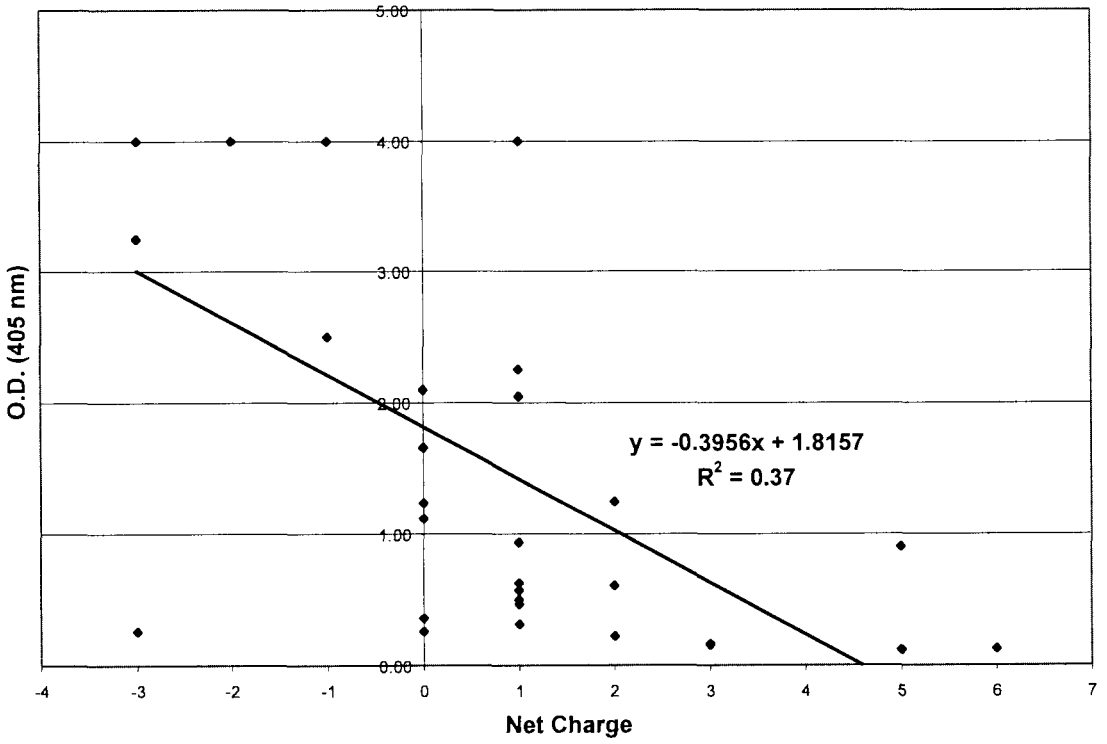


Figure 4-2: Regression of ELISA signal from 10 nM MAb CL15 on net charge of synthetic peptides.

Net charge, regression curve and R2 values were determined by ProtParam and Microsoft Excel.

To further test the specificity of the three MAbs, we studied their binding to a panel of peptides, characterized as hydrophobic to positively charged and negatively charged (see **Table 4-1**). **Figure 4-1B** shows 2F5 and 4E10 are specific for their cognate-epitope peptides relative to CL15, which is, clearly polyspecific. Statistical analysis of CL15's ELISA values revealed a correlation with net negative charge (R2=0.37; **Figure 4-2**). Thus, CL15's extensive polyreactivity appears related to a preference for net-negative charge. This is consistent with our previous studies (Menendez *et al.* 2004; Bahr 2004; Visvanathan *et al.* 2003), in which MAbs 2F5, 4E10

Table 4-1: HIV-1 MN Env (15-mer) peptides obtained from the NIH AIDS Research & Reference Reagent Program.

Peptide ID #	HIV-1 MN Env 15-mer peptide #	Peptide sequence	Net charge
1	6233	NNMVEQMHEDIISLW	-3
2	6284	CTRPNYNKRKRIHIG	5
3	6285	NYNKRKRIHIGPGRA	5
4	6298	YTTKNIIGTIRQAHC	2
5	6313	QCKIKQIINMWQEVG	1
6	6334	TKAKRRVVQREKRAA	6
7	6337	RAAIGALFLGFLGAA	1
8	6338	GALFLGFLGAAGSTM	0
9	6340	GAAGSTMGAASVTLT	0
10	6342	AASVTLTVQARLLLS	1
11	6345	LLSGIVQQQNNLLRA	1
12	6349	EAQQHMLQLTVWGIK	0
13	6351	LTVWGIKQLQARVLA	2
14	6352	GIKQLQARVLAVERY	2
15	6357	LLGFWGCSGKLICTT	1
16	6358	WGCSGKLICTTTVPW	1
17	6363	NKSLDDIWNNMTWMQ	-1
18	6365	NNMTWMQWEREIDNY	-2
19	6373	NEQELLELDKWASLW	-3
20	6374	LLELDKWASLWNWFD	-2
21	6375	DKWASLWNWFDITNW	-1
22	6376	SLWNWFDITNWLWY1	-1
23	6377	WFDITNWLWYIKIFI	0
24	6379	WYIKIFIMIVGGLVG	1
25	6381	IVGGLVGLRIVFAVL	1
26	6390	PRGPDRPEGIEEEGG	-3
27	6401	YHHRDLLLIAARIVE	0
28	6402	DLLLIAARIVELLGR	0
29	6416	VIEVLQRAGRAILHI	1
30	6417	LQRAGRAILHIPTRI	3
31	6418	GRAILHIPTRIRQGL	3

The peptides vary in chemical composition from hydrophobic to positively charged and negatively charged (as defined by the net charge of the peptides.) The sequences of the peptides bearing the 2F5 and 4E10 epitopes (ID # 19 and # 22, respectively) are shown in red.

and CL15 were used to screen phage-displayed, random peptide libraries. The behavior of each MAb was polyreactive to different degrees. All 2F5-selected peptides shared a consensus sequence (D(K/R)W), and half of 4E10-selected peptides shared > 3 residues in the (N/S)WF(N/D)IT core; however, CL15 selected peptides with completely unrelated sequences, indicating that it binds a very wide range of sequences.

4.3.2 CL15 neutralization assay

From above comparison studies, it is quite clear that 2F5 and 4E10 reactivity is different from CL15. Since 2F5 and 4E10 are bNt MAbs, it would be interesting to see if CL15 neutralizes HIV-1 at all. Thus, we sent out CL15 IgG to our collaborator (The Scripps Research Institute, USA) for neutralization assay. The method is described in section **4.2.3**. Interestingly, although CL15 binds more strongly to the 4E10-MPER peptide than does 4E10, it does not neutralize HIV-1, as shown in **Figure 4-3**.

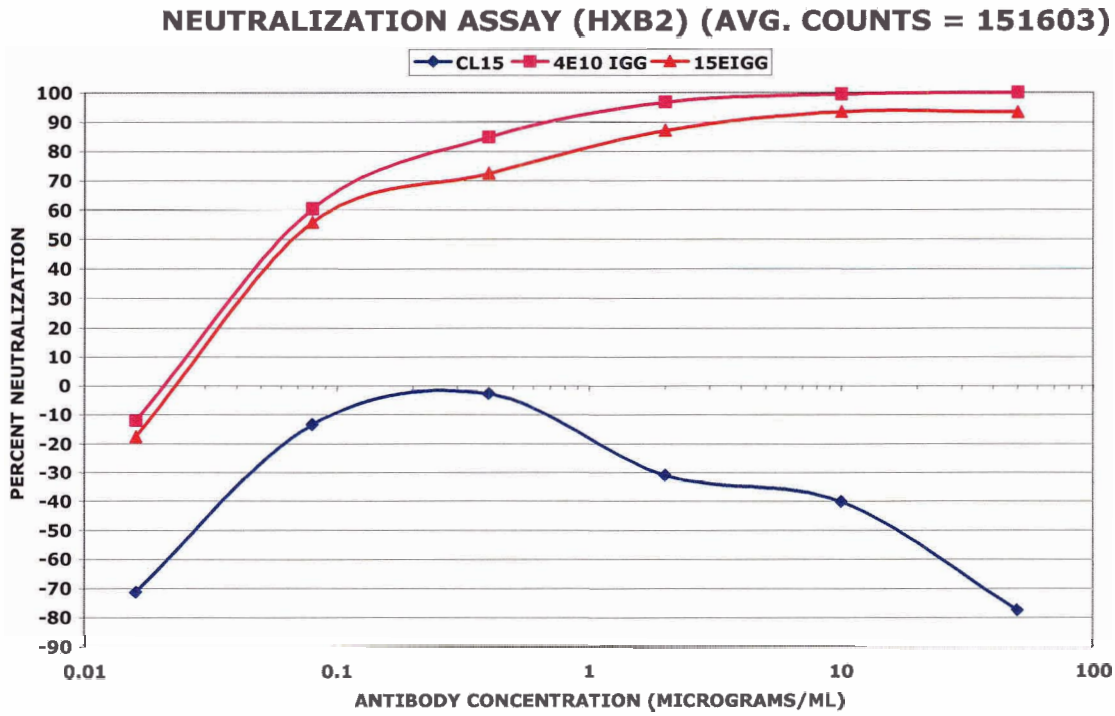


Figure 4-3: Neutralization of pseudotyped HIV-1HxB2 by MAbs CL15, 4E10 and 15E.

The average counts represent the average number of cells that are infected in the absence of antibody. Note CL15 is relatively enhancing in this assay.

4.3.3 Serum CL reactivity analysis

It has been suggested that bNt MAbs 2F5 and 4E10 are autoAbs and produced by autoreactive B cell precursors that appear after HIV-1-mediated loss of tolerance (Haynes *et al*. 2005). By this logic, a significant pool of autoreactive, naive B cells should accumulate after tolerance loss, with HIV-1 antigens driving the subsequent selection of bNtAb-producing B cells from this pool. Thus, HIV-1-bNt sera should be expected to have increased levels of autoreactivity, and perhaps, polyreactivity.

To investigate the autoreactivity in HIV-1 infection, we used the CL assay used by Haynes *et al.* (2005) and CL as autoantigen to evaluate the autoreactivity in HIV-1 positive serum samples (see **Chapter 2**, section **2.3.1**). **Figure 4-4** shows the reactivity of 12 HIV+ bNt sera with small amounts of HIV-related peptides, and with "blocking" amounts of CL or "non-specific" antigens. One of the bNt sera (#6) reacted strongly with 2F5 peptide (in agreement with another, more standard, ELISA method; see **Figure 2-2**). Whereas many of the sera reacted with CL, reactivity was, on average, stronger with the "non-specific" antigens (except BSA).

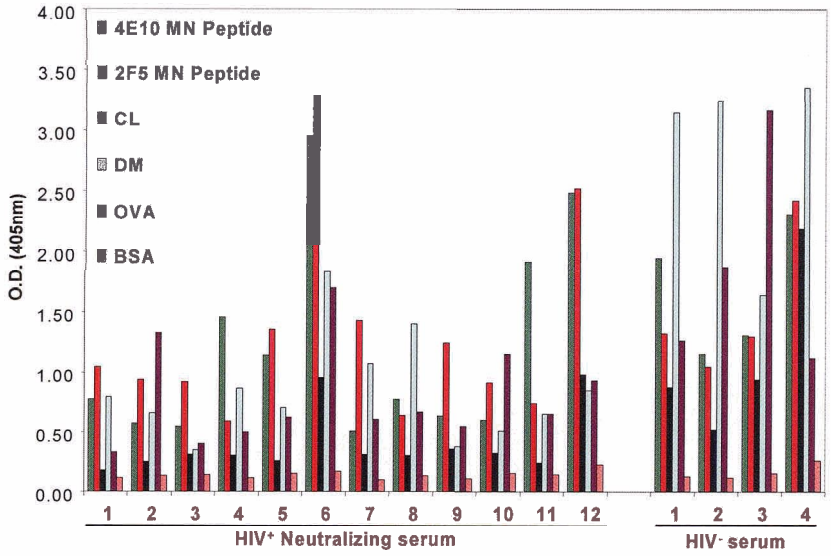


Figure 4-4: Anti-CL Abs as a marker for autoreactivity during HIV-1 infection. The assay is discribed in section 3.2.4, using sera diluted at 1:50.

To identify increases in CL reactivity over the course of HIV infection, we screened serum samples from SP, NP, RP and SC groups (**Figure 4-5**). No trend was observed to indicate stably-increasing CL reactivity in serum samples taken over time from the HIV+ individuals, or across groups, as compared to the non-specific antigens. Notably, at the serum dilution (1 in 50) used in the assay, high background (*e.g.* high signals on 4E10 and 2F5 peptides for sera that are negative in **Figure 2-2**) was observed. We then tried a different serum dilution, 1 in 250. As is shown in **Figure 4-6**, again, no significant increase of cardiolipin reactivity was observed. At the same time, peptide signals are more comparable to **Figure 2-2** now. Note that 4E10 peptide used was a different batch from the one used in **Figure 2-2,** and the average 4E10 signal was thus higher. Taken together, our results indicate that the CL reactivity of HIV^+ and uninfected people, as measured by this assay, even at dilutions that gave very high background (*e.g.*, DM, OVA, peptides, etc.) is not significant in serum samples.

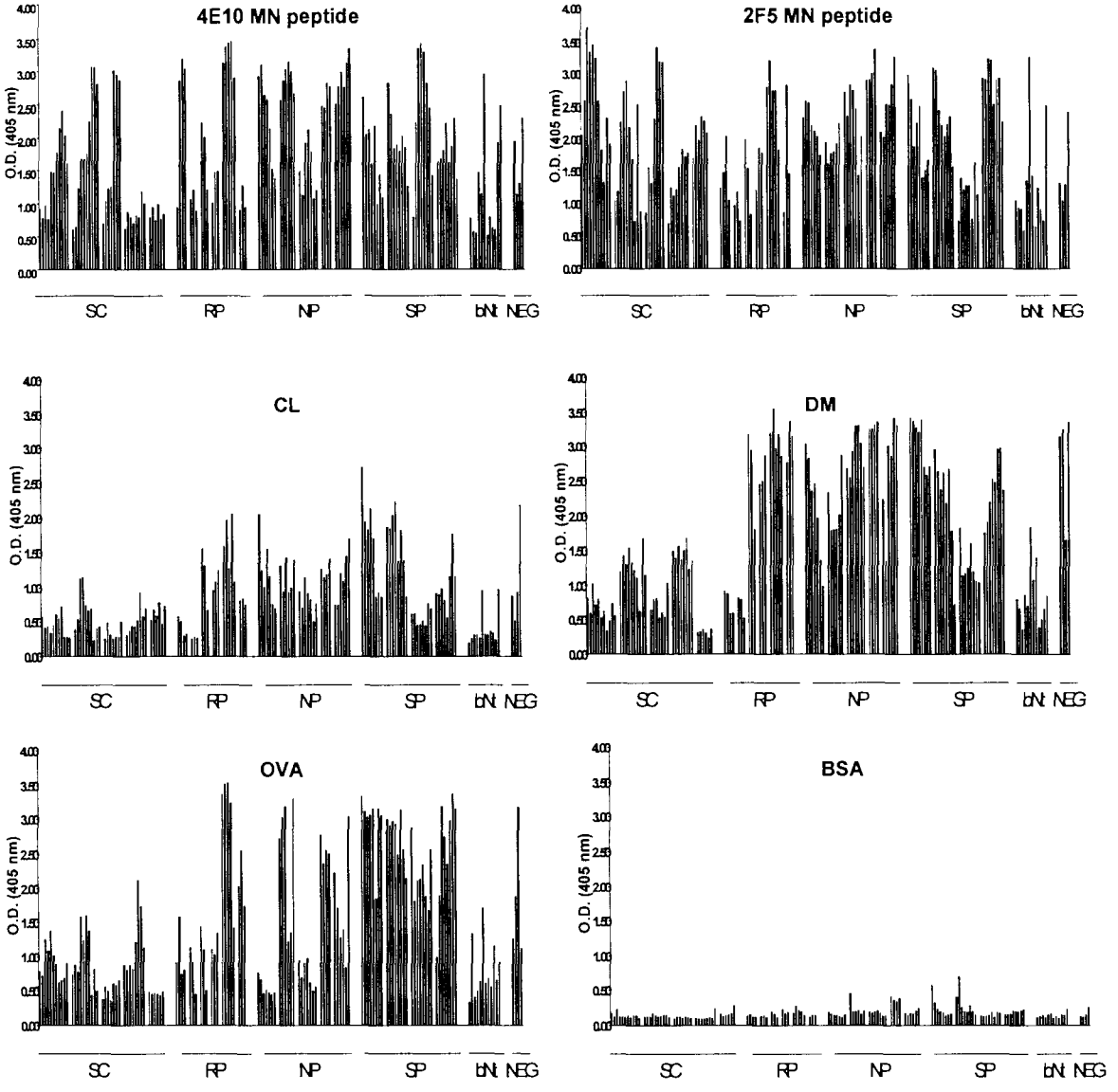


Figure 4-5: Cardiolipin reactivity in sera (1 in 50 dilution).

Serum samples from 32 HIV-1+ donors were grouped by clinical course as slow (SP), rapid (RP) and normal progressors (NP), or, covering the first 3-5 years of infection, starting at seroconversion (SC). Sera (1 in 50 dilution) were also tested from 12 HIV+ bNt donors (bNts), and 4 healthy volunteers (NEGs). Serum samples from a given HIV+ individual span over 15 years for SPs, 9 years for NPs, 4-6 years for RPs, 1-5 years for SCs, and comprise only single sample taken from the NEGs. X-axis represents patients in each group: SC1-5, RP1-6, NP1-5 and SP1-4. Sera were tested on Peptides, cardiolipin (CL), non-fat dried milk (DM), ovalbumin (OVA) and bovine serum albumin (BSA) as described in Methods 4.3.3. The Y-axis depicts the ELISA O.D. values at 405 nm.

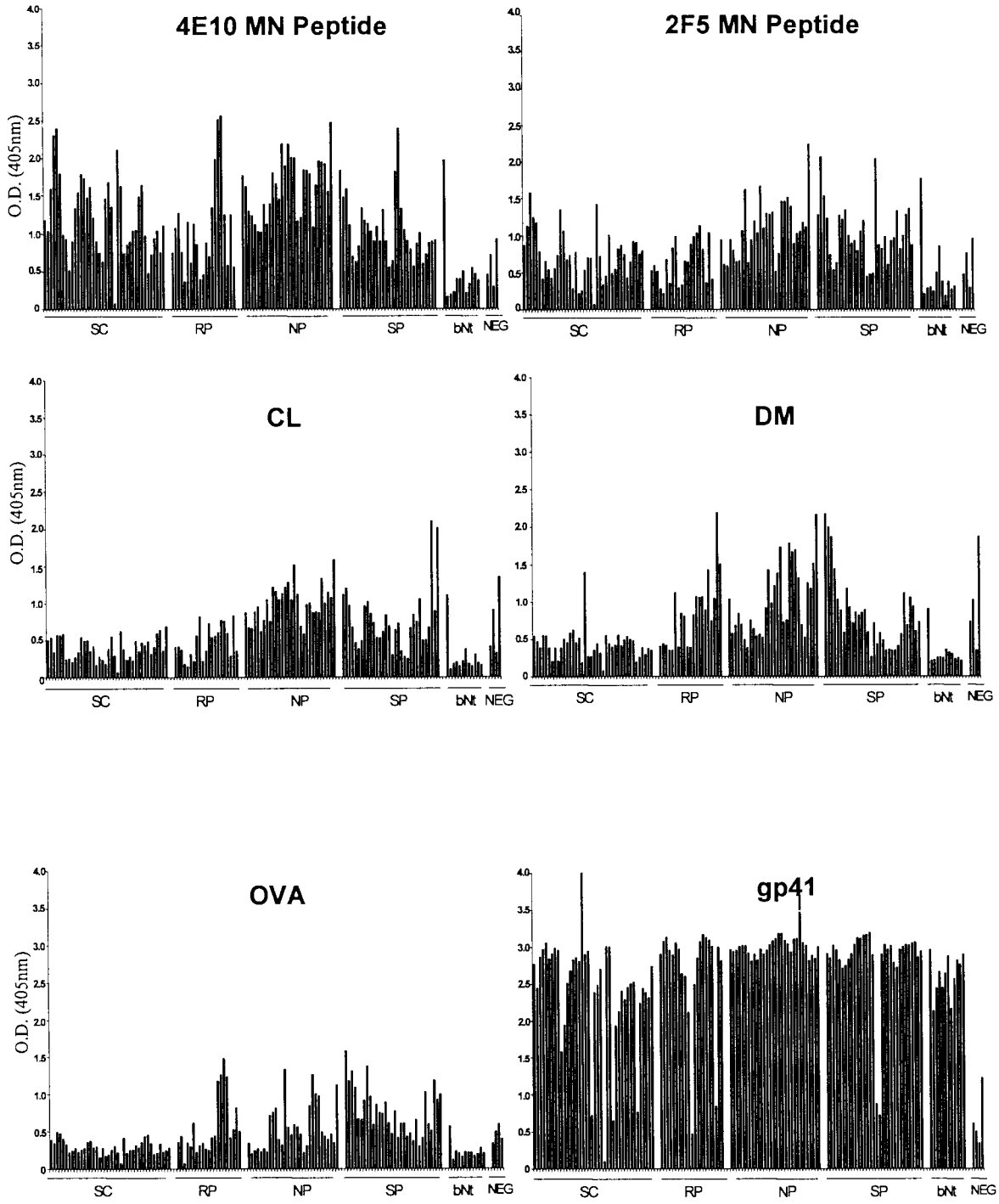


Figure 4-6: Cardiolipin reactivity in sera (1 in 250 dilution).

Sera used were 1 in 250 dilution. See **Figure 4-6** for sample and method details.

4.4 Discussion

2F5 and 4E10 were both generated in the early 1990's by electrofusion and Epstein-Barr virus transformation of B lymphocytes from HIV-1-positive donors (Buchacher *et al.* 1994). Both 2F5 and 4E10 were shown early to be able to neutralize a broad range of both laboratory-adapted and primary-isolates of HIV-1 (Conley *et al.* 1994; D'Souza *et al.* 1994). As for their neutralization capability, 2F5 is the most potent mNt Ab whereas 4E10 has the broadest cross-reactivity but is moderately potent (Binley *et al.* 2004). In passive transfer studies, 2F5 and 4E10 MAbs conferred at least partial protection against viral challenge in animal models (Conley *et al.* 1996; Gauduin *et al.* 1997; Baba *et al.* 2000; Mascola *et al.* 2000; Veazey *et al.* 2003; Haigwood *et al.* 2004). In humans, the first direct evidence that bNtAbs have the capacity to contain viremia in established HIV-1 infection was shown last year in a clinic trial with a combination of 2F5, 4E10 and another bNt MAb, 2G12 (Trkola *et al.* 2005).

In this study, we first investigated the hypothesis that 2F5 and 4E10 are autoAbs. Our results support the idea that HIV-1 bNtAbs are not unusually polyreactive or autoreactive. The reactivity of MAbs 2F5 and 4E10 with CL was insignificant compared to their HIV-related epitopes, or to a genuine APS MAb. Moreover, elevated CL reactivity was not observed in bNt sera, nor were stable increases observed during any phase of HIV-1 infection. This low CL reactivity may reflect our CL assay (Haynes *et al.* 2005), which differs from the one used clinically. Similar reactivity patterns were obtained from the sera using a more standard ELISA, indicating that the high serum concentration used (1:50) was responsible for the observed polyreactivity. At higher serum dilutions (1:250), CL reactivity should not be observed, and under those

conditions, only some sera react with DM and OVA, while binding to the 4E10 and 2F5 peptides is specific, in that it can be blocked by gp41 (**Figure 2-2** in **Chapter 2**). Firmer conclusions concerning HIV-related autoreactivity could be drawn from more comprehensive analyses (e.g., using autoantigen microarrays (Robinson *et al.* 2002)).

There have been studies showing NtAbs against MPER in natural infection (Broliden *et al.* 1992; Braibant *et al.* 2006). Broliden *et al.* first described NtAbs against MPER (Broliden *et al.* 1992), and found that neutralization activity in sera was inhibited when pre-incubated with a linear peptide QQEKNEQELLELDKW. In another recent study (Braibant *et al.* 2006), biotinylated MAb 2F5 and 4E10 were used to compete corresponding 2F5 and 4E10-like Abs (if any) in the sera. The authors stated that 2F5-like antibodies were detected in all the sera from SP, although the level is low. Note that in this study, the competition assay could only show cross-reactivity. To clearly show neutralization activity of certain serum Ab, separate the Ab from the serum, and do neutralization assay may be one of the most convincing methods. Taken together, protective NtAbs against MPER are represented in the human B-cell repertoire; future work should focus on the design of new immunogens and new approaches to induce these Abs with candidate vaccines.

CHAPTER 5: CONCLUSIONS

Developing a prophylactic vaccine offers the best hope for curbing the AIDS pandemic on a global level. Despite the success of current anti-retroviral drugs in slowing the onset of AIDS, they are not the final answer since drug-resistant isolates of HIV are becoming more and more common. In order to design a vaccine, it is important to know the type of immune response that needs to be produced for induction of protective immunity. In general, the Ab response is the predominant immunity to extracellular pathogens as opposed to cytotoxic T-cells that mediate the response against intracellular pathogens. HIV-1 is a classic intracellular pathogens, and cytotoxic T-cell responses have been shown to control the initial viremia (Pantaleo *et al.* 1994) (Koup *et al.* 1994) (Connick *et al.* 1996) (D'Souza *et al.* 1996). However, most current viral vaccines in use appear to work by eliciting NtAb responses that prevent the initial infection of cells by the viruse. This may reflect a primary role for Ab neutralization in prevention of viral disease and an important role for $CD8^+$ T cells in resolution of disease. It should be noted that most vaccines available have been developed principally by empiric methods, with an understanding of the type of effective immunity being subsequent to vaccine development.

In general, vaccines are more successful when mimicking a normally effective immune response (e.g., measles) than when trying to improve partial or absent immunity (e.g., TB, HIV) in natural infections. There is still no compelling evidence to show that the natural immune response can prevent initial HIV-1 infection. The observation that

most infected individuals maintain a healthy, asymptomatic state for many years after infection suggests that there may be immune responses that contain the infection but that eventually fail. Understanding the mechanism of such protective immunity might allow the design of a preventive vaccine.

In HIV-1 infection, Nt Abs usually appear after viremia has already decreased, suggesting that they are not responsible for the initial reduction in viral load (Pantaleo, *et al.* 2004). However, some studies have suggested that the antiviral humoral response may be more pronounced in individuals with long-term, nonprogressing infection (Pilgrim, *et al.* 1997). Neutralizing Abs mainly disrupt two stages of the HIV-1 viral life cycle, including viral attachment to target cells and membrane fusion, which are mediated by envelope proteins gp120 and gp41 separately. Specific epitopes on envelope proteins are therefore major targets for protective Abs and for epitope targeted vaccine design. One critical prerequisite for an epitope-targeted vaccine is to identify protective B-cell epitopes that are immunogenic in most humans.

In **Chapter 2**, the Ab response in HIV-1 infection was studied by linear epitope mapping. We showed that the polyclonal Ab response in natural infection is mostly directed against immunodominant regions, such as V3 region on gp120 protein and cluster I region on gp41. The Ab response is relatively stable during long-term infection compared with acute infection. Among groups with different clinical outcomes in long-term infection, rapid progressors had a broader and more vigorous Ab response than normal progressors and slow progressors. This may be a result from a higher load of replicating virus in the rapid progressors.

The Ab response against the MPER was detected in our study in serum samples from both acute and long-term infections. In **Chapter 3**, an affinity purification method was developed to purify peptide-specific Abs from sera. Unfortunately, the results from the neutralization assay for these Abs were not conclusive due to small amounts of the purified Abs. It would be interesting to purify more Abs and test for neutralization activity against more resistant primary isolates. Note that such neutralization activity in the purified Abs, if any, may represent a very small part of the neutralization activity in the serum, according to our preliminary data.

In **Chapter 4**, two bNt MAbs, 2F5 and 4E10 were tested for their reactivity properties based on a study that shows some evidence that these Abs are autoAbs binding to self-antigens. In our study, 2F5 and 4E10 are different from a typical autoAb, CL15, in both binding specificity and apparent affinity. As for autoimmunity during HIV-1 infection, there is no cardiolipin reactivity in general in the serum samples tested. No more conclusive results could be drawn under our assay conditions. Therefore, more comprehensive studies such as microarray could be used to detect autoimmunity in HIV-1 positive serum samples. Together with our collaborators, premilinary data in microarray have shown that MAbs 2F5 and 4E10 behave differrently from CL15 on chips spotted with a variety of auto-antigens (Fathman *et al.* 2005) (data not shown). Therefore, bNt MAbs 2F5 and 4E10 do not seem to be autoreactive.

Combined with our data showing that the MPER is immunogenic *in vivo*, this highly conserved region on gp41 should be further considered as a component for an HIV-1 vaccine. As for an epitope-targeted vaccine, the MPER may need to be constrained to better mimic its conformation on the virus. Identifying new Nt or non-Nt

Abs against the MPER will help our understanding of the structure of MPER and exposure of its Nt epitopes. In addition, novel vaccination approaches such as adjuvants, prime-boost strategy and new vectors will be useful in the effort to elicit bNtAbs targeting the MPER and other Nt epitopes.

REFERENCES

Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M. and Berger, E. A. (1996). "Cc Ckr5: A Rantes, Mip-1alpha, Mip-1beta Receptor as a Fusion Cofactor for Macrophage-Tropic HIV-1." Science **272**(5270): 1955-8.

Ariyoshi, K., Harwood, E., Chiengsong-Popov, R. and Weber, J. (1992). "Is Clearance of HIV-1 Viraemia at Seroconversion Mediated by Neutralising Antibodies?" Lancet **340**(8830): 1257-8.

Baba, T. W., Liska, V., Hofmann-Lehmann, R., Vlasak, J., Xu, W., Ayehunie, S., Cavacini, L. A., Posner, M. R., Katinger, H., Stiegler, G., Bernacky, B. J., Rizvi, T. A., Schmidt, R., Hill, L. R., Keeling, M. E., Lu, Y., Wright, J. E., Chou, T. C. and Ruprecht, R. M. (2000). "Human Neutralizing Monoclonal Antibodies of the IgG1 Subtype Protect against Mucosal Simian-Human Immunodeficiency Virus Infection." Nat Med **6**(2): 200-6.

Bahr, S.L. M.Sc. Thesis. *Peptide Markers for the HIV-1 Neutralizing Antibody, 4E10.* Simon Fraser University, Burnaby, BC, Canada. (2004).

Barbato, G., Bianchi, E., Ingallinella, P., Hurni, W. H., Miller, M. D., Ciliberto, G., Cortese, R., Bazzo, R., Shiver, J. W. and Pessi, A. (2003). "Structural Analysis of the Epitope of the Anti-HIV Antibody 2F5 Sheds Light into Its Mechanism of Neutralization and HIV Fusion." J Mol Biol **330**(5): 1101-15.

Belshe, R. B., Gorse, G. J., Mulligan, M. J., Evans, T. G., Keefer, M. C., Excler, J. L., Duliege, A. M., Tartaglia, J., Cox, W. I., McNamara, J., Hwang, K. L., Bradney, A., Montefiori, D. and Weinhold, K. J. (1998). "Induction of Immune Responses to HIV-1 by Canarypox Virus (ALVAC) HIV-1 and gp120 SF-2 Recombinant Vaccines in Uninfected Volunteers. NIAID AIDS Vaccine Evaluation Group." AIDS **12**(18): 2407-15.

Barre-Sinoussi, F., Chermann, J. C., Rey, F., Nugeyre, M. T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W. and Montagnier, L. (1983). "Isolation of a T-Lymphotropic Retrovirus from a Patient at Risk for Acquired Immune Deficiency Syndrome (AIDS)." Science **220**(4599): 868-71.

Berkower, I., Murphy, D., Smith, C. C. and Smith, G. E. (1991). "A Predominant Group-Specific Neutralizing Epitope of Human Immunodeficiency Virus Type 1 Maps to Residues 342 to 511 of the Envelope Glycoprotein gp120." J Virol **65**(11): 5983-90.

Binley, J. M., Jin, X., Huang, Y., Zhang, L., Cao, Y., Ho, D. D. and Moore, J. P. (1998). "Persistent Antibody Responses but Declining Cytotoxic T-Lymphocyte

Responses to Multiple Human Immunodeficiency Virus Type 1 Antigens in a Long-Term Nonprogressing Individual with a Defective P17 Proviral Sequence and No Detectable Viral Rna Expression." J Virol **72**(4): 3472-4.

Binley, J. M., Wrin, T., Korber, B., Zwick, M. B., Wang, M., Chappey, C., Stiegler, G., Kunert, R., Zolla-Pazner, S., Katinger, H., Petropoulos, C. J. and Burton, D. R. (2004). "Comprehensive Cross-Clade Neutralization Analysis of a Panel of Anti-Human Immunodeficiency Virus Type 1 Monoclonal Antibodies." J Virol **78**(23): 13232-52.

Bjorndal, A., Deng, H., Jansson, M., Fiore, J. R., Colognesi, C., Karlsson, A., Albert, J., Scarlatti, G., Littman, D. R. and Fenyo, E. M. (1997). "Coreceptor Usage of Primary Human Immunodeficiency Virus Type 1 Isolates Varies According to Biological Phenotype." J Virol **71**(10): 7478-87.

Bradney, A. P., Scheer, S., Crawford, J. M., Buchbinder, S. P. and Montefiori, D. C. (1999). "Neutralization Escape in Human Immunodeficiency Virus Type 1-Infected Long-Term Nonprogressors." J Infect Dis **179**(5): 1264-1267.

Braibant, M., Brunet, S., Costagliola, D., Rouzioux, C., Agut, H., Katinger, H., Autran, B. and Barin, F. (2006). "Antibodies to Conserved Epitopes of the HIV-1 Envelope in Sera from Long-Term Non-Progressors: Prevalence and Association with Neutralizing Activity." AIDS **20**(15): 1923-30.

Broliden, P. A., von Gegerfelt, A., Clapham, P., Rosen, J., Fenyo, E. M., Wahren, B. and Broliden, K. (1992). "Identification of Human Neutralization-Inducing Regions of the Human Immunodeficiency Virus Type 1 Envelope Glycoproteins." Proc Natl Acad Sci U S A **89**(2): 461-5.

Buchacher, A., Predl, R., Strutzenberger, K., Steinfellner, W., Trkola, A., Purtscher, M., Gruber, G., Tauer, C., Steindl, F., Jungbauer, A. (1994). "Generation of Human Monoclonal Antibodies against HIV-1 Proteins; Electrofusion and Epstein-Barr Virus Transformation for Peripheral Blood Lymphocyte Immortalization." AIDS Res Hum Retroviruses **10**(4): 359-69.

Bullough, P. A., Hughson, F. M., Skehel, J. J. and Wiley, D. C. (1994). "Structure of Influenza Haemagglutinin at the pH of Membrane Fusion." Nature **371**(6492): 37-43.

Buratti, E., McLain, L., Tisminetzky, S., Cleveland, S. M., Dimmock, N. J. and Baralle, F. E. (1998). "The Neutralizing Antibody Response against a Conserved Region of Human Immunodeficiency Virus Type 1 gp41 (Amino Acid Residues 731-752) Is Uniquely Directed against a Conformational Epitope." J Gen Virol **79 (Pt 11)**: 2709-16.

Bures, R., Morris, L., Williamson, C., Ramjee, G., Deers, M., Fiscus, S. A., Abdool-Karim, S. and Montefiori, D. C. (2002). "Regional Clustering of Shared Neutralization Determinants on Primary Isolates of Clade C Human Immunodeficiency Virus Type 1 from South Africa." J Virol **76**(5): 2233-44.

Burton, D. R. (1997). "A Vaccine for HIV Type 1: The Antibody Perspective." Proc Natl Acad Sci U S A **94**(19): 10018-23.

Burton, D. R., Stanfield, R. L. and Wilson, I. A. (2005). "Antibody Vs. HIV in a Clash of Evolutionary Titans." Proc Natl Acad Sci U S A **102**(42): 14943-8.

Caffrey, M., Cai, M., Kaufman, J., Stahl, S. J., Wingfield, P. T., Covell, D. G., Gronenborn, A. M. and Clore, G. M. (1998). "Three-Dimensional Solution Structure of the 44 kDa Ectodomain of SIV gp41." EMBO J **17**(16): 4572-84.

Calarese, D. A., Scanlan, C. N., Zwick, M. B., Deechongkit, S., Mimura, Y., Kunert, R., Zhu, P., Wormald, M. R., Stanfield, R. L., Roux, K. H., Kelly, J. W., Rudd, P. M., Dwek, R. A., Katinger, H., Burton, D. R. and Wilson, I. A. (2003). "Antibody Domain Exchange Is an Immunological Solution to Carbohydrate Cluster Recognition." Science **300**(5628): 2065-71.

Calarota, S., Jansson, M., Levi, M., Broliden, K., Libonatti, O., Wigzell, H. and Wahren, B. (1996). "Immunodominant Glycoprotein 41 Epitope Identified by Seroreactivity in HIV Type 1-Infected Individuals." AIDS Res Hum Retroviruses **12**(8): 705-13.

Cao, Y., Qin, L., Zhang, L., Safrit, J. and Ho, D. D. (1995). "Virologic and Immunologic Characterization of Long-Term Survivors of Human Immunodeficiency Virus Type 1 Infection." N Engl J Med **332**(4): 201-8.

Cardoso, R. M., Zwick, M. B., Stanfield, R. L., Kunert, R., Binley, J. M., Katinger, H., Burton, D. R. and Wilson, I. A. (2005). "Broadly Neutralizing Anti-HIV Antibody 4E10 Recognizes a Helical Conformation of a Highly Conserved Fusion-Associated Motif in gp41." Immunity **22**(2): 163-73.

Carr, C. M. and Kim, P. S. (1993). "A Spring-Loaded Mechanism for the Conformational Change of Influenza Hemagglutinin." Cell **73**(4): 823-32.

Chan, D. C., Fass, D., Berger, J. M. and Kim, P. S. (1997). "Core Structure of gp41 from the HIV Envelope Glycoprotein." Cell **89**(2): 263-73.

Chiodi, F., von Gegerfeldt, A., Albert, J., Fenyo, E. M., Gaines, H., von Sydow, M., Biberfeld, G., Parks, E. and Norrby, E. (1987). "Site-Directed Elisa with Synthetic Peptides Representing the Hiv Transmembrane Glycoprotein." J Med Virol **23**(1): 1-9.

Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P. D., Wu, L., Mackay, C. R., LaRosa, G., Newman, W., Gerard, N., Gerard, C. and Sodroski, J. (1996). "The Beta-Chemokine Receptors CCR3 and CCR5 Facilitate Infection by Primary HIV-1 Isolates." Cell **85**(7): 1135-48.

Chukwuocha, R. U., Zhu, M., Cho, C. S., Visvanathan, S., Hwang, K. K., Rahman, A. and Chen, P. P. (2002). "Molecular and Genetic Characterizations of Five Pathogenic and Two Non-Pathogenic Monoclonal Antiphospholipid Antibodies." Mol Immunol **39**(5-6): 299-311.

Cleveland, S. M., Jones, T. D. and Dimmock, N. J. (2000). "Properties of a Neutralizing Antibody That Recognizes a Conformational Form of Epitope ERDRD in the gp41 C-Terminal Tail of Human Immunodeficiency Virus Type 1." J Gen Virol **81**(Pt 5): 1251-60.

Cleveland, S. M., McLain, L., Cheung, L., Jones, T. D., Hollier, M. and Dimmock, N. J. (2003). "A Region of the C-Terminal Tail of the gp41 Envelope Glycoprotein of Human Immunodeficiency Virus Type 1 Contains a Neutralizing Epitope: Evidence for Its Exposure on the Surface of the Virion." J Gen Virol **84**(Pt 3): 591-602.

Cocchi, F., DeVico, A. L., Garzino-Demo, A., Cara, A., Gallo, R. C. and Lusso, P. (1996). "The V3 Domain of the HIV-1 gp120 Envelope Glycoprotein Is Critical for Chemokine-Mediated Blockade of Infection." Nat Med **2**(11): 1244-7.

Collis, A. V., Brouwer, A. P. and Martin, A. C. (2003). "Analysis of the Antigen Combining Site: Correlations between Length and Sequence Composition of the Hypervariable Loops and the Nature of the Antigen." J Mol Biol **325**(2): 337-54.

Conley, A. J., Kessler, J. A., 2nd, Boots, L. J., Tung, J. S., Arnold, B. A., Keller, P. M., Shaw, A. R. and Emini, E. A. (1994). "Neutralization of Divergent Human Immunodeficiency Virus Type 1 Variants and Primary Isolates by IAM-41-2F5, an Anti-gp41 Human Monoclonal Antibody." Proc Natl Acad Sci U S A **91**(8): 3348-52.

Conley, A. J., Kessler, J. A., II, Boots, L. J., McKenna, P. M., Schleif, W. A., Emini, E. A., Mark, G. E., III, Katinger, H., Cobb, E. K., Lunceford, S. M., Rouse, S. R. and Murthy, K. K. (1996). "The Consequence of Passive Administration of an Anti-Human Immunodeficiency Virus Type 1 Neutralizing Monoclonal Antibody before Challenge of Chimpanzees with a Primary Virus Isolate." J Virol **70**(10): 6751-8.

Connick, E., Marr, D. G., Zhang, X. Q., Clark, S. J., Saag, M. S., Schooley, R. T. and Curiel, T. J. (1996). "HIV-Specific Cellular and Humoral Immune Responses in Primary HIV Infection." AIDS Res Hum Retroviruses **12**(12): 1129-40.

Connor, R. I., Chen, B. K., Choe, S. and Landau, N. R. (1995). "Vpr Is Required for Efficient Replication of Human Immunodeficiency Virus Type-1 in Mononuclear Phagocytes." Virology **206**(2): 935-44.

Cotropia, J., Ugen, K. E., Kliks, S., Broliden, K., Broliden, P. A., Hoxie, J. A., Srikantan, V., Williams, W. V. and Weiner, D. B. (1996). "A Human Monoclonal Antibody to Hiv-1 Gp41 with Neutralizing Activity against Diverse Laboratory Isolates." J Acquir Immune Defic Syndr Hum Retrovirol **12**(3): 221-32.

D'Souza, M. P., Geyer, S. J., Hanson, C. V., Hendry, R. M. and Milman, G. (1994). "Evaluation of Monoclonal Antibodies to HIV-1 Envelope by Neutralization and Binding Assays: An International Collaboration." AIDS **8**(2): 169-81.

D'Souza, M. P. and Mathieson, B. J. (1996). "Early Phases of HIV Type 1 Infection." AIDS Res Hum Retroviruses **12**(1): 1-9.

Daar, E. S., Li, X. L., Moudgil, T. and Ho, D. D. (1990). "High Concentrations of Recombinant Soluble CD4 Are Required to Neutralize Primary Human Immunodeficiency Virus Type 1 Isolates." Proc Natl Acad Sci U S A **87**(17): 6574-8.

Dalgleish, A. G., Chanh, T. C., Kennedy, R. C., Kanda, P., Clapham, P. R. and Weiss, R. A. (1988). "Neutralization of Diverse HIV-1 Strains by Monoclonal Antibodies Raised against a gp41 Synthetic Peptide." Virology **165**(1): 209-15.

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R. E., Hill, C. M., Davis, C. B., Peiper, S. C., Schall, T. J., Littman, D. R. and Landau, N. R. (1996). "Identification of a Major Co-Receptor for Primary Isolates of HIV-1." Nature **381**(6584): 661-6.

Devash, Y., Matthews, T. J., Drummond, J. E., Javaherian, K., Waters, D. J., Arthur, L. O., Blattner, W. A. and Rusche, J. R. (1990). "C-Terminal Fragments of gp120 and Synthetic Peptides from Five HTLV-III Strains: Prevalence of Antibodies to the HTLV-III-MN Isolate in Infected Individuals." AIDS Res Hum Retroviruses **6**(3): 307-16.

Doranz, B. J., Rucker, J., Yi, Y., Smyth, R. J., Samson, M., Peiper, S. C., Parmentier, M., Collman, R. G. and Doms, R. W. (1996). "A Dual-Tropic Primary HIV-1 Isolate That Uses Fusin and the Beta-Chemokine Receptors CKR-5, CKR-3, and CKR-2b as Fusion Cofactors." Cell **85**(7): 1149-58.

Douek, D. C. (2003). "Disrupting T-Cell Homeostasis: How HIV-1 Infection Causes Disease." AIDS Rev **5**(3): 172-7.

Dragic, T., Litwin, V., Allaway, G. P., Martin, S. R., Huang, Y., Nagashima, K. A., Cayanan, C., Maddon, P. J., Koup, R. A., Moore, J. P. and Paxton, W. A. (1996). "HIV-1 Entry into $CD4^+$ Cells Is Mediated by the Chemokine Receptor CC-CKR-5." Nature **381**(6584): 667-73.

Earl, P. L., Moss, B. and Doms, R. W. (1991). "Folding, Interaction with GRP78-BiP, Assembly, and Transport of the Human Immunodeficiency Virus Type 1 Envelope Protein." J Virol **65**(4): 2047-55.

Eckhart, L., Raffelsberger, W., Ferko, B., Klima, A., Purtscher, M., Katinger, H. and Ruker, F. (1996). "Immunogenic Presentation of a Conserved gp41 Epitope of Human Immunodeficiency Virus Type 1 on Recombinant Surface Antigen of Hepatitis B Virus." J Gen Virol **77 (Pt 9)**: 2001-8.

Edelman, G. M. (1991). "Antibody Structure and Molecular Immunology." Scand J Immunol **34**(1): 1-22.

Elkins, M. K., Vittinghoff, E., Baranzini, S. E., Hecht, F. M., Sriram, U., Busch, M. P., Levy, J. A. and Oksenberg, J. R. (2005). "Longitudinal Analysis of B Cell Repertoire and Antibody Gene Rearrangements During Early HIV Infection." Genes Immun **6**(1): 66-9.

Emini, E. A., Schleif, W. A., Nunberg, J. H., Conley, A. J., Eda, Y., Tokiyoshi, S., Putney, S. D., Matsushita, S., Cobb, K. E., Jett, C. M., Eichbergparallel, J. W., Murthy, K. K. (1992). "Prevention of HIV-1 Infection in Chimpanzees by gp120 V3 Domain-Specific Monoclonal Antibody." Nature **355**(6362): 728-30.

Fathman, C. G., Soares, L., Chan, S. M. and Utz, P. J. (2005). "An Array of Possibilities for the Study of Autoimmunity." Nature **435**(7042): 605-11.

Feng, Y., Broder, C. C., Kennedy, P. E. and Berger, E. A. (1996). "HIV-1 Entry Cofactor: Functional cDNA Cloning of a Seven-Transmembrane, G Protein-Coupled Receptor." Science **272**(5263): 872-7.

Ferrantelli, F., Kitabwalla, M., Rasmussen, R. A., Cao, C., Chou, T.-C., Katinger, H., Stiegler, G., Cavacini, L. A., Bai, Y., Cotropia, J., Ugen, K. E. and Ruprecht, R. M. (2004). "Potent Cross-Group Neutralization of Primary Human Immunodeficiency Virus Isolates with Monoclonal Antibodies--Implications for Acquired Immunodeficiency Syndrome Vaccine." J Infect Dis **189**(1): 71-74.

Fouts, T. R., Binley, J. M., Trkola, A., Robinson, J. E. and Moore, J. P. (1997). "Neutralization of the Human Immunodeficiency Virus Type 1 Primary Isolate JR-FL by Human Monoclonal Antibodies Correlates with Antibody Binding to the Oligomeric Form of the Envelope Glycoprotein Complex." J Virol **71**(4): 2779-85.

Freed, E. O. and Martin, M. A. (1995). "The Role of Human Immunodeficiency Virus Type 1 Envelope Glycoproteins in Virus Infection." J Biol Chem **270**(41): 23883-6.

Gallaher, W. R. (1987). "Detection of a Fusion Peptide Sequence in the Transmembrane Protein of Human Immunodeficiency Virus." Cell **50**(3): 327-8.

Gallaher, W. R., Ball, J. M., Garry, R. F., Griffin, M. C. and Montelaro, R. C. (1989). "A General Model for the Transmembrane Proteins of HIV and Other Retroviruses." AIDS Res Hum Retroviruses **5**(4): 431-440.

Garrity, R. R., Rimmelzwaan, G., Minassian, A., Tsai, W. P., Lin, G., de Jong, J. J., Goudsmit, J. and Nara, P. L. (1997). "Refocusing Neutralizing Antibody Response by Targeted Dampening of an Immunodominant Epitope." J Immunol **159**(1): 279-89.

Gauduin, M. C., Parren, P. W., Weir, R., Barbas, C. F., Burton, D. R. and Koup, R. A. (1997). "Passive Immunization with a Human Monoclonal Antibody Protects Hu-PBL-SCID Mice against Challenge by Primary Isolates of HIV-1." Nat Med **3**(12): 1389-93.

Geffin, R. B., Scott, G. B., Melenwick, M., Hutto, C., Lai, S., Boots, L. J., McKenna, P. M., Kessler, J. A., 2nd and Conley, A. J. (1998). "Association of Antibody Reactivity to ELDKWA, a Glycoprotein 41 Neutralization Epitope, with Disease Progression in Children Perinatally Infected with HIV Type 1." AIDS Res Hum Retroviruses **14**(7): 579-90.

Gnann, J. W., Jr., Schwimmbeck, P. L., Nelson, J. A., Truax, A. B. and Oldstone, M. B. (1987). "Diagnosis of Aids by Using a 12-Amino Acid Peptide Representing an Immunodominant Epitope of the Human Immunodeficiency Virus." J Infect Dis **156**(2): 261-7.

Gorny, M. K., Conley, A. J., Karwowska, S., Buchbinder, A., Xu, J. Y., Emini, E. A., Koenig, S. and Zolla-Pazner, S. (1992). "Neutralization of Diverse Human Immunodeficiency Virus Type 1 Variants by an Anti-V3 Human Monoclonal Antibody." J Virol **66**(12): 7538-42.

Gorny, M. K., Xu, J. Y., Karwowska, S., Buchbinder, A. and Zolla-Pazner, S. (1993). "Repertoire of Neutralizing Human Monoclonal Antibodies Specific for the V3 Domain of HIV-1 gp120." J Immunol **150**(2): 635-43.

Gorny, M. K., Moore, J. P., Conley, A. J., Karwowska, S., Sodroski, J., Williams, C., Burda, S., Boots, L. J. and Zolla-Pazner, S. (1994). "Human Anti-V2 Monoclonal Antibody That Neutralizes Primary but Not Laboratory Isolates of Human Immunodeficiency Virus Type 1." J Virol **68**(12): 8312-20.

Gorny, M. K., VanCott, T. C., Hioe, C., Israel, Z. R., Michael, N. L., Conley, A. J., Williams, C., Kessler, J. A., 2nd, Chigurupati, P., Burda, S. and Zolla-Pazner, S. (1997). "Human Monoclonal Antibodies to the V3 Loop of HIV-1 with Intra- and Interclade Cross-Reactivity." J Immunol **159**(10): 5114-22.

Gorny, M. K., Williams, C., Volsky, B., Revesz, K., Cohen, S., Polonis, V. R., Honnen, W. J., Kayman, S. C., Krachmarov, C., Pinter, A. and Zolla-Pazner, S. (2002). "Human Monoclonal Antibodies Specific for Conformation-Sensitive Epitopes of V3 Neutralize Human Immunodeficiency Virus Type 1 Primary Isolates from Various Clades." J Virol **76**(18): 9035-45.

Goudsmit, J., Debouck, C., Meloen, R. H., Smit, L., Bakker, M., Asher, D. M., Wolff, A. V., Gibbs, C. J., Jr. and Gajdusek, D. C. (1988). "Human Immunodeficiency Virus Type 1 Neutralization Epitope with Conserved Architecture Elicits Early Type-Specific Antibodies in Experimentally Infected Chimpanzees." Proc Natl Acad Sci U S A **85**(12): 4478-82.

Goudsmit, J., Meloen, R. H. and Brasseur, R. (1990). "Map of Sequential B Cell Epitopes of the Hiv-1 Transmembrane Protein Using Human Antibodies as Probe." Intervirology **31**(6): 327-38.

Graham, B. S. (1994). "Serological Responses to Candidate AIDS Vaccines." AIDS Res Hum Retroviruses **10** Suppl 2: S145-8.

Haigwood, N. L., Montefiori, D. C., Sutton, W. F., McClure, J., Watson, A. J., Voss, G., Hirsch, V. M., Richardson, B. A., Letvin, N. L., Hu, S. L. and Johnson, P. R. (2004). "Passive Immunotherapy in Simian Immunodeficiency Virus-Infected Macaques Accelerates the Development of Neutralizing Antibodies." J Virol **78**(11): 5983-95.

Hanson, C. V. (1994). "Measuring Vaccine-Induced HIV Neutralization: Report of a Workshop." AIDS Res Hum Retroviruses **10**(6): 645-8.

Hartley, O., Klasse, P. J., Sattentau, Q. J. and Moore, J. P. (2005). "V3: HIV's Switch-Hitter." AIDS Res Hum Retroviruses **21**(2): 171-89.

Haynes, B. F., Fleming, J., St Clair, E. W., Katinger, H., Stiegler, G., Kunert, R., Robinson, J., Scearce, R. M., Plonk, K., Staats, H. F., Ortel, T. L., Liao, H. X. and Alam, S. M. (2005). "Cardiolipin Polyspecific Autoreactivity in Two Broadly Neutralizing HIV-1 Antibodies." Science **308**(5730): 1906-08.

Hioe, C. E., Xu, S., Chigurupati, P., Burda, S., Williams, C., Gorny, M. K. and Zolla-Pazner, S. (1997). "Neutralization of HIV-1 Primary Isolates by Polyclonal and Monoclonal Human Antibodies." Int Immunol **9**(9): 1281-90.

Ho, D. D., Sarngadharan, M. G., Hirsch, M. S., Schooley, R. T., Rota, T. R., Kennedy, R. C., Chanh, T. C. and Sato, V. L. (1987). "Human Immunodeficiency Virus Neutralizing Antibodies Recognize Several Conserved Domains on the Envelope Glycoproteins." J Virol **61**(6): 2024-8.

Horal, P., Svennerholm, B., Jeansson, S., Rymo, L., Hall, W. W. and Vahlne, A. (1991). "Continuous Epitopes of the Human Immunodeficiency Virus Type 1 (HIV-1) Transmembrane Glycoprotein and Reactivity of Human Sera to Synthetic Peptides Representing Various HIV-1 Isolates." J Virol **65**(5): 2718-23.

Huang, X. J., Hope, T. J., Bond, B. L., McDonald, D., Grahl, K. and Parslow, T. G. (1991). "Minimal Rev-Response Element for Type 1 Human Immunodeficiency Virus." J Virol **65**(4): 2131-4.

Letvin, N. L. (1998). "Progress in the Development of an HIV-1 Vaccine." Science **280**(5371): 1875-80.

Moog, C., Fleury, H. J., Pellegrin, I., Kirn, A. and Aubertin, A. M. (1997). "Autologous and Heterologous Neutralizing Antibody Responses Following Initial Seroconversion in Human Immunodeficiency Virus Type 1-Infected Individuals." J Virol **71**(5): 3734-3741.

Janeway, C. A. (2005). Immunobiology : The Immune System in Health and Disease. New York, Garland Science Publishing.

Javaherian, K., Langlois, A. J., McDanal, C., Ross, K. L., Eckler, L. I., Jellis, C. L., Profy, A. T., Rusche, J. R., Bolognesi, D. P., Putney, S. D. and Matthews, T. J. (1989). "Principal Neutralizing Domain of the Human Immunodeficiency Virus Type 1 Envelope Protein." Proc Natl Acad Sci U S A **86**(17): 6768-72.

Javaherian, K., Langlois, A. J., LaRosa, G. J., Profy, A. T., Bolognesi, D. P., Herlihy, W. C., Putney, S. D. and Matthews, T. J. (1990). "Broadly Neutralizing Antibodies Elicited by the Hypervariable Neutralizing Determinant of HIV-1." Science **250**(4987): 1590-3.

Kang, C. Y., Nara, P., Chamat, S., Caralli, V., Ryskamp, T., Haigwood, N., Newman, R. and Kohler, H. (1991). "Evidence for Non-V3-Specific Neutralizing Antibodies That Interfere with gp120/CD4 Binding in Human Immunodeficiency Virus 1-Infected Humans." Proc Natl Acad Sci U S A **88**(14): 6171-5.

Klasse, P. J., Pipkorn, R. and Blomberg, J. (1988). "Presence of Antibodies to a Putatively Immunosuppressive Part of Human Immunodeficiency Virus (Hiv) Envelope Glycoprotein Gp41 Is Strongly Associated with Health among Hiv-Positive Subjects." Proc Natl Acad Sci U S A **85**(14): 5225-9.

Koup, R. A., Safrit, J. T., Cao, Y., Andrews, C. A., McLeod, G., Borkowsky, W., Farthing, C. and Ho, D. D. (1994). "Temporal Association of Cellular Immune Responses with the Initial Control of Viremia in Primary Human Immunodeficiency Virus Type 1 Syndrome." J Virol **68**(7): 4650-5.

Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J. and Hendrickson, W. A. (1998). "Structure of an HIV gp120 Envelope Glycoprotein in Complex with

the CD4 Receptor and a Neutralizing Human Antibody." Nature **393**(6686): 648-59.

Kwong, P. D., Doyle, M. L., Casper, D. J., Cicala, C., Leavitt, S. A., Majeed, S., Steenbeke, T. D., Venturi, M., Chaiken, I., Fung, M., Katinger, H., Parren, P. W. I. H., Robinson, J., Van Ryk, D., Wang, L., Burton, D. R., Freire, E., Wyatt, R., Sodroski, J., Hendrickson, W. A. and Arthos, J. (2002). "HIV-1 Evades Antibody-Mediated Neutralization through Conformational Masking of Receptor-Binding Sites." Nature **420**(6916): 678-682.

Laver, W. G., Air, G. M., Webster, R. G. and Smith-Gill, S. J. (1990). "Epitopes on Protein Antigens: Misconceptions and Realities." Cell **61**(4): 553-6.

Leonard, C. K., Spellman, M. W., Riddle, L., Harris, R. J., Thomas, J. N. and Gregory, T. J. (1990). "Assignment of Intrachain Disulfide Bonds and Characterization of Potential Glycosylation Sites of the Type 1 Recombinant Human Immunodeficiency Virus Envelope Glycoprotein (gp120) Expressed in Chinese Hamster Ovary Cells." J Biol Chem **265**(18): 10373-82.

Liang, X., Munshi, S., Shendure, J., Mark, G., 3rd, Davies, M. E., Freed, D. C., Montefiori, D. C. and SHIVer, J. W. (1999). "Epitope Insertion into Variable Loops of HIV-1 gp120 as a Potential Means to Improve Immunogenicity of Viral Envelope Protein." Vaccine **17**(22): 2862-72.

Loomis-Price, L. D., Cox, J. H., Mascola, J. R., VanCott, T. C., Michael, N. L., Fouts, T. R., Redfield, R. R., Robb, M. L., Wahren, B., Sheppard, H. W. and Birx, D. L. (1998). "Correlation between Humoral Responses to Human Immunodeficiency Virus Type 1 Envelope and Disease Progression in Early-Stage Infection." J Infect Dis **178**(5): 1306-16.

Lu, M., Blacklow, S. C. and Kim, P. S. (1995). "A Trimeric Structural Domain of the HIV-1 Transmembrane Glycoprotein." Nat Struct Biol **2**(12): 1075-82.

Malenbaum, S. E., Yang, D., Cavacini, L., Posner, M., Robinson, J. and Cheng-Mayer, C. (2000). "The N-Terminal V3 Loop Glycan Modulates the Interaction of Clade A and B Human Immunodeficiency Virus Type 1 Envelopes with CD4 and Chemokine Receptors." J Virol **74**(23): 11008-16.

Mascola, J. R., Louwagie, J., McCutchan, F. E., Fischer, C. L., Hegerich, P. A., Wagner, K. F., Fowler, A. K., McNeil, J. G. and Burke, D. S. (1994). "Two Antigenically Distinct Subtypes of Human Immunodeficiency Virus Type 1: Viral Genotype Predicts Neutralization Serotype." J Infect Dis **169**(1): 48-54.

Mascola, J. R., Snyder, S. W., Weislow, O. S., Belay, S. M., Belshe, R. B., Schwartz, D. H., Clements, M. L., Dolin, R., Graham, B. S., Gorse, G. J., Keefer, M. C., McElrath, M. J., Walker, M. C., Wagner, K. F., McNeil, J. G., McCutchan, F. E. and Burke, D. S. (1996). "Immunization with Envelope Subunit Vaccine Products Elicits Neutralizing Antibodies against Laboratory-Adapted but Not Primary Isolates of Human Immunodeficiency Virus Type 1. The National Institute of Allergy and Infectious Diseases Aids Vaccine Evaluation Group." J Infect Dis **173**(2): 340-8.

Mascola, J. R., Stiegler, G., VanCott, T. C., Katinger, H., Carpenter, C. B., Hanson, C. E., Beary, H., Hayes, D., Frankel, S. S., Birx, D. L. and Lewis, M. G. (2000). "Protection of Macaques against Vaginal Transmission of a Pathogenic HIV-1/SIV Chimeric Virus by Passive Infusion of Neutralizing Antibodies." Nat Med **6**(2): 207-10.

Mascola, J. R., Louder, M. K., Winter, C., Prabhakara, R., De Rosa, S. C., Douek, D. C., Hill, B. J., Gabuzda, D. and Roederer, M. (2002). "Human Immunodeficiency Virus Type 1 Neutralization Measured by Flow Cytometric Quantitation of Single-Round Infection of Primary Human T Cells." J Virol **76**(10): 4810-21.

Mascola, J. R. (2003). "Defining the Protective Antibody Response for HIV-1." Curr Mol Med **3**(3): 209-16.

Matthews, T. J., Langlois, A. J., Robey, W. G., Chang, N. T., Gallo, R. C., Fischinger, P. J. and Bolognesi, D. P. (1986). "Restricted Neutralization of Divergent Human T-Lymphotropic Virus Type III Isolates by Antibodies to the Major Envelope Glycoprotein." Proc Natl Acad Sci U S A **83**(24): 9709-13.

Matthews, T. J. (1994). "Dilemma of Neutralization Resistance of HIV-1 Field Isolates and Vaccine Development." AIDS Res Hum Retroviruses **10**(6): 631-2.

McGaughey, G. B., Citron, M., Danzeisen, R. C., Freidinger, R. M., Garsky, V. M., Hurni, W. M., Joyce, J. G., Liang, X., Miller, M., Shiver, J. and Bogusky, M. J. (2003). "HIV-1 Vaccine Development: Constrained Peptide Immunogens Show Improved Binding to the Anti-HIV-1 gp41 Mab." Biochemistry **42**(11): 3214-23.

McKnight, A., Clapham, P. R., Goudsmit, J., Cheingsong-Popov, R., Weber, J. N. and Weiss, R. A. (1992). "Development of HIV-1 Group-Specific Neutralizing Antibodies after Seroconversion." AIDS **6**(8): 799-802.

Melikyan, G. B., Markosyan, R. M., Hemmati, H., Delmedico, M. K., Lambert, D. M. and Cohen, F. S. (2000). "Evidence That the Transition of HIV-1 gp41 into a Six-Helix Bundle, Not the Bundle Configuration, Induces Membrane Fusion." J Cell Biol **151**(2): 413-23.

Menendez, A., Chow, K. C., Pan, O. C. C. and Scott, J. K. (2004). "Human Immunodeficiency Virus Type 1-Neutralizing Monoclonal Antibody 2f5 Is Multispecific for Sequences Flanking the Dkw Core Epitope." J Mol Biol **338**(2): 311-27.

Menendez, A. Ph.D. Dissertation. *Epitope-targeting Strategy for a Vaccine against HIV-1: Peptide Ligands for the Broadly-Neutralizing Antibodies, b12, 2F5 and 2G12.* Simon Fraser University, Burnaby, BC, Canada. (2005).

Miller, M. D., Geleziunas, R., Bianchi, E., Lennard, S., Hrin, R., Zhang, H., Lu, M., An, Z., Ingallinella, P., Finotto, M., Mattu, M., Finnefrock, A. C., Bramhill, D., Cook, J., Eckert, D. M., Hampton, R., Patel, M., Jarantow, S., Joyce, J., Ciliberto, G., Cortese, R., Lu, P., Strohl, W., Schleif, W., McElhaugh, M., Lane, S., Lloyd, C., Lowe, D., Osbourn, J., Vaughan, T., Emini, E., Barbato, G., Kim, P. S., Hazuda, D. J., Shiver, J. W. and Pessi, A. (2005). "A Human Monoclonal Antibody

Neutralizes Diverse Hiv-1 Isolates by Binding a Critical gp41 Epitope." Proc Natl Acad Sci U S A **102**(41): 14759-64.

Montefiori, D. C., Pantaleo, G., Fink, L. M., Zhou, J. T., Zhou, J. Y., Bilska, M., Miralles, G. D. and Fauci, A. S. (1996). "Neutralizing and Infection-Enhancing Antibody Responses to Human Immunodeficiency Virus Type 1 in Long-Term Nonprogressors." J Infect Dis **173**(1): 60-7.

Moog, C., Fleury, H. J., Pellegrin, I., Kirn, A. and Aubertin, A. M. (1997). "Autologous and Heterologous Neutralizing Antibody Responses Following Initial Seroconversion in Human Immunodeficiency Virus Type 1-Infected Individuals." J Virol **71**(5): 3734-41.

Moore, J. P., McKeating, J. A., Huang, Y. X., Ashkenazi, A. and Ho, D. D. (1992). "Virions of Primary Human Immunodeficiency Virus Type 1 Isolates Resistant to Soluble CD4 (sCD4) Neutralization Differ in sCD4 Binding and Glycoprotein gp120 Retention from sCD4-Sensitive Isolates." J Virol **66**(1): 235-43.

Moore, J. P., Cao, Y., Conley, A. J., Wyatt, R., Robinson, J., Gorny, M. K., Zolla-Pazner, S., Ho, D. D. and Koup, R. A. (1994). "Studies with Monoclonal Antibodies to the V3 Region of HIV-1 gp120 Reveal Limitations to the Utility of Solid-Phase Peptide Binding Assays." J Acquir Immune Defic Syndr **7**(4): 332-9.

Moore, J. P. and Ho, D. D. (1995). "HIV-1 Neutralization: The Consequences of Viral Adaptation to Growth on Transformed T Cells." AIDS **9 Suppl A**: S117-36.

Moore, J. P., Kitchen, S. G., Pugach, P. and Zack, J. A. (2004). "The CCR5 and CXCR4 Coreceptors--Central to Understanding the Transmission and Pathogenesis of Human Immunodeficiency Virus Type 1 Infection." AIDS Res Hum Retroviruses **20**(1): 111-26.

Moreno, M. R., Pascual, R. and Villalain, J. (2004). "Identification of Membrane-Active Regions of the HIV-1 Envelope Glycoprotein gp41 Using a 15-Mer gp41-Peptide Scan." Biochimica et biophysica acta **1661**(1): 97-105.

Muhlbacher, M., Spruth, M., Siegel, F., Zangerle, R. and Dierich, M. P. (1999). "Longitudinal Study of Antibody Reactivity against HIV-1 Envelope and a Peptide Representing a Conserved Site on gp41 in HIV-1-Infected Patients." Immunobiology **200**(2): 295-305.

Muster, T., Steindl, F., Purtscher, M., Trkola, A., Klima, A., Himmler, G., Ruker, F. and Katinger, H. (1993). "A Conserved Neutralizing Epitope on gp41 of Human Immunodeficiency Virus Type 1." J Virol **67**(11): 6642-47.

Muster, T., Guinea, R., Trkola, A., Purtscher, M., Klima, A., Steindl, F., Palese, P. and Katinger, H. (1994). "Cross-Neutralizing Activity against Divergent Human Immunodeficiency Virus Type 1 Isolates Induced by the gp41 Sequence ELDKWAS." J Virol **68**(6): 4031-34.

Nabel, G. J. (2005). "Immunology. Close to the Edge: Neutralizing the HIV-1 Envelope." Science **308**(5730): 1878-79.

Nagaoka, H., Gonzalez-Aseguinolaza, G., Tsuji, M. and Nussenzweig, M. C. (2000). "Immunization and Infection Change the Number of Recombination Activating Gene (RAG)-Expressing B Cells in the Periphery by Altering Immature Lymphocyte Production." J Exp Med **191**(12): 2113-20.

Neurath, A. R., Strick, N. and Lee, E. S. (1990). "B Cell Epitope Mapping of Human Immunodeficiency Virus Envelope Glycoproteins with Long (19- to 36-Residue) Synthetic Peptides." J Gen Virol **71 (Pt 1)**: 85-95.

Norrby, E., Biberfeld, G., Chiodi, F., von Gegerfeldt, A., Naucler, A., Parks, E. and Lerner, R. (1987). "Discrimination between Antibodies to Hiv and to Related Retroviruses Using Site-Directed Serology." Nature **329**(6136): 248-50.

Ofek, G., Tang, M., Sambor, A., Katinger, H., Mascola, J. R., Wyatt, R. and Kwong, P. D. (2004). "Structure and Mechanistic Analysis of the Anti-Human Immunodeficiency Virus Type 1 Antibody 2F5 in Complex with Its gp41 Epitope." J Virol **78**(19): 10724-37.

Opalka, D., Pessi, A., Bianchi, E., Ciliberto, G., Schleif, W., McElhaugh, M., Danzeisen, R., Geleziunas, R., Miller, M., Eckert, D. M., Bramhill, D., Joyce, J., Cook, J., Magilton, W., Shiver, J., Emini, E. and Esser, M. T. (2004). "Analysis of the HIV-1 gp41 Specific Immune Response Using a Multiplexed Antibody Detection Assay." J Immunol Methods **287**(1-2): 49-65.

Page, M., Vella, C., Corcoran, T., Dilger, P., Ling, C., Heath, A. and Thorpe, R. (1992). "Restriction of Serum Antibody Reactivity to the V3 Neutralizing Domain of HIV gp120 with Progression to AIDS." AIDS **6**(5): 441-6.

Pantaleo, G., Demarest, J. F., Soudeyns, H., Graziosi, C., Denis, F., Adelsberger, J. W., Borrow, P., Saag, M. S., Shaw, G. M., Sekaly, R. P. and Fauci, A. S. (1994). "Major Expansion of CD8+ T Cells with a Predominant V Beta Usage During the Primary Immune Response to HIV." Nature **370**(6489): 463-7.

Pantaleo, G. and Koup, R. A. (2004). "Correlates of Immune Protection in HIV-1 Infection: What We Know, What We Don't Know, What We Should Know." Nat Med **10**(8): 806-810.

Parker, C. E., Deterding, L. J., Hager-Braun, C., Binley, J. M., Schulke, N., Katinger, H., Moore, J. P. and Tomer, K. B. (2001). "Fine Definition of the Epitope on the gp41 Glycoprotein of Human Immunodeficiency Virus Type 1 for the Neutralizing Monoclonal Antibody 2F5." J Virol **75**(22): 10906-11.

Parren, P. W., Burton, D. R. and Sattentau, Q. J. (1997a). "HIV-1 Antibody--Debris or Virion?" Nat Med **3**(4): 366-7.

Parren, P. W., Gauduin, M. C., Koup, R. A., Poignard, P., Fisicaro, P., Burton, D. R. and Sattentau, Q. J. (1997b). "Relevance of the Antibody Response against Human Immunodeficiency Virus Type 1 Envelope to Vaccine Design." Immunol Lett **57**(1-3): 105-12.

Pilgrim, A. K., Pantaleo, G., Cohen, O. J., Fink, L. M., Zhou, J. Y., Zhou, J. T., Bolognesi, D. P., Fauci, A. S. and Montefiori, D. C. (1997). "Neutralizing Antibody Responses to Human Immunodeficiency Virus Type 1 in Primary

Infection and Long-Term-Nonprogressive Infection." J Infect Dis **176**(4): 924-932.

Polonis, V. R., De Souza, M. S., Chanbancherd, P., Chantakulkij, S., Jugsudee, A., Loomis-Price, L. D., Vancott, T. C., Garner, R., Markowitz, L. E., Brown, A. E. and Birx, D. L. (2001). "HIV Type 1 Subtype E-Infected Patients with Broadened, Dual (B/E) V3 Loop Serology Have Increased Cross-Neutralizing Antibodies." AIDS Res Hum Retroviruses **17**(1): 69-79.

Popovic, M., Sarngadharan, M. G., Read, E. and Gallo, R. C. (1984). "Detection, Isolation, and Continuous Production of Cytopathic Retroviruses (HTLV-III) from Patients with AIDS and Pre-AIDS." Science **224**(4648): 497-500.

Putney, S. D., Matthews, T. J., Robey, W. G., Lynn, D. L., Robert-Guroff, M., Mueller, W. T., Langlois, A. J., Ghrayeb, J., Petteway, S. R., Jr., Weinhold, K. J. and Fischinger, P. J., Wong-Staal, F., Gallo, R. C., Bolognesi, D. P. (1986). "HTLV-III/LAV-Neutralizing Antibodies to an E. Coli-Produced Fragment of the Virus Envelope." Science **234**(4782): 1392-5.

Quinnan, G. V., Jr., Zhang, P. F., Fu, D. W., Dong, M. and Margolick, J. B. (1998). "Evolution of Neutralizing Antibody Response against HIV Type 1 Virions and Pseudovirions in Multicenter AIDS Cohort Study Participants." AIDS Res Hum Retroviruses **14**(11): 939-49.

Quinnan, G. V., Jr., Zhang, P. F., Fu, D. W., Dong, M. and Alter, H. J. (1999). "Expression and Characterization of HIV Type 1 Envelope Protein Associated with a Broadly Reactive Neutralizing Antibody Response." AIDS Res Hum Retroviruses **15**(6): 561-70.

Reeves, J. D. and Doms, R. W. (2002). "Human Immunodeficiency Virus Type 2." J Gen Virol **83**(Pt 6): 1253-65.

Rice, J. S., Newman, J., Wang, C., Michael, D. J. and Diamond, B. (2005). "Receptor Editing in Peripheral B Cell Tolerance." Proc Natl Acad Sci U S A **102**(5): 1608-13.

Richman, D. D., Wrin, T., Little, S. J. and Petropoulos, C. J. (2003). "Rapid Evolution of the Neutralizing Antibody Response to HIV Type 1 Infection." Proc Natl Acad Sci U S A **100**(7): 4144-9.

Rizzuto, C. D., Wyatt, R., Hernandez-Ramos, N., Sun, Y., Kwong, P. D., Hendrickson, W. A. and Sodroski, J. (1998). "A Conserved HIV gp120 Glycoprotein Structure Involved in Chemokine Receptor Binding." Science **280**(5371): 1949-53.

Robbins, J. B., Schneerson, R. and Szu, S. C. (1995). "Perspective: Hypothesis: Serum IgG Antibody Is Sufficient to Confer Protection against Infectious Diseases by Inactivating the Inoculum." J Infect Dis **171**(6): 1387-98.

Roben, P., Moore, J. P., Thali, M., Sodroski, J., Barbas, C. F., 3rd and Burton, D. R. (1994). "Recognition Properties of a Panel of Human Recombinant Fab Fragments to the CD4 Binding Site of gp120 That Show Differing Abilities to Neutralize Human Immunodeficiency Virus Type 1." J Virol **68**(8): 4821-8.

Robinson, W. H., DiGennaro, C., Hueber, W., Haab, B. B., Kamachi, M., Dean, E. J., Fournel, S., Fong, D., Genovese, M. C., de Vegvar, H. E., Skriner, K., Hirschberg, D. L., Morris, R. I., Muller, S., Pruijn, G. J., van Venrooij, W. J., Smolen, J. S., Brown, P. O., Steinman, L. and Utz, P. J. (2002). "Autoantigen Microarrays for Multiplex Characterization of Autoantibody Responses." Nat Med **8**(3): 295-301.

Saphire, E. O., Parren, P. W., Pantophlet, R., Zwick, M. B., Morris, G. M., Rudd, P. M., Dwek, R. A., Stanfield, R. L., Burton, D. R. and Wilson, I. A. (2001). "Crystal Structure of a Neutralizing Human IgG against HIV-1: A Template for Vaccine Design." Science **293**(5532): 1155-9.

Sattentau, Q. J. and Moore, J. P. (1995). "Human Immunodeficiency Virus Type 1 Neutralization Is Determined by Epitope Exposure on the gp120 Oligomer." J Exp Med **182**(1): 185-96.

Scanlan, C. N., Pantophlet, R., Wormald, M. R., Ollmann Saphire, E., Stanfield, R., Wilson, I. A., Katinger, H., Dwek, R. A., Rudd, P. M. and Burton, D. R. (2002). "The Broadly Neutralizing Anti-Human Immunodeficiency Virus Type 1 Antibody 2G12 Recognizes a Cluster of Alpha1-->2 Mannose Residues on the Outer Face of gp120." J Virol **76**(14): 7306-21.

Skehel, J. J. and Wiley, D. C. (1998). "Coiled Coils in Both Intracellular Vesicle and Viral Membrane Fusion." Cell **95**(7): 871-4.

Smith, R. S., Naso, R. B., Rosen, J., Whalley, A., Hom, Y. L., Hoey, K., Kennedy, C. J., McCutchan, J. A., Spector, S. A. and Richman, D. D. (1987). "Antibody to a Synthetic Oligopeptide in Subjects at Risk for Human Immunodeficiency Virus Infection." J Clin Microbiol **25**(8): 1498-504.

Srisurapanon, S., Louisirirotchanakul, S., Sumransurp, K., Ratanasrithong, M., Chuenchitra, T., Jintakatkorn, S. and Wasi, C. (2005). "Binding Antibody to Neutralizing Epitope gp41 in HIV-1 Subtype CRF 01_AE Infection Related to Stage of Disease." Southeast Asian J Trop Med Public Health **36**(1): 221-7.

Stamatos, N. M., Mascola, J. R., Kalyanaraman, V. S., Louder, M. K., Frampton, L. M., Birx, D. L. and VanCott, T. C. (1998). "Neutralizing Antibodies from the Sera of Human Immunodeficiency Virus Type 1-Infected Individuals Bind to Monomeric gp120 and Oligomeric Gp140." J Virol **72**(12): 9656-67.

Stanfield, R. L. and Wilson, I. A. (1995). "Protein-Peptide Interactions." Curr Opin Struct Biol **5**(1): 103-13.

Starcich, B. R., Hahn, B. H., Shaw, G. M., McNeely, P. D., Modrow, S., Wolf, H., Parks, E. S., Parks, W. P., Josephs, S. F., Gallo, R. C. and Wong-Staal, F. (1986). "Identification and Characterization of Conserved and Variable Regions in the Envelope Gene of HTLV-III/LAV, the Retrovirus of AIDS." Cell **45**(5): 637-48.

Steimer, K. S., Scandella, C. J., Skiles, P. V. and Haigwood, N. L. (1991). "Neutralization of Divergent HIV-1 Isolates by Conformation-Dependent Human Antibodies to gp120." Science **254**(5028): 105-8.

Tan, K., Liu, J., Wang, J., Shen, S. and Lu, M. (1997). "Atomic Structure of a Thermostable Subdomain of HIV-1 gp41." Proc Natl Acad Sci U S A **94**(23): 12303-8.

Tian, Y., Ramesh, C. V., Ma, X., Naqvi, S., Patel, T., Cenizal, T., Tiscione, M., Diaz, K., Crea, T., Arnold, E., Arnold, G. F. and Taylor, J. W. (2002). "Structure-Affinity Relationships in the gp41 ELDKWA Epitope for the HIV-1 Neutralizing Monoclonal Antibody 2F5: Effects of Side-Chain and Backbone Modifications and Conformational Constraints." J Pept Res **59**(6): 264-76.

Trkola, A., Kuster, H., Rusert, P., Joos, B., Fischer, M., Leemann, C., Manrique, A., Huber, M., Rehr, M., Oxenius, A., Weber, R., Stiegler, G., Vcelar, B., Katinger, H., Aceto, L. and Gunthard, H. F. (2005). "Delay of HIV-1 Rebound after Cessation of Antiretroviral Therapy through Passive Transfer of Human Neutralizing Antibodies." Nat Med **11**(6): 615-22.

Ugen, K. E., Srikantan, V., Goedert, J. J., Nelson Rp, J. r., Williams, W. V. and Weiner, D. B. (1997). "Vertical Transmission of Human Immunodeficiency Virus Type 1: Seroreactivity by Maternal Antibodies to the Carboxy Region of the gp41 Envelope Glycoprotein." J Infect Dis **175**(1): 63-9.

Vancott, T. C., Polonis, V. R., Loomis, L. D., Michael, N. L., Nara, P. L. and Birx, D. L. (1995). "Differential Role of V3-Specific Antibodies in Neutralization Assays Involving Primary and Laboratory-Adapted Isolates of HIV Type 1." AIDS Res Hum Retroviruses **11**(11): 1379-91.

Vanini, S., Longhi, R., Lazzarin, A., Vigo, E., Siccardi, A. G. and Viale, G. (1993). "Discrete Regions of Hiv-1 gp41 Defined by Syncytia-Inhibiting Affinity-Purified Human Antibodies." AIDS 7(2): 167-74.

Veazey, R. S., Shattock, R. J., Pope, M., Kirijan, J. C., Jones, J., Hu, Q., Ketas, T., Marx, P. A., Klasse, P. J., Burton, D. R. and Moore, J. P. (2003). "Prevention of Virus Transmission to Macaque Monkeys by a Vaginally Applied Monoclonal Antibody to HIV-1 gp120." Nat Med **9**(3): 343-6.

Visvanathan, S., Scott, J. K., Hwang, K. K., Banares, M., Grossman, J. M., Merrill, J. T., FitzGerald, J., Chukwuocha, R. U., Tsao, B. P., Hahn, B. H. and Chen, P. P. (2003). "Identification and Characterization of a Peptide Mimetic That May Detect a Species of Disease-Associated Anticardiolipin Antibodies in Patients with the Antiphospholipid Syndrome." Arthritis Rheum **48**(3): 737-45.

Vujcic, L. K. and Quinnan, G. V., Jr. (1995). "Preparation and Characterization of Human HIV Type 1 Neutralizing Reference Sera." AIDS Res Hum Retroviruses **11**(7): 783-7.

Wang, J. J., Steel, S., Wisniewolski, R. and Wang, C. Y. (1986). "Detection of Antibodies to Human T-Lymphotropic Virus Type Iii by Using a Synthetic Peptide of 21 Amino Acid Residues Corresponding to a Highly Antigenic Segment of Gp41 Envelope Protein." Proc Natl Acad Sci U S A **83**(16): 6159-63.

Wei, X., Decker, J. M., Wang, S., Hui, H., Kappes, J. C., Wu, X., Salazar-Gonzalez, J. F., Salazar, M. G., Kilby, J. M., Saag, M. S., Komarova, N. L., Nowak, M. A., Hahn,

B. H., Kwong, P. D. and Shaw, G. M. (2003). "Antibody Neutralization and Escape by HIV-1." Nature **422**(6929): 307-12.

Weiss, R. A., Clapham, P. R., Cheingsong-Popov, R., Dalgleish, A. G., Carne, C. A., Weller, I. V. and Tedder, R. S. (1985). "Neutralization of Human T-Lymphotropic Virus Type III by Sera of AIDS and AIDS-Risk Patients." Nature **316**(6023): 69-72.

Weissenhorn, W., Dessen, A., Harrison, S. C., Skehel, J. J. and Wiley, D. C. (1997). "Atomic Structure of the Ectodomain from HIV-1 gp41." Nature **387**(6631): 426-30.

Wilson, I. A., Skehel, J. J. and Wiley, D. C. (1981). "Structure of the Haemagglutinin Membrane Glycoprotein of Influenza Virus at 3A Resolution." Nature **289**(5796): 366-73.

Wu, T. T. and Kabat, E. A. (1970). "An Analysis of the Sequences of the Variable Regions of Bence Jones Proteins and Myeloma Light Chains and Their Implications for Antibody Complementarity." J Exp Med **132**(2): 211-50.

Wyatt, R. and Sodroski, J. (1998). "The HIV-1 Envelope Glycoproteins: Fusogens, Antigens, and Immunogens." Science **280**(5371): 1884-8.

Xu, J. Y., Gorny, M. K., Palker, T., Karwowska, S. and Zolla-Pazner, S. (1991). "Epitope Mapping of Two Immunodominant Domains of gp41, the Transmembrane Protein of Human Immunodeficiency Virus Type 1, Using Ten Human Monoclonal Antibodies." J Virol **65**(9): 4832-8.

Yuste, E., Sanford, H. B., Carmody, J., Bixby, J., Little, S., Zwick, M. B., Greenough, T., Burton, D. R., Richman, D. D., Desrosiers, R. C. and Johnson, W. E. (2006). "Simian Immunodeficiency Virus Engrafted with Human Immunodeficiency Virus Type 1 (HIV-1)-Specific Epitopes: Replication, Neutralization, and Survey of HIV-1-Positive Plasma." J Virol **80**(6): 3030-41.

Zemlin, M., Klinger, M., Link, J., Zemlin, C., Bauer, K., Engler, J. A., Schroeder, H. W., Jr. and Kirkham, P. M. (2003). "Expressed Murine and Human Cdr-H3 Intervals of Equal Length Exhibit Distinct Repertoires That Differ in Their Amino Acid Composition and Predicted Range of Structures." J Mol Biol **334**(4): 733-49.

Zhang, Y. J., Fredriksson, R., McKeating, J. A. and Fenyo, E. M. (1997). "Passage of HIV-1 Molecular Clones into Different Cell Lines Confers Differential Sensitivity to Neutralization." Virology **238**(2): 254-64.

Zhang, P. F., Chen, X., Fu, D. W., Margolick, J. B. and Quinnan, G. V., Jr. (1999). "Primary Virus Envelope Cross-Reactivity of the Broadening Neutralizing Antibody Response During Early Chronic Human Immunodeficiency Virus Type 1 Infection." J Virol **73**(6): 5225-30.

Zolla-Pazner, S. (2004). "Identifying Epitopes of HIV-1 That Induce Protective Antibodies." Nat.Rev.Immunol. **4**(3): 199-210.

Zolla-Pazner, S., Zhong, P., Revesz, K., Volsky, B., Williams, C., Nyambi, P. and Gorny, M. K. (2004). "The Cross-Clade Neutralizing Activity of a Human Monoclonal

Antibody Is Determined by the GPGR V3 Motif of HIV Type 1." AIDS Res Hum Retroviruses **20**(11): 1254-8.

Zwart, G., Langedijk, H., van der Hoek, L., de Jong, J. J., Wolfs, T. F., Ramautarsing, C., Bakker, M., de Ronde, A. and Goudsmit, J. (1991). "Immunodominance and Antigenic Variation of the Principal Neutralization Domain of HIV-1." Virology **181**(2): 481-9.

Zwart, G., Wolfs, T. F., Valk, M., Van der Hoek, L., Kuiken, C. L. and Goudsmit, J. (1992). "Characterization of the Specificity of the Human Antibody Response to the V3 Neutralization Domain of HIV-1." AIDS Res Hum Retroviruses **8**(11): 1897-908.

Zwart, G., Back, N. K., Ramautarsing, C., Valk, M., van der Hoek, L. and Goudsmit, J. (1994). "Frequent and Early HIV-1MN Neutralizing Capacity in Sera from Dutch HIV-1 Seroconverters Is Related to Antibody Reactivity to Peptides from the gp120 V3 Domain." AIDS Res Hum Retroviruses **10**(3): 245-51.

Zwick, M. B., Bonnycastle, L. L., Menendez, A., Irving, M. B., Barbas Cf, r., Parren, P. W., Burton, D. R. and Scott, J. K. (2001a). "Identification and Characterization of a Peptide That Specifically Binds the Human, Broadly Neutralizing Anti-Human Immunodeficiency Virus Type 1 Antibody b12." J Virol **75**(14): 6692-9.

Zwick, M. B., Labrijn, A. F., Wang, M., Spenlehauer, C., Saphire, E. O., Binley, J. M., Moore, J. P., Stiegler, G., Katinger, H., Burton, D. R. and Parren, P. W. (2001b). "Broadly Neutralizing Antibodies Targeted to the Membrane-Proximal External Region of Human Immunodeficiency Virus Type 1 Glycoprotein gp41." J Virol **75**(22): 10892-10905.

Zwick, M. B., Kelleher, R., Jensen, R., Labrijn, A. F., Wang, M., Quinnan Gv, J. r., Parren, P. W. and Burton, D. R. (2003). "A Novel Human Antibody against Human Immunodeficiency Virus Type 1 gp120 Is V1, V2, and V3 Loop Dependent and Helps Delimit the Epitope of the Broadly Neutralizing Antibody Immunoglobulin G1 b12." J Virol **77**(12): 6965-6978.

Zwick, M. B., Komori, H. K., Stanfield, R. L., Church, S., Wang, M., Parren, P. W., Kunert, R., Katinger, H., Wilson, I. A. and Burton, D. R. (2004). "The Long Third Complementarity-Determining Region of the Heavy Chain Is Important in the Activity of the Broadly Neutralizing Anti-Human Immunodeficiency Virus Type 1 Antibody 2F5." J Virol **78**(6): 3155-61.

Zwick, M. B. (2005). "The Membrane-Proximal External Region of HIV-1 gp41: A Vaccine Target Worth Exploring." AIDS **19**(16): 1725-37.

Zwick, M. B., Jensen, R., Church, S., Wang, M., Stiegler, G., Kunert, R., Katinger, H. and Burton, D. R. (2005). "Anti-Human Immunodeficiency Virus Type 1 (HIV-1) Antibodies 2F5 and 4E10 Require Surprisingly Few Crucial Residues in the Membrane-Proximal External Region of Glycoprotein gp41 to Neutralize HIV-1." J Virol **79**(2): 1252-61.