ANTIGENICITY AND IMMUNOGENICITY OF THE MEMBRANE PROXIMAL EXTERNAL REGION OF THE HIV-1 ENVELOPE PROTEIN GP41

by

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ABSTRACT

Two of the six HIV-1 broadly neutralizing monoclonal antibody (bNt MAbs) isolated so far bind epitopes located in the viral envelope protein gp41. 2F5 and 4E10 neutralize viruses from a broad range of different isolates. Although gp41 is a transmembrane protein, the study of its antigenicity and immunogenicity in the context of membrane has been largely ignored. The goal of this research is to develop novel immunogen(s) for the generation of protective bNtAbs that specifically mimic the biological activities of 2F5 and 4E10.

Our strategy is based on the assumption that the membrane proximal external region (MPER) interacts with the viral membrane in its native state on the viral spike, and that this interaction plays a major role in its structure and function. We hypothesized that the presentation of the "real" 2F5 and 4E10 epitopes is dependent on the interaction of MPER with the viral membrane and its structure in this context. Thus, membrane should be a key element for the proper presentation of 2F5 and 4E10 epitopes to the immune system, and for the generation of the neutralizing Abs 2F5 and 4E10. Using several DNA constructs, we evaluated the influence of membrane, as well as other regions of gp41 in the interaction of these Abs with gp41. We documented fundamental differences between the binding of 2F5 and 4E10 to the MPER in the context of membrane, which indicates that the membrane environment influences the antigenicity of this region. We have also shown for the first time that the gp41 transmembrane region is important for the interaction of 4E10 with the MPER. We used DNA immunizations to test the ability of membrane-bound MPER to induce an Ab response with 2F5- and 4E10-like activity,

and showed that a low Ab response against the 2F5 epitope can be generated in rabbits with the DNA vaccines tested. However, the Ab response generated was not HIV-1 neutralizing. Thus, although an Ab response can be generated by this method, future work needs to be done in order to increase the MPER immunogenicity.

To my dear husband, Alfredo for his unfailing love and support,
to my mother for being a source of encouragement
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LIST OF ABBREVIATIONS

Ab	Antibody
ABTS	2,2;-azino-bis(3-ethylbenzthiazoline-6) sulfonic acid
BAFF	human B-cell activating factor
bNt	broadly Neutralizing
BSA	bovine grow hormone
CDR-3	third complementary-determining region of the heavy chain
CHR	C-terminal heptad repeat
CL	cardiolipin
CT	cytoplasmic tail
DM	Non-fat dry milk
ELISA	Enzyme-linked immunosorbent assay
Env	virus envelope
FP	fusion peptide
H	Heavy chain
HA	influenza hemagglutinin
HIV-1	Human immunodeficiency virus-type-1
HRP	Horseradish peroxidase
H_2O_2	Hydrogen peroxide
IC50	Inhibitory concentration required to reduce ELISA signal to 50%
ID	immunodominant
IN	intranasal
IP	intraperitoneal
LT	*Escherichia coli* heat-labile enterotoxin
MAb	monoclonal antibody
MAP	multiple antigenic peptide
MPER	membrane proximal external region
NHR	N-terminal heptad repeat
Nt	Neutralizing
OD	Optical density
Ova	ovalbumin
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PERV	porcine endogenous retrovirus
PR	polar region
RT	Room temperature
6HB	six helix bundle
SA	Streptavidin
SC	subcutaneous
TBS	Tris-buffered saline, pH 7.5
TBS/BSA	Tris-buffered saline, 2% (w/v) bovine serum albumin
TBS/BSA/Tw	Tris-buffered saline, 2% (w/v) bovine serum albumin, 0.1% (w/v) tween-20
TCLA	T-cell laboratory adapted
TM	transmembrane domain

Tw	Tween-20
VLP	virus-like particle
VSV	vesicular stomatitis virus
WT	wild type

CHAPTER 1. Introduction

1.1. Overview of the thesis

This thesis is focused on the study of the antigenic and immunogenic properties of the membrane-proximal external region of HIV gp41 (MPER) in the context of the cell membrane. It is divided is four chapters; the first chapter is an introduction of the gp41 protein structural and functional domains, with more emphasis on the MPER features, since that region involves the core of this work. Challenges in the field of HIV-1 vaccine development are presented as well as new directions to convey the challenges imposed by this complex virus.

The second and third chapters present the results of the research work; Chapter 2 covers the generation of DNA constructs, their expression and antigenicity studies. Twentynine DNA constructs were made and the expression of recombinant proteins on the cell surface was evaluated, as well as their interaction with the HIV-1 Nt MAbs 2F5 and 4E10. Chapter 3 is devoted to studies of the immunogenicity of selected constructs used as DNA vaccines and the analysis of the Ab responses they generated.

Chapter 4 comprises a unified discussion of the results described in Chapters 2 and 3, and proposals for the future directions of this research.

1.2. HIV-1 vaccine: the challenges

According to the Joint United Nations Programme on HIV/AIDS statistics, at the end of 2006 more than 25 million people had died of Acquired Immunodeficiency Syndrome (AIDS) since 1981 (http://www.unaids.org). The disease is caused by infection

with the Human Immunodeficiency Virus type 1 or 2 (HIV-1 or HIV-2), although HIV-1 is responsible for the majority of infections. HIV-1 is transmitted by sexual contact or contact with bodily fluids (*e. g.*, transfusion of infected blood or sharing contaminated needles between intravenous drug users), and represents a serious health problem with a current estimate of 39.5 millions people living with HIV/AIDS worldwide.

HIV-1 is a retrovirus from the genus *Lentivirus*. As any other retrovirus, its genome is composed of two copies of positive-stranded RNA, which encodes several structural, regulatory and accessory genes (**Figure 1-1**). Its life cycle is characterized by synthesis of viral DNA by a virally-coded reverse transcriptase that uses the two genome RNA molecules as templates. The newly-synthesized DNA is integrated into the host cell genome. Once inserted, the viral DNA is either transcribed and translated leading to the production of new viral progeny, or it remains functionally latent for long periods of time until it is reactivated and the host cell becomes a producer cell. HIV-1 entry into host cells is mediated by the virus envelope (Env) comprising the surface (gp120) and the membrane-anchored (gp41) glycoproteins. They are produced as a highly-glycosylated precursor (gp160), which is processed by a host protease into the two subunits (104, 271). These two proteins remain associated by non-covalent interactions, and form the hetero-trimeric spikes in the viral surface (66, 141). Env is the only viral protein expressed in the viral membrane, as well as in the membrane of infected cells.

The infection process is initiated by binding of gp120 to the primary receptor, CD4, on the target cell (54), which induces conformational changes in gp120 and allows the exposure or formation of a binding site for a secondary receptor

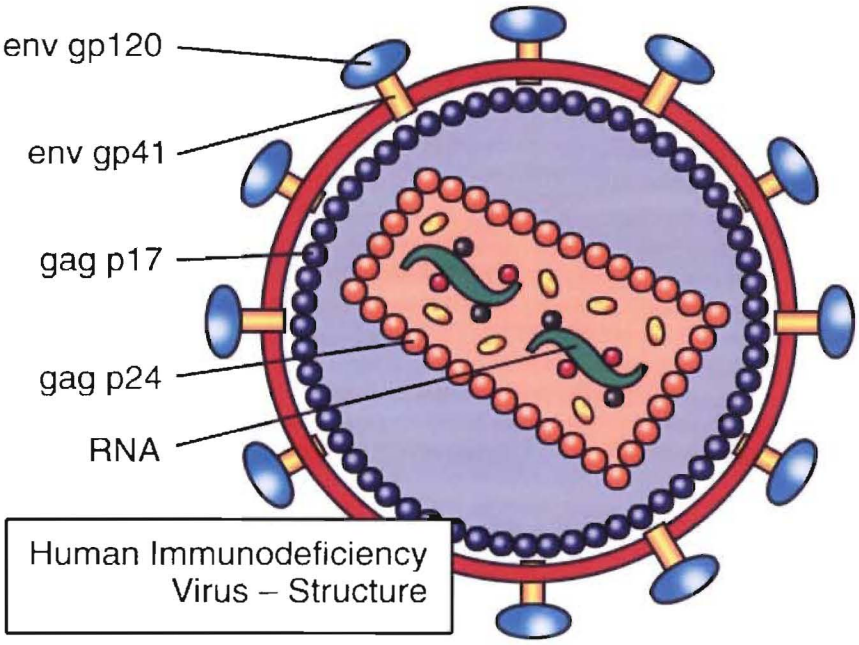


Figure 1-1. Schematic representation of the Human Immunodeficiency Virus. HIV is a complex enveloped retrovirus. The viral spike is composed of a heterotrimer made from two proteins, the Env surface protein (gp120) and the Env transmembrane protein gp41. Figure reproduced with permission from AVERT.

(*e. g*, CCR5 or CXCR4) (14, 238). Interaction with the co-receptor, in turn, triggers several conformational changes in gp41, which lead to the fusion of viral and host membranes and pore formation, followed by the release of the viral nucleocapsid core into the cell (39, 80, 263).

Both gp120 and gp41 are the targets of antibody (Ab)-mediated virus neutralization (187, 193). Large amounts of Ab are elicited during the course of natural infection; but neutralizing Abs (Nt Abs) levels are generally low, and/or isolate-specific (167). HIV-1 has several immune evasion strategies, such as a low number of viral spikes, gp120 shedding and conformational masking of Nt epitopes; these minimize its recognition by the immune system and the production of Nt Ab. Normally, the trimeric and glycoslated nature of the spike results in the shielding and low immunogenicity of some regions of the protein. The shedding of gp120 is a particularly effective mechanism of diverting the Ab response since loss of gp120 from the viral spike exposes regions of the protein that are highly immunogenic, but irrelevant to neutralization since they are normally occluded in the neutralization-sensitive spike. Also, shedding of gp120 produces a "naked" membrane-anchored gp41, which is highly immunogenic, but still irrelevant to the Nt Ab response. A similar problem is encountered by recombinant envelope vaccines, which typically produce strong Ab responses with a very limited neutralization capacity (76, 158, 221). The persistent nature of HIV-1 infection, linked to high replication and mutation rates of the virus, favor the appearance of escape mutants and the generation of quasi-species against which the immune system has no a pre-formed response. The large number of quasi-species in fact act as decoys for the immune system. This constant interplay between the virus and the host immune response results in

the inability of the immune system to clear an established infection (201). The unprecedented variability of HIV-1 probably stands as the single most challenging aspect for vaccine development, since an effective vaccine must protect against a large number of divergent viral strains circulating in the human population.

Nevertheless, broadly (b)Nt Abs are sometimes generated (i.e. are capable of Nt a broad range of primary isolates). So far, only six bNt monoclonal (M) Abs have been isolated from HIV-1-infected donors; they are all directed against Env. The bNt MAbs b12, 447-52D and 2G12 bind to gp120 (25, 49, 242), whereas; 2F5, Z13 and 4E10 recognize the membrane-proximal external region of gp41 (MPER) (174, 292). These bNt MAbs inhibit cellular HIV-1 infection by multiple genetic clades *in vitro*, and prevent experimental infections in animal models with viruses bearing the envelope proteins from primary HIV-1 isolates (17, 152, 153).

With the discovery of bNt MAbs and their characterization came the possibility of targeting their production *in vivo* by active immunization. This has proven difficult due to a number of factors, particularly the structural complexities of the epitopes targeted by these Abs. For example, b12 is directed against a complex discontinuous epitope that overlaps the CD4 binding site of gp120, whereas 2G12 recognizes the termini of several oligosaccharide chains on the highly-glycosylated face of gp120. MAbs 2F5 and 4E10 bind adjacent linear epitopes located in the MPER; however, it is thought that those linear regions do not completely represent their full immunogenic epitopes. In addition to the complexities of their epitopes, the bNt MAbs have several unusual structural features, such as high levels of somatic mutation, very long hypervariable H3 loops (b12, 2F5, 447-52D, Z13 and 4E10) (37, 183, 213, 226), and VH domain swapping (2G12) (34). In

spite of these difficulties, elicitation of bNt Ab continues to be crucial for HIV-1 vaccine development, although it is generally accepted that an efficient anti-HIV-1 vaccine should involve both the cellular and humoral arms of the host immune response. However, in order to develop an anti-HIV vaccine, the process of viral infection, as well as the structures of the viral spike required for Nt activity should be better understood.

1.3. The functional and structural domains of gp41

In comparison with gp120, gp41 is more conserved and does not contain clearly defined variable regions; it consists of ~345 amino acids with a molecular weight of 41 kDa, divided into three major domains (81): the extracellular region (aa 512-683); (numbering based on HIV-1HXB2 (127), unless specified), also called the ectodomain, the transmembrane domain (TM, aa 684-705), and the cytoplasmic tail (CT, aa 705-856) **Figure 1-2**. The ectodomain contains several distinct functional determinants involved in fusion of viral and host cell membranes: *i*) a N-terminal hydrophobic region that functions as a fusion peptide (FP, aa 512-527); *ii*) a polar region (PR, aa 525-543); *iii*) two alpha-helix repeat regions referred as the N-terminal (aa 559-587) and C-terminal (aa 638-676) heptad repeats (NHR and CHR, respectively); *iv*) a disulfide-bridged, hydrophilic loop connecting the two HRs, also known as the connecting loop, the cluster I epitope or the ID loop (ID, aa 598-604) and *v*) a tryptophan-rich region close to the membrane and known as the MPER (aa 660-683), **Figure 1-2**.

Available crystal structures of the HIV-1 gp41 ectodomain core do not contain the FP and the ID loop, and have deletions of the MPER (39, 234, 263). These studies revealed gp41 as a trimer which core forms a six helix bundle (6HB), which is considered

to be the post-fusion structure of the ectodomain and different from the native metastable structure of gp41 in the viral spike. Recently, two different structural models for gp41 have been described based on cryoET data for the viral spike of the whole virion (282, 285). Zhu *et al.* (285) proposed that the MPER and TM 'stalk' of each trimer is composed of three separate legs that project obliquely from the trimer head as a tripod-like structure, whereas the structural model of Zanetti *et al.* (282) shows the TM domain of SIV gp41 as a stem in the viral surface. The tripod-like model structure correlates with the current view of possible membrane interaction with the gp41 MPER. Nevertheless, the structure of the native gp41 protein in the viral spike remains an open question to be revealed by future studies.

The Fusion Peptide: As shown in **Figure 1-2**, the FP corresponds to the first 15 amino acids of gp41 and is followed by a polar region. It is believed that in the native (*i.e.*, nonfusogenic) state, the FP is buried in the gp120/gp41 quaternary complex and is only exposed transiently for interaction with the host cell-membrane after gp120 binding to the CD4 receptor. The involvement of the FP in triggering virus-cell membrane fusion has been confirmed by mutational studies (21, 79, 82, 216). It has been proposed that the FP is inserted obliquely into the cell membrane, causing membrane destabilization and promoting membrane fusion (24). The proposed model for HIV-1 infection is shown in **Figure 1-3**.

The functional structure of the FP has been very controversial. For example, an α-helical structure has been proposed by Martin and colleagues determined with a16-mer synthetic peptide interacting with a phospholipid bilayer, however, the peptide mainly

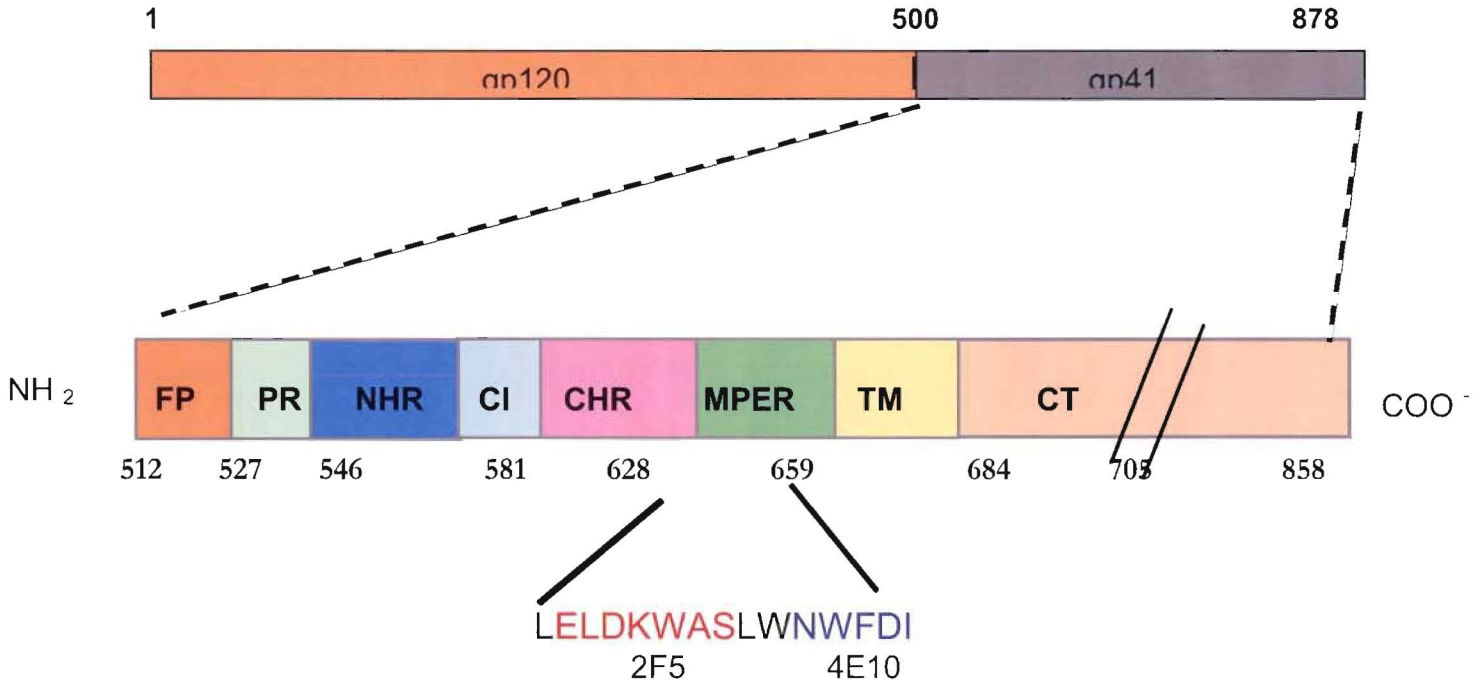


Figure 1-2. Schematic representation of the HIV-1 env gene products and gp41 functional regions. The HIV-1 Env protein is synthesized as a precursor (gp160) that is cleaved by the host protease into the two Env proteins, gp120 (surface protein) and gp41 (transmembrane protein). Fusion peptide (FP); Polar region (PR); N-heptad repeat (NHR); Custer I (CI); C-heptad repeat (CHR); membrane proximal external region (MPER); transmembrane region (TM); cytoplasmic domain (CT).

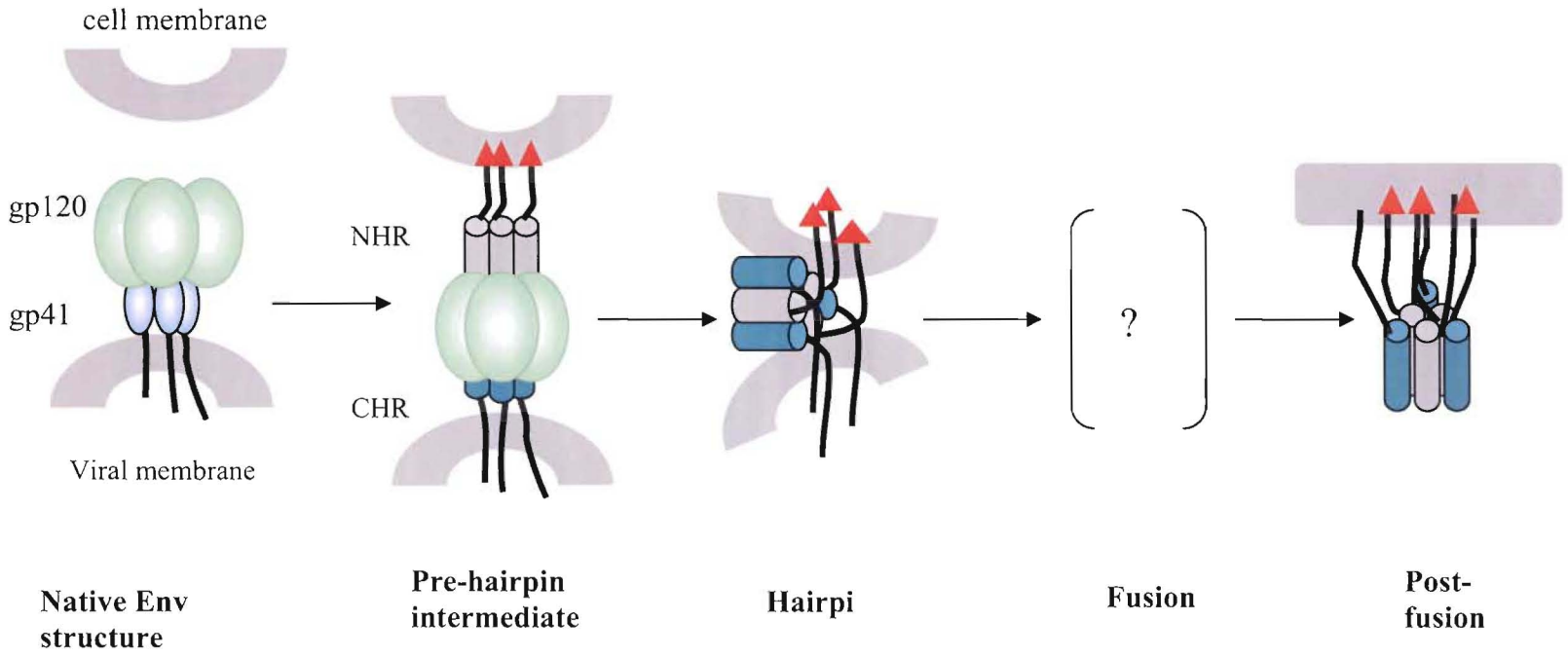


Figure 1-3. Model of HIV gp41-mediated membrane fusion. In the viral spike (native conformation) the fusion peptide is not exposed. After interaction of gp120 (light green) with its receptor and coreceptor, the spike undergoes conformational changes that allow the exposure of the fusion peptide (red arrow) and its insertion on the target cell membrane. The NHRs form a trimeric coiled coil, known as the pre-hairpin intermediate. The pre-hairpin structure evolves to a stable structure in which the three NHRs form a core bundle in parallel, with the three CHRs associated antiparallel to the NHR bundle; this structure is known as the hairpin or six-helix bundle (6HB). It is believed that the formation of the 6HB causes the apposition of the two membranes. However is it not clear how the fusion process occurs (pore formation and expansion). NHR: N-Heptad Repeat, CHR: C-Heptad Repeat, (39).

adopts a β-sheet conformation in the presence of lipids (148-150). Other studies with a 23-mer peptide have suggested that the FP is a β-pleated structure (180, 181, 188-191, 232, 276). More recently, the structure of the FP was evaluated using solid-state NMR spectroscopy (284), this study revealed that the structure adopted by the FP after interaction with membrane could be influenced by the cholesterol concentration in the model vesicles used. Helical structure for FP in DPC micelles was recently reported by Li and co-workers, using solution NMR. They proposed that the α-helix is the most likely the physiologically-active conformation of the FP during HIV-1 infection (134). The differences between studies could be explained by the differences in peptide length, lipid composition of the membrane model, and/or methods used. Thus, although the role of the FP in the fusion process is well established, the structure of the region in the native conformation of gp41 or in the fusogenic state is not known.

The N-terminal and C-terminal Heptad Repeats: The presence of the NHR and CHR in the HIV-1 gp41 ectodomain is a feature shared with the transmembrane envelope proteins of other retroviruses (for review see (222)). It is believed that these regions play a key role in virus-host membrane fusion, with gp41 undergoing drastic conformational changes that result in the formation of the 6HBs. This is a stable structure (a trimer of hairpins) in which three NHRs form a core bundle in parallel, with three CHRs associated antiparallel to the NHR bundle (**Figure 1-3)** Interfering with the formation of the 6HB using NHR or CHR synthetic peptides can inhibit HIV-1 infection and cell-cell fusion at peptides nM concentrations (39, 116, 266, 267), indicating that it is a key process for viral infectivity. The formation of 6HBs was originally accepted as the process that brings the viral and the cellular membranes together, and allows the aggregation of

several activated Env complexes to form a pore, leading to entry of the nucleocapsid into the cell (80, 161, 263) (for a review of gp41 structure and fusion see (261, 291)).

There is no consensus on a model explaining the events leading to viral entry; two entirely different propositions have been made concerning the timing of 6HB formation. Some studies indicate that the transition of gp41 into 6HBs drives membrane fusion by bringing the two membranes in close proximity (80, 88), whereas others propose that the 6HBs are formed after the pore formation (146, 161). It has also been suggested that two parallel pathways of gp41 conformational rearrangement may coexist, one leading to 6HB formation, and the other leading to the generation of hairpin monomers (33). A couple of studies have supported this model (61, 125), thus fusion is considered a very dynamic process during which different conformations of gp41 may coexist at the same time.

The Transmembrane Region: The TM is 22 amino acids long, and anchors Env into the lipid bilayer. It is highly conserved among different HIV-1 isolates, and is believed to play a direct role in viral fusion, since substitution of the TM region for a covalently-linked lipid anchor does not support fusion (209, 262). Direct involvement of the gp41 TM in the formation of the fusion-competent Env protein has been recently proposed (264). In contrast, Wilk *et al.* (268) reported that substitution of the gp41 TM by the TM of the cellular protein CD22 did not affect infectivity, suggesting that the gp41 TM is not required for fusion. Other studies support the impairment of fusion when the gp41 TM was replaced by the TM domain from glycoporin A, vesicular stomatitis virus G (165), and more recently, by the TM domain from influenza hemagglutinin (HA) (137). Important roles of theTM in fusion have also been described for other viral proteins (137,

236). The length and the amino acid composition of membrane-spanning regions may determine the localization of proteins in specific regions of the plasma membrane that have the appropriate lipid composition; this phenomenon is known as "the hydrophobic matching principle" (for review see (114)). Modification of those parameters probably has a direct influence on TM activity (for review see (182)) and most likely, the overall protein function. As solving the structure of a TM protein is usually very difficult, the full structure gp41 including the TM region is not known. However, early computer modeling suggested that as is typical for TM domains, the gp41 TM region adopts an alpha helical conformation (83).

The Cytoplasmic Tail: The gp41 cytoplasmic domain is unusually long for a transmembrane protein (~150 aa); its exact functions are not clearly understood, although it is believed to have important functions *in vitro*. Mutagenesis studies of the CT have suggested an involvement in Env incorporation into the virus (192, 280), decreased virus infectivity (272), and structural perturbations of gp120 leading to increased sensitivity to Ab-mediated neutralization (68). Other functions have also been indicated for the CT, including interaction with the viral matrix protein (50, 64, 71, 280), targeting to vesicles (59, 138, 206) as well as interaction with other proteins (112)Kliger, 1997 #72; Wyss, 2001 #790; (15, 164, 184, 273). The presence of an endocytic motif YXXΦ in CT (aa 712-715) has been reported (59, 138, 139); in fact, substitution of the Tyr_{712} for Ser increased the expression of the Env proteins in the cell surface, by reducing endocytosis of Env (28). The structure of the CT region is not known, but a computer prediction model indicates the presence of three aliphatic regions called lentivirus lytic peptides: LLP-2 (aa 768-788), LLP3 (aa 788-814) and LLP-1 (aa 828-855) (69, 101, 102, 123,

163). Several studies with these peptides have shown that they bind to membranes (124, 224, 244), perturb membranes (8, 40, 48), and also bind to calmodulin (223, 237). Structural modeling has described gp41 as a type I single pass membrane protein (83); however, the work of Dimmock and colleagues with a MAb targeting the intracellular CT suggested that the protein could have other membrane spanning domains, and accordingly, they have proposed a model for a possible structure that shows the CT doubling back through the membrane surface of the virus and back inside, possibly, exposing the Kennedy epitope (46).

1.4. The MPER structure and function

The MPER includes the last 24 C-terminal amino acids of the gp41 ectodomain, LLELDKWASLWNWF(N/D)ITNWLWYIK (aa 660-683) (288); it is a highly-conserved region (174, 197, 210, 290). The most interesting feature of this region, and what makes it so attractive for HIV-1 vaccine development, is that three HIV-1 bNt MAbs (2F5, 4E10 and Z13) recognize epitopes located in the MPER (174, 290).

The actual structure of the MPER is the subject of an ongoing debate; the region has been described as an α-helix in some studies, and as an extended β-turn in others. It was originally predicted that the MPER has an α-helical structure (83), and this is supported by crystallographic structures of this region by three independent studies. These show that the region immediately N-terminal to the MPER, and partially overlapping the 2F5 epitope, exists as an α-helix, at least in the fusogenic state of the virus (39, 234, 263). Moreover, a NMR structure of the19-mer peptide, KWASLWNWFNITNWLWYIK (aa 665-683), shows that the MPER adopts an α-helical

conformation in dodecylphosphocholine micelles in which the aromatic and polar residues are distributed around a helical axis. The peptide interacts with the H_2O-DPC interface of the micelles, and it was found that all the "aromatic" groups of Trp and Tyr residues in the MPER are positioned in the same plane (219). Likewise, another structural study has shown that the 2F5 epitope adopts a helix conformation (18). In this case, the structure of a 42-residue peptide, NN-T-20-NITN (YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF, aa 638-673), was analyzed using heteronuclear 2D and 3D NMR. The secondary structure under near-physiological conditions showed that the NN-T-20-NITN peptide is comprises of a mostly unstructured N-terminal region and a helical region beginning at the center of T-20 and extending toward the C-terminus. Thus, it was generally accepted that the MPER functional structure is in helical conformation.

More recently, other structural data have raised the possibility of alternative MPER models. Contrary to a helical structure for the MPER, structures of the 2F5 Fab bound to peptide from within the MPER revealed a different conformation. The first structure of 2F5 in complex with a 7-mer peptide, ELDKWAS (185), and a second structure with a 17-mer peptide, EKNEQELLELDKWASLW (aa 654-670) (183), revealed that this region of the MPER is not a helix, but an extended conformation with a distinct β-turn at the DKW in the core of the peptide epitope. As the DKW core is buried in the Fab interface, it is thought that these residues are exposed on the Env trimer prior to fusion. In contrast, the epitope bound by the 4E10 Fab (aa 670-678), which is adjacent to the 2F5 epitope, adopts a helical structure (37). It is believed that the structural differences observed for the MPER, could be related to the overall gp41 structural

changes that occur during fusion, and raises the possibility that a structural transition of the MPER occurs during fusion from an extended structure to a helical one (11).

Intriguingly, the structure of the MPER in micelles shows the residues that directly contact the 4E10 MAb paratope pointing into the micelle (219). This supports the possible interaction of those residues with membrane, due to their proximity to the membrane. Similarly, it has been observed that 4E10 and Z13, which bind overlapping epitopes, interact with two different faces of the MPER helix (177). This recent report delineated the Z13 epitope using Ala-substitution studies (177). Taken together, these observations support the flexible nature of the region and its possible neutralization by different mechanisms, perhaps during the fusion event. However, it also raises the possibility that immunogens with different orientations of the MPER helix, or even with extended structures can be useful for generation of Nt Abs. Moreover, the structural flexibility of the region might contribute to the low immunogenicity of the MPER and the lack of Nt Ab produced by immunization studies. In support of the idea that the MPER is flexible, Granseth *et al.* (92) showed that the membrane proximal regions for membrane-inserted proteins are unusually enriched in irregular structure and in interfacial helices, and that they run roughly parallel with the membrane surface, whereas β-strands are extremely rare. In this region, hydrophobic and aromatic residues tend to point into the membrane and charged/polar residues tend to point away from it. This analysis is very interesting considering our current knowledge of the gp41 MPER, for which the existing data do not support a single static structure. Whether the α-helical structure of the MPER is present in the native pre-fusion state of gp41, or is induced upon binding of the virus to its receptor(s) has yet to be determined.

The HIV-1 membrane is considered to be highly-ordered, and it is believed that lipids play a role in infection by affecting Env structure and function. In fact, viral infectivity is impaired by modification of the lipid content, such as increasing the temperature or treating the virus with 50% ethanol (4). The HIV-1 membrane contains a very high ratio of cholesterol to phospholipids, > 1.00 (200). Cholesterol plays an important role in viral infection since its depletion ablates infection (214, 217). Moreover, the cholesterol-binding motif, LWYIK (aa 679-683) has been found in the MPER,(251). Saez-Cirion *et al.* studied the ability of the interfacial sequence preceding the TM, DKWASLWNWFNITNWLWYIK (aa 664-683), to interact with membrane, and showed that it forms lytic pores in liposomes composed of the main lipids occurring in the HIV-1 envelope: 1-palmitoyl-2-oleoylphosphatidylcholine, sphingomyelin, and cholesterol (1:1:1 molar ratio) (208). These results suggest that the MPER sequence may participate in the clustering of gp41 monomers within the HIV-1 envelope, and may destabilize the bilayer architecture at the locus of fusion. The authors hypothesized that the interfacial MPER sequence behaves as a signal for lipid domain targeting, and proposed several mechanisms by which the presence of lipid domains could influence the fusion process: (*i*) by supporting clustering of the MPER, and further activation of fusion activity; (*ii*) lipid domain targeting by the MPER might cause surface aggregation of gp41 trimers, thereby assisting the formation of oligomeric complexes involved in fusion pore opening; and (*iii*) the creation of membrane projections (nipples) enriched in cholesterol/sphingolmyelin by MPER. The interaction of the MPER with membrane was also demonstrated by studies in which the peptide, DKWASLWNWFNITNWLWYIK (aa 664-683), mediated membrane partitioning, fusion and permeability (232, 233).

It has been well established that the MPER plays a role in fusion of the viral and cell membranes, however, the exact nature of that role has yet to be defined. This region contains numerous hydrophobic residues and is unusually rich in Trp. Substitution of some of these residues dramatically affects the efficiency with which gp41 is incorporated into virions, and (possibly as a consequence) inhibits viral entry into target cells (210). Salzwedel *et al.* showed that deletion of 17-amino acids in the MPER (aa 666-682) completely abrogated the ability of Env to mediate both cell-cell transmission and viral entry; however, virus maturation, and binding to CD4 were not affected. This study concluded that the MPER is essential for fusion activity and for the incorporation of Env into virions (210). In addition, the sequence LLELDKWASLW (aa 660-670) is important for fusion, since its deletion impaires syncytia formation (194). An additional study by Muñoz-Barroso *et al.* using a cell-cell fusion assay allowed the classification of MPER mutants to three different phenotypes: those showing reduced activity, defective variants that are unable to mediate fusion, and mutants that assemble non-expanding fusion pores (171). These results have lead further support to a role for the MPER in the fusion process. Besides this analysis, there is no model explaining the mechanism by which the MPER mediates fusion, even though a 36 amino-acid synthetic peptide (YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF, aa 638-673) derived from the CHR (also called DP-178, T-20, Enfuvirtide and FuzeonTM) (267), is currently used as new class of antiviral drug and works by inhibiting fusion. This peptide sequence overlaps with the sequence recognized by 2F5 MAb (121, 132). The role of the MPER in fusion is further supported by three bNt MAbs (2F5, 4E10, and Z13) that recognize

epitopes in this region; thus, binding to the MPER blocks infection, presumably by interfering with some critical step(s) required for viral entry.

A recent study used two different modes of viral transmission, cell-cell and virus-cell fusion to evaluate the crucial role of the FP-proximal PR and the MPER during HIV-1 infection, (12). Interestingly, Ala substitution of Trp_{672}, Trp_{666} Phe_{673} and Ile_{675} in the MPER reduced viral entry potential by ~120 fold without affecting cell-cell fusion. However, a single Ala substitution in the MPER, in combination with that of Leu_{537} in the polar region, inhibited cell-cell fusion and viral entry. Therefore, the authors proposed that the MPER might have distinct roles during the different steps of the fusion process.

To test the contribution of individual MPER residues on Nt activity, Zwick *et al.* generated Env mutants by replacing MPER residues with Ala (288). The mutant Envs were used to make pseudoviruses, which were tested in neutralization assays with 2F5 and 4E10. The results of the study are in agreement with the critical binding residues required for interaction of 2F5 with synthetic peptides bearing the 2F5 epitope sequence (196, 240). However, the situation is somewhat different for 4E10, for which the residues critical for binding and for Nt activity did not fully coincide. 4E10 critical residues for binding to epitope peptide were Trp_{672}, Phe_{673}, and Thr_{676} (27, 37), whereas residues critical for Nt activity were Trp_{672}, Phe_{673}, and Trp680. It was suggested that these differences might be related to the use of the MPER in two different settings (synthetic peptide *vs.* membrane-attached region). Taken together, these studies confirm the role of the MPER during viral transmission, and support current efforts to target this region for neutralization of HIV-1.

HIV-1 infection occurs mainly *via* mucosal transmission, thus it must be understood how Abs neutralize the virus at the epithelial surface. Bomsel *et al.* showed that HIV-1-infected cells that are in direct contact with the apical surface of an epithelial cell can generate virus that crosses the tight epithelial barrier by transcytosis. This event could be blocked by IgM or dimeric IgA generated against Env (3, 20) suggesting that induction of mucosal Abs against Env can block HIV-1 mucosal transmission. In addition, this group showed that HIV-1 binds to epithelial cells using an alternative receptor, glycosphingolipid galactosyl ceramide. This virus-receptor interaction is mediated by the conserved ELDKWA epitope on gp41, since S-IgA from the colostrum and cervicovaginal secretions from several HIV-1^+ patients were capable of blocking epithelial HIV-1 transcytosis by binding to the ELDKWA epitope. This finding further supports the potential of an MPER HIV-1 vaccine, since it could be used to elicit protective Abs at mucosal sites (3).

1.5. Immunogenicity of gp41 in natural infection

Although gp41 is mostly occluded by gp120 on the native viral spikes, shedding of gp120 can expose gp41 to the immune system. In addition, gp41 may be exposed in different structures during the several stages of the fusion process. Reflecting this, the Ab response during HIV-1 natural infection typically includes strong reactivity against gp41 (111). There are several immunogenic regions on gp41, including the PR that follows the FP, the NHR, the ID loop, the MPER, and to a lesser extent, the CT.

A few human MAbs have been isolated against the polar region, AAGTMGAASMTLTVQARQ (aa 525-543), and Abs against the NHR have been

reported in sera from HIV-1^+ donors, indicating their immunogenic nature (29, 195). The ID loop (also known as epitope cluster I) has been known as the primary ID region on gp41 since the earliest serum mapping studies (42, 86, 87). Although most of the Abs against this region are not Nt, one MAb has been shown to have Nt activity to both diverse T-cell laboratory adapted (TCLA) viruses from clade B, and three primary isolates from group O (51, 74). Interestingly, some MAbs generated against this region show an infection-enhancing activity (203). Epitopes in the CHR (aa 521-663) are only available after interaction of gp120 with CD4; these regions are probably masked by gp120 (215). However, a relatively ID region, immediately N-terminal to the 2F5 epitope (ELDKWA) has been described and named epitope cluster II (644-663) (275). In addition, it is believed that the CT of gp41 is contained by the viral membrane, and therefore not exposed to the immune system, there are several MAbs that recognize a hydrophilic region in the so-called "Kennedy epitope" (PRGPDRPEGIEEEGGERDRDRS) at the N-terminus of the CT (aa 724-745) (30, 45). Two possible structures have been proposed for the CT, with each being adopted as part of drastic conformational changes produced by gp41 during viral infection (41, 62). Thus, regions within the CT could interact with membrane (LLP-3, LLP-2 and LLP-1), and perhaps cross it, to be exposed and form immunogenic epitopes on the viral surface (*e.g.*, the Kennedy epitope).

1.6. Prevalence and nature of the Ab response against the MPER

In contrast to the NHR and ID loop, the MPER is not strongly immunogenic, yet three bNt MAbs have been discovered against this region: 2F5, 4E10 and Z13 (29, 174,

290). Both 2F5 and 4E10 neutralize a broad range of both laboratory-adapted and primate isolates of HIV-1 (17, 49, 231). Of the two, 2F5 is the most potent Nt Ab, whereas 4E10 neutralizes a broader range of HIV-1 isolates, as shown by pseudovirus studies using an extensive panel of Env derived from primary isolates (17, 160). A number of studies have reported results from "serum mapping" using synthetic linear peptides covering the Env sequence to detect Ab reactivity in serum samples (23, 26, 35, 84, 111, 170). Using this approach, some studies have detected Ab against the MPER, indicating that it is immunogenic during natural infection. The work of Broliden and colleagues (26) revealed the presence of cross-reactive Abs with the peptide, QQEKNEQELLELDKW (aa 652-666) with a frequency of 56%, among samples from 50 HIV-1 positive subjects. Abs to the region upstream the 2F5 epitope have also been detected by others. Ugen *et al.* (246) investigated 20 sera from HIV-1^+ mothers by screening with the peptide QNQQEKNEQELLEL (aa 650-663) and found that 70% of the samples reacted with this peptide. In another study, a set of overlapping peptides covering the MPER was tested against sera, and it was found that the region adjacent to the C-terminus of ELDKWA was ID. In addition, more than 30% of the sera were reactive to ELDKWA peptides (35). A peptide, ELLELDKWAS (aa 659-668) was used by Vanini *et al.* to affinity-purify and quantify epitope-specific Abs from HIV-1^+ sera, they found that 12 out of 48 sera had reactivity against the peptide (250)). In addition Geffin *et al.* showed an association between Ab reactivity to the ELDKWA epitope peptide and disease progression in 29 children who had been perinatally infected with HIV-1. Approximately 50% of infected individuals displayed a detectable Ab response against the peptide (84). More recently, Srisurapanon and coworkers studied the serum

Ab reactivity against the peptide ELDKWA in HIV-1 infected subjects, and showed that its frequency was low (15-35%), and that Ab titers to this epitope in sera from AIDS patients were significantly lower than those in sera from asymptomatic subjects (225). Braibant and colleagues used competition assays to determine the presence of 2F5 and 4E10-like Abs in HIV-1^+ sera. Sera were used to compete with biotinylated MAbs 2F5 and 4E10 for binding to Env captured on microplates (23). They detected 2F5-blocking Abs in all the sera from long-term non-progressors, although the levels were low. They also analyzed the binding of Abs to the MPER peptide by direct ELISA, and showed that 60 % of sera were positive for Ab against this region. This study demonstrated that almost all long-term non-progressors develop MPER-binding Abs, albeit at very low levels, but did not address the question of whether these Abs were Nt. There exists the possibility that Nt Abs against the MPER are at such low titer or low affinity in a serum that they are not sufficient to inhibit HIV-1 infection *in vitro*, and are therefore undetectable in neutralization assays.

Recently, a new approach based on neutralization rather than binding, has been developed for detecting the prevalence of epitope-specific Nt Abs, in HIV-1^+ sera, especially against the 2F5 and 4E10 epitopes. The epitopes for these two MAbs were grafted into the MPER of SIV (281) or HIV-2 (Shaw, *et al.*, CROI, 2006), replacing homologous SIV and HIV-2 sequences with that of HIV-1. The chimeric pseudoviruses were used to screen 96 HIV-1^+ for Nt Abs. None of the sera neutralized chimeric SIV bearing the 2F5 epitope, and only one neutralized SIV chimeric virus bearing the 4E10 epitope (281). Similarly, bNt sera from three asymptomatic patients (two from clade B and the other from clade C) also failed to neutralize the chimeric viruses (60). In another

study, the neutralization activity of 14 sera from donors infected with clade C viruses were evaluated for the presence of 2F5- and 4E10-like Nt Abs using the HIV-2 chimeric pseudoviruses. MPER-specific Nt Abs were detected in ~28 % of the people within the first year of infection (94), even though clade C viruses have been reported to be insensitive to 2F5 Nt activity (17, 31, 93).

From these studies, it can be concluded that Abs against the MPER are detected in a significant proportions of HIV-1^+ sera by conventional ELISA, but it not known if those Abs are Nt. However, studies using chimeric pseudoviruses with epitopes in a context that is more closely related to the MPER structure, support the notion that MPER-specific Nt Abs are rare or absent during natural infection. It is not known if the lack of Nt activity shown by MPER-binding sera is due to low titers of Nt Ab, or if Nt Abs are completely absent. This issue of quantity *vs.* quality has not been fully resolved, yet it has significant implications for vaccine design.

There have been some reports on the Ab response against the MPER in natural infection (see below); but most serum Ab responses to this region are not as bNt as 2F5 or 4E10 in neutralization assays (23, 26, 35, 84, 111, 170). This is not surprising as the Nt activity in many sera appeared to be restricted to gp120 (60).

1.7. MAb 2F5 and its epitope

The 2F5 epitope was first mapped to a linear sequence (ELDKWA, aa 662-667) on the MPER (174). This epitope has since been confirmed by others, and has been expanded from the original 6-mer to a longer 17-mer linear epitope (aa 655~671) (11, 49, 162, 186, 240). Mutagenesis studies have demonstrated that the central residues DKW

are critical for Ab binding and neutralization (49, 162, 196, 288). Consistent with this, peptides bearing the DKW motif are repeatedly selected from phage-displayed peptide libraries (49, 162, 245), as well as a phage-displayed gp160 gene fragment library (290).

However, Neurath *et al.* (178) showed that 2F5 reacts with sequences from two partially overlapping peptides from CT in addition to the ELDKWA epitope (PTPRGPDRPEGIEEEGGERDRDRSIRLV, aa 722-749), and (GGERDRDRSIRLVNGSLALIWDDLRSLC, aa 737-764). These two peptides do not show any homology with (aa 638-675), and did not block the reactivity of 2F5 with peptides from that region; peptide (aa 722-749) inhibited 2F5 binding to HIV-1 by 63 % while peptide (aa 737-764) didn't have a measurable effect. The authors suggested that the epitope for 2F5 is discontinuous and that the ELDKWA (aa 662-667) segment represents only a portion of the epitope, providing a major contribution to the gp41-binding capacity of the MAb. This is an isolated result that requires confirmation. Since (aa 722-764) is located in the CT of the protein and presumably is not exposed, it seems unlikely that this domain could play a role in the binding of the Ab. However, the work of Dimmock *et al.* proposed that gp41 has more than one transmembrane domain and this implies that regions thought to be on the inside are actually exposed on the virion surface (46), (for review see (62)).

The crystal structure of the 2F5 Fab in complex with a series of short synthetic peptides or an elongated 17-mer peptide, EKNEQELLELDKWASLW (aa 654-670), has been resolved by two different laboratories (183, 185). Both studies showed an extended β-turn conformation of the peptide in the region, DKW. One interesting characteristic of the longer peptide is that only 41% of it (the charged face) is bound by 2F5 Ab, while the

other hydrophobic face is unbound. This suggests that the non-2F5-bound face is occluded by other portions of the envelope protein, and/or is buried in the plasma membrane.

Another striking feature of the 2F5 Fab-peptide complex is its unusually long CDR-H3. Curiously, of the 22 amino acids, only 10 at the base of CHR interact with the peptide, whereas the apex remains largely unbound. Yet, mutagenesis studies showed that substitution of the Trp at the hydrophobic tip of 2F5's long CDR-H3 significantly decreases the binding affinity of 2F5 to both gp41 and its epitope peptide, and also decreases neutralization activity (289). This suggests that the tip of the long CDR-H3 is involved in further interaction, and/or that the hydrophobic tip is required for the Ab to maintain its overall structure and thus, its MPER-binding paratope. Given the hydrophobicity of the CDR-H3 and the proximity of the 2F5 epitope to the viral membrane, the long CDR-H3 may also contact the membrane directly to facilitate MPER binding (183).

A recent study by Julien *et al.* (Keystone Symposia, HIV Vaccine, 2007) revealed a new X-ray structure for the 2F5 Fab bound to an elongated peptide. It showed that the structure at the N-terminus of the DKW core and the β-turn motif of the core as in previous structures, whereas the residues at the C-terminus of the epitope did not adopt any specific conformation. The new structures also revealed that the CDR-H3 loop does not adopt a single conformation, indicating that the interaction between Ab and peptide does not lock the H3 loop into a single conformation. These results complement those of Menendez *et al.* who showed that 2F5 can bind to a variety of sequences C-terminal to the DKW core, and that critical binding residues could be identified within those regions

(162). Lorizate *et al.* have proposed that the N-terminal region of gp41 is part of the 2F5 epitope (140). In their model, the MPER and the FP are adjacent, and may form the entire epitope. It is generally accepted that the natural epitope of 2F5 includes more than the core epitope (ELDKWA). Moreover, viruses bearing the DKW core may still be resistant to neutralization by 2F5 (31), which suggests that other regions of the protein are involved in the formation of the neutralization-sensitive epitope.

A study from Haynes *et al.* indicates that bNt MAbs 2F5 and 4E10 are polyspecific as they bind self-antigens (whole cells and cardiolipin (CL)) (105), and could be considered auto-Abs. Yet, CL is not a predominant lipid in the HIV-1 membrane (5), and is not found in high levels in the plasma membrane. In a recent study they compared the CL reactivity of 4E10 and 2F5 with two human thrombogenic anti-CL MAbs using a BIAcore instrument (1). They showed that the bNt Abs bind with similar affinities to CL; however, they observed a difference in the kinetics of binding of 2F5 and 4E10 to their protein epitopes conjugated to liposomes *vs.* the protein epitopes alone. The binding of 2F5 and 4E10 to their respective epitope-peptides conjugated to lipid was better defined by a two-step model, whereas the interaction of the MAbs with linear peptide followed a Langmuir model. The authors concluded from this that 4E10 and 2F5 interact with membrane and MPER in a two-step model involving an initial encounter followed by conformational docking. In further support of the Haynes hypothesis, the study of Sanchez-Martinez *et al.* (211) shows that CL-containing liposomes inhibit the neutralization activity of 2F5 MAb. However, the 2F5 interaction with liposomes does not necessarily mean that the liposomes mimic the 2F5 MPER epitope. Alternately, liposomes may interact with 2F5 by a different mechanism. Different mechanisms of

interaction of MAbs with different Ags have been confirmed before for bNt MAbs b12 and 2G12 (162, 212, 287). Considering these results, it would be interesting to clarify whether mutations in the 2F5 MAb that abrogate binding to the MPER also affect binding to CL. Nevertheless, the biological consequences of the CL reactivity of 4E10 and 2F5 are unclear.

Importantly, when HIV-infected individuals were passively immunized with 2F5, and 4E10, no immunopathological side effects were reported (119, 243). Trkola *et al.* evaluated passive transfer of 2F5 and 4E10 in combination with bNt Ab 2G12 in HIV-1-infected individuals (243). These studies raised concerns about the *in vivo* activity of 2F5 and 4E10, since the viral rebound observed in several patients occurred along with the emergence of 2G12 resistant virus with no resistance emerging to the anti-MPER MAbs. However, a new study from the same group, showed a different picture after performing a more in-depth analysis of the virus *in vivo* and *in vitro* (145). No mutations in the MPER were found from viruses obtained from patients. In contrast, if the viruses isolated from the same patients were cultured under high levels of Nt MAbs, neutralization-resistance viral variants emerged, indicating that they comprised a sub-dominant subset of the total virus *in vivo*. The mutations that supported escape from 2F5 and 4E10 were in residues in the MPER previously defined as critical for Nt of the virus (mutations D664N and F673L). Moreover, it was noted that viral escape mutants have reduced infectivity, which could account for the reduced frequency of these viruses *in vitro*. This new study also supports the potential of the MPER as a target for HIV-1 vaccine development.

1.8. MAb 4E10 and its epitope

The 4E10 epitope was first mapped to AEGTDRV (aa 823-829), located in the cytoplasmic tail of gp41 (29). The intracellular localization of the 4E10 epitope resulted in 4E10 being ignored as a bNt Ab for several years. Zwick *et al.* and Stiegler *et al.* resurrected interest in 4E10 after they mapped the epitope to the linear sequence, NWFDIT (aa 671-676), just C-terminal to the 2F5 epitope in the MPER (231, 290), also helped by a study of Binley *et al.* who showed that 4E10 was the most broadly-Nt Ab known so far (17). As with 2F5, there is also conflicting information regarding the "full" 4E10 epitope, as a new study suggests that 4E10 binds both FP at the N-terminus of gp41 and the MPER epitope (103). The authors hypothesized that the full 4E10 epitope in previous studies was missing because soluble gp41 does not include the FP. Recent mutagenesis of the 4E10 peptide has shown that Trp_{672}, Phe_{673}, and Thr_{676} are essential for 4E10 binding (27), whereas Trp_{672}, Phe_{673} and Trp_{680}, located C-terminal to the core epitope (7 aa away), are important for 4E10-mediated neutralization (10) Thus, similar to the epitope of 2F5, the 4E10 epitope seems to involve other residues than those originally mapped in the core epitope.

The crystal structure of 4E10 in complex with MPER peptide, WNWFDITNW (aa 670-678), reveals several interesting features (37). First, the 4E10 epitope peptide adopts an unusual helical conformation when bound; yet typically, β-turns are the predominant secondary structure of Ab-bound peptides (227). The importance of the helical epitope conformation for 4E10 binding has been supported by the study of Zwick *et al.* in which binding of the Ab was reduced by denaturation of recombinant gp41 (290). In addition, the crystal structure of the Fab 4E10-peptide complex illustrates that the core sequence, WFXIT, makes the greatest number of selective contacts with 4E10,

with Trp672 being the most highly contacted residue. The WFXI (T/S) motif, in which the X residue does not play a major role in 4E10's binding, appears to be highly conserved among all HIV-1 isolates, thus explaining 4E10 ability to neutralize a wide range of HIV-1. Ala substitution studies of the MPER support the crucial role of the conserved Trp672 in virus infectivity (210). Consistent with this, more sequence variation occurs on the opposite side of the helical epitope flanking the conserved WFXIT where there are fewer contacts with Ab. The Ab combining site of 4E10 is remarkably hydrophobic, due in part to its CDR-H3 loop, as well as an unusually hydrophobic CDR-H2 loop, making this region considerably more hydrophobic than that of most Abs. The CDR-H3 of 4E10 is also relatively long (18 aa); yet only two residues at the base of the CDR-H3 contact the C-terminal region of the peptide epitope, with the apex of the loop bending away from the peptide. The tip of the CDR-H3 loop of 4E10 is composed of mainly non-polar residues that form a very hydrophobic flat surface, which has been suggested to interact with the viral membrane (see details below). Since it does not directly bind to the peptide epitope, the importance of this long CDR-H3 in neutralization is still unknown since a mutational analysis of the 4E10 Ab is not available.

In a recent study from Cardoso *et al.* (36) a series of peptides were developed to further characterize the epitope recognized by 4E10 MAb. The crystal structures of 4E10 in complex with peptides that contained residues that increased helical character or length, were shown to positively influence binding of 4E10 to its epitope. An extended and modified core epitope for 4E10 with a sequence motif of WFX(I/L)(T/S)XX(L/I)W was deduced. This type of study is of significance since vaccines could be designed, in

which residues that are not critical for 4E10 binding (X) are replaced with those that impose helix-promoting constraints. Such vaccines would promote a 4E10-binding structure and may be more likely to produce Nt Abs.

1.9. MAb Z13 and its epitope

The Fab Z13 was isolated by screening a phage-displayed Fab library prepared using the bone marrow cells of the patient, FDA-2, an HIV-1^+ individual with whose serum is bNt (72, 78, 252). The libraries were screened using with synthetic peptide, LLELDKWASLWNWFDITNWLW (aa 660-680), from the HIV-1_{MN} isolate. Positive phage clones were identified by ELISA with the same peptide used for the screening, and then the soluble Fab was produced and analyzed in binding studies. Among the Fabs selected, the Z13 Fab showed strongest reactivity with the peptide. Interestingly, the whole HIV-1_{MN} virus also was used for the library screening, and a similar Ab to Z13 was selected, indicating that the epitope recognized by the Fab Z13 is actually exposed in the viral surface.

Using a competition ELISA, it was determined that the epitope recognized by Z13 overlapped the region recognized by the 2F5 and 4E10 Abs; however, the majority of the overlap was with 4E10 epitope, since 4E10 inhibited the binding of Z13 to the peptide more strongly than 2F5 (> 90% and >70 %, respectively). Using different lengths of peptides covering the 4E10 and 2F5 epitopes from different HIV-1 isolates, it was determined that the core region of the MPER was recognized by both Z13 and 4E10. Both Abs recognize a peptide as short as NWFDITK (aa 671-677). Interestingly, Z13 did not bind to gp41 from the HIV-1 isolate IIIB; which has a different amino acid

sequence (NWFNIT), indicating the importance of D674 for Z13 interaction, and further supporting that the epitope recognized by Z13 overlaps the one recognized by 4E10. However, the Nt capacity of Z13 is weaker than those of 2F5 and 4E10, even though it neutralizes primary isolates as well as T-cell adapted laboratory adapted (TCLA) ones (290).

Recently, an improved version of Z13 Ab was developed by Nelson *et al.* (177) by generating a phage-displayed Fab library containing random mutations in the CDR-L3 of the Z13 coding sequence. A Fab with higher MPER affinity was selected (Z13e1), which binds gp41 and MPER peptide ~35-fold more strongly than Z13. Interestingly, the Z13e1 Ab binds better to a peptide that is elongated at the N-terminus (LLELDKWASLWNWFDITNWLWYIKKKK, aa 660-683), thus, the authors proposed that the optimized Z13e1 Ab has a core epitope located N-terminal to the core epitope recognized by 4E10 Ab, and C-terminal to the 2F5 epitope. The newly optimized Z13e1 Ab showed an increased affinity for the MPER, and an increase in its neutralization potency.

The critical residues recognized by Z13e1 Fab were mapped using Ala substitutions in the MPER, and revealed that the most important residues for Z13e1 binding are Asn_{675} and Asp_{678} (177). Although the structure of Z13e1 in complex with its epitope is not available, a hypothetical model of the MPER sequence (DKWASLWNWFDITNWLW, aa 664-680) as an ideal α-helix was created. According to this model, the critical MPER-contacting residues for the 4E10 and Z13e1 Abs are located on opposing faces of the helix, placing the Z13e1 epitope on the "non-Nt" face of the helix (27, 37). This could explain the lower neutralization potency of Z13e1

compared to 4E10 and 2F5. It also reflects the fact that the MPER is flexible and probably changes its conformations to bind 4E10, Z13, and perhaps 2F5 during infection. Thus, each of the three Abs may bind the MPER at different points during the conformational transition to pore formation and fusion.

1.10. Vaccines targeting the MPER

The isolation of three bNt Abs whose epitopes are located in the MPER couple with the conserved nature of the MPER in HIV-1 isolates from different clades makes the MPER a key target for HIV-1 vaccine development. However, as with all the bNt MAbs, 2F5 and 4E10 have been isolated a single time from infected donors, and Z13 was selected from a phage displayed Fab library, indicating that the Nt epitopes on the MPER are poorly immunogenic in natural infection. Consistently with this, the prevalence of MPER Nt Ab in sera from infected patients has proved to be very low (see above). As explained in Section 1.2, gp41 is a very complex protein (*i.e.*, it adopts different conformations during the infection process), and the gp41 structure required for infection remain controversial. The situation with the MPER structure in particular is not very different; α- helical and β-extended structures have been reported for it. 2F5 and 4E10 were isolated by the Katinger laboratory in 1993 (174) and very early on mapped to short linear sequences on gp41 (49, 186, 240). Thus, most of the effort involved in eliciting an anti-MPER Nt response has been based on the use of short, linear epitopes as immunogens in various formats. Moreover, since the 4E10 epitope was originally mapped to the CT region of gp41, most immunogenicity studies done so far have targeted the elicitation of 2F5-like Abs, directed against the ELDKWA epitope.

Chen and co-workers published three different immunization studies using the 2F5 epitope peptide conjugated to carrier proteins in three different formats, all these studies reported elicitation of an epitope-specific immune response, however, no neutralization activity was reported. Lu *et al.* developed multiepitope vaccines using three "Nt" epitopes (GPGRAFY, **ELDKWA** and RILAVERYLKD) corresponding to the gp120 V3 loop epitope, and two gp41 epitopes. The PI C-G-(**ELDKWA**-GPGRAFY)2-K and PII (CG-GPGRAFY-G-**ELDKWA**-G-RILAVERYLKD) peptides were synthesized and conjugated to bovine serum albumin (BSA); and both vaccines induced high levels of epitope-specific Abs to the three Nt epitopes in rabbits (142).

Similarly, Liao *et al.* conjugated the peptide, P2 (Env IIIB aa646-674: C-TSLIHSLIEEQNQQEKNEQELL**ELDKWA**) from gp41 to a linker peptide KG ((KGGG)7-K) chemically linked to BSA. Immunization studies in rabbits and mice elicited an anti-2F5 epitope Ab response. Another study from the same group evaluated the immunogenic properties of a ELDKWA-tetramer epitope vaccine (C-(**ELDKWA**G)4-BSA), (136). In this context a strong Ab response to ELDKWA-peptide was obtained after immunization of rabbits and mice (274). Ni *et al.* used the conserved DP178 peptide sequence to develop a monosaccharide-centered multivalent immunogen. A tetravalent format of saccharide-centered peptides (MVP-1) induced titers of anti peptide Ab, reaching 1.2 $x10^6$ that recognized the DP178 peptide and cross-reacted with gp41 (179).

In an effort to induce protective mucosal Nt Abs against the 2F5 epitope, Decroix *and collaborators* evaluated the conjugation of the 2F5 epitope peptide to tetanus toxoid (TT). A 17-residue peptide containing the 2F5 epitope sequence

(EKNEQELL**ELDKWAS**LWC) was coupled to TT. BALB/c mice immunized with the conjugate induced low levels of IgG and IgA to the immunizing peptide in various mucosal tissues. (57). The same group also evaluated the antigenicity of the 2F5 epitope (**ELDKWAS**LW) fused to an unnatural peptide sequence (PADRE), which is a T-helper-cell epitope (pan DR) capable of binding most major histocompatibility complex class II molecules of different haplotypes in mice and humans, and to increase Ab responses. The Ab response elicited by IM immunization against ELDKWA peptide was mainly of the IgA isotype, and occurred selectively in mucosa. Although the immunization was performed IM, the authors clearly showed elicitation of mucosal Ab response. Selection of peptide "mimotopes" for 2F5 from phage-displayed peptide libraries Ab have been reported by Tumanova *et al.* those peptides were immunogenic in rabbits and induced a gp41 cross-reactive response (245), however the Nt activity of the sera was not reported. Since the Nt activity of the sera was not reported for any of the above studies, it is not known whether these approaches induced any significant Nt response; however, they demonstrated that the 2F5 linear epitope is immunogenic in experimental animals.

All these immunization studies have in common the use of 2F5 core epitope originally described by Katinger's group. However other studies using longer peptides, indicated that the epitope recognized by 2F5 is larger than core ELDKWA, and other residues located within the MPER are important for the 2F5 binding to these region (162, 186). Thus, the use of only of the core epitope recognized by 2F5 more likely will induce an specific anti-peptide Ab response, mainly due to the features of the peptide, bearing hydrophobic and charged residues, which are known to be critical in the Ab response elicited by peptides (228). The elicitation of an antipeptide response may explain the

failure in eliciting Nt Ab, since the peptide probably did not recreated all full epitope or structural features recognized by the bNt Ab 2F5.

Joyce *et al.* selected the conserved sequence of the HIV-1 fusion inhibitor peptide DP178 (AC-YTSLIHSLIEESQNQQEKNEQ**ELDKWAS**LWNWF-NH2), which overlaps the 2F5 epitope, to develop immunogens. They tested whether an increase in the peptides helicity would enhance the interaction of 2F5 with its epitope, and if these constructs could induce Nt Abs. N-terminal extensions, derived from either gp41 or the yeast GCN-4 leucine zipper dimerization domains, were used to increase the α helical content of DP178 peptide (120). The selected structured peptides with improved alpha helix as well as the DP178 wt were conjugated to keyhole limpet hemocyanin (KLH) to produce immunogens and used to immunize guinea pigs. Although high titers of peptide-specific Ab were achieved, the sera were not Nt.

McGaughey *et al.* tested the elicitation of 2F5-like Ab using cyclic, beta turn-constrained peptides, by incorporation of a side-chain to side chain lactam bridge (159). Longer peptides bearing 13 residues from the MPER maximized MAb 2F5 binding. Two variants of the longer peptides were conjugated to the outer membrane protein from *Neisseria meningitides* and used for immunization studies in guinea pigs. Although a high level of 2F5 crossreactive Ab epitope peptide was elicited, the antisera failed to demonstrate any viral Nt activity (159). The authors concluded that although the structure of the conjugate can be important for binding, it is not a prerequisite for inducing Nt Ab, or that the structure of the peptide changed after the conjugation to the carrier protein.

Ho *et al.* evaluated the possibility of targeting 2F5 epitope directly to the antigen presenting cells by fusing the 2F5 epitope (ELDKWA) into different permissible sites on

an anti-HLA-DR Ab (108). Interestingly, the 2F5 epitope was recognized by the Ab in three out four constructs in which the epitope was incorporated into a β-turn. However, none of the five constructs were the 2F5 epitope was incorporated into surface exposed helical region have reactivity with 2F5 Ab. In a subsequent study Ho *et al.* confirmed by BIAcore that the proteins displaying 2F5 into the site of a β-turns bind 2F5 with up to 10-fold higher affinity than their unconstrained counterpart. Immunogenicity studies in rabbits showed that gp160 cross-reactive Abs were induced, but virus neutralization was not detected. Thus, they concluded that factors other than 2F5 binding affinity might have a critical role to play in the design of a 2F5-based vaccine (109). Hoever, it could be possible that the region of the MPER was to short to support the whole epitope structure.

In a similar study Coffier *et al.* fused the 2F5 epitope (ELDKWA) into permissive sites on the maltose binding protein of *Echerichia coli,* and the antigenic properties of the hybrid proteins were studied using BIAcore. Increasing the length of the inserted epitopes, as well as inserting multiples copies of the epitope increase 2F5 reactivity. Intraperitoeal immunization (IP) of mice with the recombinant proteins elicited Abs against a synthetic peptides containing ELDKWA and correlation between antigenicity and immunogenicty was observed. However, none of the hybrid proteins elicited Nt Abs, even for the HIV-1 laboratory-adapted isolates MN and FR (47).

The immunogenic properties of the 2F5 epitope have also been evaluated using the HIV-1 envelope protein gp120 as a carrier. Jian *et al.* modified the gp120 from the HIV-1_{IIIB} and by inserted the peptide (LL**ELDKWAS**L), into the V1, V2, V3, or V4 loops. Guinea pigs and mice were immunized with DNA plasmids that expressed the hybrid proteins. Differences between the Ab responses to the 2F5 epitope were observed

for these two animal models. All the mice elicited an anti gp120 Ab response yet only the mice immunized with the V1 and V3 mutants induced cross-reactivity with 2F5 epitope. . In guinea pigs, an anti- peptide response was obtained only for the animals immunized with the V2 mutants. Other fourteen variants of the 2F5 epitope insertion in the V2 loop were developed and evaluated in mice. Interestingly, in this case some insertions induced cross-reactivity with 2F5 epitope, indicating that the immunogenicity of the epitope was dependent upon its conformation at the different positions tested, nevertheless, none of the sera produced HIV-1 Nt activity (115).

The grafting of MPER epitopes into gp120 has been recently revisited by Law *et al.* (131). Several insertions of the MPER in the V1/V2 region of gp120 were tested, including the 2F5 and the 4E10 epitopes. The 4E10 epitope was flanked with helical domains and the helices were manipulated by sequential deletions of one residue at a time, N-terminal to the 4E10 epitope, with the objective of rotating the α-helix, and allow the exposure of the critical binding residues in the 4E10 epitope. Using this approach, the most antigenic 4E10 variant was identified. A DNA-prime immunization followed by protein-boost IM injection procedure was used to immunized mice and rabbits. In contrast to Jiang *et al.* (115), no Ab response to either 2F5 or 4E10 epitopes was detected; instead, the response was exclusively directed against the carrier protein.

Matoba *et al.* produced 46 residues from the gp41 (aa 649-684) fused to the cholera toxin B subunit in *E. coli* and showed in mice the elicitation of Ab that blocked transcytosis of HIV-1 (156). In a second study from the same group, the same immunogen was evaluated in by prime-boost studies in mice to optimize the production of Ab that block mucosal HIV-1 transcytosis. The immunogenicity of the protein was

also evaluated by immunization of guinea pigs in combined IN and IP routes. It was shown that the regimen where mucosal priming with adjuvant followed by systemic boosting exhibit the best Ab response in both compartments (systemic and mucosal) (155). However the Nt activity of the sera was not reported.

Another vaccine strategy involved chimeric viruses bearing the 2F5 epitope. Muster *et al.* developed the first chimeric virus carrying the 2F5 epitope (173), by grafting ELDKWAS into an antigenic loop of the influenza virus hemaglutinin. The resulting chimeric virus elicited IgG and IgA systemically in mice after a combined oral/IP immunization regimen. In addition, the antisera neutralized the laboratory-adapted HIV-1 isolates MN, RF, and IIIB. Another immunization study was also using chimeric virus bearing 2F5 epitope fusion to the influenza hemagglutinin, but the Nt activity of the sera was not reported (73).

Similarly, Eckhard *et al.* investigated the immunogenic potential of the MAb 2F5 epitope (ELDKWA) and two of its major escape variants (ELNKWA and ELDNWA) as internal fusions to the hepatitis B virus (HBV) surface Ag (HBsAg (67). Recombinant HBsAg-HIV proteins produced in the methylotrophic yeast, *Pichia pastoris,* self-assembled into 22-nm lipoprotein particles. Mice immunized with these particles elicited an anti-HBsAg Ab response in the range considered to be protective against HBV infection in humans. In spite of extremely high titers of Abs reactive with the 2F5 epitope fused to GST, gp160 and gp41 in ELISA, the sera failed to neutralize HIV-1 *in vitro.* These results indicated that elicitation of Nt Ab to the 2F5 epitope depends on the molecular context in which it is presented.

Following a similar approach, Marusic *et al.* expressed the ELDKWA sequence as an N-terminal fusion to the potato virus X (PVX) coat protein. Mice immunized with chimeric virus particles IP or intranasal (IN) produced strong anti-2F5 epitope IgG titers, as well as low levels of IgA, as shown by binding to a synthetic peptide containing the ELDKWA sequence in ELISA. Sera from two out of six mice immunized IN, neutralized HIV-1 strain IIIB at low serum dilutions. The chimeric virus particles also were evaluated in severe combined immunodeficient mice reconstituted with human peripheral blood lymphocytes (hu-PBL-SCID), in this case the animals were immunized with autologous dendritic cells (DCs) pulsed with the viral particles; the serum from one of the animals showed Nt activity tested by syncytium neutralization assay (151).

Zhang *et al.* inserted the 2F5 epitope (ELDKWA) in a flexible and exposed loop at the N-terminus of the viral capsid protein, L1, of bovine papillomavirus type I. (BPV-1). The chimeric virus was produced in insect cells and oral and IM administration of mice with chimeric virus-like particles (VLPs) induced an anti-ELDKWA Ab response. Although both mucosal and systemic Abs were induced, their titers were low since the mice were only immunized twice. Interestingly, sera at a 1:20 dilution from 4 of 8 mice neutralized HIV-1 Bal and MN weakly, but not the primary isolate HIV-1_{Ada} (283). In a recent study, Kusov *et al.* produced a live chimeric virus in which the 2F5 epitope replaced the 2A C-terminal extension of the major viral structural protein, VP1, of hepatitis A virus (128). Two different kinds of immunogens bearing the 2F5 epitope VP1 chimeric protein were prepared; mature (live vaccine) and empty VLPs. The live virus was used for immunization of non-human primates (marmosets), whereas the chimeric VLPs were used to immunize guinea pigs. Twenty days after the immunization, the live

virus was shown to be infectious after its purification from the feces of marmosets, and the serum from one of two marmosets showed weak binding to synthetic 2F5 peptide at a serum dilution of 1/50. Guinea pigs immunized three times with the VLPs emulsified in complete Freund's adjuvant for the first immunization also produced weakly detectable serum Ab responses to the 2F5 peptide (titers were not reported). Only two out of four guinea pigs immunized with the VLPs induced crossreactivity with 2F5 epitope peptide and one serum neutralized the HIV-1_{LAI} isolate. Nt activity was not detected for any of the marmosets sera.

A Nt Ab directed to the MPER from another retrovirus has been isolated; it targets the E2 region (FEGWFN) of the PE15 protein of a porcine endogenous retrovirus (PERV), homologous to the region of the 4E10 epitope (WNFIT) on gp41 (75). The envelope proteins of PERV are structurally and functionally comparable to those of HIV-1, in which gp70 and p15E are the counterparts of gp120 and gp41, respectively. P15E, like gp41, is a trimer and can form the 6HB. Considering this analogy between the two viruses, Luo *et al.* used the HIV-1 MPER (aa 665-682) to replace a homologous region on the P15E protein from PERV (143). Two different fusion proteins were evaluated, one in which the E2 region was replaced by the MPER and another where the HIV-MPER was fused to the C-terminus of E2. The genes encoding for these proteins were transferred to a vesicular stomatitis virus (VSV) from which VSV recombinants were prepared. The fusion proteins were also expressed and purified from a baculovirus expression system. The immunogenic capacity of recombinant VSV and fusion proteins was evaluated in rabbits by a VSV- prime and protein-boost immunization procedure. An anti-MPER Ab response was elicited, and three out nine animals showed low (1/20)

Nt titers. This is a very interesting result, since VSV is an enveloped virus and the MPER may be associated with membrane. The low Nt titers most likely are related to the use of recombinant soluble protein for boosting, a response originally generated with a membrane-tethered MPER. Interestingly, in spite of the fact that the hypothesis for substituting the MPER was based in part by the homology between the E2 region and the 4E10 gp41 epitope, the recognition of the VSV or the recombinant P15E-MPER immunogens by the 4E10 MAb was not shown.

Lenz *et al.* produced a gene encoding a fragment of gp41 consisting of the CHR-MPER and TM region; however, they were not able to express the protein in bacteria, most likely because of protein toxicity. Only a spontaneous mutant clone that evolved with a protein with an 18-residue addition from the vector was isolated and, with the use of detergents, the gp41 fragment was purified. The recombinant protein by itself as well as a reconstituted in proteoliposomes, were recognized by the 2F5 and 4E10 MAbs, and the proteoliposomes were able to inhibit viral infection. Immunization studies in mice elicited very low Ab titers for the recombinant protein and moderate titers by the proteoliposomes; however, no Nt activity was detected. Thus, although the membrane presentation on the MPER was used, this study did not demonstrate an improvement in the quality of the elicited Abs. Only two immunizations were given to the animals and the doses used were not indicated. Thus, it is not clear whether a low concentration of the immunogens was the reason for the low Ab titers. Furthermore, no dissection of the Ab response was done with the aid of peptides, and is it unknown whether specific reactivity against the 4E10 or 2F5 epitopes was elicited (133).

Kim *et al.* investigated the elicitation of 2F5 and 4E10 Abs with the MPER in the context of biomembranes. HIV-1 gp41 derivatives in a pre-fusion state on the surface of VLPs were produced in Sf9 cells and used for immunization of guinea pigs with or without *E. coli* heat-labile enterotoxin (LT) as adjuvant. High titers to the VLPs were obtained; however, binding to the MPER peptide was so low that the titers were not measured, and only a low, non-Nt Ab response to gp41 was elicited. Dissection of the Ab response by ELISA and western blot indicated that all the sera elicited Ab to the epitope located in the CHR domain, but not any reactivity with an MPER peptide covering the 2F5 and 4E10 epitopes. Low response to the gp41 resulted probably to the low level expression of gp41 relative to other proteins from the host cells that were also incorporated onto the VLPs.

Interestingly, and in agreement with the results of Kim *et al.*, a recent paper from Adam *et al.* suggests that the presence of the ID epitope in the region corresponding to the CHR (previously defined as the cluster II (epitope), can mask the Ab response elicited against the MPER (1). After immunization with gp140 in mice, two MAbs (5A9 and 13H11) isolated that bind to CHR epitopes and partially cross-blocked 2F5 Ab binding to Env. Using competition ELISA they also showed the presence of Ab with CHR reactivity in 83 % of sera from HIV-1^{+} donors. They hypothesized that B-cells make ID non-Nt Abs to the CHR that block les prevalent B-cells clones from recognizing the 2F5 epitope region.

Ye *et al.* used a DNA immunization approach to test the immunigenicity of the protein elicited by a DNA vector expressing the fusion protein HA-gp41in the context of membrane (278). Their goal was to recreate the appropriate structure of gp41, and

particularly the MPER, by replacing gp120 with the HA1 domain of the influenza HA protein. Since HA1, is smaller than gp120, it should preserve gp41, in its prefusogenic structure, and at the same time, permit the exposure of epitopes typically occluded by gp120. Interestingly, 2F5 and 4E10 showed better binding to the fusion protein than to the native Env trimer, tested in a cell-based ELISA; this indicates a better antigenicity of the MPER in the context of the HA-gp41 fusion protein. However, it was not shown whether similar amounts of HA/gp41 and native Env trimers were present on the cell surface. Mice immunized IM with DNA constructs expressing the HA-gp41 fusion elicited Abs with gp41 reactivity and the pooled sera neutralized the laboratory-adapted HIV-1_{SF162} at a 1/40 dilution. No epitope mapping studies for dissecting the elicited Ab response were performed; thus, it is impossible to assess whether the Nt activity observed is due to the elicitation of 2F5- or 4E10-like Abs, or Abs to other epitope(s).

Presentation of the HIV-1 Env in the context of membrane to the immune system has been evaluated using the HIV-1 VLPs. VLPs are usually non-replicating, (empty) particles that are assemblies of a Gag protein core surrounded by cellular membrane, with or without the Env proteins. Interestingly, different variations of Env can be embedded into the VLP membranes. Among the variants evaluated in VLPs so far are full-length Env gp160 (cleaved or cleavage-defective), gp140 (cleavage or cleaved-defective), gp41, and also gp41 derivatives in a prefusogenic state (122, 199).

McBurney *et al.* generated HIV-1 VLPs bearing cleaved gp160 and compared them, with VLPs without envelope, and with soluble gp140 and gp160 proteins in immunization studies in mice. It was found that membrane-embedded envelope elicited a broader Ab response than the soluble proteins, and IgA and IgG Abs were generated after

oral immunization with the VLPs. Interestingly, only the sera produced by the VLP immunizations had Nt Abs (157). The authors speculate that the presentation of the trimeric protein in an environment more closely related to the natural viral spike in the VLPs, has a positive influence in the response, since it probably induced conformational dependent Abs that recognize epitopes only present on the native virion-associated envelope.

Another approach for presenting membrane-associate proteins to the immune system is the production of proteoliposomes. Grundner *et al.* generated proteoliposomes using Env gp140 cleavage-defective trimers, and demonstrated that the proteins are more immunogenic when presented to the immune system in a solid phase (97). Immunization study in rabbits with this type of immunogen induced Nt Ab response, however whether this activity was related to the MPER was not determined.

Since it is believed that the HIV-1 Env proteins undergo several conformational changes during infection, some studies testing the immunogenic properties of HIV Env proteins have evaluated fusion complexes. Zipeto *et al.* prepared different fusion complexes by using different temperatures and fixation conditions. For these experiments, CHO cells expressing His-tagged CD4 and CCR5 were co-cultivated with CHO cells expressing HIV-gp120/gp41 for 4 hours at different temperatures. Fusion complexes prepared with different fixative reagents were purified and used to immunized mice. The fusion complexes prepared at 21°C, 30 °C or 37 °C were immunogenic in mice and induced Nt Abs against both R5 and X4 HIV-1 heterologous isolates; however, complexes prepared at 37°C induced the highest titers of Nt Ab and the use of different fixative agents did not affect the Nt titers induced except for glutaraldehyde, which was

ineffective. Unfortunately no epitope mapping of the induced Nt sera was reported, thus it is not known whether these fusion complexes are suitable for inducing an MPER-specific Nt response.

The failure to elicit Nt Abs with 2F5 peptides, indicated that those peptides did not recreated the complete epitope recognized by 2F5 Ab. Immunogenicity studies using the whole MPER suggest a dominant effect of the 2F5 epitope over the 4E10 epitope, which can be explained by their location and possible exposure during the viral infection process. It is known that the 2F5 and 4E10 Abs compete for binding to the MPER (290), which can be totally visualized considering the proximity of the two epitopes. Also, the 4E10 epitope is probably associated with membrane as a helix, this structure probably more difficult for an Ab to bind than the 2F5 epitope with it central turn. New current MPER vaccine research suggests that priority should be given to answering the following questions; (*i*) Can the presence of the 2F5 epitope next to the 4E10 epitope mask 4E10 and inhibit or affect the elicitation of an Ab response to the 4E10 epitope? (*ii*) Does elicitation of these Abs require different structural conformations of the MPER?. More importantly, (*iii*) Can Abs different from 2F5 and 4E10 with broadly Nt activity be generated against the MPER?

HIV-1 vaccine research faces the challenge of developing a vaccine to a structurally unknown immunogen. Without a clear understanding of gp41 structural changes and events that lead to virus infection, it is a matter of trial and error to generate an MPER-targeted vaccine. In addition, if we consider that gp41 undergoes drastic structural changes during infection, then the immune system maybe constantly exposed to the diverse structures of the MPER during natural infection. Such structures may or may

not be Nt-sensitive, thus making the overall response to the different epitopes located in the MPER very low, and could explain the low immunogenic nature attributed to the neutralization-sensitive sites on the MPER.

1.11. DNA immunization, a novel approach for vaccine development

The pioneering study of Wolff *et al.* which reported the long-term expression of chloramphenicol acetyl transferees (CAT), luciferase, and β-galactosidase (β-gal) in mouse skeletal muscle after injection with purified, naked plasmid DNA (269), opened the door to the concept of DNA immunization. The protective response obtained by Ulmel and collaborators using DNA encoding the influenza A conserved nucleoprotein (N1) antigen, established this new vaccination procedure as a very attractive one (247). DNA vaccines are usually circular plasmids that carry a gene encoding the target Ag (or antigens) under the transcriptional control of a promoter region active in eukaryotic cells. The coding region of the inserted gene is followed by transcription termination and polyadenylation signals. To permit selection of plasmid-containing bacteria during DNA production, the plasmid also contains an antibiotic resistance gene and a bacterial origin of replication. DNA vaccination permits the expressing Ags *in vivo* to stimulate both the humoral and cellular immune responses.

Different delivery routes have been explored for DNA immunization. Direct IM or intradermal injections introduces the DNA into myocytes, dendritic cells, and/or keratinocytes, and uses either a needle and syringe or a needle-free injector such as a Gene gun, which administers DNA fixed to gold beads, and leads to keratinocyte and dendritic cell transduction (106, 235), and electropolation (154). Delivery of DNA

vaccines to the gastrointestinal or respiratory mucosal epithelia has been explored by several formulations, including administration of DNA associated with liposomes (230), chochelates (anionic lipid and DNA condensed in the presence of calcium) (144), DNA mixed with poly-(lactide-co-glycolide) (118) and attenuated live *Salmonella typhimurium* (*aroA* auxotrophic mutant, which can not grow *in vitro*) transformed with the plasmid (55).

Several advantages in immunizing with DNA *versus* protein have been proposed. First the purity of the DNA used for immunization can be more pure than the counterpart protein or whole pathogen. Second, since only a small fragment of the coding region required for a pathogen reproduction is used for immunization, there is no danger to the body. Third, several different genes can be mixed and injected at the same time, making it possible to vaccinate against different proteins or different variants of a pathogen, or against several different pathogens, at the same time. Fourth, DNA is generally less costly to produce than the whole pathogen; moreover, the methods for producing clinical grade DNA are significantly cheaper than peptide synthesis or protein production and purification. Fifth, once produced, DNA vaccines don't require refrigeration, and can be stored for years under a variety of conditions. This made them advantageous for use in remote regions, particularly undeveloped countries. And finally, DNA vaccines may produce immunomodulators or adjuvants beside their targeted protein.

The mechanisms underlying the induction of of the immune response after DNA immunization, are unclear; however, three different mechanisms have been suggested; (*i*) direct priming by somatic cells (monocytes, keratinocytes or cells lacking MHC class II molecules), (*ii*) direct transfection of APCs, and (*iii*) cross-priming in which plasmid

DNA transfect a somatic cells and/or professional APC and the secreted proteins are taken up by other professional APC and presented to T cells (99).

Several cells have been involved in the development of an immune response after DNA immunization. These include myocytes, which express low levels of MHC I and no MHC II or costimulatory molecules, such as B7, and therefore are probably not responsible for the induction of cytotoxic T-cells or Ab responses. However, transfected myocytes may act merely as a source of Ag and priming occurs in the draining lymp nodes. In this case, a better immune activation will occur if the Ag is released from the myocyte by secretion or after cell damage. The APC can also be transfected at low levels and at the site of injection, and these cells can migrate to the lymphoid tissue and present Ag to the T and B cells (113).

In general, all the mechanisms explaining the Ab response to DNA vaccinations are based on the response to soluble proteins. However, the use of DNA immunization for eliciting Abs against membrane-attached protein is very attractive, since problems have been encountered in producing Abs to membrane-attached proteins. It is difficult to raise Abs against membrane-attached proteins in their native form; and it has proven difficult to purify transmembrane proteins while retaining their native conformation (91). Furthermore, production of recombinant proteins in a "foreign" cell (probably non-mammalian cells), does not guarantee native structure, nor its posttranslational modifications. In contrast, DNA immunization could be a rapid way to achieve the production of the desired Ag in the right conformation.

Several reports are available in which DNA plasmids carrying a gene for membrane-anchored proteins have been used for immunization. For example,

Chowdhury *et al.* developed a single-chain Ab (ScFv) library after immunizing mice with a plasmid DNA carrying the coding sequence for mesothelin, a GPI-linked, membrane-associated differentiation antigen that is over-expressed on the surface of ovarian cancers. The screening of the single chain Ab library allowed the selection of a scFv with high affinity for the mesothelin (44). Interestingly, high levels of Ab were also elicited in rabbits after immunization with a plasmid expressing mesothelin (43).

Production of scFV after immunization with DNA encoding for membrane-attached protein has been also reported in other studies (207). The pDisplay vector from Invitrogen, carries the coding sequence for the platelet-derived grow factor transmembrane region (PDGFR-TM). This expression vector was designed for the expression of a protein tethered to the cell surface. A MAb against the dengue virus protein, NSI was isolated after immunization of mice with a pDisplay carrying the NS1 gene fused to the PDGF-TM coding sequence (198). Fornst *et al.* expressed the Hepatitis C virus E2 protein intracellular or targeted to membrane using the pDisplay vector (77), and this vaccine induced a strong response in mice and macaques. Induction of Ab responses have also been reported for membrane attached protein from parasites such as *Shistosoma japonicum* and S. *mansoni* (53, 253). However, the question of whether secreted or membrane-attached immunogens are more efficient in eliciting an immune response has been widely debated. Some studies suggest that Ag attached to the cell surface (77), are often more immunogenic than if it is expressed intracellularly or in secreted form, whereas others have claimed the opposite result (22). Boyle *et al.* evaluated the effect of the cellular localization of a protein on the immune response after DNA immunization of mice with three forms of ovalbumin (Ova) (22). The highest titers

of IgG1 were obtained with the secreted version of Ova, indicating that membrane or intracellular localization limits the availability of Ag for B- cell priming, which, in turn affects the magnitude and form of the Ab response.

Several safety issues have been investigated in clinical models. These include, integration of the plasmid into cellular DNA, development of autoimmunity, elicitation of anti-DNA Abs, development of tolerance to the Ag being expressed and development of antibiotic resistance. However, although intensive testing has been done to investigate these issues, none of the studies have detected possible dangers associated with the use of DNA vaccines (168),

Recently, positive results were announced for a influenza H5N1 (bird flu) DNA vaccine in humans, which was shown to be immunogenic (65). Moreover, two DNA vaccines have been approved for veterinary use, one that protects horses from West Nile virus and one that protecs farm-raised salmon from infectious haematopoietic necrosis virus (110). As of today, DNA vaccination is still an experimental procedure for humans. Some DNA vaccines have been evaluated on humans and proven to be harmless; however, only the recent study by Drape *et al.* has shown that the immunogenic effect can be of practical use (65).

Besides the advantages of DNA over protein immunization stated above, differences in the immune response generated by the two types of vaccine are evident (99). In mice, the amount of protein produced after DNA immunization is very low compared to the μg doses used for protein immunization; thus the Ag produced is often not enough to mount a strong Ab response. To increase the Ab response several approaches have been suggested, including the use of DNA prime/protein boost

strategies. Protein boost can be done with different forms of immunogens, including soluble proteins, VLPs and whole cells (175). However, if the structure of the antigen is very different from the one in used in the priming, or contains too many new epitopes *e. g*, VLPs) a possible generation of a new immune Ab response can occur.

Adjuvants are defined as substance that, while non-immunogenic when used alone, can speed, enhance, prolong, or direct the quality and/or quantity of the immune response to a specific Ag (129). Molecular or genetic adjuvants have also been used to increase the immune response to Ag in DNA immunization. In the case of DNA vaccines, DNA encoding cytokines or other molecules capable of activating the innate immune response have been evaluated for use in vaccine formulations. The coding region for the selected adjuvant can be cloned in the same or in a separate plasmid, and used simultaneously for immunization. Some molecular adjuvants being investigated are the cytokines IL-2, IL-4, IL-7, IL-12, IL-15, IL-18, GMCSF and TNF-α (reviewed by (107, 260), the complement fragment C3d (95) and the *Salmonella typhimurium* FliC gene (7).

Codon usage of HIV-1 genes is very different from that of mammalian cells, thus viral proteins are typically expressed at very low levels *in vivo*. This is considered to be mechanism for the virus to avoid the immune response by decreasing Ag availability. Thus, to increase viral protein expression *in vivo* in DNA immunization studies, codon optimization of the viral gene is recommended. Several studies have shown that a better expression of a recombinant protein influences the Ag availability in circulation and its recognition by APC and ultimately, influences or modulates the immune response. Thus, optimization of DNA codon usage constitutes another approach used to increased immunogenicty of protein produced by DNA immunization.

1.12. Objectives of this work

HIV gp41 is a transmembrane protein that is the target of neutralizing antibodies and the characterization of its antigenicity and immunogenicity in the context of membrane has been limited. The goal of this research is to develop novel immunogen(s) for the generation of protective bNtAbs that specifically mimic the biological activities of MAbs 2F5 and 4E10. Our strategy was based on the assumption that the MPER interacts with the viral membrane in its native state on the viral spike, and that this interaction plays a major role in the structure of the neutralization-sensitive epitopes as well as the structure of epitopes that can elicit Nt Abs. We hypothesized that the presentation of the "real" 2F5 and 4E10 epitopes depends upon the interaction of the MPER with the viral membrane and its structure in this context. Thus, the membrane should be a key element for the proper presentation of 2F5 and 4E10 epitopes to the immune system, and for the generation of 2F5- and 4E10-like Nt Abs.

The specific objectives of this work were a follows:

1. Determine the contribution of the cell membrane and other regions of gp41 to the antigenicity of the gp41 MPER.
2. Generate immunogens that present the gp41 MPER to the immune system in the context of plasma membranes.
3. Study the immunogenicity of the MPER in the context of plasma membranes by characterizing the Ab response to the MPER after DNA immunization and evaluating the Abs for HIV-1 neutralization.

CHAPTER 2. Antigenicity of the gp41 membrane-proximal external region (MPER) displayed on the surface of COS-7 cells.

2.1. Introduction

During HIV natural infection, a significant antibody (Ab) response is generated against gp41, a component of the viral spike protein involved in membrane fusion. Although this Ab response is essentially non-neutralizing, three of the five anti-HIV-1 broadly-neutralizing antibodies (bNt Abs) known to date bind to the membrane proximal external region (MPER) of gp41, (197, 290) making gp41 a key protein for the development of an antibody-based protective vaccine. 2F5 and 4E10 monoclonal antibodies (MAbs) bind to "linear" epitopes (aa 662-667, ELDKWA and aa 671-676, NWF(D/N)IT) respectively, on the MPER. They neutralize a broad spectrum of primary HIV-isolates at low concentrations *in vitro*; 2F5 has the greatest potency whereas 4E10 is more effective against isolates from multiple viral clades (17). In addition, passive immunization with these antibodies protected macaques from SHIV infection (152, 153). The neutralizing ability of MAbs 2F5 and 4E10 indicates the potential of the humoral response in protecting against HIV-1.

The low prevalence of bNt Abs against the MPER in HIV-1 infected subjects (60, 281) suggests that neutralizing epitopes in the MPER are poorly immunogenic or transiently exposed in either the native viral spike or during the infection process. The low frequency of 2F5 and 4E10 isolation events also indicates that. A role of the MPER in membrane fusion has been suggested (171, 210, 233). A recent study by Crooks *et al.* (52) suggests that 2F5 and 4E10 can exert neutralization activity after gp120-CD4

engagement, indicating that the MPER has a major role in late events of fusion of viral and host cell membranes.

The term antigenicity refers to the ability of an Ag to interact specifically with the paratope (*i.e.* functional binding site) of an Ab. Studies of antigenicity often probe the structural and molecular basis of these interactions, and provide information on what is required of an Ag to support Ab binding. The HIV-1 vaccine field faces the particular challenge of developing a vaccine to very dynamic and structurally undefined antigens. There is no clear understanding of gp41 structural changes during virus infection; therefore, there is not a known, predetermined structure to which Ab binding is sought in order to achieve neutralization. At present, the usual approach is still trial and error.

In this chapter, we describe the generation and characterization of a group of genetic construcs that express the gp41 MPER in the context of cellular membranes. The cell type chosen was COS-7. This approach attempts to recreate the native environment of this important fragment of gp41, under the assumption that the replication of that environment may propitiate the elicitation of bNt Ab against this region. Here we established the experimental conditions to evaluate Ab binding to the MPER, in the context of membrane, and also investigated the antigenicity of the constructs for three known bNt Ab against this region, in order to determine the contribution of membrane to the MPER antigenicity.

2.2. Materials

The pDisplay vector was purchased from Invitrogen, Burlington, ON. This mammalian expression vector permits display of proteins on the cell surface. Proteins

expressed from this vector are fused at the N-terminus to the murine Ig κ-chain leader sequence, which directs the protein to the secretory pathway, and at the C-terminus to the platelet derived growth factor receptor transmembrane domain (PDGFR-TM), which anchors the protein to the plasma membrane, displaying it on the extracellular surface. The vector also encodes a hemagglutinin A epitope, Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala (HA-tag) at the N-terminus, that allows an easy detection of the recombinant proteins. It carries the human cytomegalovirus (hCMV) promoter and the bovine growth hormone (BGH) polyadenylation signal (poly A).

A plasmid carrying the coding sequence for the full HIV-1 Env precursor gp160 from the primary isolate JR-CSF (220) was a gift from D. R. Burton (The Scripps Research Institute, La Jolla, CA. The DNA vaccine vector pW4303 (265) was a kind gift from S. Lu (University of Massachusetts, Worcester). The vector contains a combination of the hCMV IE promoter and Intron A sequence for efficient expression of recombinant proteins, and the BGH poly A signal. The tissue plasminogen activator (tPA) leader sequence allows the translocation of the recombinant proteins to the secretory system. The vector contains the ampicillin resistant gene (β-lactamase) for selection in media with antibiotic and the pUC origin of replication for replication in prokaryotic cells.

The *Escherichia coli* strains, TOP10F1 (recombination (*recA*) and endonuclease (*endA*) deficient) and DH5α (Invitrogen) were used for DNA amplification and cloning, and the XL-1 blue (Stratagene, CA) was used for the amplification of recombinant DNA from mutagenesis reactions.

The following kits for DNA preparation from Qiagen (Valencia, CA) were used: the Qiagen Endo-free purification kit (Cat. No.12362) was used for the purification of

DNA used for mammalian cells transfection and DNA immunization experiments. Preparation of PCR fragments and vector DNA fragments was performed with the QIAQuick PCR purification kit (Cat. No. 28104) and QIAquick® Gel Extraction Kit (Cat. No. 28704), respectively. All the miniprep DNA preparations were performed using the GenElute™ Five-Minute Plasmid Miniprep Kits (Cat. No. PFM250-1KT, Sigma-Aldrich, Oakville, ON).

MAbs 2F5 and 4E10 were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Germantown, Maryland). Murine MAb IgG 17/9 was a gift from I. Wilson (The Scripps Research Institute). The MAb Z13e1 was a kind gift of M.B. Zwick (The Scripps Research Institute). The FDA-2 and AD18 HIV+ IgGs purified on Protein A/G resin were a kind gift from P. Parren (The Scripps Research Institute). Anti-Human IgG labelled with Alexa Fluor-488 was purchased from Invitrogen. The chromogen 2.2'-azino-bis(3-ethylbenzthiazoline-6) sulfonic acid (ABTS) and bovine grow hormone (BSA) were from Sigma-Aldrich. The non-fat dry milk (DM) was from Bio-Rad (Cat. No.170-5016).

2.3. Methods

2.3.1. Construction of plasmids encoding fragments of gp41

The plasmid encoding the Env sequence for the HIV-1 primary isolate JR-CSF (220) was used as a template for PCR amplification of the coding regions for different fragments of gp41. The primers used for the PCR are shown in **Table 2-1**. Different

DNA fragments were PCR amplified, digested with BglII and BsmI, and ligated to pDisplay vector DNA (Invitrogen) digested with similar restriction enzymes **Figure 2-1**. The coding cassettes for each of the recombinant proteins were PCR amplified and transferred to (NheI-BamHI) digested pJW4303 plasmid, The primers used for transferring the coding cassette from the pDisplay vector to the pJW4303 are also shown in **Table 2-1**. All the recombinant clones were initially selected based on restriction analysis and verified by DNA sequence analysis.

The codon optimized gp41 gene from JR-CSF was synthesized by GENEART (Regensburg, Germany). For making the constructs bearing the TM1, TM2 and CT regions, the PCRs fragment were digested with NheI and BamHI and ligated to similarly digested pJW4303 plasmid. Primers used for making the new constructs are shown in **Table 2-2**. The HA-tag was not included in these new constructs. Plasmid pNHR-PDGFR-TM was the first construct made. Briefly, the coding region for gp41 and the PDGFR-TM were PCR amplified and digested NheI-BsmI, and BsmI-BamHI, respectively. The two PCR products were ligated to NheI and BamHI digested pJW4303. The recombinant plasmid pNHR-PDGFR was used as a template for the amplification of the fragments expressed by pCHR-PDGFR and pMPER-PDGFR plasmids.

Table 2-1. Primers used for PCR amplification of the wild type gp41 coding regions cloned into the pDisplay and pJW4303 vectors.

Primers for making the constructs in the pDisplay vector	
Primer	Sequence
FIII-NHR	5'-CGGCCAGATCTGGTATAGTGCAACAGCAAAAC-3'
FII-CHR	5'-CGGCCAGATCTTACACCCTAATTGAAGAATCGCAG-3'
FI-MPER	5'-CGGCCAGATCTTGGAATACTAGTTGGAGTAATAAATC-3'
RI-MPER	5'-CCGGCAGCATTCTTTATATACCACAGCCATTTTGTTAT-3'
RII-TM	5'-CCGGCTCTCGAGCTATGCTGGGAGGAGGGTCTGAAA-3'
Primers for transferring the coding region to the pJW4303	
Primer	Sequence
Fw-HA	5'-GCGGTGGCTAGCTATCCATATGATGTTCCAG-3
Rv-PDGF	5'-GCGGCGGATCCCTAACGTGGCTTCTTCTG-3'

Table 2-2. Primers for making the constructs with the codon-optimized gp41 gene.

Primer	Sequence
Fw-MPER	5'-CGGCCGCTAGCTACACCCTGATCGAGGAAG-3'
Fw-CHR	5'-CGGCCGCTAGCTGGAACACCAGCTGGTC-3'
Fw-NHR	5'-CGGCCGCTAGCGGCATCGTGCAGCAGC-3'
Fw-PDGFR-	5'-CGGCCGAATGCTGTGGGCCAGGA-3'
Rv-TM1	5'-CGGCCGGATCCTCATCAGCCCTCGGGCCGGTC-3'
Rev-TM2	5'-CGGCCGGATCCTCATCACAGCCGGTGGTAGCTG-3'
Rev-CT	5'-GCGCGGGATCCTTGCCACCCGG-3'
Rev-PDGFR-	5'-CGGTGGGATCCCTACTAACGTGGCTTCTTCTG-3'

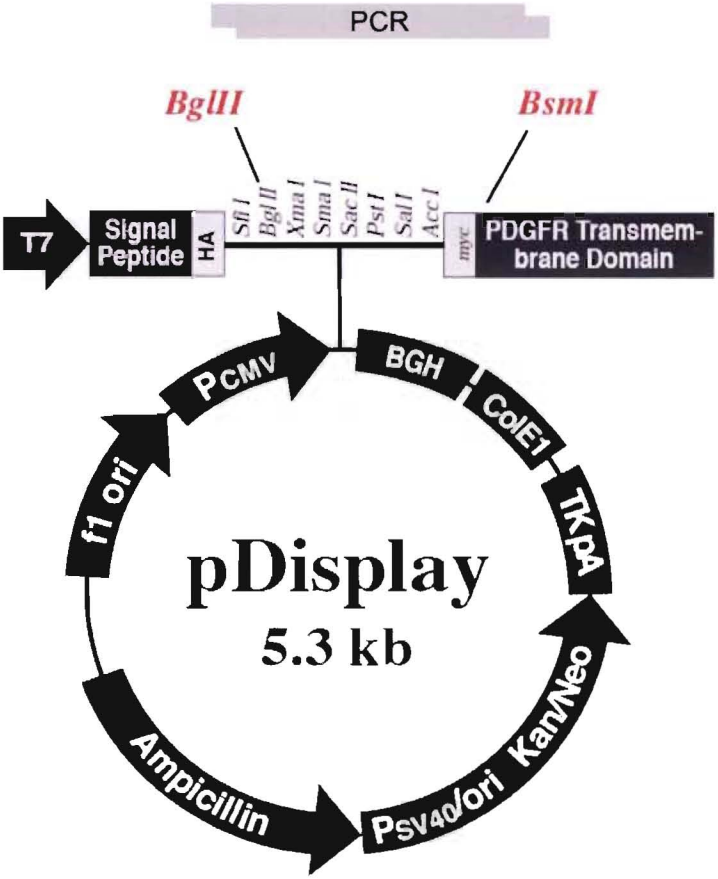


Figure 2-1. Schematic representation of the pDisplay vector. Three PCR-amplified gp41 fragments were inserted into the vector digested with BglII-BsmI to create the pDisp-MPER, pDisp-CHR and pDisp-NHR. Figure modified from the pDisplay manual, with Invitrogen's permission.

2.3.2. Construction of the MPER alanine substitutions

Mutagenesis to introduce single alanine substitutions into the MPER were performed using the Quick Change Site-Directed Mutagenesis Kit (Stratagene), following the manufacturer's recommendations for primers design and experimental conditions. Mutagenesis primers were synthesized to replace each amino acid of the MPER with Ala. In the case of Ala_{667}, the primers were designed to substitute this residue with a Gly. Briefly, the mutagenesis reactions were done in a 50µl final volume, using Pfu polymerase, 50 ng template DNA (pMPER-TM1), 50 pmoles of each primer (sense and antisense) and 1µ dNTP mix. The mutagenesis reactions were performed for 16 to 18 cycles, according to the number of mutations, 1-2 or 3 respectively. The DNA sequence of the primers used for the PCR amplifications are shown in **Table 2-3**. The reactions were incubated with DpnI to digest the methylated DNA and used to transform XL-1blue competent *E. coli* cells. Colonies were selected from LB plates containing 100 µg/ml of Ampicillin (LBA). Single colonies were grown in 2 ml LBA media at 37 ° C for 14-16 hours, and cells used to purify DNA. Positive clones were chosen based on DNA sequence (Macrogene, Korea) using BGH-rev primer.

Competent *E. coli* DH5-α cells were used for the amplification of all the constructs DNA. All plasmids used for mammalian cell transfections were prepared from overnight cultures from a single ampicillin resistant colony in LBA media, and processed using Endo-free Midi Kits following manufacture's recommendations.

Table 2-3. Primers used for construction of the alanine-substituted MPER mutants.

Mutation		*Primer sequence* [a]
E662A ----------	(S)	5'-GAGCAGGAACTGCTGGCCCTGGACAAGTGGGCC-3'
	(A)	5'-GGCCCACTTGTCCAGGGCCAGCAGTTCCTGCTC-3'
L663A ----------	(S)	5'-GCAGGAACTGCTGGAAGCCGACAAGTGGGCCAGCCTG-3'
	(A)	5'-CAGGCTGGCCCACTTGTCGGCTTCCAGCAGTTCCTGC-3'
D664A ----------	(S)	5'-GAACTGCTGGAACTGGCCAAGTGGGCCAGCCTG-3'
	(A)	5'-CAGGCTGGCCCACTTGGCCAGTTCCAGCAGTTC-3'
K665A ----------	(S)	5'-CTGCTGGAACTGGACGCCTGGGCCAGCCTGTGG-3'
	(A)	5'-CCACAGGCTGGCCCAGGCGTCCAGTTCCAGCAG-3'
W666A ----------	(S)	5'-GCTGGAACTGGACAAGGCCGCCAGCCTGTGGAAC-3'
	(A)	5'-GTTCCACAGGCTGGCGGCCTTGTCCAGTTCCAGC-3'
A667G ----------	(S)	5'-GGAACTGGACAAGTGGGGCAGCCTGTGGAACTGG-3'
	(A)	5'-CCAGTTCCACAGGCTGCCCCACTTGTCCAGTTCC-3'
S668A ----------	(S)	5'-CTGGAACTGGACAAGTGGGCCGCCCTGTGGAACTGGTTC-3'
	(A)	5'-GAACCAGTTCCACAGGGCGGCCCACTTGTCCAGTTCCAG-3'
L669A ----------	(S)	5'-CTGGACAAGTGGGCCAGCGCCTGGAACTGGTTCGACATC--3'
	(A)	5'-GATGTCGAACCAGTTCCAGGCGCTGGCCCACTTGTCCAG-3'
W670A----------	(S)	5'-GGACAAGTGGGCCAGCCTGGCCAACTGGTTCGACATCAC-3'
	(A)	5'-GTGATGTCGAACCAGTTGGCCAGGCTGGCCCACTTGTCC-3'
N671A ----------	(S)	5'-GTGGGCCAGCCTGTGGGCCTGGTTCGACATCACC-3'
	(A)	5'-GGTGATGTCGAACCAGGCCCACAGGCTGGCCCAC-3'
W672A ----------	(S)	5'GTGGGCCAGCCTGTGGAACGCCTTCGACATCACCAAGTGG-3'
	(A)	5'-CCACTTGGTGATGTCGAAGGCGTTCCACAGGCTGGCCCAC-3'
F673A ----------	(S)	5'-CCAGCCTGTGGAACTGGGCCGACATCACCAAGTGG-3'
	(A)	5'-CCACTTGGTGATGTCGGCCCAGTTCCACAGGCTGG-3'
D674A ----------	(S)	5'-CTGTGGAACTGGTTCGCCATCACCAAGTGGCTG-3'
	(A)	5'-CAGCCACTTGGTGATGGCGAACCAGTTCCACAG-3'
I675A ----------	(S)	5'-CTGTGGAACTGGTTCGACGCCACCAAGTGGCTGTGGTAC-3
	(A)	5'-GTACCACAGCCACTTGGTGGCGTCGAACCAGTTCCACAG-3
T676A ----------	(S)	5'-GGAACTGGTTCGACATCGCCAAGTGGCTGTGGTAC-3
	(A)	5'-GTACCACAGCCACTTGGCGATGTCGAACCAGTTCC-3
K678A ----------	(S)	5'-GGAACTGGTTCGACATCACCGCCTGGCTGTGGTACATCAG-3
	(A)	5'-CTTGATGTACCACAGCCAGGCGGTGATGTCGAACCAGTTC-3
W679A ----------	(S)	5'-GGTTCGACATCACCAAGGCCCTGTGGTACATCAAGATTTTC-3
	(A)	5'-GAAAATCTTGATGTACCACAGGGCCTTGGTGATGTCGAACC-3
L680A ----------	(S)	5'-GGTTCGACATCACCAAGTGGGCCTGGTACATCAAGATTTTC-3
	(A)	5'-GAAAATCTTGATGTACCAGGCCCACTTGGTGATGTCGAACC-3
W681A ----------	(S)	5'-CGACATCACCAAGTGGCTGGCCTACATCAAGATTTTCATC-3
	(A)	5'-GATGAAAATCTTGATGTAGGCCAGCCACTTGGTGATGTCG-3
Y682A ----------	(S)	5'-CACCAAGTGGCTGTGGGCCATCAAGATTTTCATCATG-3
	(A)	5'-CATGATGAAAATCTTGATGGCCCACAGCCACTTGGTG-3
I683A ----------	(S)	5'-ACCAAGTGGCTGTGGTACGCCAAGATTTTCATCATGATC-3
	(A)	5'-GATCATGATGAAAATCTTGGCGTACCACAGCCACTTGGTG-3
K684A ----------	(S)	5'-AAGTGGCTGTGGTACATCGCGATTTTCATCATGATCGTG-3
	(A)	5'-CACGATCAT GATGAAAATCGCGATGTACCACAGCCACTT-3
I685A ----------	(S)	5'-TGGCTGTGGTACATCAAGGCTTTCATCATGATCGTGGC-3
	(A)	5'-GCCACGATCATGATGAAAGCCTTGATGTACCACAGCCA-3
F686A ----------	(S)	5'-CTGTGGTACATCAAGATTGCCATCATGATCGTGGCCGG-3
	(A)	5'-CCGGCCACGATCATGATGGCAATCTTGATGTACCACAG-3

[a] S, sense primer; A, antisense primer

2.3.3. Transfection of COS-7 cells and lysate preparation

DNA transfection was performed using Lipofectamine 2000 (Invitrogen), following the manufacture's recommendation. Briefly COS-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% bovine calf serum (Hyclone) and 1mM L-glutamine. The cells were incubated at 37 °C and 5% CO2 under humidified conditions. Forty eight hours after transfection, the cells were washed three times with 1X HyQ Phosphate buffered saline without calcium and magnesium (1X PBS (-/-) HyClone) and recovered from the plates using 1X PBS, containing 1mM EDTA followed by centrifugation for 5 min at 1000 X g. The pellet was resuspended in 500 µl lysis buffer (0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl), supplemented with a broad specificity protease inhibitor cocktail (Roche Diagnostic GmbH), then mechanically disrupted by passage through a 22-gauge needle, followed by sonication with a Virsonic sonicator (VirTis, Gardiner, NY). The lysate was aliquoted and stored at -20 °C.

2.3.4. Whole cells Enzyme-linked immunosorbent assay (ELISA)

COS 7 cells were seeded on 24 well plates (Corning) at $2x10^5$ cells/well. Twenty-four hours later, the cells were transiently transfected using Lipofectamine. Briefly, 0.8 µg of DNA and 2 µl of Lipofectamine were separately diluted in 50 µl each of DMEM without supplements. The two samples were mixed and incubated for at RT for 5 minutes and added to cells. Twenty-four hours after transfection the media was removed and the plates were washed 3 times with 1X PBS containing Calcium and Magnesium (PBS+/+). The cells were fixed with 3.5% of paraformadehyde (Sigma-Aldrich) for 20

min at 37 °C. After several washes with 1X PBS+/+, the slides were incubated for 1h at RT with MAb 2F5 and/or 4E10 Abs (10 nM) diluted in 1X PBS+/+ containing 5% DM. Bound MAb was detected using goat anti-human IgG conjugated to horseradish peroxidase (HRP) (Pierce Chemical Co., Rockford, IL), followed by incubation with the colorimetric substrate 3,3', 5,5'-Tetramethylbenzidine (TMB). The product was detected using a fluorometer plate-reader (TECAN, Germany).

2.3.5. Cell lysate ELISA

High-binding microtiter plates (Corning Inc., Corning, NY) were used for ELISA of all cell lysate. The microwells were coated overnight at 4 °C with 10 µl of the cell lysate plus 25 µl of TBS (Tris-buffered saline, pH 7.4, 150 mM NaCl). Recombinant gp41 (Cedarlane) was used as a positive control at 50 ng/well and ovalbumin (Ova) (1µg/w), 2% bovine serum albumin (BSA) and 5% non-fat dried milk (DM) were used as negative controls. Microwells were then blocked for 1 h at room temperature (RT) with TBS 2% BSA, washed six times with TBS containing 0.1 % Tween 20 (TBS/Tw), and incubated with either human MAbs (2F5, 4E10, Z13e1), murine MAb 17/9, or human sera diluted in TBS/Tw containing 1% BSA (TBS/BSA/Tw) for 2 h at RT, (for some experiments the sera and the Abs were diluted in TBS/Tw containing 5% DM). After six washes, the microwells were incubated for 1 h at RT with Protein A/G or IgG specific secondary Ab conjugated to HRP, and diluted in TBS/Tw. Plates were washed six times, and bound HRP was detected by addition of ABTS solution (400 µg/ml 2'2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid in citrate/phosphate buffer containing 0.03% (v/v)

H_2O_2). Absorbance at 405 nm and 490 nm was measured using a Versamax microplate reader (Molecular Devices, Sunnyvale, CA) and reported as $OD_{405\text{-}490}$.

2.3.6. Cell surface staining, immunofluorescence and confocal microscopy

COS 7 cells were seeded in 24-well plates on top of microscope cover glass (Cat. no. 12-545-81, Fisher Scientific) at a density of $2x10^5$/well. Twenty-four hours later, the cells were transiently transfected using Lipofectamine as described above. Twenty-four hours after transfection, the media was removed and the plates were washed 3 times with PBS+/+. The cells were fixed with 3.5% paraformadehyde for 20 min at 37 °C. After several washes with 1X PBS+/+, the slides were incubated for 1h at RT with 10 nM 2F5 MAb diluted in 1X PBS+/+ containing 5% DM. Goat anti- rabbit IgG conjugate to Alexa-Fluor®488 was used as a secondary Ab for fluorescence microscopy. After incubation and washing, slides were mounted using Prolong with DAPI (Invitrogen). Images were collected using a Zeiss LSM 410 confocal laser scanning microscope with a 63X/1.5 NA oil immersion objective, (Zeiss LSM 410), and a fluorescence Olympus Motorized Inverted Microscop model IX81, OLYPUS, Tokio, Japan) All images were analyzed using NIH ImageJ confocal software.

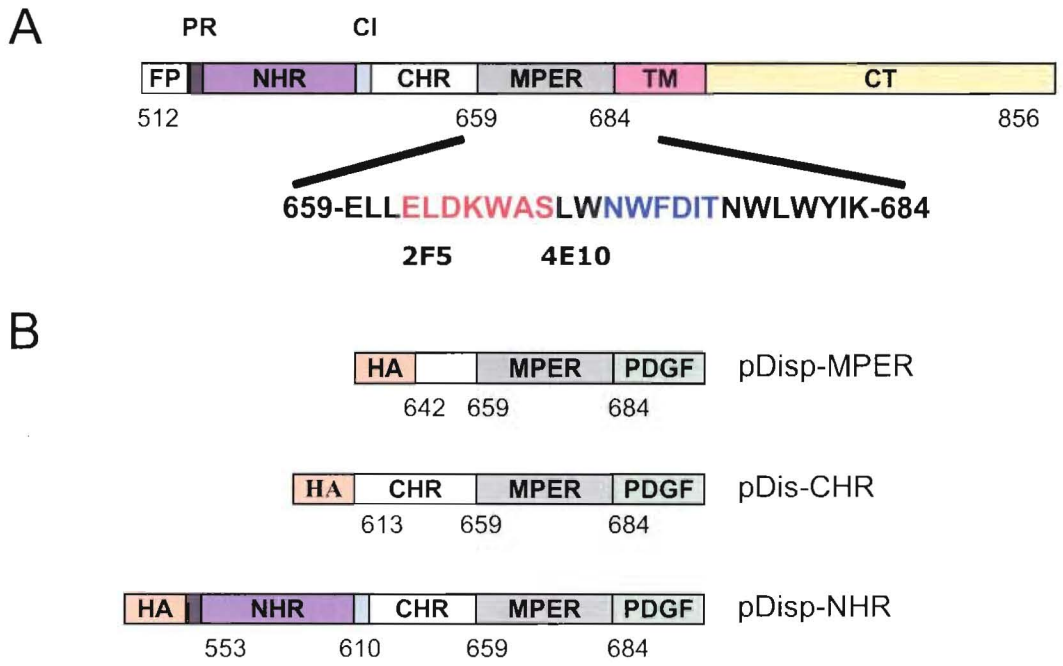
2.4. Results

2.4.1. Expression of the gp41 ectodomain tethered to the PDGFR transmembrane region

HIV-1_{JR-CSF} is a primary isolate from clade B, which is neutralized by the bNt Mabs 2F5 and 4E10. It was used as template for PCR amplification of several fragments of the gp41 gene, including the MPER. These PCR fragments were inserted into the pDisplay vector. This mammalian expression vector encodes the transmebrane region of the PDGF receptor (PDGFR-TM), which permits display of proteins fused to it on the cell surface; it has been successfully used to display proteins on the cell plasma surface (77, 241, 277). **Figure 2-1** shows a schematic representation of the pDisplay vector and the strategy used for cloning the DNA encodes fragments of gp41 into it. The fragments to be expressed were selected considering the known gp41 structural data. **Figure 2-2** shows a schematic representation of the three DNA constructs made in pDisplay and the aminoacid sequences of the corresponding proteins.

The three constructs (pDisp-MPER, pDisp-CHR, pDisp-NHR) as well as the mock vector pDisplay were used to transiently transfect COS-7 cells. The cells were harvested 48 hours after transfection and the cell lysates were used as antigens in ELISA to test the expression of the recombinant proteins. As shown in **Figure 2-3**, the three constructs with the MPER tethered to the PDGFR-TM expressed proteins that are recognized by the anti-HIV-1 Nt MAbs 2F5 and 4E10, as well as the murine MAb 17/9 which recognizes the HA-tag at the N-terminus of each protein. However the binding was very weak, indicating a low level of expression.

Since expression of the gp41 fragments was negligible from the pDisplay, we decided to test a different vector backbone. The plasmid pJW4303 has been successfully used for DNA immunizations (REF); in this vector, recombinant proteins are expressed under a combination of CMV promoter and Intron A sequence, and uses the tPA signal



pDisp-MPER
YPYDVPDYA**GAQPARS**YTLIEESQIQQEKNEQELLELDKWASLWNWFDITKWLWYIKN
AVGQDTQEVIVVPHSLPFKVVVISAILALVVLTIISLIILIMLWQKKPR*

pDisp-CHR
YPYDVPDYA**GAQPARS**WNTSWNKSLDSIWNNMTWMEWEKEIENYTNTIYTLEESQIQ
QEKNEQELLELDKWASLWNWFDITKWLWYIKNAVGQDTQEVIVVPHSLPFKVVVISAIL
ALVVLTIISLIILIMLWQKKPR*

pDisp-NHR
YPYDVPDYA**GAQPARS**GIVQQQNNLLRAIEAQQHMLQLTVWGIKQLQAVLAVERYLKD
QQLMGIWGCSGKLICTTAVPWNTSWNKSLDSIWNNMTWMEWEKEIENYTNTIYTLIEE
SQIQQEKNEQELLELDKWASLWNWFDITKWLWYIKNAVGQDTQEVIVVPHSLPFKVVVI
SAILALVVLTIISLIILIMLWQKKPR*

Figure 2-2. Schematic representation of the coding region in the DNA constructs and amino acid sequence of the proteins expressed from the pDisp-MPER, pDisp-CHR and pDisp-NHR. (A) Env gp41 transmembrane protein and its different domains. (B) Diagram illustrating the three different DNA constructs made using PCR amplified fragments from Env wild type gene of HIV-1_{JR-CFS}. FP, fusion peptide; PR, polar region; NHR, N-heptad repeat; CI, cluster I or immunodominant region; CHR, C-terminal heptad repeat; MPER, membrane proximal external region; TM, gp41 transmembrane region; CT, cytoplasmic region; HA, Hemagglutinin A tag; PDGF, platelet derived grow factor receptor transmembrane region. (C) Amino acid sequences of recombinant proteins, the HA-tag sequence is labelled in red, the spacer sequence is in bold black, in black regular, the gp41 fragments and in blue the PDGF-TM region. The same expression cassettes are included in the pJW-MPER, pJW-CHR and pJW-NHR. respectively.

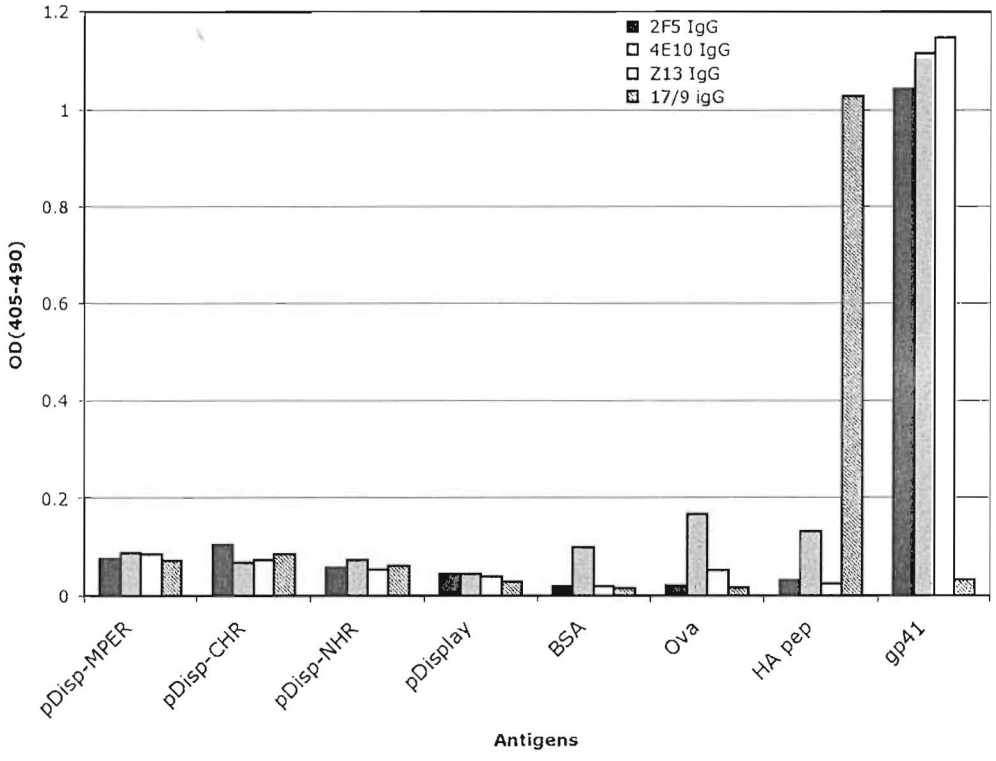


Figure 2-3. Protein expression of PDisplay constructs detected by ELISA. Cells were transfected with the DNA constructs or the pDisplay vector alone and lysates used to detect expression of Ag recognized by MAbs. The antibody binding is expressed as OD(405-490).. Data are representative of three different experiments.

peptide. The expression cassette for each of the three constructs, including the PDGF-TM region were transferred to the pJW4303 vector by PCR amplification from the three constructs in pDisplay. The amplified DNA fragments were digested NheI-BamHI and ligated to the pJW4303 digested with similar enzymes. Thus, the coding regions inserted into pDisplay and pJW4303 are identical (**Figure 2-2**). The new constructs were named pJW-MPER, pJW-CHR and pJW-NHR respectively.

The expression of recombinant proteins from the plasmids pJW-MPER, pJW-CHR and pJW-NHR was evaluated by cell lysate ELISA. Each plasmid and the mock vector were introduced into COS cells, which were harvested 48 h after transfection. Cell lysates were prepared, and used as Ag in ELISA as described above. As shown in **Figure 2-4**, the three constructs expressed proteins that were recognized by the MAb17/9, as well as MAbs 2F5, 4E10 and Z13e1. MAb 17/9 recognized all the samples with similar ELISA signals, indicating that no gross differences in protein expression existed between the three constructs. However, the reactivity of the anti-MPER MAbs was different between constructs and antibodies (**Figure 2-4**). 2F5 IgG bound better to the construct expressing the MPER only, and its reactivity decreased with the increase in protein size. Z13e1 and 4E10 IgGs reactivity patterns with the MPER was not influenced by the size of the proteins, however, a lower reactivity of 4E10 was observed in comparison with the other MAbs. In general 2F5 bound better to the MPER in these constructs than 4E10 and Z13. The difference in binding between 2F5 and 4E10 was statistically significant indicating clear differences in 2F5 and 4E10 epitopes antigenicity.

The pJW-constructs were also tested for their ability to be recognized by antibodies from HIV-1 positive donors. As shown in **Figure 2-5**, the Nt serum FDA-2

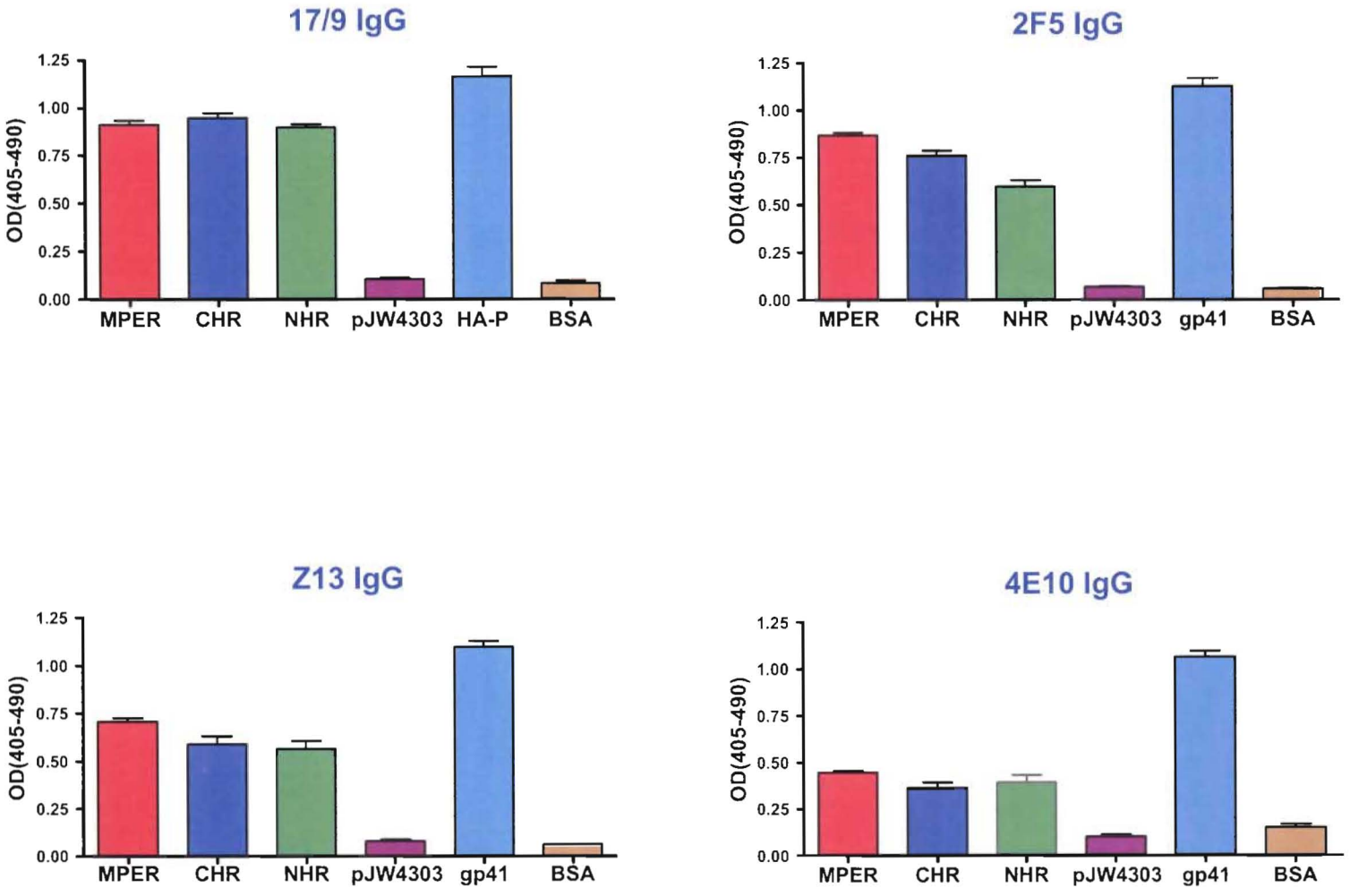


Figure 2-4. Expression of gp41 fragments from the pJW4303 vector, detected by cell lysate ELISA. The plasmids pJW-MPER, pJW-CHR, pJW-NHR and pJW4303 were transiently transfected in COS-7 cells and the cell lysates were used as antigens in direct ELISA. All the MAbs were used at 5 nM. HA-P: HA peptide. Error bars show standard error.

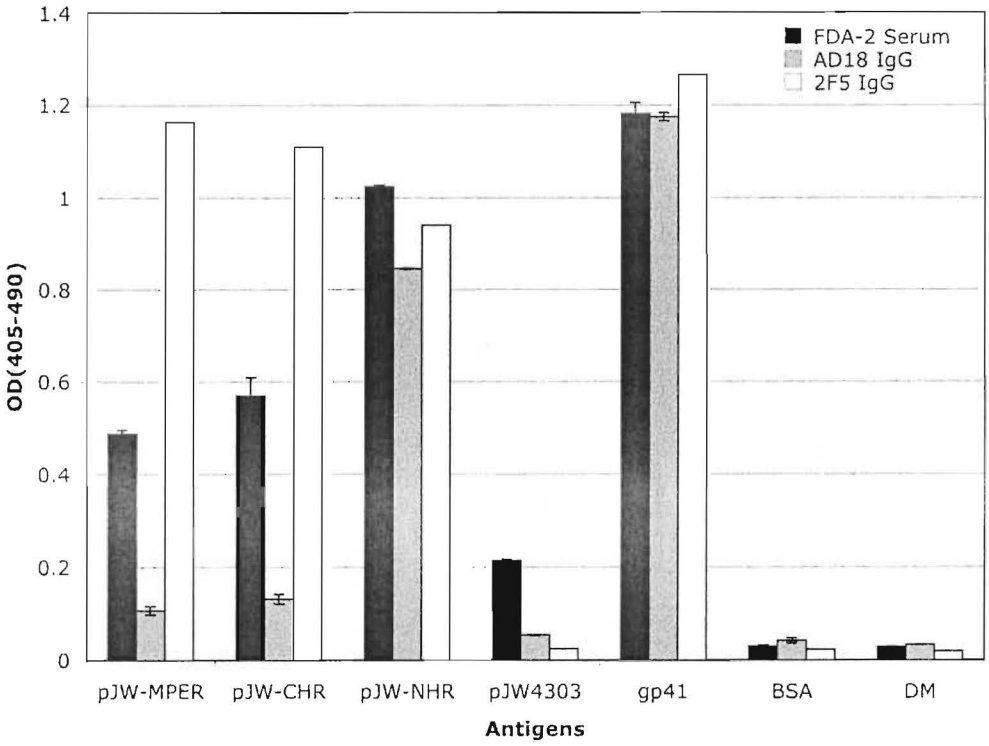


Figure 2-5. Recognition of recombinant gp41 fragments by sera from HIV positive donors in cell lysate ELISA. The FDA-2 serum was used at 1/250 dilution and the AD18 IgG was used at 100 nM. Data are representative of different experiments.

(252), and purified IgG from AD18 recognized the recombinant proteins, this suggested that the gp41 epitopes present in these recombinant proteins resemble those that elicit Ab responses during natural infection. Only the HIV-1 positive sample FDA-2 bound to the three proteins, whereas AD18 IgG bound preferentially to the protein elicited by the longer pJW-NHR. These data indicate that an anti-MPER Abs were present in the FDA-2 serum.

Since fragments of gp41 were fused to the PDGFR-TM it was expected that the recombinant proteins would be expressed on the cell surface. COS cells were grow in microscopy slides, then, transfected with the pJW constructs and the mock vector. Exposure on the plasma membrane was explored by confocal microscopy of intact, non-permeabilized cells probed with MAb 2F5 and goat-anti-human IgG conjugated to Alexa-Fluor 488. Cell staining in **Figure 2-6** demonstrate cell surface exposure of the MPER regions expressed by pJW-MPER, pJW-CHR and pJW-NHR. No reactivity was observed for cells transfected with the mock vector **Figure 2-6**.

2.4.2. Optimization of gp41 codon usage for expression in mammalian cells

The differences in codon usage between the HIV-1 and mammalian genomes has been reported in several studies (100, 126, 130, 229). The HIV-1 genes are characterized by a high A/U content, which diverges significantly from the high content of G/C characteristic of mammalian genes. Accordingly, the expression of HIV-1 genes is limited *in vivo* by the composition of the tRNA pool of its host. It is believed that by having a different codon usage, HIV-1 ensures low expression of its viral proteins, facilitating escape from immune surveillance. To increase the expression of the gp41

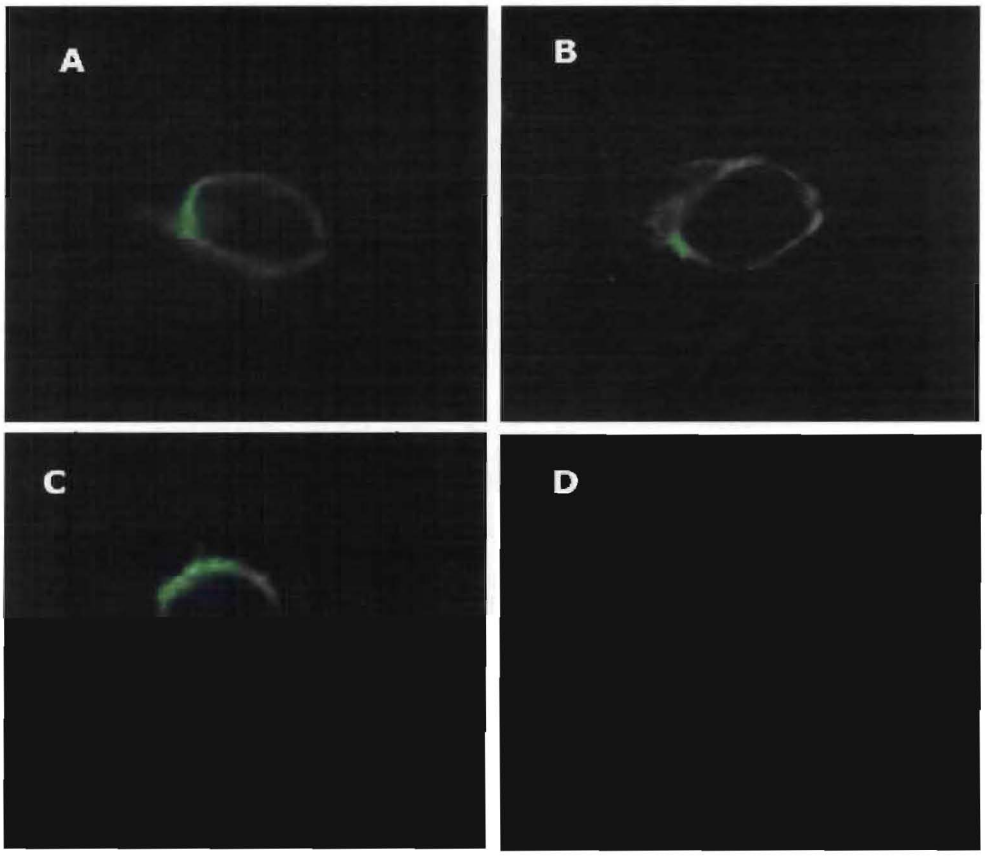


Figure 2-6. Confocal microscopy of non-permeabilized COS 7 cells transfected with the pJW-constructs. pJW-MPER (A), pJW-CHR (B), pJW-NHR (C) and pJW4303 (mock, D). Expression on the cell surface was visualized by staining with MAb 2F5 and Goat anti-human IgG Alexa 488 conjugate.

recombinant fragments in mammalian cells, a synthetic gene from HIV-1_{JR-CSF} gp41 was made, with the codon composition adjusted to that of humans, following the work of Andre *et al.* (6). The DNA sequence was synthesized by GENEART, which also performed a further analysis of secondary structure to the sequence, to avoid the formation of structures that could make the DNA unstable *in vivo*. The sequence for the gp41 optimized gene and its translated protein are shown in **Figure 2-7**.

The gp41protein has a long cytoplasmic domain, which is believed to modulate the protein surface expression. In the cytoplasmic region close to the membrane, a Tyr-based YXXϕ endocytosis motif (in which ϕ represents a hydrophobic amino acid with a large aliphatic side chain) is highly conserved on the envelope proteins of HIV-1, HIV-2, and SIV, as well as glycoproteins from other retroviruses (147). It is thought that the YXXϕ motif functions as a potent endocytosis signal through its interaction with the cellular clathrin adaptor protein AP-2, which has been shown to bind specifically to this motif (19). Recently Bu *et al.* showed that mutation of the Tyr to Ser in this YXXϕ motif increased the immunogenicity of the HIV-1 Env protein, in DNA immunization studies (28). Thus, we decided to incorporate this substitution in the codon-optimized synthetic gp41 gene.

2.4.3. Antigenicity of MPER proteins tethered to the gp41 transmembrane region

Primers for PCR amplification were designed to make constructs similar to the ones with the gp41 wild type gene and the PDGFR-TM. Primers were designed to encode a gp41 fragments including the gp41 transmembrane region and parts of, or the entire gp41 cytoplasmic domain (CT). The gp41 TM and CT regions were included in these

```
1/1                                                 31/11                                                 61/21
GCC GGA GCC CAG CCC GCC AGA TCT GCC GTG GGC ATC GGC GCC CTG TTT CTG GGC TTC CTG GGA GCC GCC GGA AGC ACC ATG GGA GCC GCC
A   G   A   Q   P   A   R   S   A   V   G   I   G   A   L   F   L   G   F   L   G   A   A   G   S   T   M   G   A   A

91/31                                               121/41                                                151/51
AGC ATG ACC CTG ACC GTG CAG GCC CGG CAG CTG CTG TCC GGC ATC GTG CAG CAG CAG AAC AAC CTG CTG CGG GCA ATC GAG GCC CAG CAG
S   M   T   L   T   V   Q   A   R   Q   L   L   S   G   I   V   Q   Q   Q   N   N   L   L   R   A   I   E   A   Q   Q

181/61                                              211/71                                                241/81
CAC ATG CTG CAG CTG ACC GTG TGG GGC ATC AAG CAG CTG CAG GCC AGG GTG CTG GCC GTG GAG AGA TAC CTG AAG GAC CAG CAG CTG ATG
H   M   L   Q   L   T   V   W   G   I   K   Q   L   Q   A   R   V   L   A   V   E   R   Y   L   K   D   Q   Q   L   M

271/91                                              301/101                                               331/111
GGC ATC TGG GGC TGC AGC GGC AAG CTG ATC TGC ACC ACC GCC GTG CCC TGG AAC ACC AGC TGG TCC AAC AAG AGC CTG GAC AGC ATC TGG
G   I   W   G   C   S   G   K   L   I   C   T   T   A   V   P   W   N   T   S   W   S   N   K   S   L   D   S   I   W

361/121                                             391/131                                               421/141
AAC AAC ATG ACC TGG ATG GAA TGG GAG AAA GAG ATC GAG AAC TAC ACC AAC ACC ATC TAC ACC CTG ATC GAG GAA AGC CAG ATC CAG CAG
N   N   M   T   W   M   E   W   E   K   E   I   E   N   Y   T   N   T   I   Y   T   L   I   E   E   S   Q   I   Q   Q

451/151                                             481/161                                               511/171
GAA AAG AAC GAG CAG GAA CTG CTG GAA CTG GAC AAG TGG GCC AGC CTG TGG AAC TGG TTC GAC ATC ACC AAG TGG CTG TGG TAC ATC AAG
E   K   N   E   Q   E   L   L   E   L   D   K   W   A   S   L   W   N   W   F   D   I   T   K   W   L   W   Y   I   K

541/181                                             571/191                                               601/201
ATT TTC ATC ATG ATC GTG GGC GGC CTG ATC GGC CTG CGG ATC GTG TTC AGC GTG CTG TCC ATC GTG AAC AGA GTG CGG CAG GGC AGC AGC
I   F   I   M   I   V   G   G   L   I   G   L   R   I   V   F   S   V   L   S   I   V   N   R   V   R   Q   G   S   S

631/211                                             661/221                                               691/231
CCC CTG AGC TTC CAG ACC CTG CTG CCC GCC ACC AGA GGC CCC GAC CGG CCC GAG GGC ATC GAG GAA GAG GGC GGC GAG CGG GAC AGG GAC
P   L   S   F   Q   T   L   L   P   A   T   R   G   P   D   R   P   E   G   I   E   E   E   G   G   E   R   D   R   D

721/241                                             751/251                                               781/261
AGA TCC GGC CAG CTG GTG AAC GGC TTC CTG GCC CTG ATC TGG GTG GAC CTG CGG AGC CTG TTC CTG TTC AGC TAC CAC CGG CTG CGG GAC
R   S   G   Q   L   V   N   G   F   L   A   L   I   W   V   D   L   R   S   L   F   L   F   S   Y   H   R   L   R   D

811/271                                             841/281                                               871/291
CTG CTG CTG ACA GTG ACC CGG ATC GTG GAG CTG CTG GGC AGG CGG GGC TGG GAG ATC CTG AAG TAC TGG TGG AAC CTG CTG CAG TAC TGG
L   L   L   T   V   T   R   I   V   E   L   L   G   R   R   G   W   E   I   L   K   Y   W   W   N   L   L   Q   Y   W

901/301                                             931/311                                               961/321
TCC CAG GAA CTG AAG AAC AGC GCC GTG AGC CTG CTG AAC GCC ACC GCC ATC GCC GTG GCC GAG GGC ACC GAC CGG ATC ATC GAG GTG GTG
S   Q   E   L   K   N   S   A   V   S   L   L   N   A   T   A   I   A   V   A   E   G   T   D   R   I   I   E   V   V

991/331                                             1021/341                                              1051/351
CAG CGG GTG TAC AGA GCC ATC CCC CAC ATC CCC ACC CGG ATT CGG CAG GGC CTG GAA CGG GCC CTG CTG TGA TGA GGT ACC GGG TGG CAA
Q   R   V   Y   R   A   I   P   H   I   P   T   R   I   R   Q   G   L   E   R   A   L   L   *   *
```

Figure 2-7. The codon optimized gp41 gene (JR-CSF).

constructs to determine whether those regions contribute to the antigenicity of the MPER in the context of the cell surface. The primers used for making the constructs with the optimized gp41 coding sequence are shown in **Table 2-2**. The restriction sites NheI and BamHI were used for cloning of the gp41 gene fragments into the pJW4303 plasmid digested with similar enzymes. **Figure 2-8** shows a schematic representation of the coding regions present in the constructs made with the optimized gp41 gene. For the expression of proteins with the gp41 TM region, two different sets of constructs were generated. The TM1 series carry the gp41 TM region and 27 amino acids from the gp41 cytoplasmic region, whereas the TM2 series carry the gp41 TM region and 66 amino acids from the gp41 cytoplasmic region. A third series of constructs (CT) carrying the whole gp41 cytoplasmic region was also made. Restriction analysis was used to select plasmids bearing PCR fragments of correct size, and positive clones were further verified by DNA sequencing .

COS cells were transfected with the TM1 and TM2 constructs series under similar conditions, and cell lysates were prepared as described in section 2.3.4. To assess whether the TM1 and TM2 proteins where exposed in the cell surface, we performed whole cell ELISAs on paraformaldehyde-fixed, non-permeabilized cells. As shown in **Figure 2-9**, intact cells reacted with both antibodies, indicating that the proteins are expressed in the cell surface. Surprisingly, and different from the proteins tethered by PGDFR-TM, 2F5 and 4E10 reacted equally well to each of the proteins produced by the TM1 or the TM2 constructs (**Figure 2-4** *vs.* **Figure 2-9**). Since the antigenicity of the PDGFR-TM constructs for 2F5 and 4E10 was not assayed on whole cell ELISA, we

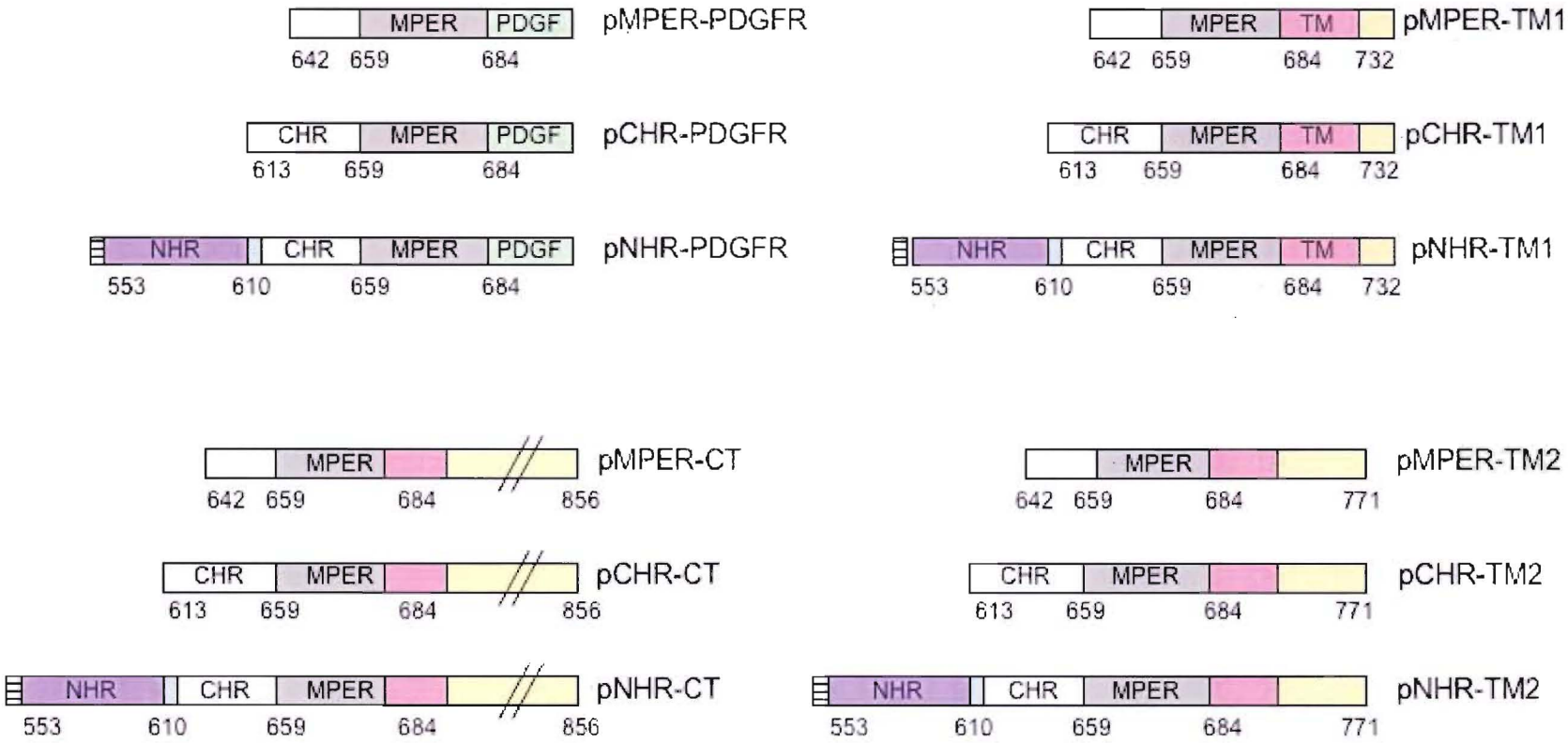


Figure 2-8. Schematic representation of gp41 fragments constructs bearing the codon optimized gp41 gene sequence. NHR: N-heptad repeat; CI: cluster I or immunodominant region; CHR: C-terminal heptad repeat; MPER:membrane proximal external region; TM: gp41 transmembrane region; TM1(aa 684-732); TM2 (aa 684-771); CT: cytoplasmic region (aa 684-878). PDGF, platelet derived grow factor-TM region. PCR fragments were cloned into pJW43-3.

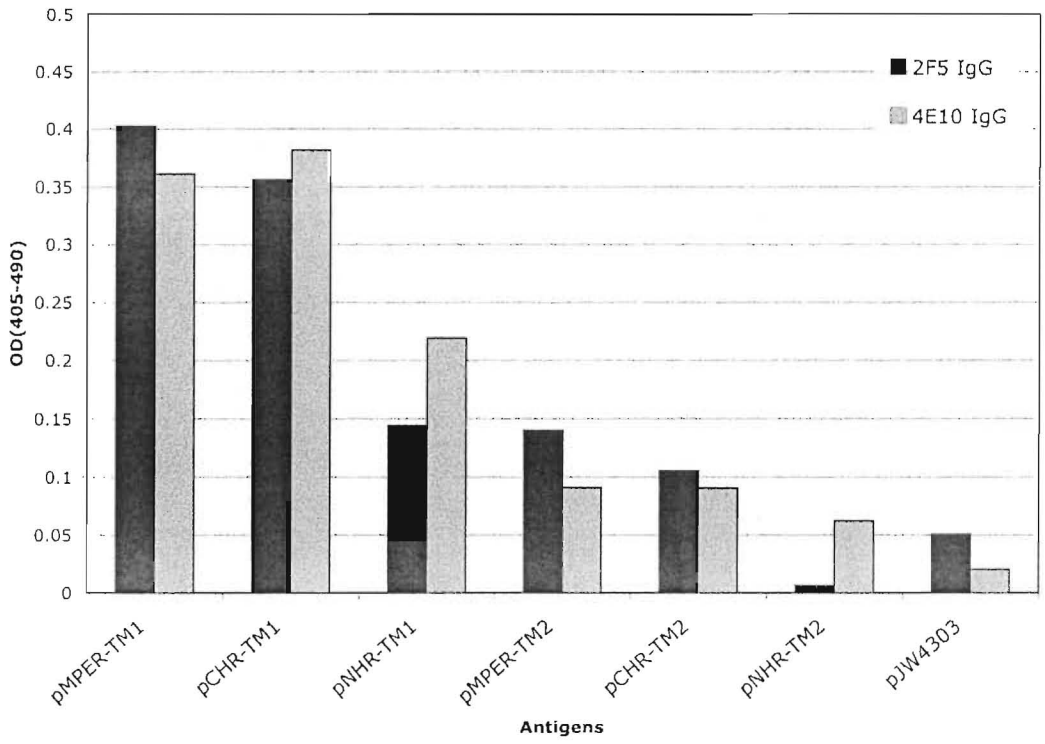


Figure 2-9 Antigenicity of recombinant proteins bearing TM1 and TM2 regions in a whole-cell ELISA. COS-7 cells were transfected with each of the plasmids and 48 h after transfection cells were fixed with 3.5 % paraformaldehyde, washed and probed with 10 nM 2F5 and 4E10 IgG. The results are representative of three experiments.

also performed cell lysates ELISAs on the TM1 and TM2 constructs, to rule out differences determined by the type of assay. The binding of the MAbs to the expressed proteins in cell lysate ELISA is shown in **Figure 2-10**; in agreement with the whole cell ELISAs, 2F5 and 4E10 reactivity against each independent protein was very similar. Since 2F5 and 4E10 reactivity with constructs bearing the TM2 region was low, TM2 DNA constructs were not further characterized. Next, we explored whether the same situation would be observed for proteins carrying the whole gp41 cytoplasmic region (CT constructs). The binding of 4E10 and 2F5 to proteins bearing the PDGFR-TM, the gp41 TM1 and the whole gp41 CT region was compared side-by-side in cell lysate ELISAs. In agreement with results in **Figures 2-4**, 2F5 bound better than 4E10 to the proteins bearing the PDGFR-TM. However, such a difference was not observed for the constructs with TM1 or CT regions (**Figure 2-11**); instead, 4E10 binding reached the same or better levels than those of 2F5. These results demonstrate that the transmembrane region exerts some influence on the antigenicity of the 4E10 epitope in the context of cell surface. Since the relative expression of the constructs could not be normalized, comparisons between constructs were not made.

The expression of the proteins produced by the TM1 and CT constructs was also assessed by fluorescence microscopy, and their cell surface localization was investigated by confocal microscopy. Fluorescence images of non-permeabilized cells transfected with the pMPER-TM1 construct, and stained with 2F5 are shown in **Figure 2-12**. As seen in the image about 50 % of the cells are positive for staining with 2F5, but unstained cells are also present, indicating that the antibody binds in a specific manner. Confocal

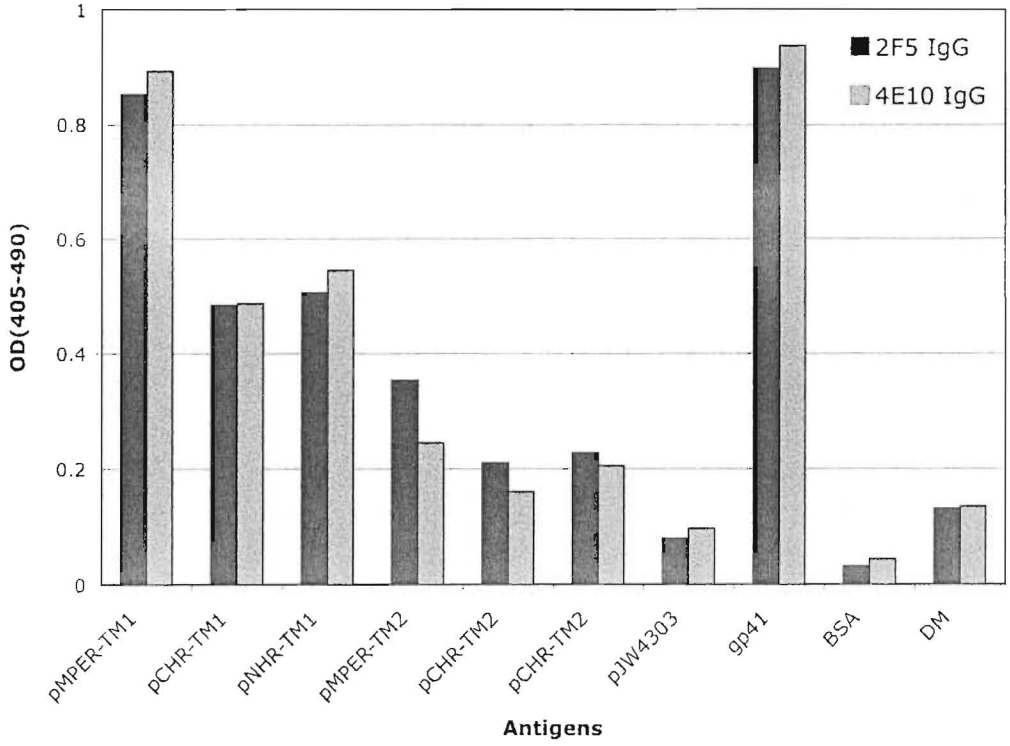


Figure 2-10. Antigenicity of recombinant proteins bearing TM1 and TM2 in cell lysate ELISA. The genes carrying the TM1 or TM2 fragments were introduced into COS-7 cells and the lysates used as Ag in ELISA.

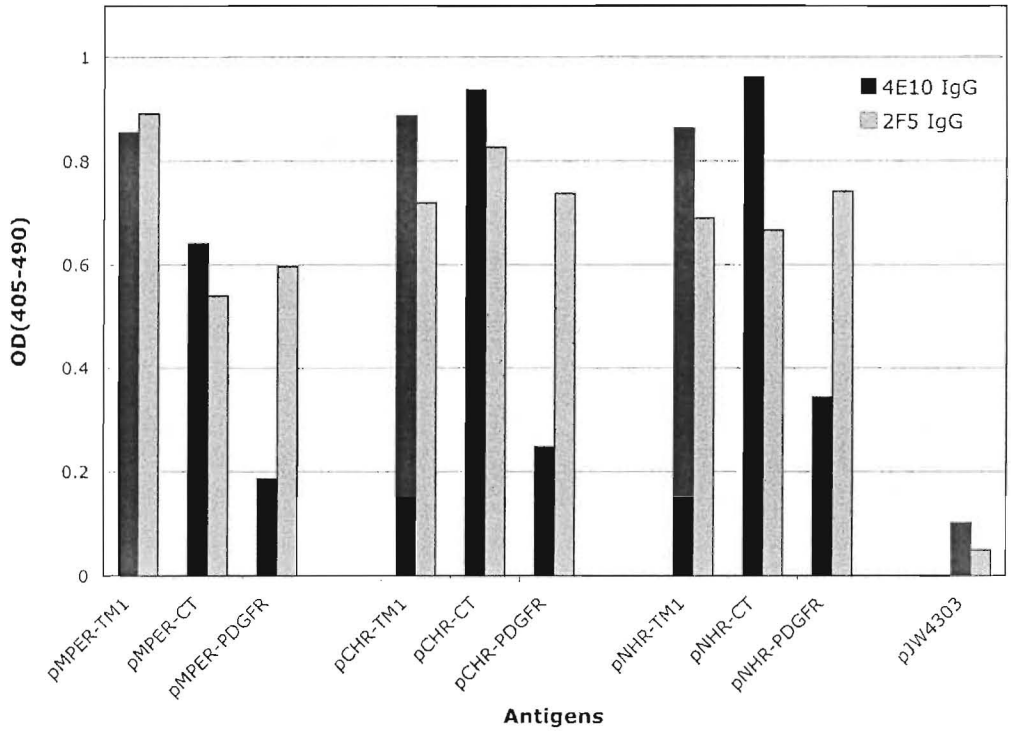


Figure 2-11. Reactivity of MAbs 2F5 and 4E10 with proteins bearing the PDGFR-TM, gp41 TM1 and gp41CT regions. COS-7 cells were transfected with plasmids carrying three different MPER fragments (MPER, CHR or NHR), each fused to three alternative C-terminal regions (TM1, CT or PGDFR). Total cell lysates were probed with 10 nM 4E10 IgG or 10 nM 2F5 IgG. Results are representative of three assays.

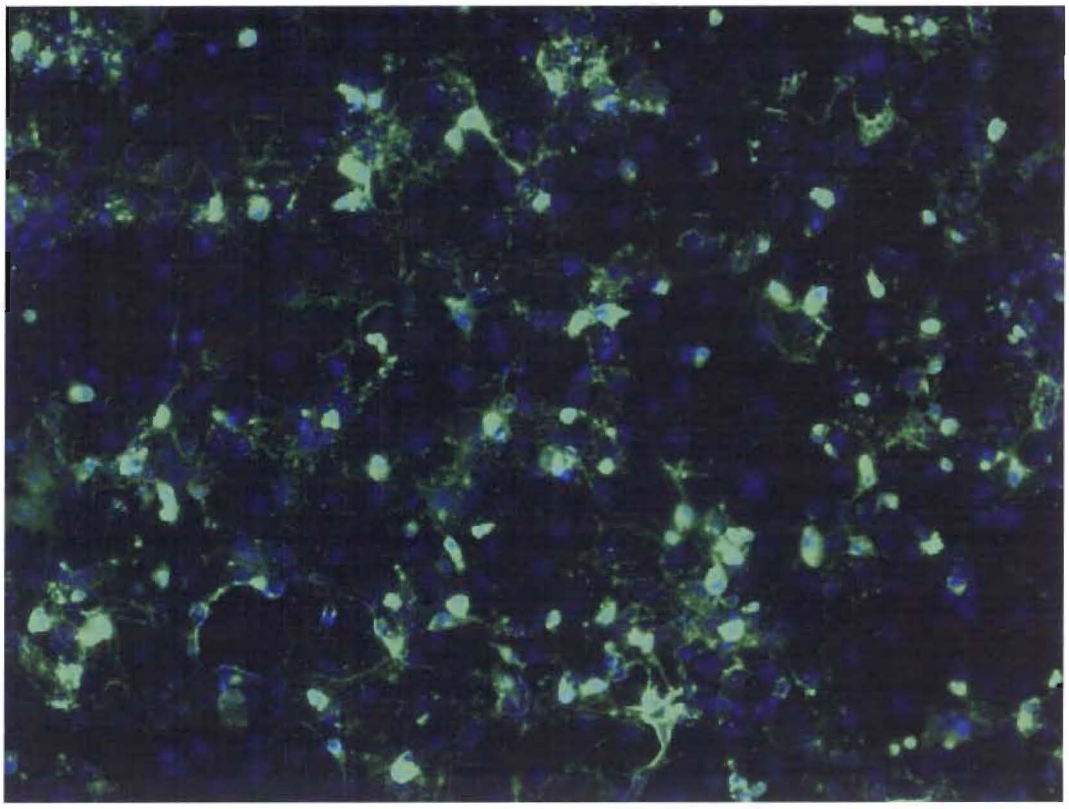


Figure 2-12. COS-7 cells expressing pMPER-TM1, fluorescence microscopy. Cells were transiently transfected with pMPER-TM1 DNA construct. Fixed, non-permeabilized cells were stained with 5 nM 2F5 IgG, followed by anti-human IgG Alexa Fluor 488 conjugate. Cells were visualized by fluorescence microscopy. MPER is shown green and cell nuclei in blue (DAPI). Around ~50% of the cells are expressing the MPER.

microscopy (**Figure 2-13**) on cells expressing proteins with the gp41 TM1 region (pMPER-TM1, pCHR-TM1, pNHR-TM1) showed that the proteins produced from these three constructs localized to the cells surface. Cells transfected with the mock vector showed no staining with 2F5 (data not shown).

Expression of the proteins from constructs pMPER-CT, pCHR-CT and pNHR-CT was visualized by fluorescence microscopy using MAb 4E10. **Figure 2-14** shows four different fields; cells expressing the desired protein (pNHR-CT) as well as untransfected cells are visible in the same field, confirming the specificity of 4E10 interaction with cells expressing the gp41 MPER region. As seen in **Figure 2-15**, the pattern of cell staining seen by confocal was similar to the one by immunofluorescence, and very different from that observed for the proteins bearing TM1 region. The staining of the CT proteins localized to the cell cytosol, even though the cells were not permeabilized. This indicates that the presence of the full gp41 CT region promotes membrane permeabilization, which allows access of the antibody to the cytosolic protein pool. In addition, we did not determine whether the proteins also localized to the cell membrane. Thus, we have two different patterns of protein localization with the constructs carrying the gp41 TM, which depended on the presence of the full gp41 CT region.

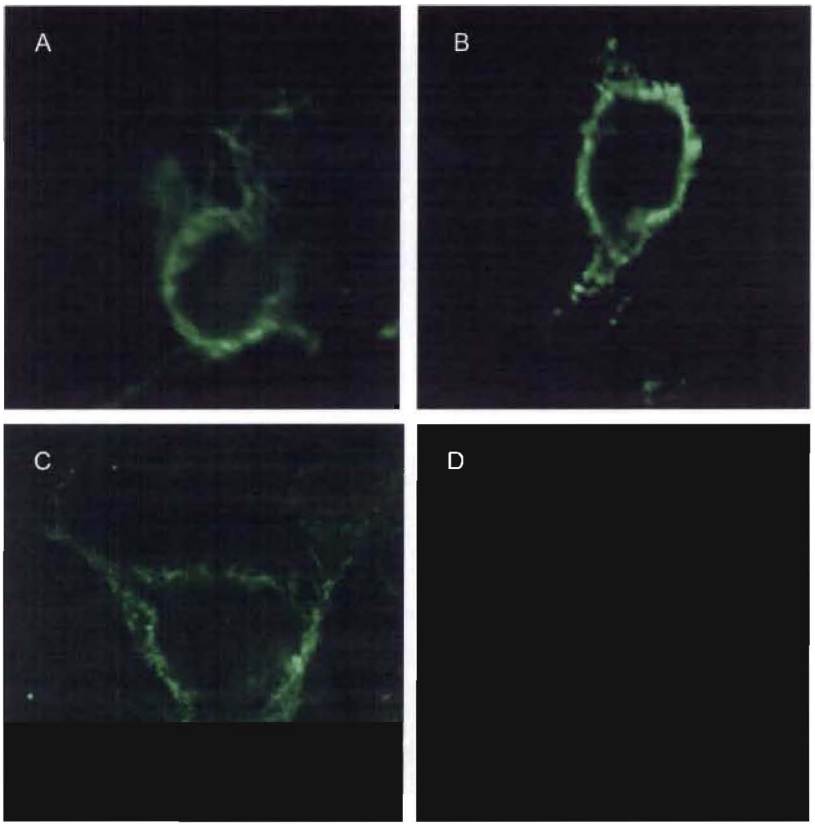


Figure 2-13. COS-7 cells expressing TM1 proteins. Cells were transiently transfected with pMPER-TM1 (A), pCHR-TM1 (B), pNHR-TM1 (C) and pJW4303 (D) DNA constructs. Non-permeabilized cells were stained with 5 nM 2F5 IgG, and visualized by confocal microscope. The bund 2F5 Ab was detected with anti-human IgG conjugate to Alexa fluor 488.

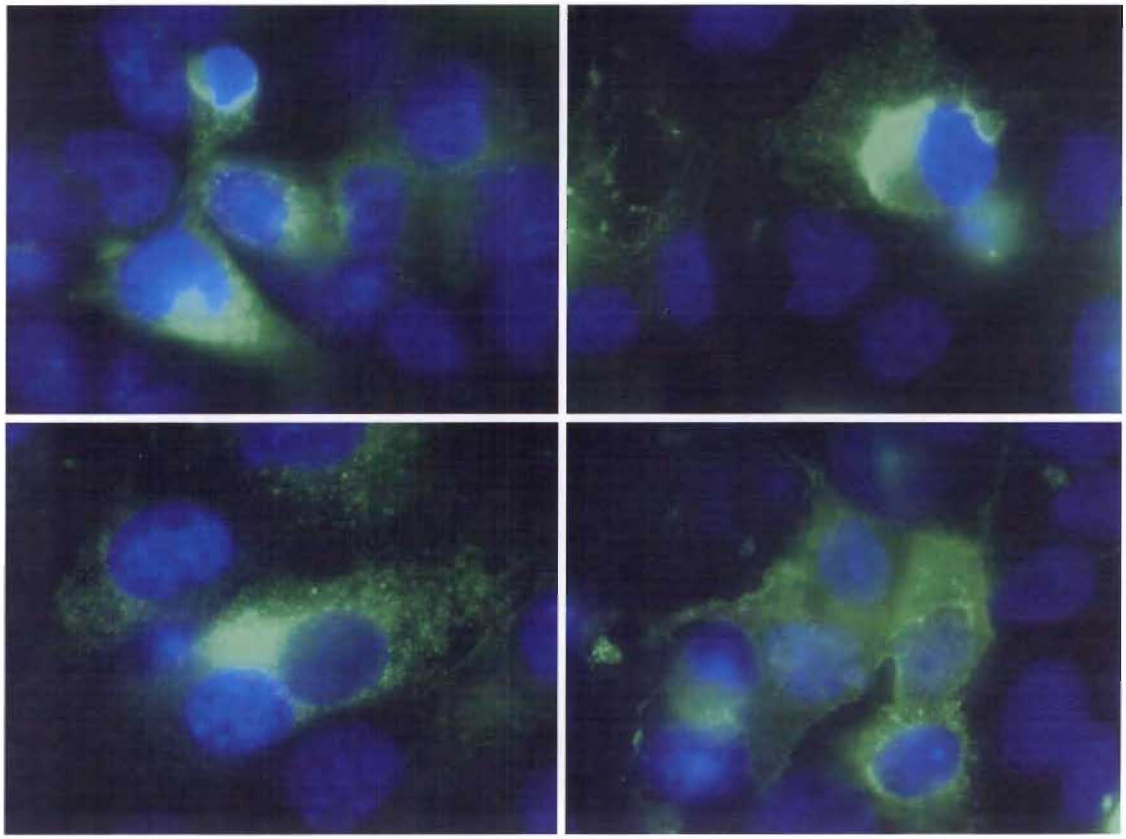


Figure 2-14. COS-7 cells expressing pNHR-CT, fluorescence microscopy. Cells were transiently transfected with pNHR-CT DNA construct. Non-permeabilized cells were stained with 5 nM 4E10 IgG, and visualized by fluorescence microscopy. MPER (green) cell nuclei (blue). Four different fields are shown.

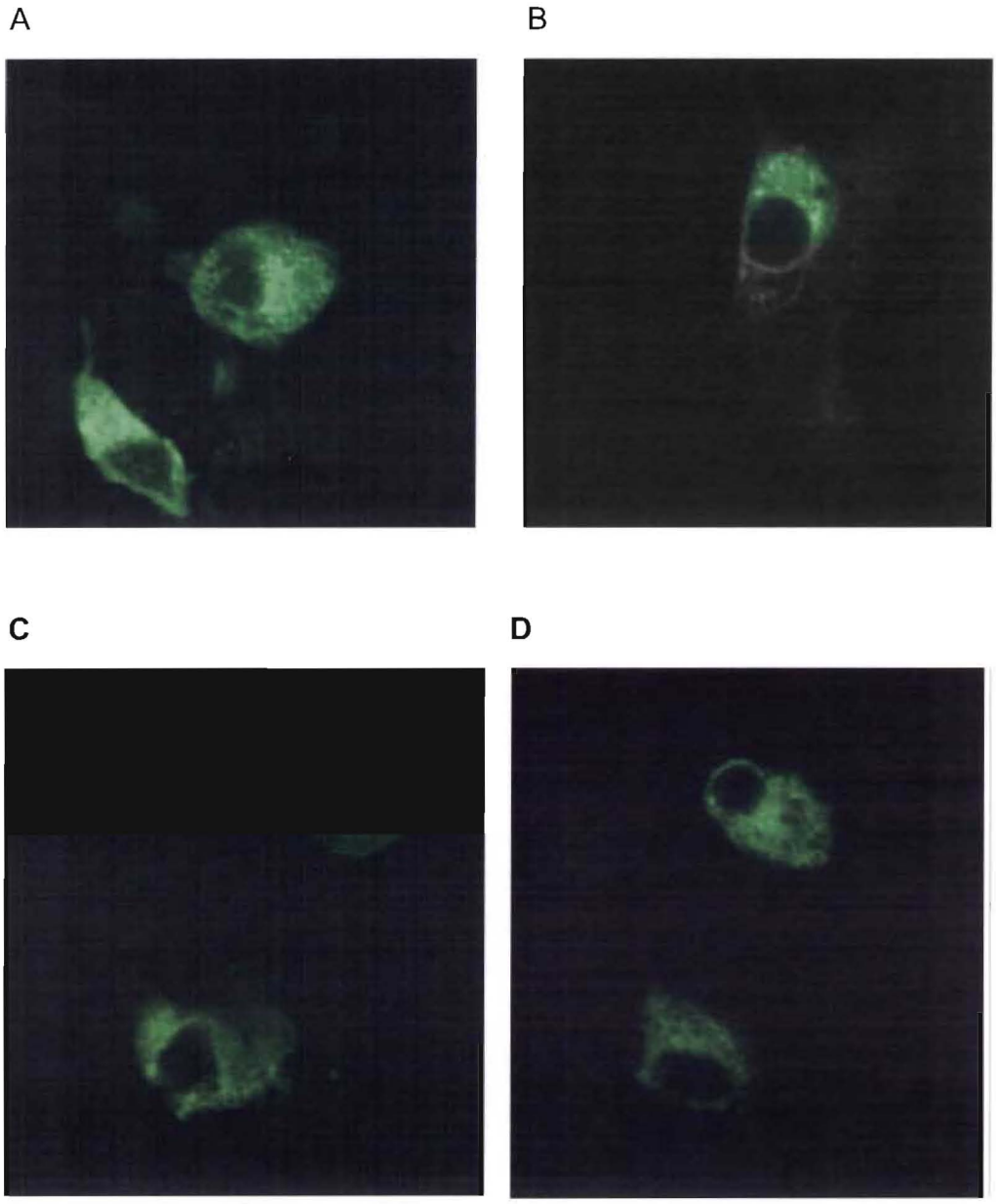


Figure 2-15. COS-7 cells expressing CT proteins, confocal microscopy. Cells were transiently transfected with pMPER-CT (A), and pCHR-CT (B), pNHR-CT (C, D). DNA constructs. Non-permeabilized cells were stained with 5 nM 2F5 IgG, and visualized by confocal microscopy.

2.4.4. Alanine scanning mutagenesis of the MPER

Alanine scanning mutagenesis is an important tool for studying antigenicity. It allows the identification of critical or important residues for the interaction between Ab and its epitope. Alanine is the amino acid of choice for substitutions since it eliminates the side chains beyond the β-carbon, without altering the main-chain conformation and it does not impose extreme electrostatic or steric effects.

Amino acids involved in critical contacts between MAbs 2F5 and 4E10 and their cognate epitopes in the MPER, have been inferred from the crystal structures of each Fab with synthetic peptide epitopes (37, 183). Critical binding residues in the epitopes have been determined by functional assays using Ala-substituted synthetic peptides (27, 177, 240), and sensitivity to neutralization by MAb 2F5 and 4E10 in pseudovirus carrying mutated MPERs (288). To verify that the interaction of the 2F5 and 4E10 with their epitopes in the MPER expressed in the cell surface occurs through the contacts previously identified, a set of Ala substitutions was generated in the MPER using the plasmid pMPER-TM1 as template.

Primers were designed to create 22 independent mutations in the MPER (aa 662-684), and two other corresponding to the first two amino acids of the gp41 TM region (aa-685-686). The primers used for this experiment are shown in **Table 2-3**. Plasmids bearing the mutation were independently introduced into COS cells. Cell lysates were prepared for ELISA, including lysates from pMPER-TM1 and the mock vector, which served as a positive and negative control in ELISA, respectively. The MPER mutants were tested for their ability to react with the MAbs, 2F5, 4E10 and Z13e1 (**Figure 2-16**).

In general, the reactivity patterns for 2F5, 4E10 and Z13e1 with the mutants were

similar to the reactivity reported for each Ab with alanine substitution on synthetic peptides. However, the data could not be normalized to account for potential differences in expression between the different constructs. Even with this limitation, it is clear that for most clones at least one MAb appeared to bind at high levels, suggesting that for most clones there were no gross differences in protein expression. As reported by others (196, 240), 2F5 reactivity was very sensitive to substitution of the DKW (663-665) residues; it was also low for mutants in Trp_{670}, Leu_{679} and Trp_{680} (although these three mutants showed reduced binding with all three antibodies, suggesting the possibility of a lower expression level). 4E10 was sensitive to mutations in the previously reported CBR identified with synthetic peptides (Trp_{672}, Phe_{673} and Thr_{676}) (27). 4E10 was also sensitive to Ala substitution of residue Trp680, described as an important for 4E10 Nt activity (288). In the case of Z13e1, several residues were important for binding including Trp_{670}, Asp_{671}, Trp672, Phe_{673}, Asp_{674} and Thr_{676}. These data are in general agreement with previous MPER Ala-mutagenesis studies using synthetic peptides (177). Interestingly, Z13e1 is also sensitive to the mutation of the Lys_{683}, a residue not reported before. The substitution of the first two residues of gp41 TM does not appear to affect the interaction of 2F5, 4E10 or Z13e1 with the MPER (**Figure 2-16**).

E	L	D	K	W	A	S	L	W	N	W	F	D	I	T	K	W	L	W	Y	I	K	I	F
661	662	663	664	665	667	668	669	670	671	672	673	674	675	676	677	678	679	680	681	682	683	684	685

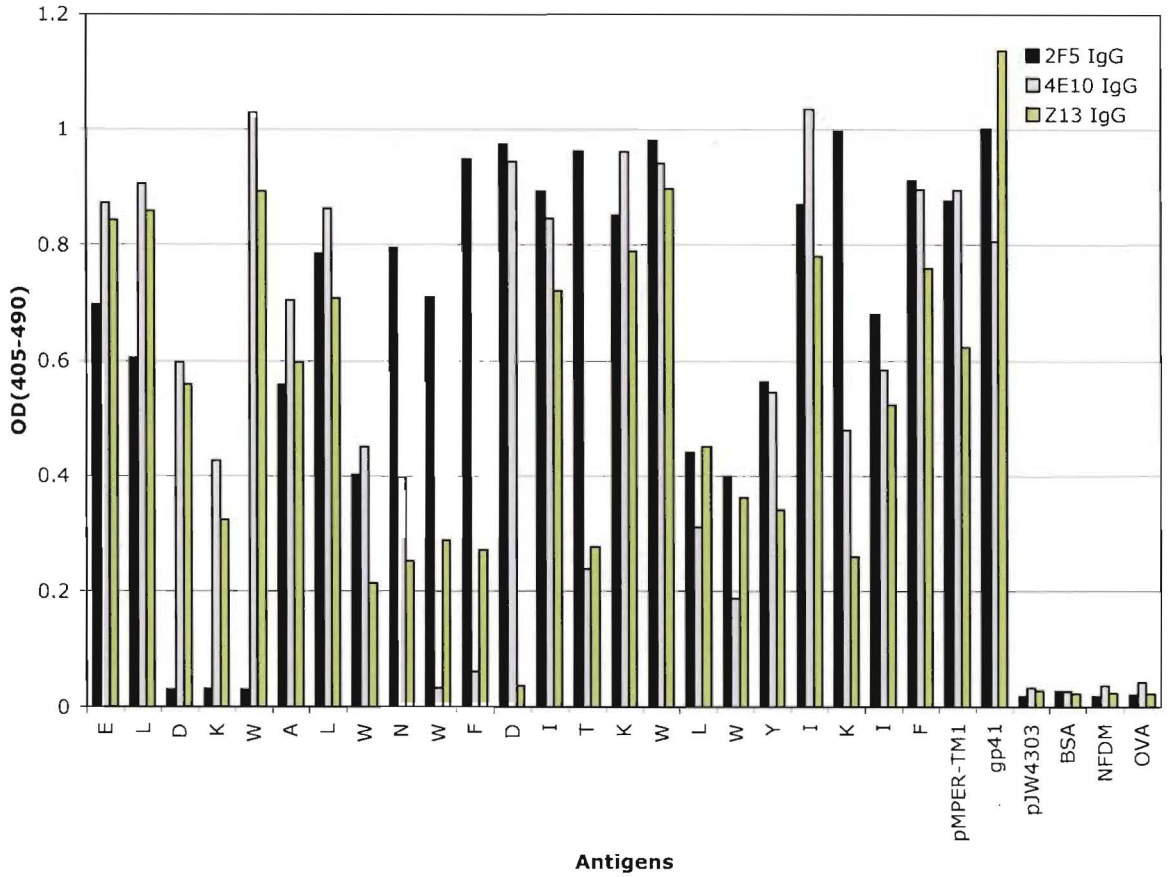


Figure 2-16. Reactivity of MAb 2F5, 4E10 and Z13 with the MPER Ala-substituted mutants in cell lysate ELISA. Substitution of Ser_{668} was not included in this experiment.

2.5. Summary

This chapter describes studies on the antigenicity of the gp41 MPER anchored to the cell membrane by a TM region, and exposed on the cell surface. As part of the characterization of 2F5 and 4E10 epitopes on the MPER, we tested the influence of other regions of gp41, different transmembrane regions and membrane proximity on the antigenicity of the MPER. For this study, we generated multiple sets of DNA constructs using either wild type or a codon-optimized gene of gp41, in combination with the PDGFR-TM region, the gp41 TM region, three fragments of the gp41 ectodomain and three fragments from the gp41 CT region. We showed by confocal microscopy and whole cells ELISA that the proteins produced by most constructs were displayed on the cell surface. The proteins were recognized by the bNt MAb 2F5, 4E10 and Z13e1. We found that increasing the size of the ectodomain towards the N-terminus decreased the recognition of the MPER by 2F5 when the MPER was anchored to the membrane by the PDGFR-TM. Furthermore, a significantly lower binding was observed for 4E10 relative to 2F5 in all constructs tested. However, all gp41 fragment fused to the gp41 TM (TM1 or CT) were recognized by MAb 4E10 at the same level of 2F5, indicating that the TM region is important for optimal binding of 4E10 to the MPER. This is a previously unreported finding, which may reflect the need of specific structure and/or positioning of the MPER in the vicinity of membrane for 4E10 binding, and might has serious implications for vaccine design.

Another finding is the fact that the full gp41 CT region to promoted cell membrane permeability, evidenced by the MAbs accessibility to the cytoplasmic compartment of MPER-CT expressing cells. Since naturally-infected cells produce gp41,

this finding may reflect an uncharacterized membrane disruption function of the CT region of gp41, with potential implications in membrane fusion, virus assembly and virus cytotoxicity.

In this study a set of Alanine substitution mutants was generated within the MPER, and validated with 2F5, 4E10 and Z13. These mutants are useful tools for characterization of Ab responses against this important region of gp41 (see Chapter 3), and provide antigenicity information that complements the data generated with the use of synthetic peptides.

2.6. Appendix 1

The **Table 2-4** show all the DNA constructs made in this study, icluding some preliminary constructs made in pDisplay vector, which are described in this section. *Constructs using the pDisplay vector:* Originally, a set of 14 DNA constructs were made in the pDisplay vector, and their expression evaluated in COS-7 cells. The protein expression for the different constructs was shown by western blot (WB) using the 2F5, 4E10 and 17/9 MAb. In **Figure 2-17** a schematic representation of each of the constructs, and a summary of their recognition by western blot is presented. Two constructs (2 and 5) carrying the gp41 TM region (aa 685-705) did not express the recombinant protein (this was tested in several experiments). A negative influence of the FP region (hydrophobic region) of gp41 on the expression was observed (constructs 7, 9,13, and 14). For the generation of constructs 11, 12, 13 and 14, the loop region (Cys-5aa-Cys, aa 598-604) was replaced by a Gly linker. **Figure 2-18 s**hows a WB using cell lysate prepared after transfection with some of the constructs. An analytical digestion of

all the generated plasmids is also presented in **Figure 2-19**. Only three of these constructs were examined further in this thesis work, these are constructs pDisp-MPER (construct number 1), pDisp-CHR (construct number 4) and pDisp-NHR (construct number 10).

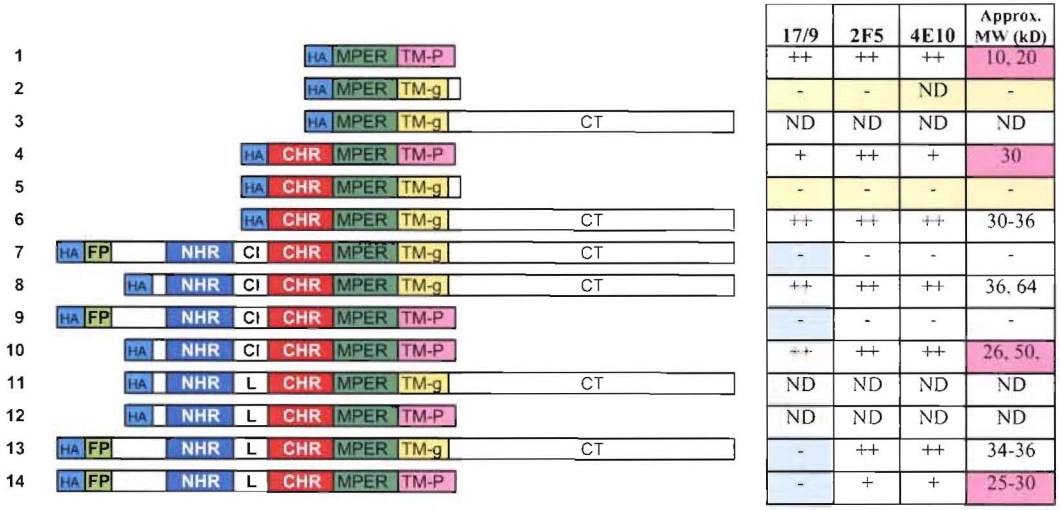
Western blot: Cell lysates were resuspended in Buffer Laemmli and analyzed by SDS-PAGE. Proteins were transferred from the gels to a Polyvinylidene Difluoride (PVDF) membrane (BioRad) using semi-dry transfer apparatus (BioRad). The membranes were blocked with TBS containing 5% DM, ON at 4 °C. Primary Ab was diluted in TBS/Tw containing 5% DM. The membranes were incubated with the primary Ab for 2 h at RT, followed by 3 washes with TBS/Tw. Goat anti-human Ab HRP conjugate was used to detect the bound Ab, followed by detection using substrate from Western LightningTM Chemiluminescence Reagent Plus (PerkinElmer LAS, Inc). Bands were visualized in medical X-ray films expose directly against the blot membranes.

Table 2-4. Summary of all the constructs generated for this work.

#	Name	Vector	gp41 region	TM region	Remarks
1	pDisp-MPER	pDisplay	HA-MPER	PDGFR	*Env* wild type
2	pMM-2	pDisplay	HA-MPER-TM	gp41	*Env* wild type
3	pMM-3	pDisplay	HA-MPER-TM-CT	gp41	*Env* wild type
4	pDisp-CHR	pDisplay	HA-CHR-MPER	PDGFR	*Env* wild type
5	pMM-5	pDisplay	HA-CHR-MPER-TM	gp41	*Env* wild type
6	pMM-6	pDisplay	HA-CHR-MPER-TM-CT	gp41	*Env* wild type
7	pMM-7	pDisplay	HA-FP-NHR-CI-CHR-MPER-TM-CT	gp41	*Env* wild type
8	pMM-8	pDisplay	HA-NHR-CI-CHR-MPER-TM-CT	gp41	*Env* wild type
9	pMM-9	pDisplay	HA-FP-NHR-CI-CHR-MPER	PDGFR	*Env* wild type
10	pDisp-NHR	pDisplay	HA-NHR-CI-CHR-MPER	PDGFR	*Env* wild type
11	pMM-11	pDisplay	HA-NHR-L-CHR-MPER-TM-CT	gp41	*Env* wild type
12	pMM-12	pDisplay	HA-NHR-L-CHR-MPER	PDGFR	*Env* wild type
13	pMM-13	pDisplay	HA-FP-NHR-L-CHR-MPER-TM-CT	gp41	*Env* wild type
14	pMM-14	pDisplay	HA-FP-NHR-L-CHR-MPER	PDGFR	*Env* wild type
15	pJW-MPER	pJW4303	HA-MPER	PDGFR	*Env* wild type, used for immunization
16	pJW-CHR	pJW4303	HA-CHR-MPER	PDGFR	*Env* wild type, used for immunization
17	pJW-NHR	pJW4303	HA-NHR-CI-CHR-MPER	PDGFR	*Env* wild type, used for immunization
18	pMPER-TM1	pJW4303	MPER-TM1	gp41	*Env* codon optimized
19	pCHR-TM1	pJW4303	CHR-MPER-TM1	gp41	*Env* codon optimized
20	pNHR-TM1	pJW4303	NHR-CI-CHR-MPER-TM1	gp41	*Env* codon optimized
21	pMPER-TM2	pJW4303	MPER-TM2	gp41	*Env* codon optimized
22	pCHR-TM2	pJW4303	CHR-MPER-TM2	gp41	*Env* codon optimized
23	pNHR-TM2	pJW4303	NHR-CI-CHR-MPER-TM2	gp41	*Env* codon optimized
24	pMPER-CT	pJW4303	MPER-TM-CT	gp41	*Env* codon optimized
25	pCHR-CT	pJW4303	CHR-MPER-TM-CT	gp41	*Env* codon optimized

#	Name	Vector	gp41 region	TM region	Remarks
26	pNHR-CT	pJW4303	NHR-CI-CHR_MPER-TM-CT	gp41	*Env* codon optimized
27	pMPER-PDGF	pJW4303	MPER	PDGFR	*Env* codon optimized
28	pCHR-PDGF	pJW4303	CHR-MPER	PDGFR	*Env* codon optimized
29	pNHR-PDGFR	pJW4303	NHR-CI-CHR-MPER	PDGFR	*Env* codon optimized

HA, hemaglutinin tag
FP, fusion peptide
NHR, N-terminal heptap repead
CHR, C-terminal heptap repead
CI, cluster I region
L, Linker of 7 Gly, that substituted the inverted region or the loop (CSGKLIC) in the gp41 immunodominant epitope
MPER, membrane proximal external region
TM, gp41 transmembrane region
TM1, gp41 TM plus 27 aa from CT
TM2, gp41 TM plus 66 aa for TM
CT- gp41 cytoplasmic tail
PDGFR, platelet derived grow factor receptor transmembrane region.



	17/9	2F5	4E10	Approx. MW (kD)
1	++	++	++	10, 20
2	-	-	ND	-
3	ND	ND	ND	ND
4	+	++	+	30
5	-	-	-	-
6	++	++	++	30-36
7	-	-	-	-
8	++	++	++	36, 64
9	-	-	-	-
10	++	++	++	26, 50,
11	ND	ND	ND	ND
12	ND	ND	ND	ND
13	-	++	++	34-36
14	-	+	+	25-30

Figure 2-17. Schematic representation of constructs made in the pDisplay vector. All the constructs were made by PCR amplification from gp41 wild type gene from the HIV-1 isolate JR-CSF. The table summarizes data from western-blot, and shows arbitrary band intensities. ND: not done.

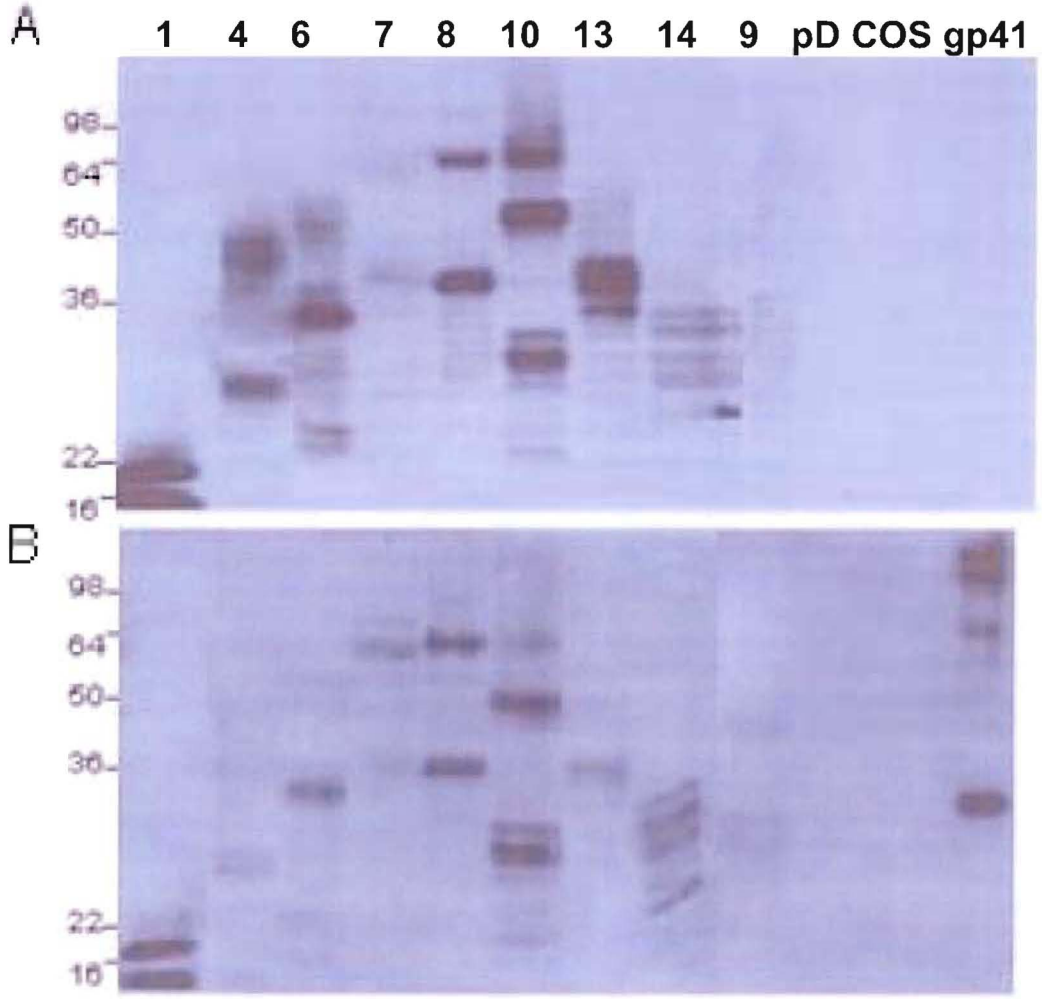


Figure 2-18. Western blot with cell lysates show protein expression form gp41 pDisplay constructs. The numbers represent different cell lysates prepared with the plasmids (the constructs are explained in Table 2-4); pD: cell lysate prepared using pDisplay (mock), COS: cell lysate from non-transfected cells. (A) 2F5 IgG (10nM), (b) 4E10 IgG (5nM).

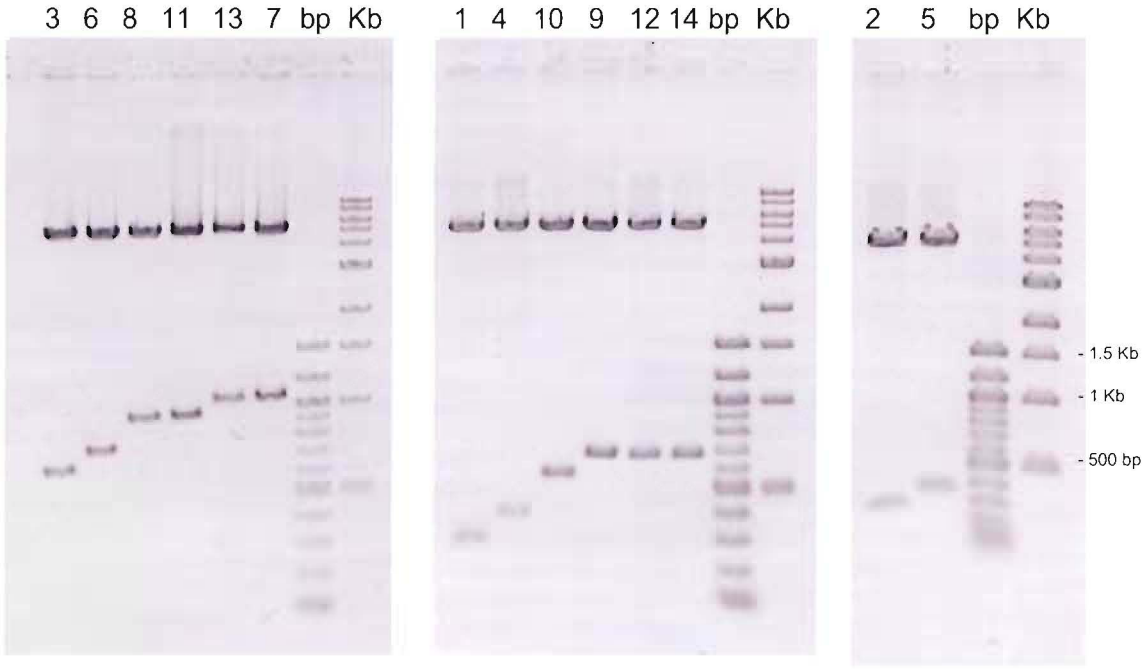


Figure 2-19. Digestion of gp41 DNA constructs in pDisplay vector. Plasmids were digested with *Bgl*II-*Xho*I to illustrate the different fragments cloned into pDisplay. The samples were run in a 1 % TBE agarose gel. The NEB DNA markers 100 bp (bp) and 1 Kb (Kb) were included. The numbers represent the number corresponding to each of the constructs described in Table 2-4.

CHAPTER 3. Immunogenicity of the MPER anchored to the cell membrane

3.1. Introduction

The strongest Nt-sensitive sites on gp41 have been mapped to the MPER (REF). Multiples approaches have been evaluated to induce a neutralizing Ab response against the MPER using the linear epitope sequences. The production of 2F5-like Ab has been evaluated using fusion proteins (125, 128, 133, 239, 278), conjugating the peptide epitope to carrier proteins (120, 136, 142, 159, 274), and by grafting the epitope onto recombinant proteins or other viral scaffolds (47, 67, 70, 73, 109, 135, 151, 172, 173, 283). The situation with 4E10 has been less well explored in part because the epitope was mapped more recently, (290), and it has proven to be less immunogenic. Recent studies have evaluated the induction of Abs to the whole MPER (131, 155).

However, to date, immunization has not generated NtAb comparable to the anti-gp41 neutralizing MAb such as 2F5 or 4E10. Structural and functional features on both the bNt MAbs and the MPER can probably account for the failure of these approaches. First, 2F5 and 4E10 have undergone a high level of somatic mutation and possess a long CDR-H3. Such long H3 sequences are seen in humans among immature B cells population in bone marrow, but are rare in B-cell population in the periphery (259), is not clear in which conditions there are developed; suggesting that these MAbs are rare, and/or difficult to elicit. This feature imposes some species constraints for immunization experiments: mice, the most characterized and cheapest animal model,can not be used since they do not produce antibodies with long H3 (270). Second, in strcutures of 2F5 and 4E10 complexed with their peptide epitopes (37, 183, 185), parts of their H3 are not

in direct contact with the peptide, thus, it has been suggested that they might contact the cell membrane directly, raising the possibility of membrane involvement in the formation of the native epitopes (37, 98, 183). Third, the structure of the MPER at each of its different functional stages (or the one required for eliciting bNt Abs) is not known. Therefore, it is very likely that the neutralization-competent structure of the MPER has not been faithfully mimicked by any approach used to date, and that the real neutralizing epitopes are more complex than the known linear sequences, opening the possibility that other regions of gp41, as well as the viral membrane, can be important to fully form the immunogenic epitopes of these two bNt Abs.

The idea of presenting the MPER in the context of membrane for producing bNt Abs arose in part, due to the failure of previous approaches, as well as from a body of biochemical and structural data (see Chapter 1). Some previous incursions into this field have been made with the synthesis of proteoliposomes, in which Env protein was trapped on liposomes using antibodies (98), and with virus like particles (VLP), which are viral particles that can be assemble and secreted to the media (279). These VLP are engineered to expose selected proteins in their surface. Both of these procedures have limitations; the major are being that the presence of the whole Env protein recapitulates the problem created by Env in natural infection, that is, to skew the immune response towards non-neutralizing epitopes.

In Chapter 2, we described the construction and characterization of several DNA constructs that express truncated versions of gp41 carrying the MPER. We have demonstrated that these recombinant proteins are anchored to the cell membrane, and exposed on the cell surface (see Chapter 2). They were antigenic (to various degrees)

towards the anti-MPER Nt MAbs 2F5, 4E10 and Z13e1. In this Chapter, we have the immunogenicity of some of those genetic constructs (pJW-MPER, pJW-CHR, pJW-NHR) *in vivo* in rabbits using DNA immunization, to investigate whether membrane-anchoring of the MPER, as well as other selected regions of gp41 are required to produce HIV-1-neutralizing Ab that target the gp41 MPER.

3.2. Materials

A set of eighty five15-mer synthetic peptides that correspond to the HIV-1 transmembrane protein $gp41_{MN}$, and overlap by four residues, was obtained from the NIH AIDS Research and Reference Reagent Program. The influenza virus hemagglutinin A peptide (YPYDVPDYA) was a kind gift from Robyn Stanfield (The Scripps Research Institute, La Jolla, CA). The B2.1 peptide ((NH_3)-HERSYMFSDLENRCIAAEKK-($CONH_2$)), the bio-2F5 peptide (H-EQELLELDKWASLWSGK(biotin)GC-NH_2, bio-4E10 peptide (H-SLWNWFDITNWLWYISGC(biotin) and bio-Z13 peptide (H-DKWASLWNWFDITNW (biotin)GC-NH_2 were synthesized by NeoMPS, Inc. (San Diego, CA).

New Zealand White female rabbits were purchased from Millbrook Breeding Labs (Amherst, MA). Animals were housed in the facility managed by the Department of Animal Medicine at the University of Massachusetts Medical School (Worcester, MA), in accordance with an IACUC-approved protocol. The 1.0 μm gold microcarrier particles used for immunizations were from Hercules, CA, Cat. No. 165-2263).

The secondary reagents used for ELISAs, including goat anti-human IgG (Fab)-Horseradish perosidase (HRP) (Cat. No. 31482), goat anti-rabbit IgG (Fc)-HRP (Cat. No.

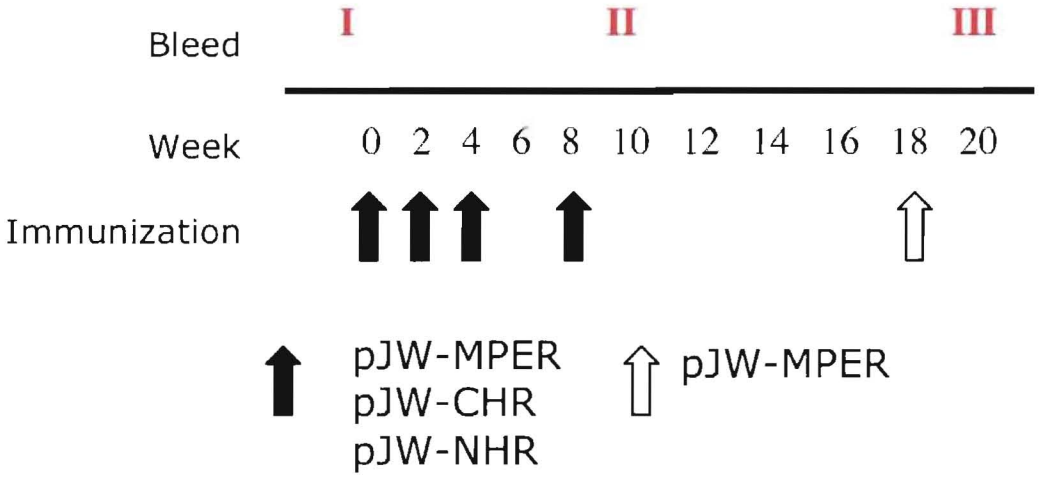
31463), goat anti-mouse IgG (Fc)-HRP (Cat. No. 31439) and Protein A/G-HRP (Cat. No. 32490) were purchased from Pierce (Nepean, ON, CA). Dynabeads® MyOneTM Streptavidin C1 (Invitrogen, Burlington, ON, CA Cat No. 650.01) were used for serum antibody (Ab) affinity purification.

3.3. Methods

3.3.1. DNA immunizations

The DNA immunizations were performed by Shan Lu and Shixia Wang (Laboratory of Nucleic Acid Vaccines, Department of Medicine, University of Massachusetts Medical School, Worcester). New Zealand White female rabbits were immunized with plasmid DNA. Briefly, plasmid DNA was absorbed onto 1.0 μm gold beads at 2 μg DNA/mg. Each animal received 36 non-overlapping shots (1 μg of DNA/shot) at each immunization at the surface of shaved abdominal skin using a Bio-Rad Helios gene gun. Each of the DNA plasmids (pJW-MPER, pJW-CHR, pJW-NHR and pJW4303 (mock)) was used to immunize two rabbits, as shown in **Figure 3-1A**. Rabbits were given in total five DNA immunizations at weeks 0, 2, 4, 8 and 18. **Figure 3-1B** shows that the first four immunizations were done with the DNA samples mentioned above, whereas the final boosting immunization (week 18) was done using the DNA plasmid pJW-MPER, with the exception of the control animals immunized with the mock vector pJW4303, which received the same mock vector. Serum samples were collected prior to the first immunization, and two weeks after the fourth and fifth gene-gun immunizations **Figure 3-1**

A



B

Rabbits	Plasmids used	
	Immunization (1-4)	**Immunization 5**
R1, R2	pJW-MPER	pJW-MPER
R3, R4	pJW-CHR	pJW-MPER
R5, R6	pJW-NHR	pJW-MPER
R7, R8	pJW4303	pJW4303

Figure 3-1. Schedule for DNA immunizations. Eight New Zealand white rabbits were immunized via gene gun. The rabbits received 36 μg of DNA per dose.

3.3.2. Titration on gp41 protein ELISA

For titration ELISA experiments, 96-wells plates Easy Wash; (COSTAR, Corning Incorporte, NY, USA, Cat. no. 3369) were coated with 50 ng/well of recombinant gp41 (Cedarlane) and incubated overnight at 4°C. Other Ags, including BSA, nonfat dry milk and Ova, were used as negative controls. After blocking for 2 h at RT with 2% w/v BSA in TBS, plates were washed three times with TBS containing 0.1 % Tween-20; TBS/Tw. Serial dilutions of sera or Abs on TBS/Tw containing 2.5 % DM (blotto), 0.1 % Tw20 were added to each well (final volume 35 µl). The plates were then incubated with the sera or Ab for 2 h at RT. After six washes with TBS/Tw, wells were incubated with the appropriate secondary Ab or Prot-A/G conjugated to HRP. Plates were washed six times, and the bound HRP was detected by addition of ABTS solution (400 µg/ml 2'2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) in citrate/phosphate buffer containing 0.03 % (v/v) H_2O_2). Absorbance at 405 nm and 490 nm was measured using a Versamax microplate reader (Molecular Devices, Sunnyvale, CA) and reported as $OD_{(405-490)}$.

3.3.3. Direct peptide mapping ELISA

Peptides were coated onto microwells at 400 ng per well in TBS, and dried at 37 °C for 4 h. Next, other wells were coated with the negative (2% BSA) and positive (gp41, 50 ng in 35µl per well) controls. The plates were incubated at 4 °C overnight, then blocked with 2 % BSA in TBS for 1h at 37 °C. After three washes, serial dilutions of rabbit sera or Ab controls were added. The plates were incubated for 2 h at RT, and then washed six times with TBS/Tw. HRP conjugated to goat-anti-rabbit IgG (diluted 1:1000) was added to the plates and incubated for 45 minutes at RT. After six washes with

TBS/Tw, bound HRP was detected by addition of ABTS.

3.3.4. Competition ELISA

For Ab competition ELISA, rabbit serum was added as competitor, and MAbs 4E10 were used as the detection Abs. Cell lysate was prepared after transient transfection of COS-7 cells with the plasmid pMPER-TM1 (see Chapter 2, Section 2.3.4). The plates were coated with the cell lysate as described in Chapter 2, Section 2.3.4). The lysate from COS-7 cells transfected with the mock vector was used as negative control. Others antigens (Ags) such as gp41 and BSA were used as controls. The plates were incubated overnight at 4 °C and then washed three times with TBS/Tw. Diluted rabbit sera were mixed with two different concentrations of the competitor 4E10 IgG (2.5 nM and 5 nM) or 4E10 Fab (25 nM and 50 nM). Control 4E10 Ab alone and rabbit sera alone were also included. After 2 h incubation at RT, plates were washed and bound human MAb (2F5 or 4E10) was detected using goat anti-human IgG-HRP conjugate. The concentrations of the 4E10 Ab used for the experiment were defined based in previous titration experiments of 4E10 in pMPER-TM1 lysate. The bound rabbit Ab was detected using goat anti-rabbit IgG-HRP.

3.3.5. Affinity purification of Ab

Peptide-specific Abs were purified from rabbit sera following the procedure of Wang (258). Five hundred µl SA-coated magnetic beads (1 µM) were added to a 1.5 ml Eppendorf tube, and pelleted by centrifugation for 5 min at 5000 rpm, and the supernatant (SN) was aspirated. Beads were washed once in 500 µl PBS, and then 500 µl PBS

solution containing 40 μg 2F5 peptide was added to beads and incubated for 1 h at RT on a rocker. Beads were pelleted and place in a magnet apparatus (MagnaRak, Invitrogen, Cat. no. CS15000). Subsequently, 100 μl 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 pelleted, and the SN was aspirated. After a wash in PBS, beads were resuspended in 500 μl PBS and stored t at 4°C. For affinity purification of peptide-specific serum IgG, the SA-coated magnetic beads were first equilibrated to RT, washed and then incubated in 200 μl serum, and incubated for 1 h at RT. The beads were then pelleted and the SN (unbound serum, or flow through; FT) was transfered to a fresh tube. The beads were then washed twice and bound Ab was eluted by addition of 500 μl glycine-HCl buffer (100 mM glycine, pH 2.6) followed by incubation for 20 min at RT. Beads were pelleted and the SN (containing peptide specific Ab) was transferred to a tube containing 150 μl 1M Tris HCl pH 7.5 to neutralize the pH, also 9 μl of FBS was added to purified Ab. The pH-neutralized Ab fraction was washed three times with 2 ml of PBS using a Microcon centrifugal filter YM-10 (Cat. no. 42407, Millipore, Billerica, MA, USA), and concentrated to a small volume.

3.3.6. Neutralization assays

Neutralization assays were performed by Monogram Bioscience (San Fransisco, CA), using a single-round infection with Env-pseudotyped virus as described in (201). Briefly, pseudotyped virus particles were produced by co-transfection of a cell line with an Env negative HIV genomic vector, carrying the reporter gene of firefly luciferase and a plasmid carrying the HIV envelope gene. Virus particles contain Env in the surface and

are infective. Cell infection by the viruses can be quantified using a luciferase assay. Pseudovirions carrying the Env from different HIV isolates are incubated for 1 h with serial 4-fold dilutions of sera or Ab solutions before infection of U87-derived target cells that express CD4 plus the CCR5 and CXCR4 co-receptors. HIV-1 pseudovirons carrying Env from the MN, SF162, 92HT594, JRCSF and NL43 were used in this study. Virions pseudotyped with Env from amphotropic murine leukemia virus (aMLV) were used as negative control. The HIV-neutralizing sera Z23, was used as a positive control. Sera from rabbits after the 5^{th} DNA immunizations, as well as affinity-purified rabbit IgG specific for 2F5 peptide, were tested for neutralization activity. Nt activity was defined as the percent inhibition of viral infection (luciferase activity) at each Ab dilution, compared with an Ab-negative control. Titers were calculated as the reciprocal of the serum dilution conferring 50 % inhibition (IC_{50}). Thus, the IC_{50} corresponds to the concentration of the Ab or dilution of the sera that inhibited 50 % of virus infection.

3.4. Results

3.4.1. The pJW-MPER, pJW-CHR and pJW-NHR plasmids are immunogenic in rabbits

The immunogenicity of the MPER constructs (pJW-MPER, pJW-CHR and pJW-NHR, **Figure 2-2**) was evaluated by DNA immunization in rabbits. The immunization and bleed schedules are shown in **Figure 3-1**. Eight New Zealand rabbits were DNA immunized by gene gun administration; two rabbits were immunized with each of the plasmids tested, pJW-MPER, pJW-CHR, pJW-NHR, and pJW4303 (mock vector). An

Ab response against an Antigen (Ag) is usually mounted seven days after the Ag administration; however, the type of Ab response, and its magnitude are related to the nature of the Ag used; with proteins typically being more immunogenic than carbohydrates. In the case of DNA immunization with plasmid DNA (DNA vaccines), the DNA is taken up by host cells and the desired Ag is produced by the host cells *in vivo*, in comparison with immunization with proteins, the Ab response to a protein Ag after DNA immunization takes a longer period to be elicited and requires several rounds of immunization (Dr. Shan Lu, personal communication). Accordingly, we tested the elicited Ab response after immunizations with the DNA constructs only after the rabbits received four doses of DNA.

Sera from HIV-1 infected patients as well as MAbs including bNt Ab 2F5 and 4E10 bind to the recombinant gp41 ectodomain in ELISA. Thus, cross-reactivity with gp41 is an indication that the Ab response generated in these DNA immunizations, behaves similarly to the Abs elicited during natural infection. We performed serum titration ELISA experiment on recombinant gp41. The ELISA results summarized in **Table 3-1** show that sera R1 to R8 react with gp41, the data presented in **Table 3-1** correspond to end point Abs titers on gp41. We choose end point titers instead of half max titers, since they are more commonly used in the published literature and the titration curves for the different serum had very similar shape. The end point titers were calculated based on the reactivity of the sera to the negative control Ag, BSA, as the serum dilution producing an ELISA signal twice the value of the BSA. Since Ab reactivity to gp41 was present in the pre-bleed sera, the gp41 titers elicited by immunization were calculated as the differences in end point titers between the bleed III (after the four immunization) and bleed I (the

pre-bleed).

Reactivity against gp41 was observed for all the sera in the bleed I (prebleed) before the first immunization, the highest reactivity being observed for serum R2. Interestingly, the R2 serum did not show a gp41 cross-reactive Ab response after the fourth round of DNA immunization. The highest gp41 reactive titers were induced in rabbits (R5 and R6), which were immunized with the pJW-NHR plasmid, with an endpoint titer of 250,000. This pJW-NHR encodes for the largest gp41 fragment, thus its larger immunogenic region probably induced stronger Ab response. Marked differences in immune response were also observed for rabbits R3 and R4 immunized with the pJW-CHR plasmid, with R4 showing almost twice the level of gp41 Ab reactivity of R3. Thus, the proteins produced by pJW-MPER, pJW-CHR and pJW-NHR are immunogenic, and induce a gp41-crossreactive Ab response in rabbits.

Table 3-1. Anti gp41 response in rabbits immunized with pJW-MPER, pJW-CHR and pJW-NHR DNA constructs.

		Serum end point titers[1]		
Rabbit serum	Plasmid	Bleed I[2]	Bleed II[3]	Bleed II-Bleed I[4]
R1 serum	pJW-MPER	4,800	20,000	15,200
R2 serum	pJW-MPER	10,000	10,000	0
R3 serum	pJW-CHR	4,800	10,000	5,200
R4 serum	pJW-CHR	4,800	20,000	15,200
R5 serum	pJW-NHR	4,800	300,000	295,200
R6 serum	pJW-NHR	4,800	300,000	295,200
R7 serum	pJW4303	4,800	4,800	0
R8 serum	pJW4303	4,800	4,800	0

[1]Titers presented here are the end point titers (defined as reciprocal of the dilution with values twice over the background values (i.e. binding of the sera to the negative control BSA)
[2]value of the pre-bleed sera titers (at week 0).
[3]Value of the Bleed II titers after the 4th DNA immunization (at week 10)
[4] Value of the Bleed II titers minus the pre-bleed titers (I)

3.4.2. DNA immunized rabbits elicited an MPER specific Ab response

We evaluated the sera from immunized animals for the presence of Abs that react with the protein expressed by each of the plasmids *in vitro*. Cell lysates were prepared after transfection of COS cells with each of the DNA constructs as well as the mock vector. The reactivity of the sera from bleeds after the 4th DNA immunization (bleed II) is shown in the **Table 3-2**. Sera from rabbits immunized with plasmids that encode gp41 fragments (R1 to R6) reacted with the cell lysates prepared using transfected COS cells with pJW-MPER, pJW-CHR and pJW-NHR, but not with the mock vector. However, sera R7 and R8 from rabbits immunized with the mock vector did not bind to the lysate containing gp41 fragments. The sera from rabbits R1 to R6 bind to the lysate generated with the pJW-MPER, suggesting that an MPER specific reactivity was generated; however, that conclusion was confounded by the fact that this assay also detected Abs against the highly immunogenic HA-tag at the N-terminus of all of the proteins. In addition, a peptide corresponding to the sequence of the PDGFR-TM, was not available, thus, we could not assess whether cross-reactive Abs were also generated to this region.

Since the goal was evaluate serum reactivity to the MPER, yet all the constructs had the HA-tag at the N-terminus and the PDGFR-TM, we decided to test the sera reactivity with a cell lysate produced from pMPER-TM1-transfected cells. pMPER-TM1 (**Figure 2-8**) produces a protein with the same MPER fragment as the pJW-MPER, but with no HA-tag. It also carries a different TM region (the native gp41 TM region), thus any cross-reaction would be based on the MPER alone. Cell lysates were prepared using pMPER-TM1 and pJW4303 (mock) and used in an ELISA. **Figures 3-2A**, **B**, and **C** show that sera from rabbits R1 to R6 have reactivity with MPER, however, no similar

Table 3-2. Rabbit sera reactivity against MPER proteins expressed by the MPER DNA constructs. Sera were obtained after the fourth immunization (bleed II).

Rabbit sera/MAb	pJW-MPER*	pJW-CHR	pJW-NHR	pJW4303
R1 (pJW-MPER)	**1.25**	1.36	0.97	0.26
R2 (pJW-MPER)	**1.05**	1.36	0.70	0.14
R3 (pJW-CHR)	0.68	**1.37**	0.90	0.24
R4 (pJW-CHR)	1.08	**1.49**	1.36	0.25
R5 (pJW-NHR)	0.66	1.26	**1.38**	0.70
R6 (pJW-NHR)	0.66	1.49	**1.40**	0.18
R7 (pJW4303)	0.13	0.10	0.11	0.16
R8 (pJW4303)	0.11	0.09	0.11	0.12
2F5 IgG (10nM)	0.83	0.80	0.59	0.06

* Whole cell lysates were prepared from COS-7 cells transfected with plasmids, and were and used as Ag for ELISA. The sera were used at 1/ 500 dilution. The data presented is $OD_{(405-490)}$. Highlighted values are those of the serum reactivity to the lysate produced with the same plasmid used to immunize those animals.

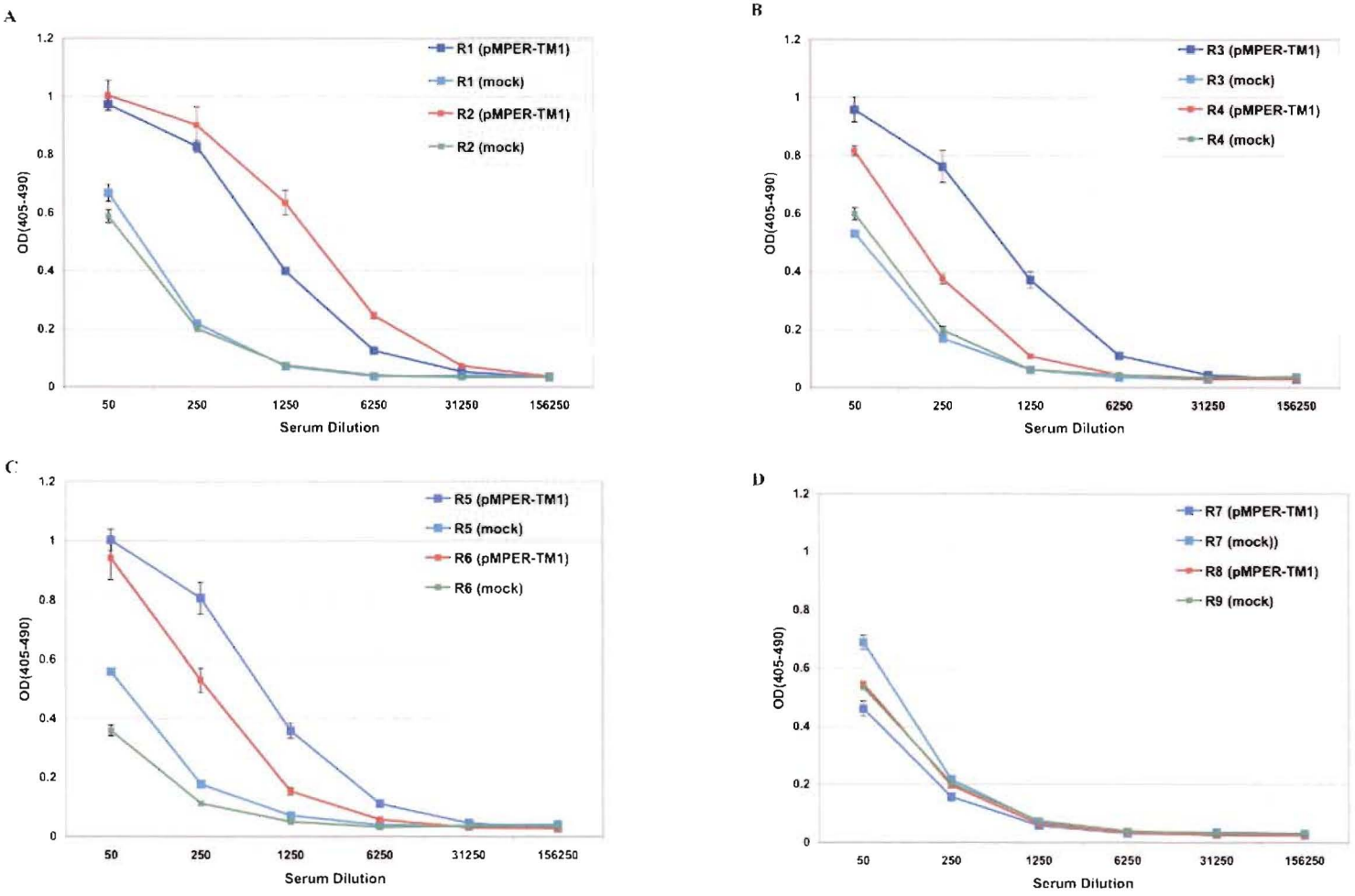


Figure 3-2. Sera from bled II binding to MPER-TM1 protein in cell lysates ELISA. Sera were used 1/500 dilution. (A) Sera R1and R2; (B) Sera R2 and R3; (C) Sera R5and R6 and (D) Sera R7and R8. The pJW4303 (mock) was included..

sera reactivity was observed for sera from rabbits R7 and R8 (**Figure 3-2D**). All the rabbits sera have similar level of background binding to COS cells transfected with mock vector (negative control). These results show that MPER-specific response was elicited after DNA immunization with DNA constructs pJW-MPER, pJW-CHR and pJW-NHR. In general, the response against gp41 and MPER did not correlate very well, for example, drastic differences in reactivity to gp41 were noticed in ELISA for rabbits R1 and R2 (**Table 3-2**), but no such differences were observed for the MPER reactivity in cell lysate ELISA (**Figure 3-2**), indicating that the two assays can assess different types of Ab response.

3.4.3. DNA boosting can focus the Ab response against the MPER

The Ab response elicited after four DNA immunizations has a specific reactivity with the MPER. To determine if there was an increase in reactivity against the MPER after the fifth immunization with pJW-MPER, we compared the reactivity of the sera after the fourth and fifth immunizations, using a cell-lysate ELISA. To truly assess MPER reactivity, cell lysates produced by the DNA construct pMPER-TM1 were used. As shown in **Figure 3-3** there was no increase in the reactivity to the MPER Abs for rabbit R1, and only a small increase in MPER reactivity was observed for rabbit serum R2, suggesting that Ab levels reached after four immunizations were probably the maximum that can be obtained with this type of immunization procedure. In contrast with these results differences between the two sera were observed for their reactivity with recombinant gp41gp41. Interestingly, this assay also showed that the R2 prebleed serum has little reactivity with the MPER; thus, reactivity of R2 serum with gp41 shown in

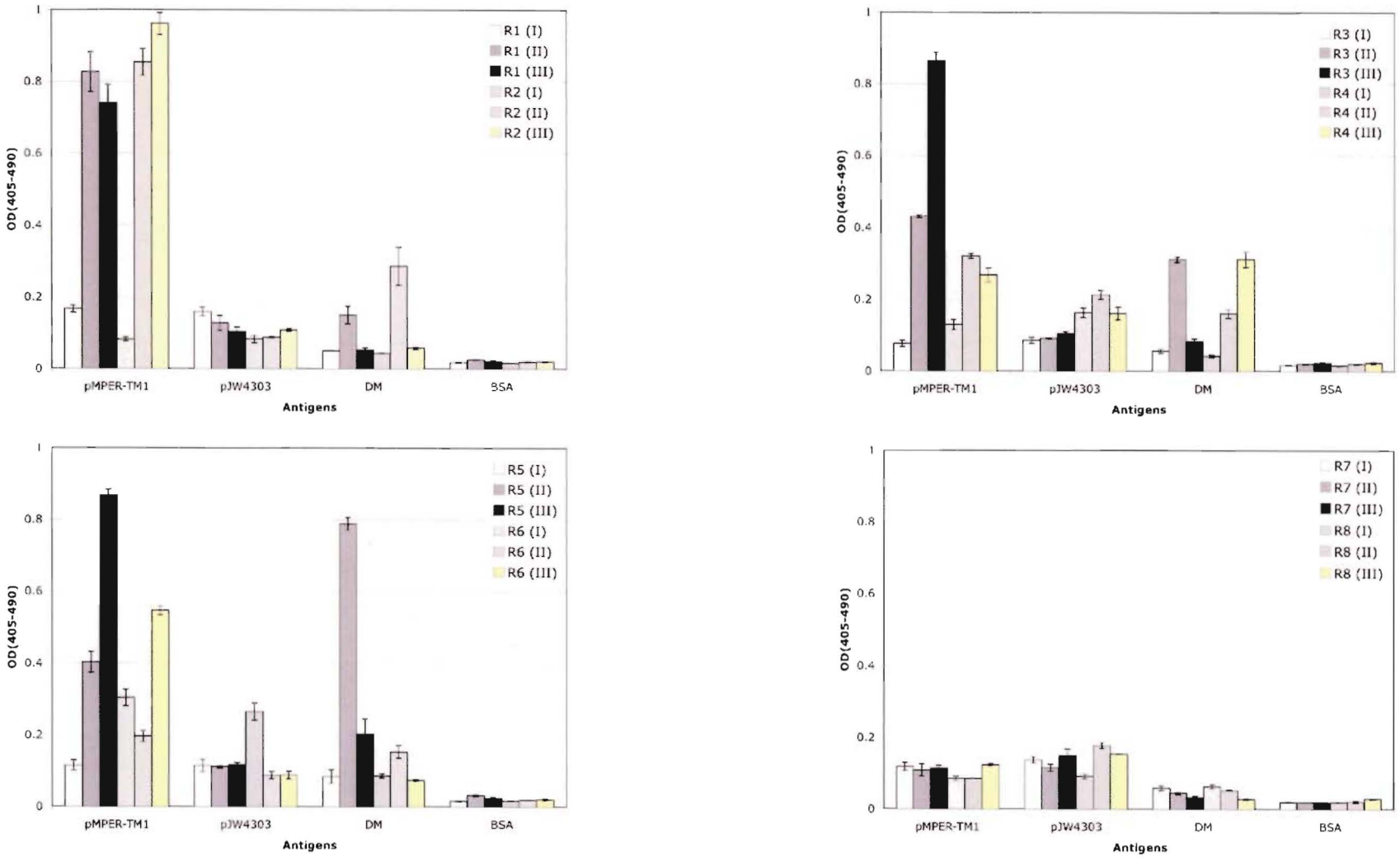


Figure 3-3. A comparison of the binding of rabbit sera after four (II) and fifth DNA immunizations (III), in cell lysate ELISA. Cell lysate was prepared using pMPER-TM1. Sera were used at a 1/500 dilution. The pre-bleed (I) was also included for reference

Table 3-2 is probably non-specific. Binding of the sera R3 increased after the fifth immunization, however, such an increased was not observed for the R4 serum, which showed higher than R3 serum titers to the gp41 (see **Table 3-2**). Rabbits R5 and R6 increased their reactivity to the MPER after the fifth immunization (**Figure 3-3**). The reactivity of rabbits R7 and R8 sera with the MPER was also included as a control for this experiment. None of the bleeds from rabbits R7 and R8 showed significant binding to the MPER. Thus, four out of six rabbits increased their Ab response against the MPER after a fifth immunization with pJW-MPER, indicating that the anti-MPER Ab response was refocused after this immunization.

3.4.4. Peptide mapping of immunized rabbit sera reveals reactivity with the 2F5 epitope

The rabbit immunizations with the pJW constructs elicited serum Ab response, which cross-reacted with gp41 and more importantly with the MPER. However, to further characterize the nature of the Ab response elicited after the fourth (bleed II), and fifth (bleed III) DNA vaccinations, peptide mapping ELISAs were performed on the rabbits sera using the NIH Reagent Bank set of overlapping peptides, covering the gp41 protein from HIV-1_{MN} isolate, as well as the HA-tag synthetic peptide. The non-related peptide B2.1 was also added as a negative control for the assay. **Table 3-3** shows the amino acid sequence of the gp41 peptides used for the mapping experiments.

Table 3-3. Overlapping HIV-1_{MN} gp41 peptides.

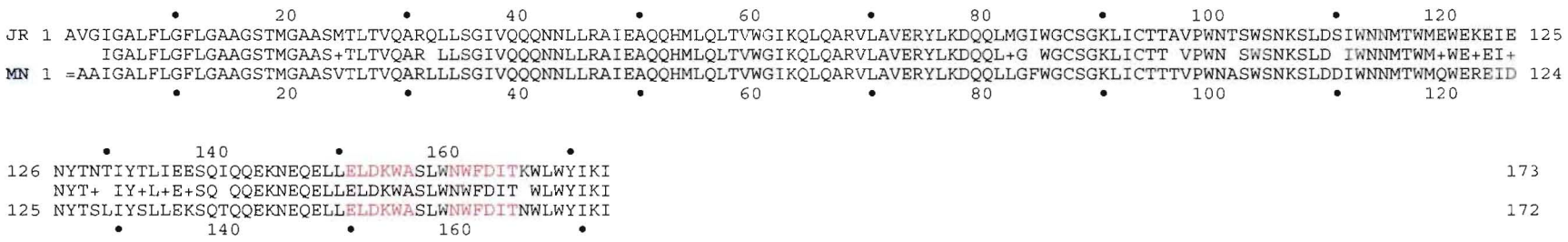
Cat.No.	Peptide sequence	Amino acid number[a]	Cat. No.	Peptide sequence	Amino acid number
6342	AASVTLTVQARLLLS	533-547	6362	ASWSNKSLDDIWNNM	613-627
6343	TLTVQARLLLSGIVQ	537-551	6363	NKSLDDIWNNMTWMQ	617-621
6344	QARLLLSGIVQQQNN	541-555	6364	DDIWNNMTWMQWERE	621-635
6345	LLSGIVQQQNNLLRA	545-559	6365	NNMTWMQWEREIDNY	625-639
6346	IVQQQNNLLRAIEAQ	549-563	6366	WMQWEREIDNYTSLI	629-643
6347	QNNLLRAIEAQQHML	553-567	6367	EREIDNYTSLIYSLL	633-647
6348	LRAIEAQQHMLQLTV	557-571	6368	DNYTSLIYSLLEKSQ	637-651
6349	EAQQHMLQLTVWGIK	561-575	6369	SLIYSLLEKSQTQQE	641-655
6350	HMLQLTVWGIKQLQA	565-589	6370	SLLEKSQTQQEKNEQ	645-659
6351	LTVWGIKQLQARVLA	569-583	6371	KSQTQQEKNEQELLE	649-663
6352	GIKQLQARVLAVERY	573-587	6372	QQEKNEQELLELDKW	653-667
6353	LQARVLAVERYLKDQ	577-591	6373	NEQELLELDKWASLW	657-671
6354	VLAVERYLKDQQLLG	581-595	6374	LLELDKWASLWNWFD	661-675
6355	ERYLKDQQLLGFWGC	585-599	6375	DKWASLWNWFDITNW	665-679
6356	KDQQLLGFWGCSGKL	589-603	6376	SLWNWFDITNWLWYI	669-683
6357	LLGFWGCSGKLICTT	593-607	6377	WFDITNWLWYIKIFI	673-687
6358	WGCSGKLICTTTVPW	597-611	6378	TNWLWYIKIFIMIVG	677-691
6359	GKLICTTTVPWNASW	601-615	6379	WYIKIFIMIVGGLVG	681-693
6360	CTTTVPWNASWSNKS	605-619	6380	IFIMIVGGLVGLRIV	685-697
6361	VPWNASWSNKSLDDI	609-623			

These peptides are part of the peptide set Cat no. 6451, lot no. 12, from the NIH Reagent Bank.
[a] Numbering according to the HBX2 isolate.
The most frequently detected peptides in our sera mapping study are highlighted in blue.

Alignment between the HIV-1 gp41 proteins from isolates JR-CSF and MN is presented in **Figure 3-4** to show differences between the two isolates, which might have impact on the serum reactivity. The major difference in sequence between the two isolates is found in the 2F5 epitope N-terminal flanking region.

The peptide epitope mapping data from bleed II are shown in **Figures 3-5** to **3-7** and for bleed III in **Figures 3-8** to **3-10**. The bNt 2F5 IgG binds to three overlapping peptides (6372, 6373 and 6374), but the stronger interaction is observed for peptide 6373 in which the core 2F5 epitope ELDKWA is located in the middle of the peptide sequence. The sera binding to peptides corresponding to 2F5 epitope peptide is summarized in **Table 3-4**. Two values for each serum correspond to the OD values obtained for bleed II and Bleed III respectively; the following representation of the OD values was adopted: < 0.1 (-), > 0.1< 0.2 (+/-), > 0.2 <0.4 (+), > 0.4 < 0.8 (+ +) and > 0.8 (+ + +). All the sera showed some reactivity with the 2F5 epitope peptides after four DNA immunizations (bleed II). However, after the fifth immunization a general drop in the reactivity for the 2F5 epitope peptides was observed for all the sera.

The general data from the peptide mapping ELISA are presented as histograms for each individual serum tested, after the fourth and fifth immunizations (**Figures 3-5** to **3-10**). **Table 3-5** presents a summary of the most important results for an easy comparison; peptides to which the sera bound with a signal higher than 2-fold over the unrelated B2.1 peptide were considered as positives, and included in the table. First of all, a consistent response against the MPER was observed after the fourth immunization (**Table 3-5A**), regardless of the immunogen used. This response is concentrated against peptides 6372,



% Identity = 88.4 (153/173) % Homology = 5.2 (9/173) % Total = 93.6 (162/173)

Figure 3-4. Alignment between gp41 from HIV-1 isolates MN and JR-CSF. Alignment was done using DNA Strider. The epitopes for MAbs 2F5 and 4E10 are in red.

Table 3-4. Summary of sera reactivity with 2F5 epitope.

		R1 (pJW-MPER)		R2 (pJW-MPER)		R3 (pJW-CHR)		R4 (pJW-CHR)		R5 (pJW-NHR)		R6 (pJW-NHR)	
Peptide epitope	**Peptides #**	**II**	**III**	**II**	**III**	**II**	**III**	**II**	**III**	**II**	**III**	**II**	**III**
2F5	6372	+ *	++	+/-	-	+/-	+/-	+/-	+/-	++	++	-	+
2F5	6373	+++	++	+	-	+	+/-	++	+/-	+++	-	+/-	+
2F5	6374	+	-	+/-	-	+/-	-	+/-	-	+	-	-	+

* The sera reactivity with peptides is determined by the optical density value (OD) determined by ELISA
OD values > 0.1 < 0.2 are represented as (+/-)
OD values > 0.2 < 0.4 are represented as (+)
OD values > 0.4 < 0.8 are represented as (+ +)
OD values > 0.8 are represented as (+++)

Table 3-5A. Peptides positive for reactivity with rabbit sera, after the fourth immunization (bleed II) with MPER constructs.

Rabbit	NHR	CI	CHR	MPER
R1	na	na	na	6372, 6373*, 6374, **6378**
R2	na	na	na	6373, **6378**
R3	na	na	6362, 6364, 6366	6373*, **6378**
R4	na	na	6362*, 6363, 6364*, 6365*	6372, 6373*, 6374, **6378**
R5	6345, 6347, **6349***, 6351, 6352, 6355	**6357, 6358**	6365, 6371*	6372*, 6374, 6376*, **6378***
R6	**6349**, 6350*	**6357, 6358**	no reactivity	6372, **6378**

NHR (N-heptad repeat) was considered to be covered by peptides 6342-6354, CI (cluster I) by peptides 6356 to 6359, CHR (C-heptad repeat) by peptides 6360 to 6371 and MPER (membrane-proximal external region) by peptides 6372 to 6378.
na indicates not applicable, these are regions that were not present in the corresponding immunogen, therefore the response against the region was not evaluated.
* Denotes peptides with strong reactivity, at least 4 times over unrelated B2.1 (see Figures 3-5 to 3-7).
Peptides in bold face are of frequent reactivity.

Table 3-5B. Peptides positive for reactivity with rabbit sera, after the fifth immunization (bleed III) with MPER constructs.

Rabbit	NHR	CI	CHR	MPER
R1	na	na	na	6372, 6373
R2	na	na	na	no reactivity
R3	na	na		6372, 6378
R4	na	na	6360, 6362, 6363, 6365, 6366	6372, 6373
R5	**6349**	6359	no reactivity	6372
R6	**6349**, 6350	no reactivity	no reactivity	6370, 6372, 6373

Peptides in bold face are of frequent reactivity.

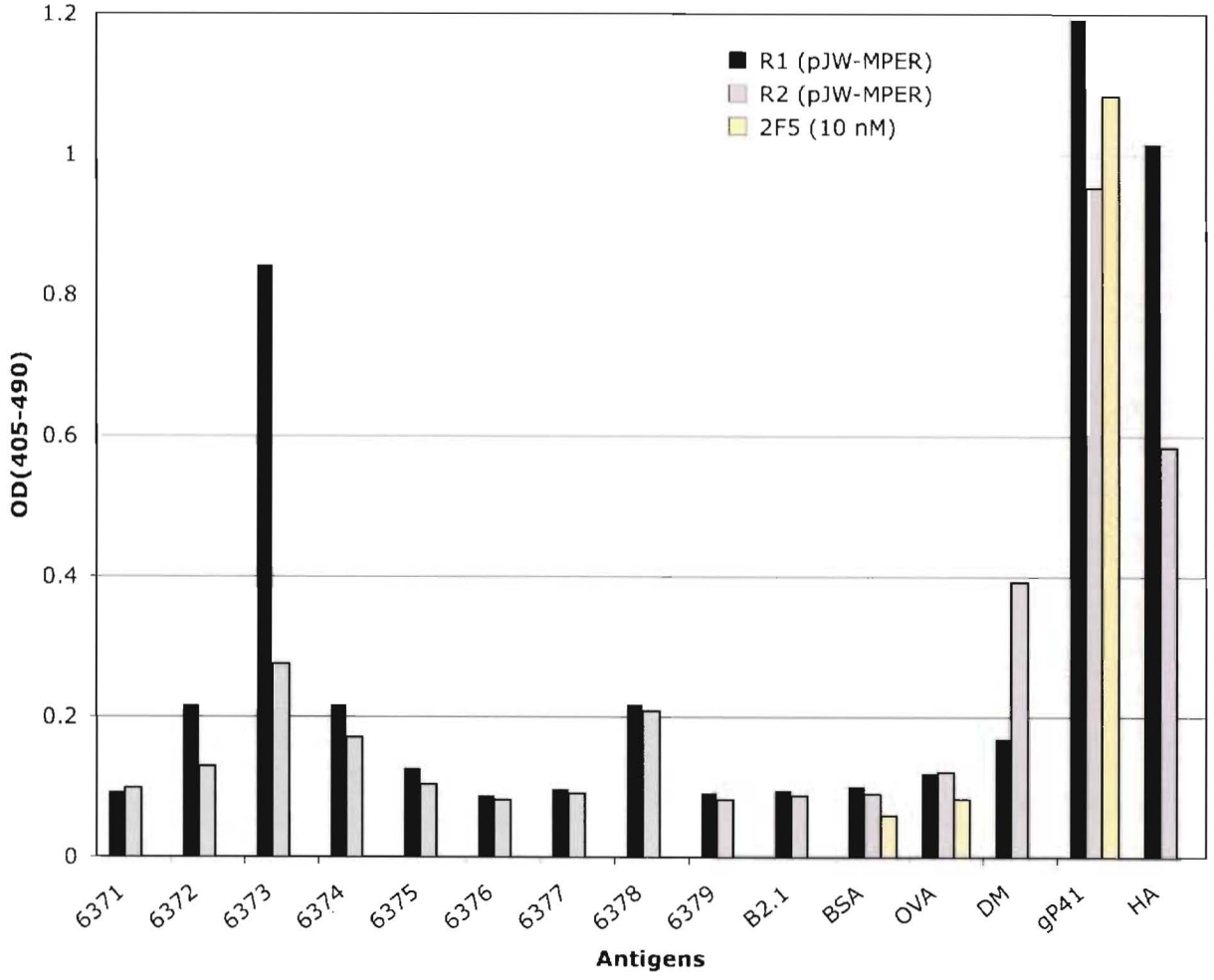


Figure 3-5. Sera R1 and R2 peptide mapping (bleed II). The peptides were directly captured on ELISA plates and the sera were used at 1/600 dilution. Bound rabbit IgG was detected using goat anti-rabbit IgG HRP-conjugate.

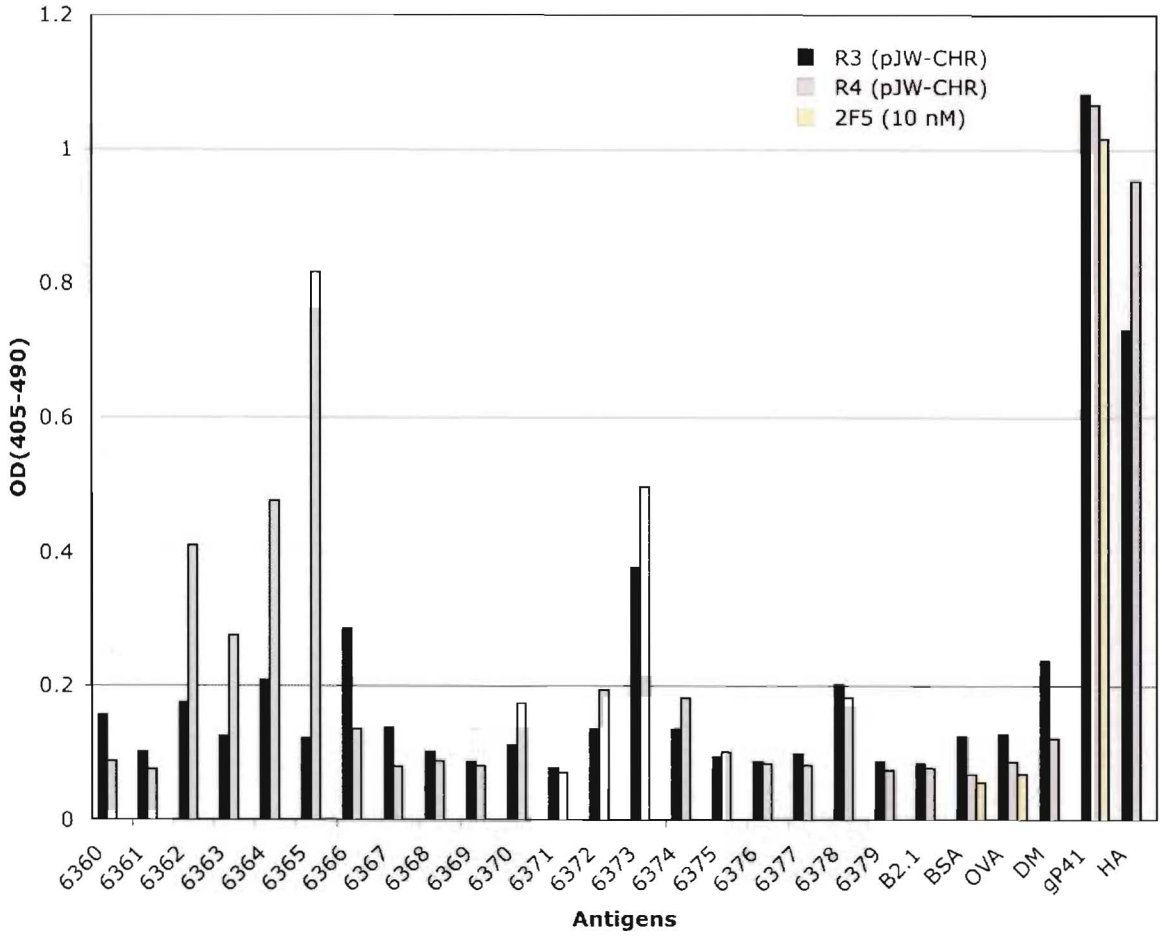


Figure 3-6. Sera R3 and R4 peptide mapping (bleed II). The peptides were directly captured on ELISA plates and the sera were used at 1/600 dilution. Bound rabbit IgG was detected using goat anti-rabbit IgG HRP-conjugate.

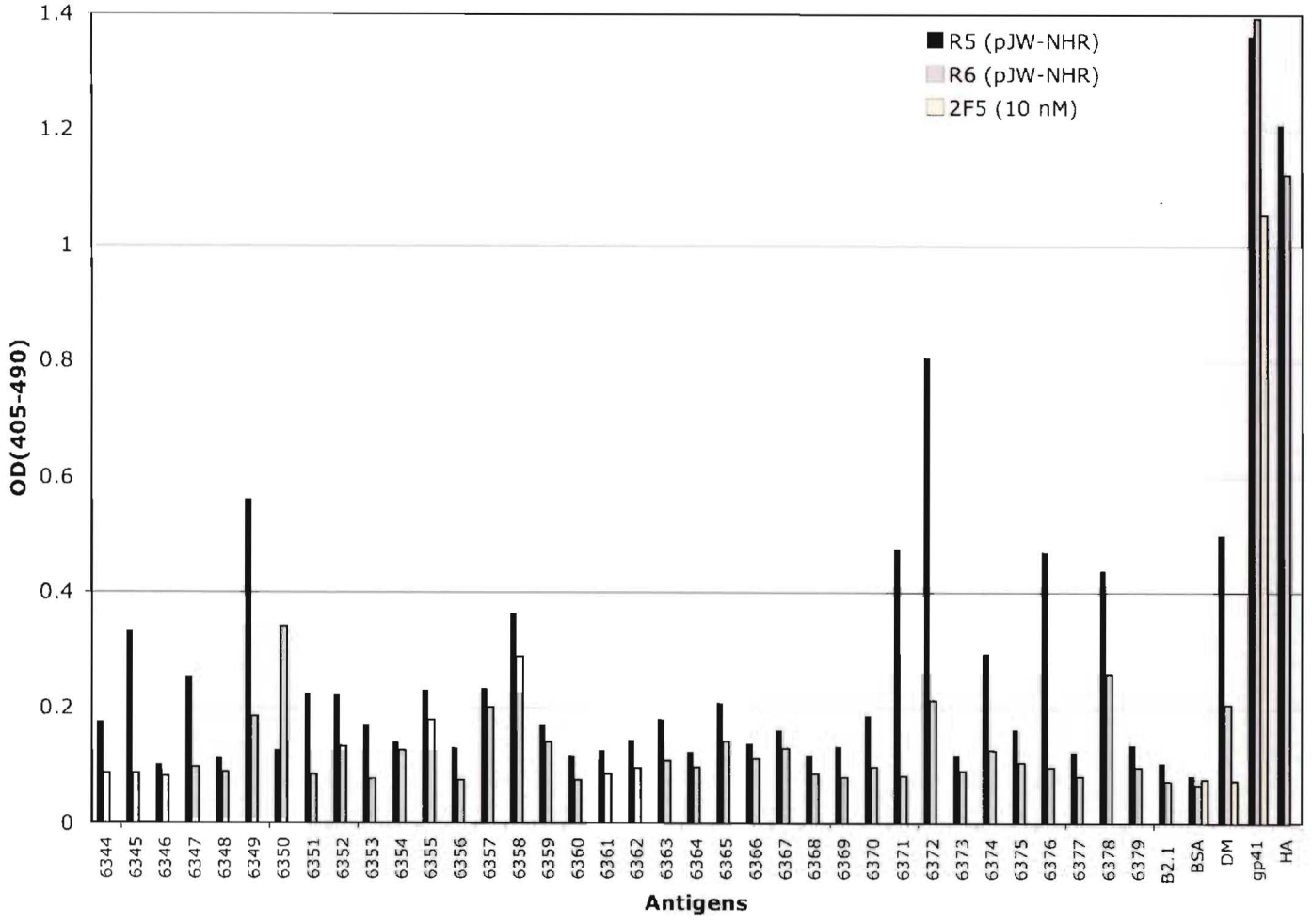


Figure 3-7. Sera R5 and R6 peptide mapping (bleed II). The peptides were directly captured on ELISA plates and the sera were used at 1/600 dilution. Bound rabbit IgG was detected using goat anti-rabbit IgG HRP-conjugate.

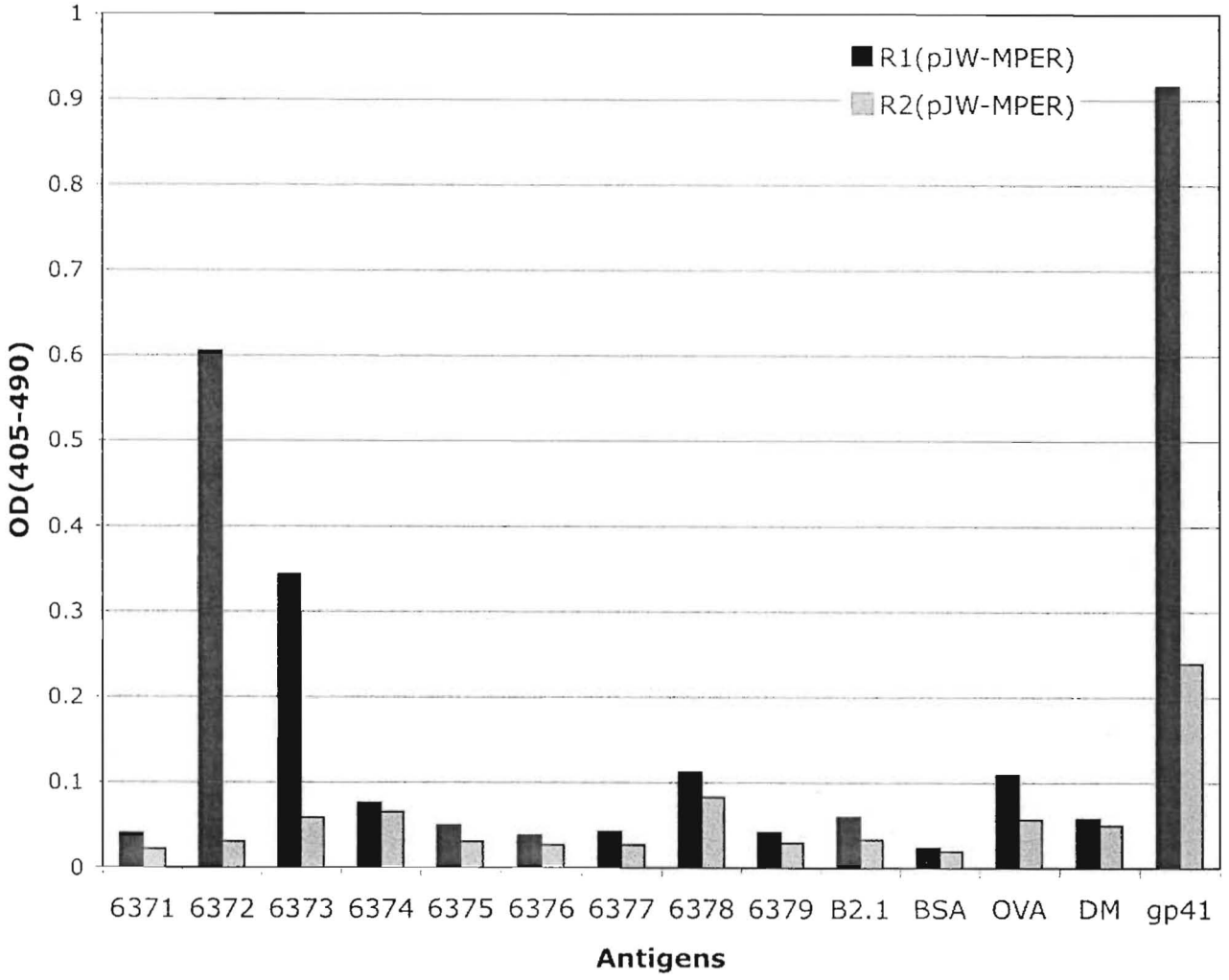


Figure 3-8. Sera R1 and R2 peptide mapping after the fifth DNA immunization (bleed III). The peptides were directly captured on ELISA plates and the sera were used at 1/600 dilution.

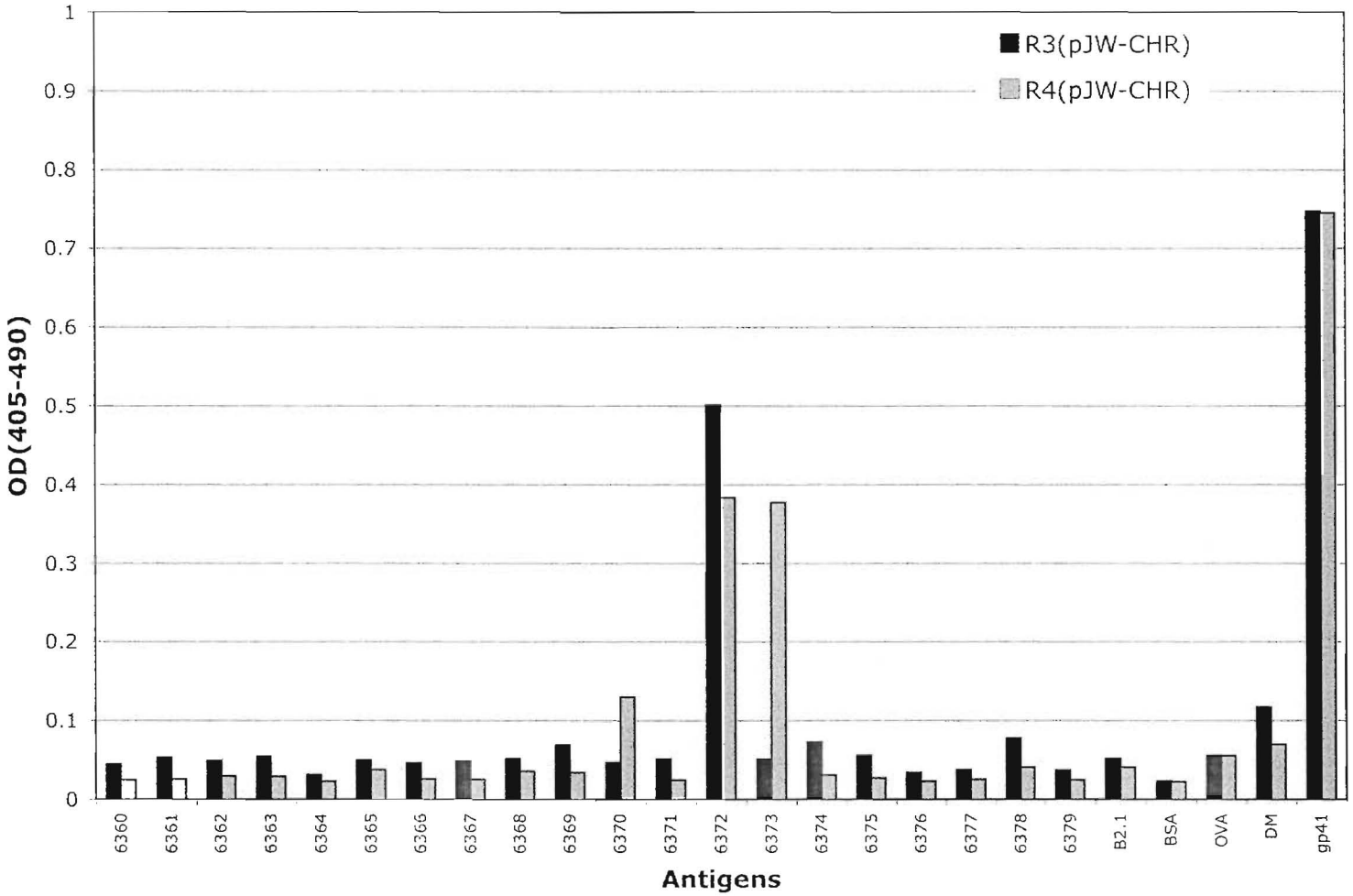


Figure 3-9. Sera R3 and R4 peptide mapping after the fifth DNA immunization (bleed III). The peptides were directly captured on ELISA plates and the sera were used at 1/600 dilution. Bound rabbit IgG was detected using goat anti-rabbit IgG HRP-conjugate.

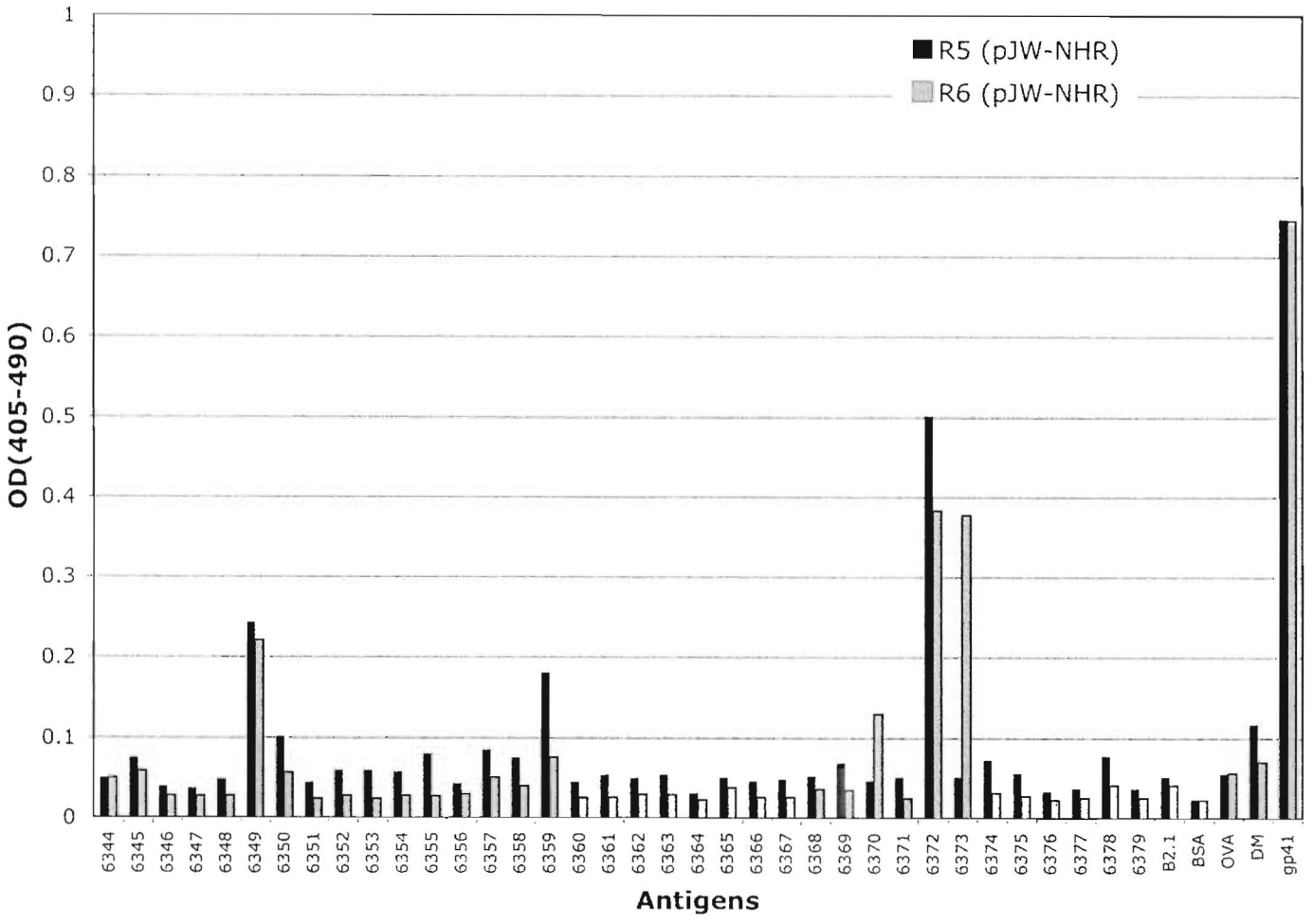


Figure 3-10. Sera R5 and R6 peptide mapping after the fifth DNA immunization (bleed III). The peptides were directly captured on ELISA plates and the sera were used at 1/600 dilution. Bound rabbit IgG was detected using goat anti-rabbit IgG HRP-conjugate.

6373 and 6374, which cover the sequence QQEKNEQELL**ELDKWA**SLWNWFD. This fragment contains the canonical epitope for MAb 2F5 (underlined). The peptide mapping also identified an apparently immunodominant site on the MPER contained in peptide 6378 (TNWLWYIKIFIMIVG), however the sequence covering this peptide included the residues, IFIMIVG which are part of gp41 TM, not included in the DNA constructs evaluated in this immunization. Thus, most likely the observed Ab reactivity is directed to the N-terminal half of the peptide (TNWLWYIK).

Some reactivity was observed with peptides from the CHR, mainly to the region covered by peptides 6362 to 6365 (ASWSNKSLDDIWNNMTWMQWERE), and to the NHR region around peptide 6349 (EAQQHMLQLTVWGIK). In general, low reactivity, confined to peptides 6357 and 6358 (LLGFWGCSGKLICTTTVPW) (corresponding to Cluster I region) was observed with rabbit sera R5 and R6 immunized with pJW-NHR plasmid, which coded for this region. This is interesting, given the immunodominant nature of the region in humans, especially peptide 6359 (GKLICTTTVPWNASW), which is recognized by most sera from HIV-1 positive donors (42, 86, 87). Low reactivity was also observed to the peptide 6353 which included the AVERY epitope. No binding to Z13/4E10 epitopes (peptides 6375 and 6376) was detected except some weak binding for R5 serum. All the sera bound to the HA-tag.

Reactivity after the fifth immunization (**Table 3-5B**) showed a different pattern. A general drop in the number of reactive peptides, as well as in the binding to the 2F5 epitope peptides, was observed for all the sera. This contrasts with the results observed in ELISA using cell lysates from MPER-expressing cells, which showed in general a better response against the MPER region. This discrepancy may indicate that the

boosting of the anti-MPER response achieved with the fifth immunization was skewed towards antibodies recognizing epitopes that are not present on the linear peptides used in the mapping (*i. e.*, conformational epitopes).

The peptide mapping assay was also performed using the HIV^+ broadly neutralizing sera FDA-2. This experiments was done to show that the low reactivity of the rabbits sera after the DNA immunizations observed in the peptide mapping assays presented above, were not due to the low amount of peptides available on the ELISA plate well. As shown in **Figure 3-11**, high reactivity with the peptides (6358 and 6359) corresponding to the cluster I region was observed. Interesting a high reactivity to the MPER peptides specifically to the peptides (6375 and 6376) was observed. These peptides correspond to the core epitope recognized by the bNt MAb Z13, which was selected from a Fab antibody library generate using immunoglobulin genes amplified form B- cells isolate from FDA-2 patient (32, 252). Interestingly, high reactivity with the peptide 6378 was observed for the FDA sera, indicating a very immunogenic region. Thus, we concluded that the low reactivity of the sera observed in our mapping assay is more likely related to low prevalence of Ab with specific peptide reactivity in the rabbit sera.

After analyzing the Ab response elicited by our DNA constructs in three different ELISA formats, using different kind of Ag (gp41, peptides, and cell lysates with MPER attached to the membrane), we found different patterns of reactivity of the sera, indicating that the assay used for the analyses of the Ab response can have a strong influence in the results. We observed a clear discordance between the data evaluated in the different formats, no clear reactivity with peptides was observed in the mapping

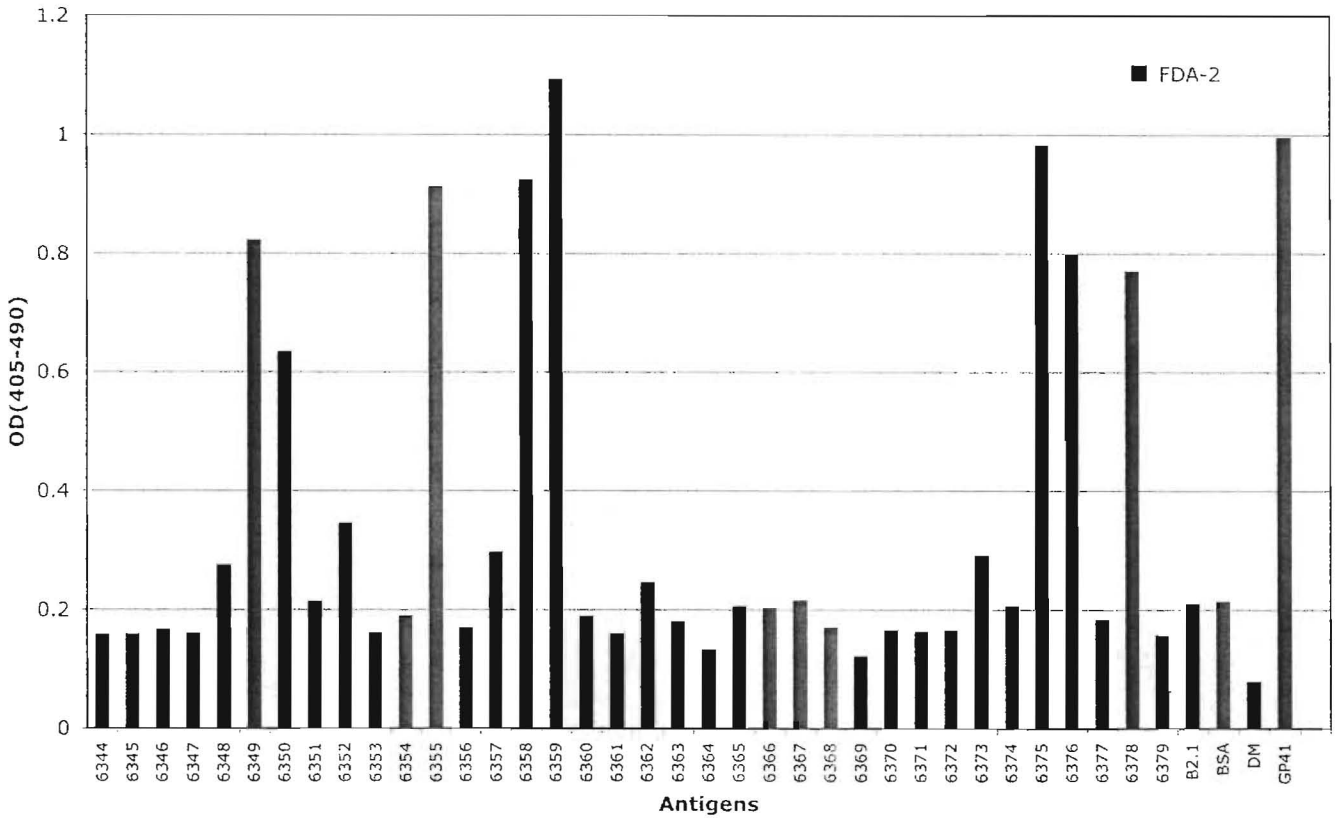


Figure 3-11. Peptide mapping of purified IgG from HIV^{+} donor FDA-2. The human IgG was used at 40 nM (6 μg/ml), bound human Abs were detected using goat anti-human IgG HRP conjugate.

experiment; however, those same sera showed reactivity to the MPER in cell lysate ELISA. While the peptide mapping assay can determine the prevalence of Ab that can bind to linear epitopes, the cell lysate indicate the presence of anti-MPER Abs that bind to longer fragments of the MPER, suggesting that an important fraction of the antibody response recognizes complex or discontinuous epitopes in the MPER, whose structure may be determined by membrane interaction.

3.4.5. Affinity purification of 2F5 epitope cross-reactive Ab from rabbit sera

The elucidation of the mechanism of interaction between Abs elicited after MPER immunization and their interactions with the MPER is one of the goals of this study. Since the proteins expressed by the DNA constructs used for immunizations are displayed on the cell surface, the ideal Ag for this type of study should be the same Ag also displayed on the cell plasma membrane. Thus, the cell lysate ELISA format on which the Ag generated *in vitro* after mammalian cell transfection with our constructs, is more likely to recreate the Ag produced *in vivo* after the DNA vaccination. To further map epitopes on the MPER in the context of cell membrane we used a set of alanine substituted MPER.

Direct ELISA using cell lysates as Ag in combination with gp41 and BSA was used for testing the reactivity pattern of the rabbits sera R1 to R6 with the MPER Ala mutants, however, no difference was observed in the reactivity pattern between the wild type (wt) and mutant MPER. This is probably due to the polyclonal nature of the response, *i.e.*, while a single mutation can probably affect the reactivity of a subset of Ab, there can still be enough of other reactivities in the pool that produce a strong binding signal and mask

the effect of the mutation. Thus, we affinity purified the fraction of Abs from the sera that bind to the 2F5 epitope using a 2F5 epitope synthetic peptide. The R1 serum, which had strong reactivity with the 2F5 epitope, was used for purification, as described in the Methods (Section 3.3.5). **Figure 3-12** shows the reactivity of Abs purified from serum R1 (R1PurAb) with 2F5 peptide, as well as the R1 whole serum and the flow through from the purification (FTR1); the 2F5 MAb is also included as a control. The R1PurAb retained binding activity to 2F5 peptide and the FTR1 had very little reactivity with this peptide, indicating that most of the 2F5 reactivity was removed from the serum.

The specific reactivity of the R1PurAb with the MPER on the cell was tested by fluorescence microscopy. The R1PurAb was used to detect protein expressed after transfecting COS cells with pMPER-TM1. **Figure 3-13** shows that the R1PurAb bound to MPER expressed by the plasmid pMPER-TM1. In the fluorescence microscopy capture presented in **Figure 3-13A**, we see two cells expressing the MPER (labeled in green), while several other cells in blue can be seen in the same capture; this is consistent with the specific interaction of the R1PurAb with a subset of cells that express the recombinant protein. The **Figure 3-13B** is the same field but only illuminated with the UV lamp, allowing blue stain (DAPI) of the cell nuclei to be visualized.

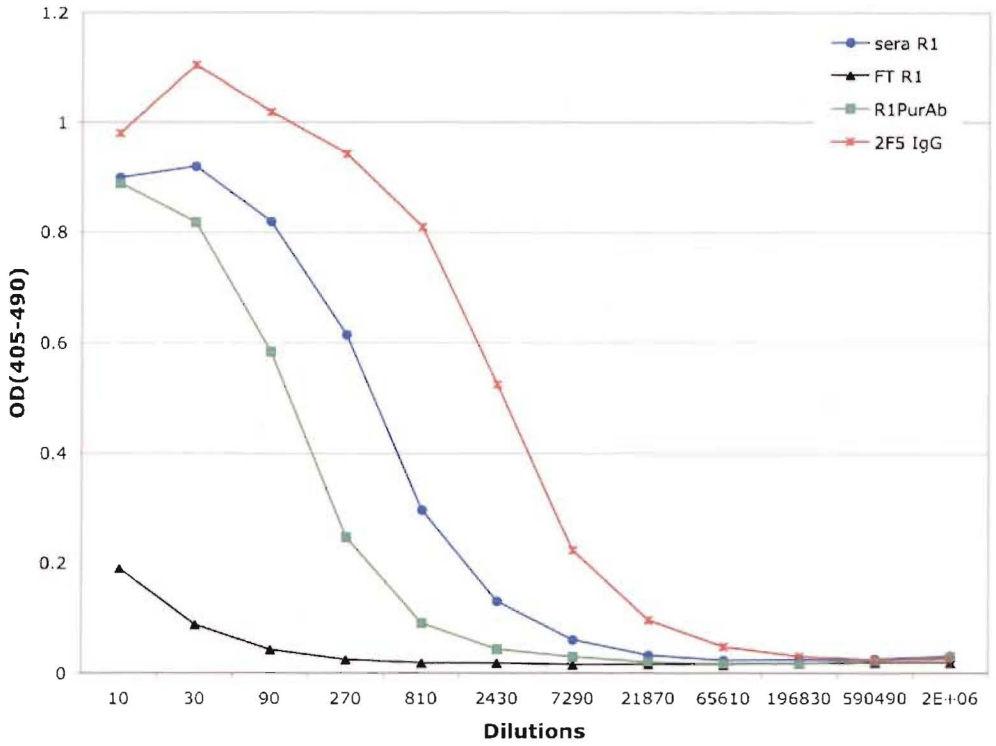


Figure 3-12. Reactivity of affinity-purified Ab with the 2F5 peptide epitope. The peptide was adsorbed to the ELISA plate. The 2F5 Ab was used at serial dilution starting with a concentration of 20 nM. The flow through (FTR1) from the purification was also included. The bound rabbit Ab was detected using goat anti-rabbit IgG HRP-conjugate.

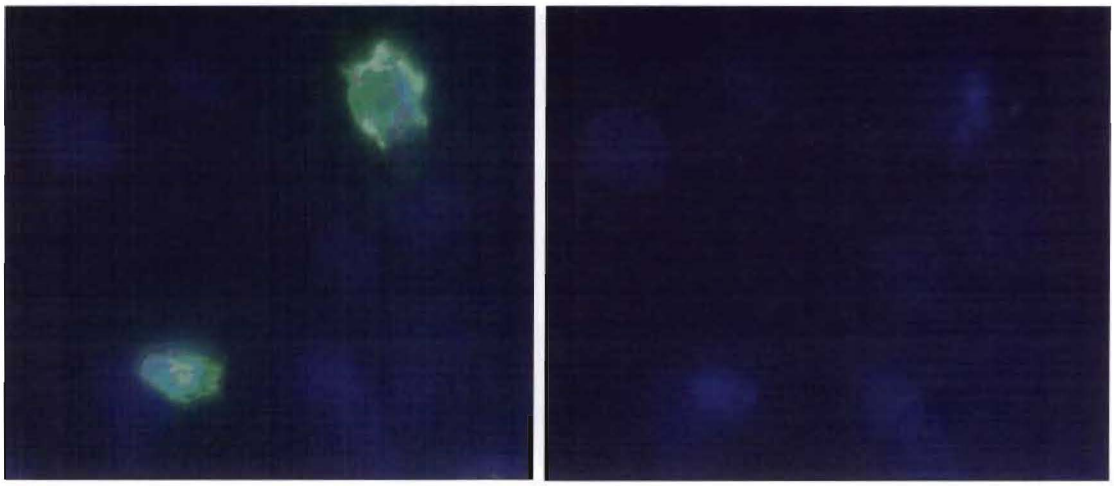


Figure 3-13. Recognition of membrane anchored MPER by 2F5 peptide affinity-purified Ab from serum R1 by fluorescence microscopy. COS cells were transfected with pMPER-TM1 DNA construct. The purified Ab (from bleed III) was used at a concentration of 10 nM, and visualized with goat anti-Rabbit Ab conjugated to Alexa-Fluor 488 (green staining). DAPI was used in the mounting media to label the cells nuclei (blue). (A). DAPI and Alexa Fluor detection. (B). DAPI detection. Fluorescence microscopy image captured using Olympus Motorized Inverted Microscope.

3.4.6. 2F5 epitope cross-reactive Abs from sera R1 and MAb 2F5 have different reactivity patterns with Ala-substituted mutants of the MPER

The reactivity of the R1PurAb with Ala-MPER mutants in the 2F5 epitope was tested in comparison with 2F5 IgG in cell lysate ELISA. For this experiments COS cells transfected with pMPER-TM1 were used as Ag, and cells transfected with the mock vector were used as a negative control. **Figure 3-14** shows that the substitution of the DKW amino acids for alanine drastically decreased the interaction of MAb 2F5 with the MPER in the cell lysate assay. This is in agreement with the previously defined critical binding residues for the 2F5 MAb in the MPER (49, 186, 240). Interestingly, the interaction of the Ab purified from rabbit 1 serum after the fourth immunization showed a very different reactivity pattern. Only two residues in the canonical 2F5 epitope (ELDKWASLW) were critical for the interaction of the R1PurAb with the Ala-substituted mutants. Similar to MAb 2F5, the binding of R1PurAb is sensitive to mutations D_{664}, K_{665} and W_{670}; however, in striking contrast to 2F5, R1PurAb binding was insensitive to mutations in W_{666}. The purified R1PurAb was more sensitive than 2F5 to mutation in W_{670}. These results suggest that the critical binding residues for 2F5Ab and the R1PurAb are different, and likely have different mechanisms of interaction with the MPER. The reactivity of the 4E10 IgG with the MPER Ala mutants was also probed in this experiment (**Figure 3-15**) and used to verify the relative presence of same amount of protein in each of the samples tested. This is an important control for the data presented here, since as was stated before in Chapter 2 (Section 2.3.5), we do not have a region in our wt and ala-mutants that is different from the MPER (*e.g.* and HA-tag

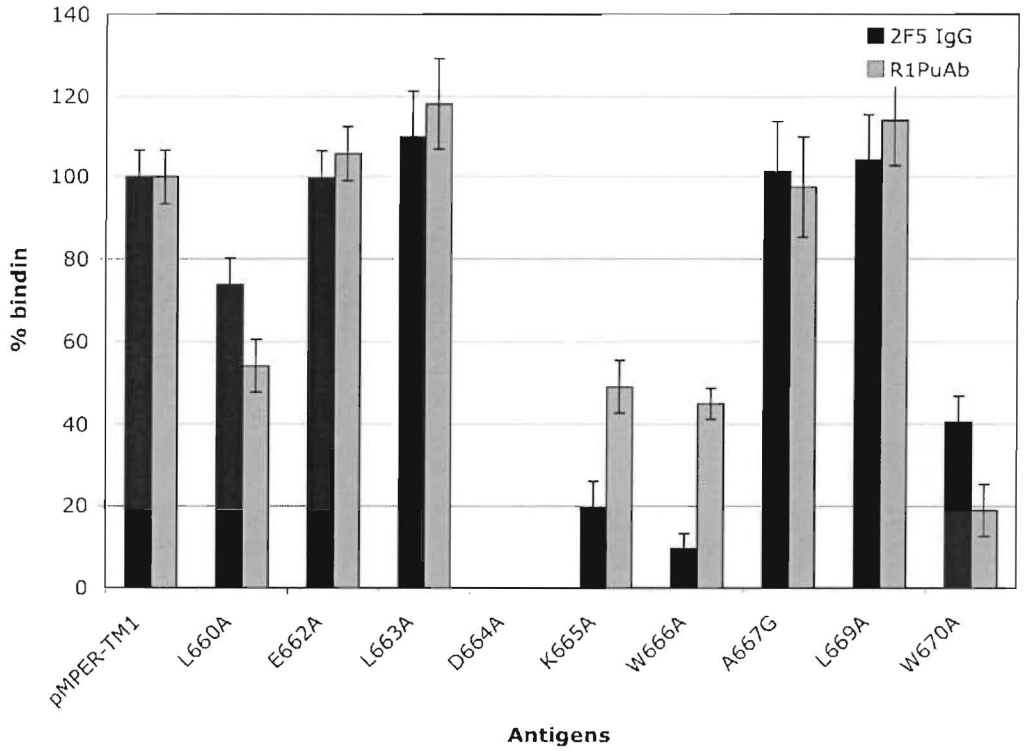


Figure 3-14. Reactivity of the R1PurAb with the 2F5 epitope MPER Ala-mutants. COS cell lysate was used as Ag in ELISA, the lysates were prepared after transient transfection of the cells with each of the mutant plasmids. 2F5 IgG was used at 10 nM and the PurAb also at 10 nM, based on the data from the Figure 3-13. Values expressed as % of wt binding. S668A (not done).

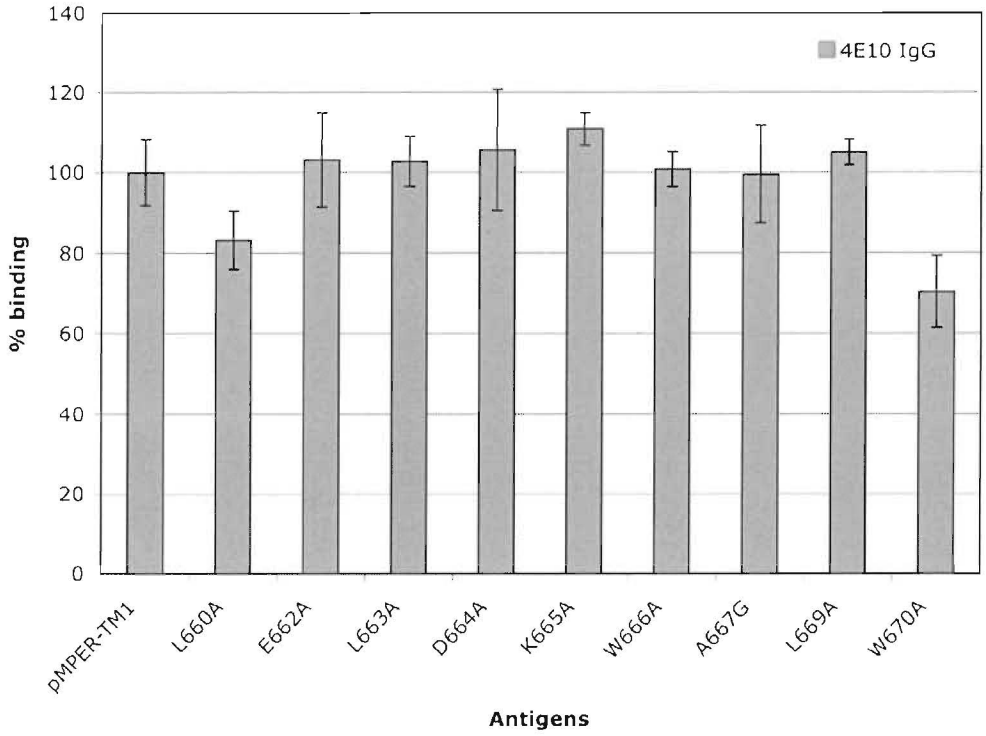


Figure 3-15. Reactivity of 4E10 with the MPER mutants. COS cell lysate was used as Ag in ELISA, the lysates were prepared after transient transfection of the cells with each of the mutant plasmids. 4E10 IgG was used at 10 nM and detected with ProtA/G-HRP. Values expressed as % of wt binding.

that can be used for normalizing the data (*i.e.*, to show the presence of similar amounts of protein in each sample tested).

3.4.7. Immunization with pJW-MPER, pJW-CHR and PJW-NHR do not induce 4E10 peptide reactive Abs

We showed in the peptide mapping experiments (Section 3.4.4) that none of the immunized rabbit sera reacted with the 4E10 epitope peptide 6376 (SLWNWFDITNWLWYI). Since there were discrepancies in the binding of sera to 2F5 peptide epitopes and membrane-anchored MPER, we decided to test whether the lack of reactivity with the 4E10 peptide was determined by the assay used. First, we tested the reactivity of the sera from the fifth immunization, with biotinylated 4E10 peptide epitope captured on Streptavidin (SA), instead of adsorbed directly to the plate, using two different dilutions of the sera (1/250, 1/500). As seen in **Figure 3-16**, while sera R1 bound to the 2F5 epitope in this type of ELISA assay, no reactivity was found for the epitope of 4E10. Similar results were obtained for other sera (not shown). Thus, the capturing conditions did not affect our data with 4E10 peptide. This strongly suggests that no Abs that react with the 4E10 epitope were present in the rabbits sera.

Second, we performed a competition ELISA. For this assay, it was important to find the right concentration of both Abs to be competing to each other, since 4E10 has a nanomolar affinity for its epitope (27) it may be difficult for lower affinity Ab in the sera to compete with 4E10 for the interaction with its epitope. Thus, it was necessary to find a concentration of 4E10 that would give a half-maximal signal of what can be reached in the assay.

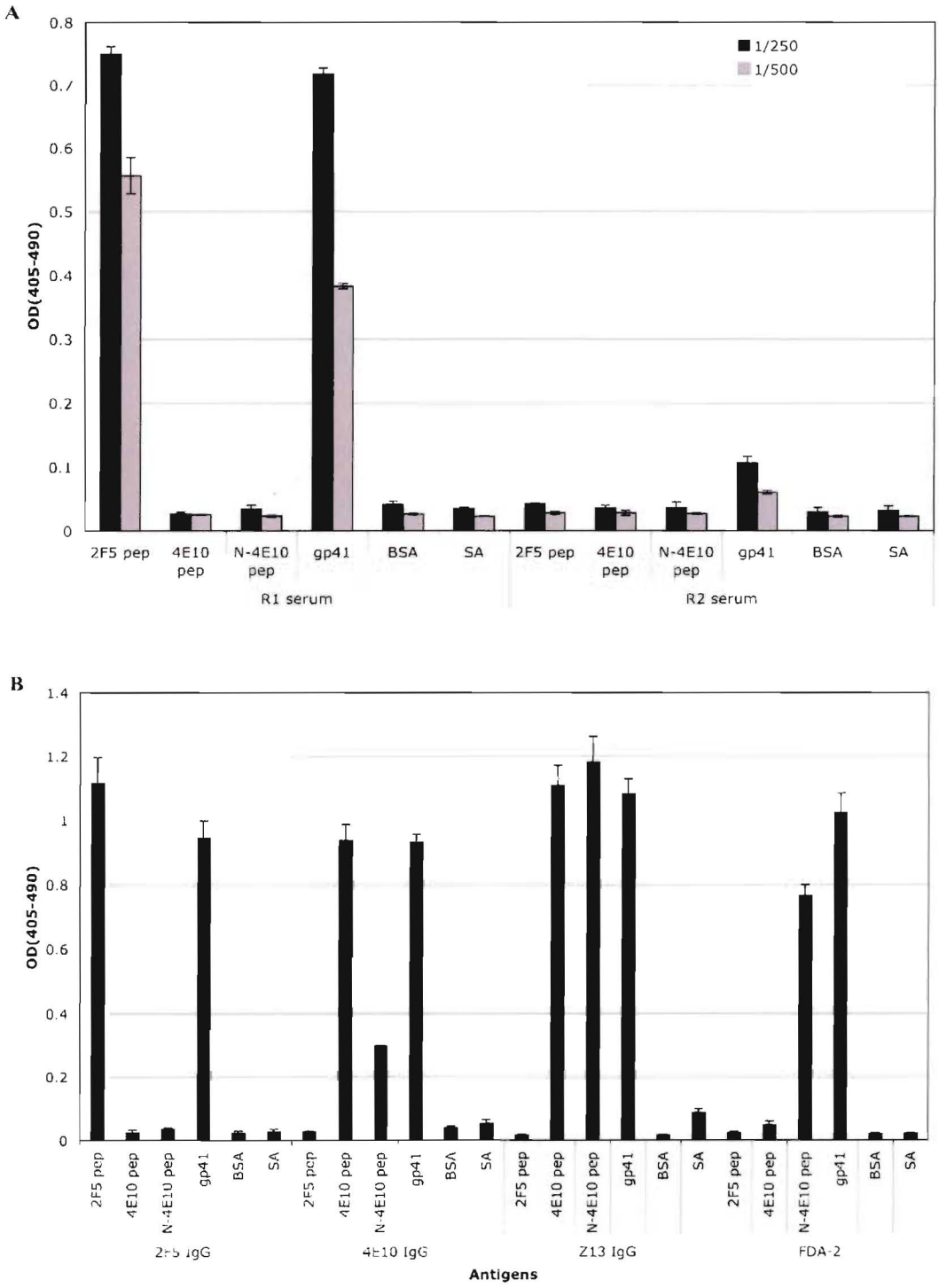


Figure 3-16. Reactivity of sera from Rabbits 1 and 2 with biotinylated 2F5 and 4E10 peptide epitopes captured on streptavidin. (A) R1 and R2; (B) MAbs and FDA-2 controls. N-4E10 pep (peptide epitope recognized by Z13 MAb).

For this experiment we used four different serum dilutions: 1/50, 1/100, 1/200 and 1/300. Another key issue of this assay was to define whether the secondary Ab used for detection of one Ab, does not react with the other one. We verified that the anti-human HRP conjugate did not bind to rabbit Abs (the secondary Ab reactivity is also included in the experiment as an internal control). We tested competition of the sera R1 and the FTR1 with 4E10 Ab in two different formats, as an IgG (10nM) and as Fab at two different concentrations (25 nM and 50 nM). The FT was used because it is depleted of 2F5 epitope binding Ab (see **Figure 3-12**), but it should still have other specificities. The competition ELISA was done using cell lysates as Ag, and gp41 included as a control. **Figure 3-17** shows the competition assay using 4E10 Fab at 25 nM with R1 and FTR1 sera. Binding of 4E10 with the MPER (in the cell lysate ELISA using pMPER-TM1) was not affected by the presence of any of several concentrations of the R1 or FTR1 sera tested. The secondary anti-human Ab bound specifically only to 4E10. The reactivity of the R1 and FTR1 with the Ag were also included as controls. Thus while the R1 and the FTR1 bind to the MPER and to gp41, the epitopes recognized by both samples do not overlap with the 4E10 epitope. Similar results were obtained for the 4E10 Fab at 50 nM (not shown). Competition of the 4E10 IgG with the rabbit R1 and FTR1 sera is shown in **Figure 3-18**. No competition of the sera with 4E10 Ab binding to the MPER was observed for any of the sera tested. However, some competition of the sera with 4E10 IgG (10nM) was observed in gp41 reactivity at R1 sera dilutions 1/50 and 1/100 dilutions, probably non-specific as it did not occur with the MPER; this was not observed for the FTR1 serum. Since competition between 2F5 and 4E10 Abs for reactivity

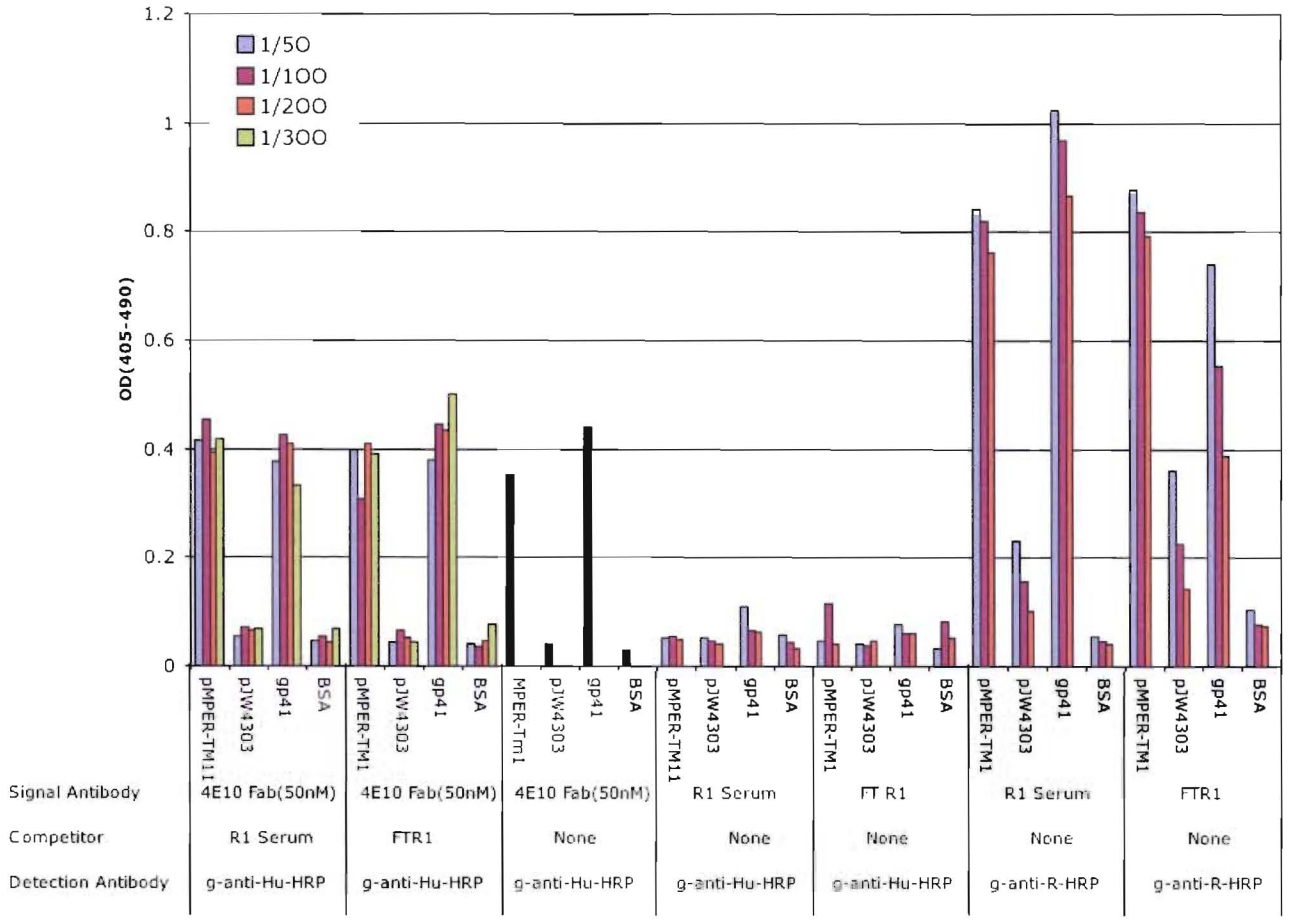


Figure 3-17. Serum R1competition ELISA with 4E10 Fab (25 nM). Several dilutions of the sera R1 and R1 FT were tested to compete 4E10 Fab. Bound 4E10 was detected with goat anti-human IgG HRP conjugate. The pMPER-TM and pJW43-3 represent cell lysates prepared with these plasmids. The black columns represent the 4E10 Fab reactivity.

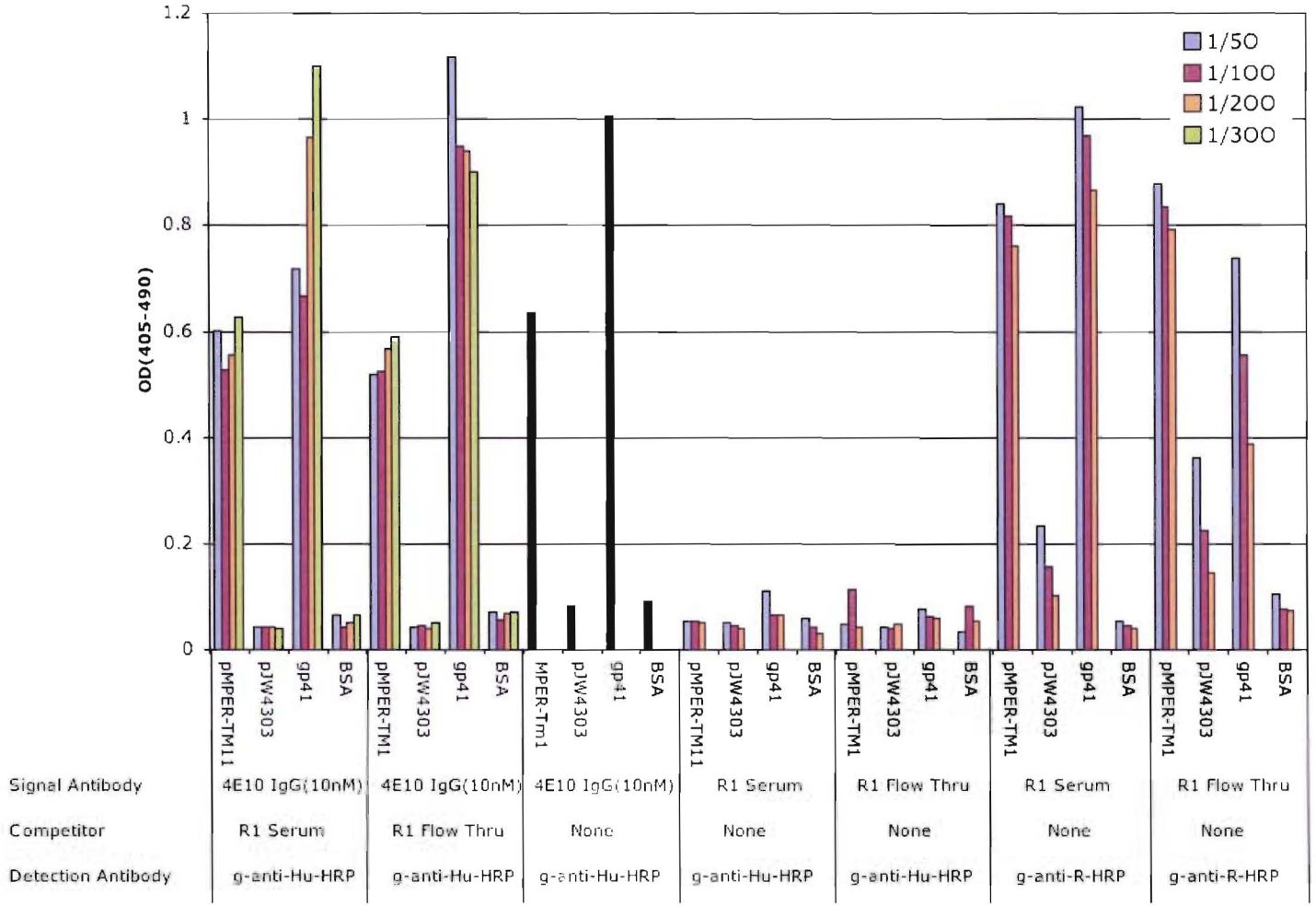


Figure 3-18. Sera R1competition ELISA with 4E10 IgG (10 nM). Several dilutions of the sera R1 and R1 FT were tested to compete 4E10 IgG. The bound human Ab was detected with goat anti-human IgG HRP conjugate. The pMPER-TM and pJW43-3 represent cell lysates prepared with these plasmids. The black columns represent the 4E10 IgG reactivity

with their epitopes in gp41 has been previously reported (290) and the serum R1 has significant reactivity with the 2F5 epitope, we believe that this 2F5 reactivity may be responsible for the observed competition of the R1 serum with 4E10 IgG. Thus, the use of a different capturing procedure for the peptide epitope in ELISA, and the competition ELISA confirmed that the sera do not contain Abs reactive with the 4E10 epitope, and therefore indicate that those Ab were not elicited by DNA immunization with the MPER DNA constructs.

3.4.8. HIV-1 neutralization activity of immunized rabbit sera

The HIV-1 neutralization capacity of the sera elicited after the fifth DNA immunization (bleed III) with the pJW-MPER, pJW-CHR and pJW-NHR was tested *in vitro*, using pseudotyped HIV-1. Representative samples used for these experiments included sera R1 (pJW-MPER), R4 (pJW-CHR), R5, R6 (pJW-NHR) and R8 (negative control).

Ab from bleed III sera R3, R4, R5 and R6 were affinity-purified as described in section 3.3.5 and titrated on 2F5 peptide, by ELISA (**Figure 3-19**). These purified Abs samples were tested for neutralization activity. Also, the FTR1 after depletion of the 2F5 epitope cross-reactive fraction was tested for binding to MPER-TM in the cell lysate ELISA; it was observed that the FTR1 still interacted specifically with the MPER and gp41 (**Figure 3-20**), indicating the presence of other MPER-reactve Ab. Thus, we decided to test the FTR1 for HIV-1 neutralization activity also.

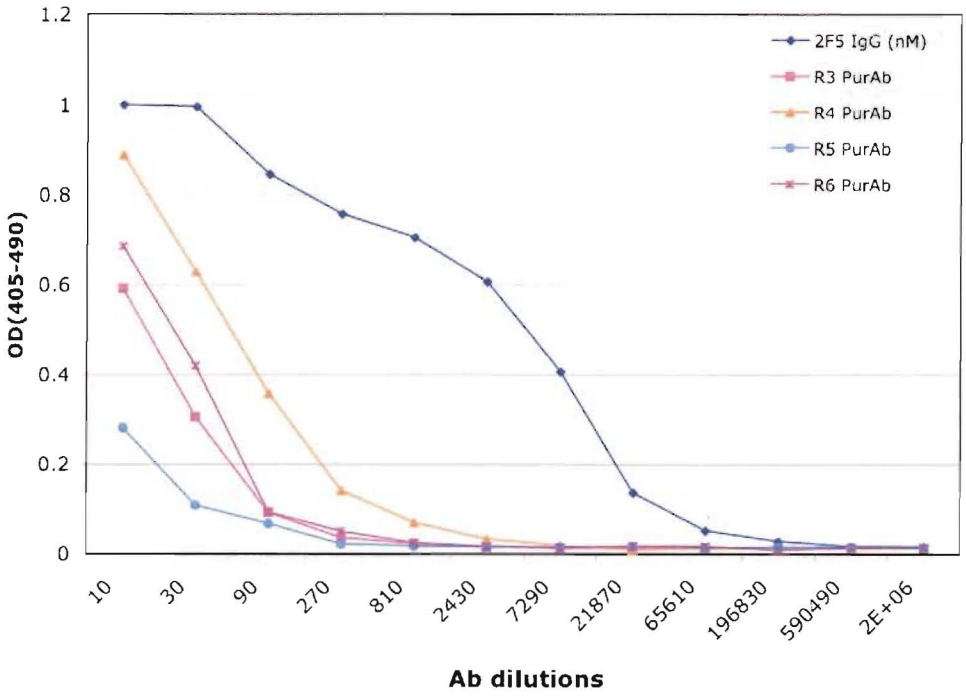


Figure 3-19. Affinity-purified Ab from sera R3, R4, R5, and R6. Purified Abs were titrated on 2F5 synthetic peptide. 2F5 IgG was used as a control starting at 20 nM and diluted 1:4.

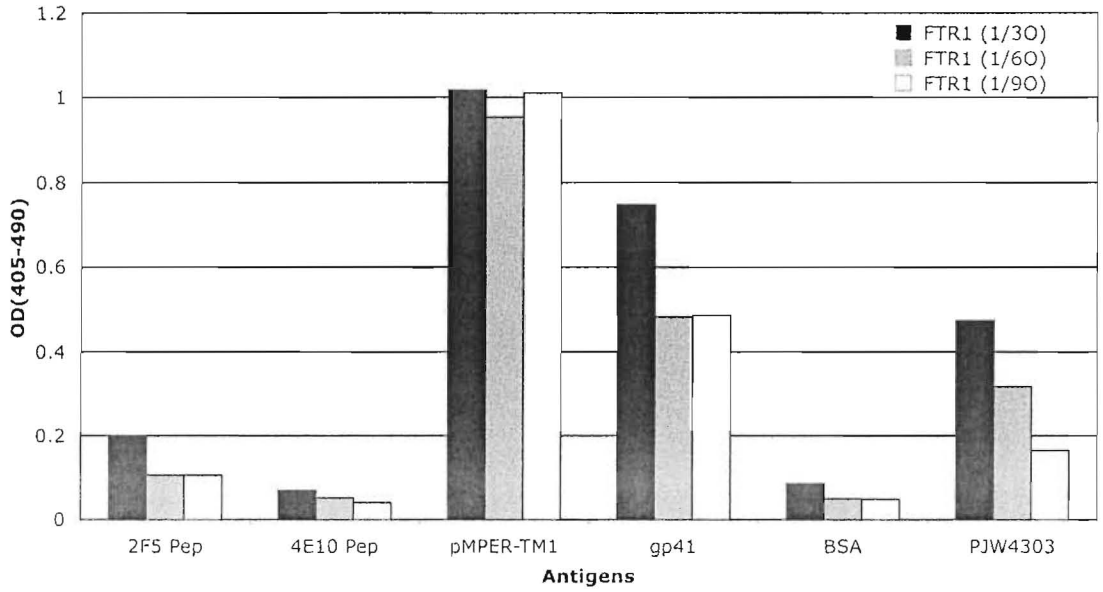


Figure 3-20 Reactivity of FTR1 with the MPER. Serial dilutions of the FTR1 were titrated for binding to several Ag in ELISA including cell lysates prepared with pMPER-TM1,and pJW4303 (mock) plasmids.

The samples were tested against several HIV-1 isolates from clade B, including the tissue culture laboratory adapted (TCLA) isolate MN, and the primary isolates JRCSF, 92HT594, NL143 and SF162. The envelope from amphotropic murine leukaemia virus (aMLV) was used as a negative control. The HIV-1 broadly neutralizing plasma, Z23 was used as a positive control in the neutralization assay. **Table 3-6** summarizes the neutralization results. Nt activity is displayed as the percent inhibition of viral replication at each Ab dilution in respect to an Ab-negative control. For the sera, the data is presented as the serum dilution that neutralizes 50 % of pseudovirions infectivity, for the purified Ab, the IC_{50} were calculate based on the IgG concentration used. For example, in the case of sera R1 less than 1/20 dilution of the sera is required to obtain 50 % of inhibition of the luciferese activity. However, similar concentration of serum also neutralized the negative control virus aMLV. No neutralization activity was detected for any of the serum or IgG samples tested. In this experiment we tested a pseudovirus carrying the Env protein from the isolate JR-CSF, which is the same envelope protein produced by our DNA constructs. However, no neutralization of that virus was observed. For the purified Ab, it was not possible to test all the pseudovirions, due to small sample volume. The Ab concentration at which PurAbR1, PurAbR3, PurAbR4 and PurAbR6R7 neutralized the negative control virus (aMLV) was less than twice of the one required for neutralization of the HIV-1 isolate, indicating that this samples are not very neutralizing. Thus, no significant HIV-1-neutralization activity was found in any of the samples. A more detailed presentation of the data, including the IC_{50} curves are presented on Appendix 2.

Table 3-6. HIV-1 neutralization activity of rabbit sera and antibodies affinity-purified on the 2F5 peptide epitope. Data shown are IC_{50} represented as inverse of the dilution for the sera and FTR1, and as Ab concentration for the purified Ab.

Serum dil/ Ab (μg/ml)	92HT594	MN	SF162	JRCSF	NL43	aMLV
R1 serum	<20	<20	<20	<20	<20	<20
FTR1	<10	<10	<10	<10	<10	<10
R4 serum	<10	<10	<10	<10	<10	<10
R5 serum	<10	<10	<10	<10	<10	<10
R6 serum	<10	<10	<10	<10	<10	<10
R8 serum	<10	<10	<10	<10	<10	<10
PurAbR1	<0.59	0.63	1.97	nd[a]	nd	0.2
PurAbR3	1.60	1.09	12.15	nd	nd	0.59
PurAbR4	0.40	0.55	2.78	nd	nd	0.33
PurAb(R6/R7)[b]	>10.5	0.30	1.83	nd	nd	0.14
Z23 ($^{+}$ control)[c]	1206	15613	67007	683	7549	<100

[a] nd, no determined

[b] Purified Abs from sera R5 and R6 (rabbits immunized with pJW-NHR) were mixed

[c] from Monogram Bioscience

3.5. Summary

In this study we have evaluated the immunogenic activity of the gp41 MPER after DNA vaccinations using three different DNA constructs: pJW-MPER, pJW-CHR and pJW-NHR. These three plasmids express the MPER attached to the cell membrane through the PDGFR-TM region. The immunogenicty of the proteins produced by these three constructs *in vivo* was evaluated in rabbits, after immunization with DNA by delivery *via* gene gun. In total, eight rabbits were immunized, two with each of the three constructs, and two other rabbits with the mock vector pJW4303. The animals received in total five DNA immunizations, four doses with one of the three plasmids, and a final dose with the pJW-MPER plasmid. Control animals received five mock vector immunizations.

We showed that the DNA constructs producing gp41 fragments were immunogenic, since Abs binding to recombinant gp41 and to MPER were detected in sera of immunized rabbits, by ELISA. We performed epitope mapping assay of the immune sera using a set of overlapping synthetic peptides, and showed reactivity of some sera with the 2F5 peptide epitope. However, no reactivity with the 4E10 peptide epitope was detected in ELISA, neither by direct adsorption of the peptide to the plate nor by capture of biotinylated peptide on streptavidin. In addition, competition experiments also failed to reveal any reactivity against the 4E10 in membrane-anchored MPER. These results indicate that the 4E10 epitope is not immunogenic in the MPER context tested, and are in agreement with the reported low immunogenicity of this epitope (131).

We have clearly documented discrepancies in the results from different procedures used to evaluate the anti-MPER response the sera of vaccinated animals. For example, an

increase in the anti-MPER Ab response from the fourth to the fifth immunization was observed in cell lysate ELISA but this finding could not be confirmed by the peptide mapping. Also, binding to gp41 did not always correlate with binding to the membrane-anchored MPER. We believe that cell lysate ELISA with membrane-anchored MPER can detect Abs to conformational epitopes that cannot be detected in synthetic peptide ELISAs, and not even with recombinant gp41.

Serum Abs having specific reactivity with the 2F5 epitope peptide were affinity purified using a biotinylated 2F5 synthetic peptide epitope captured on SA-coated magnetic beads. We analyzed the reactivity of the purified Ab from R1 with a set of alanine-substituted mutants of the 2F5 epitope on the membrane-anchored MPER, using the cell lysate ELISA. This study suggested that Ab against the 2F5 epitope present in the sera, reacted with the MPER by a mechanism different from 2F5, indicating that the Ab response elicited against this epitope was qualitatively different from 2F5.

No HIV-1 neutralization activity was detected for any of the sera or affinity purified Abs tested. However, we cannot rule out the possibility that Abs with Nt activity are presented in the elicited response, but at quantities that are to low to detect. The constructs carrying the gp41 TM regions should be tested since we have documented an influence of this domain in the antigenicity of the MPER towards 4E10, thus it is possible that the TM region may also influence the quality of the Ab response. In addition, other immunization approaches such as a protein boost after the DNA immunization, the use of molecular adjuvants or the use of membrane preparations as immunogens should be considered for future studies.

3.6. Appendix 2

Neutralization study: All the neutralization curves, from which the data presented in **Table 3-6** originated, are shown in the following figures. The sera from the rabbits R1, R3, R5, R6 and R8 and the flow through after affinity purification of the 2F5 reactive fraction from sera R1 (FTR1) were evaluated in the neutralization assay. The serum R8 corresponds to one of the animals immunized with the pJW4303 (mock), and is used as negative control. The HIV-1 positive plasma Z23 is the Monogram Bioscience positive control of neutralization. The amphotropic murine leukaemia virus (aMLV) was used as a negative control virus. Serial dilutions of the sera were evaluated in the assay. No neutralization activity was observed for any of the sera tested, however, all the affinity purified Ab show neutralization activity to all the HIV-1 isolates tested, which was also observed for the negative control virus (aMLV). Thus, the observed neutralization activity for the purified antibodies is non-specific to HIV-1, suggesting either that the purified Ab are qualitatively different from 2F5 or that they are somehow denatured, since some acidification was noticed (data not shown).

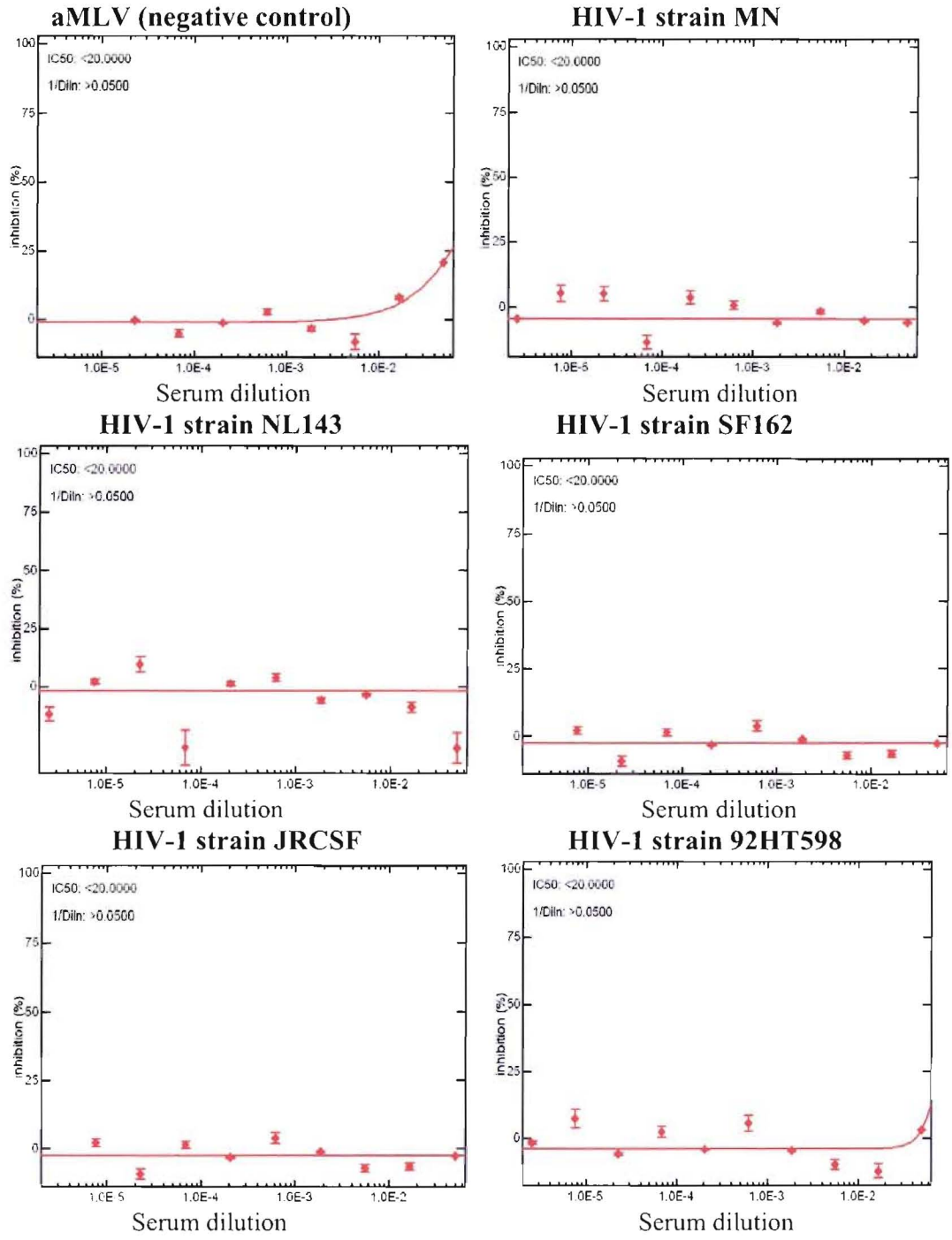


Figure 3- 21. Neutralization data for serum R1. The assay was done with pseudovirion bearing the MN (TCLA), and 92HT594 (primary isolate), NL143, SL162, JRCSF envelope proteins. The aMLV was the negative control virus, and HIV positive plasma Z23. All sera samples were tested in a 18-hour incubation with the viruses before the addition of cells.

Flow through from R1 (FTR1)

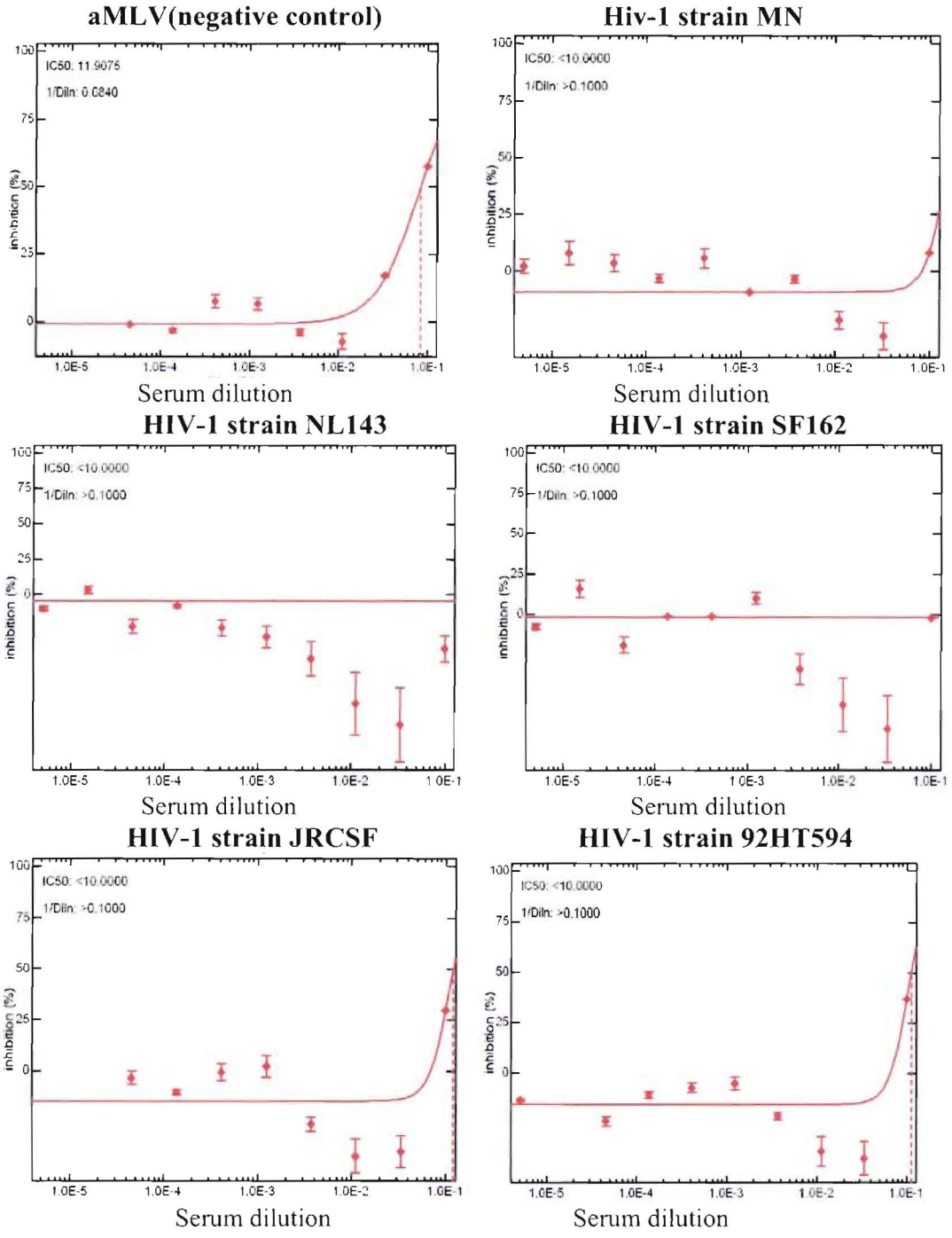


Figure 3-22. Neutralization data for the FTR1. The assay was done with pseudovirion bearing the MN (TCLA), and 92HT594 (primary isolate), NL143, SL162, JRCSF envelope proteins. The aMLV was the negative control virus, and HIV positive plasma Z23.

R4 serum

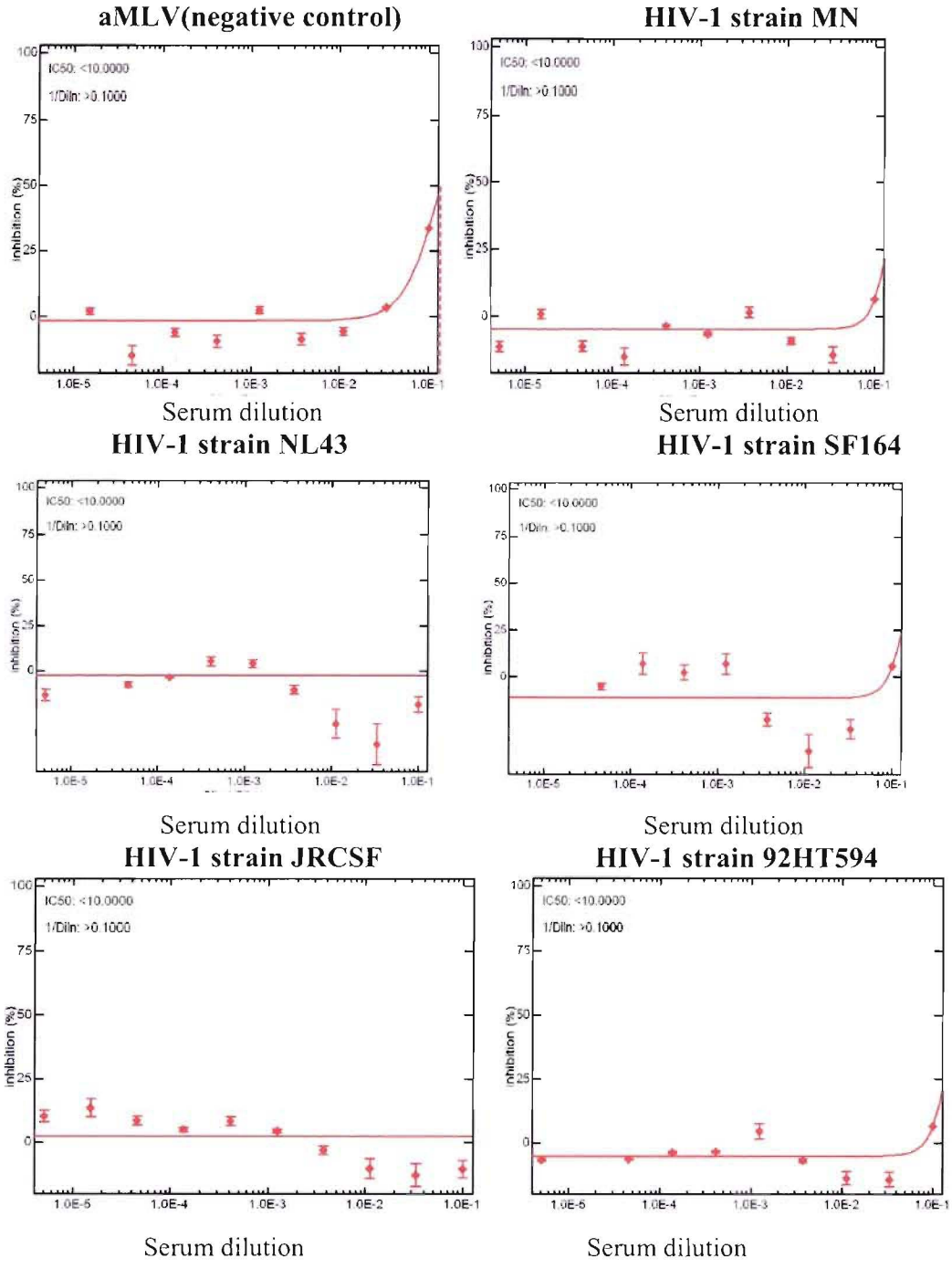


Figure 3-23. Neutralization data for the serum R4. HIV-1 The assay was done with pseudoviruses bearing the MN (TCLA), and 92HT594 (primary isolate), SL162, NL143, JRCS envelope proteins. The aMLV was the negative control virus, and HIV positive plasma Z23 was the positive serum sample.

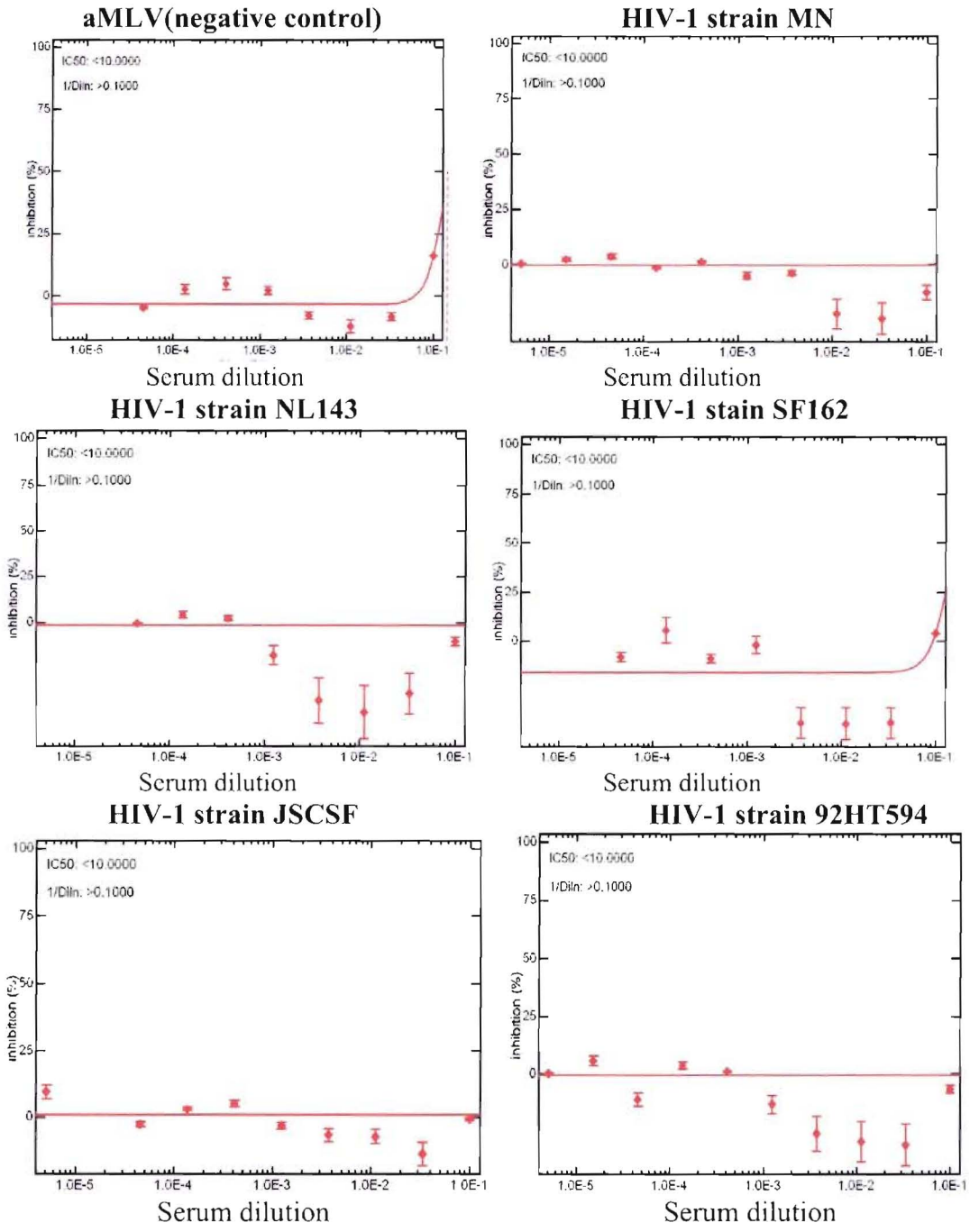


Figure 3-24. Neutralization data for the serum R5. HIV-1 The assay was done with pseudoviruses bearing the MN (TCLA), and 92HT594 (primary isolate), SL162, JRCS envelope proteins. The aMLV was the negative control virus, and HIV positive plasma Z23 was the positive serum sample.

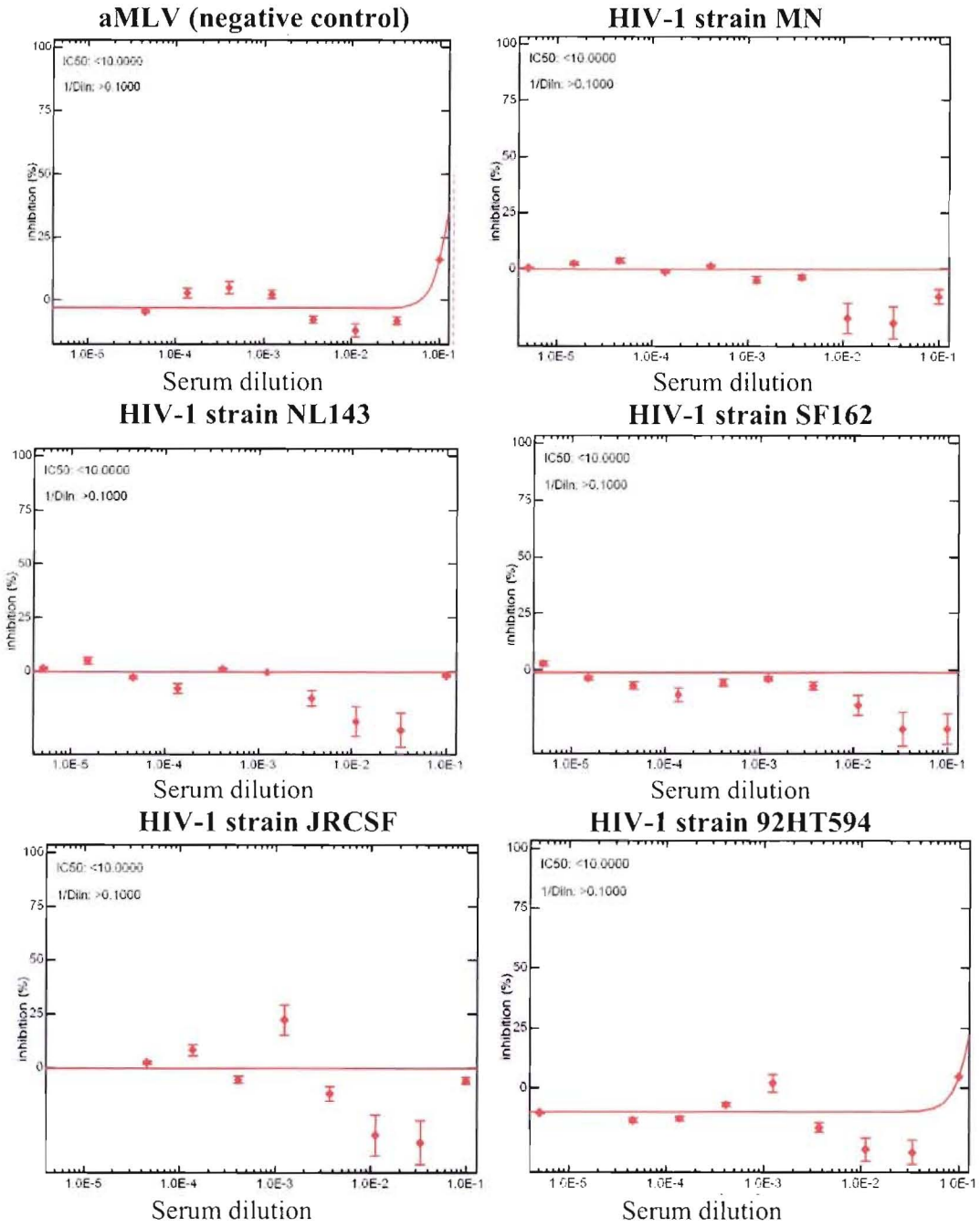


Figure 3-25. Neutralization data for the serum R6. The assay was done with pseudoviruses bearing the MN (TCLA), and 92HT594 (primary isolate), SL162, JRCS envelope proteins.

R8 serum

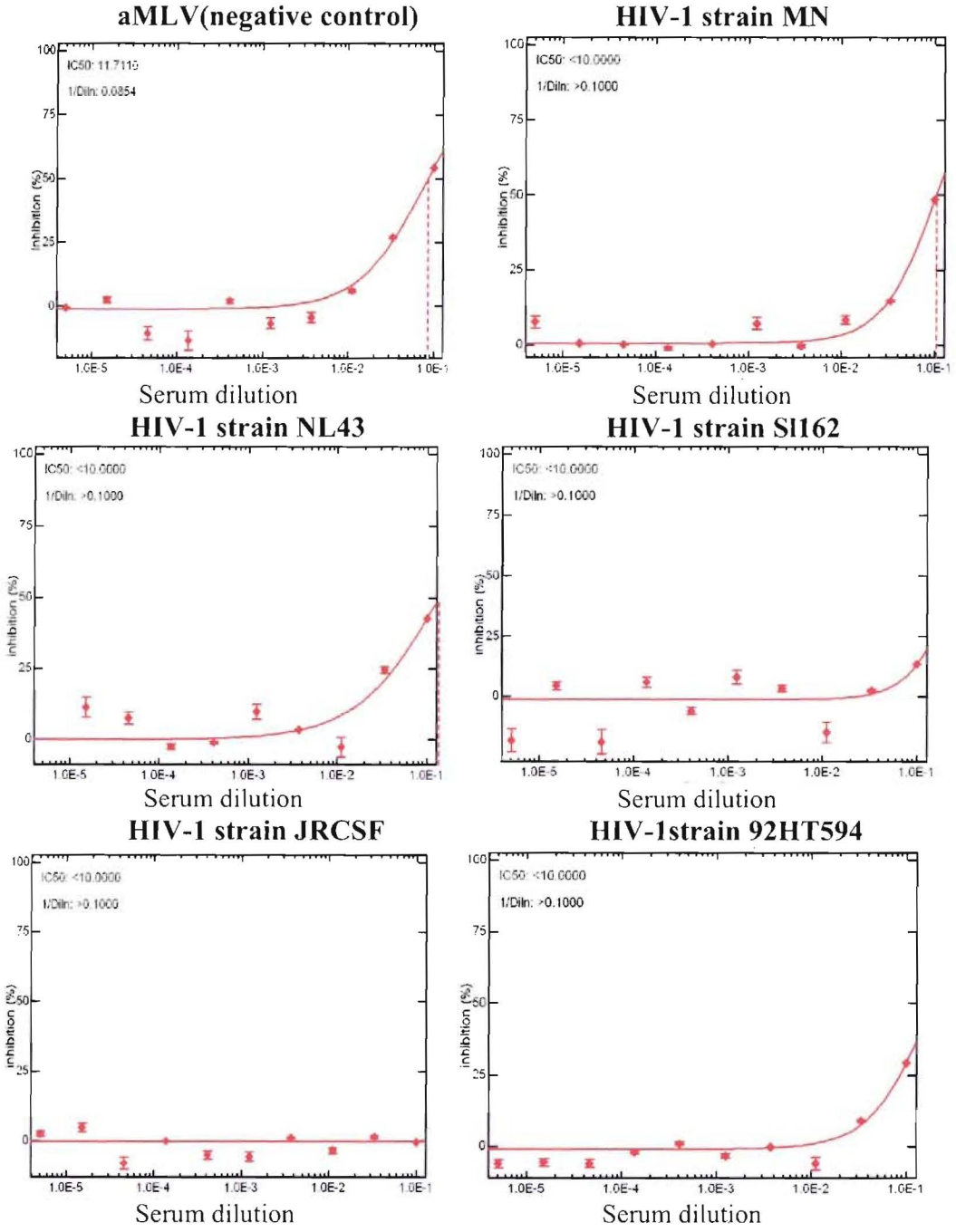


Figure 3-26. Neutralization data for the serum R8 (negative control serum). The assay was done with pseudoviruses bearing the MN (TCLA), and 92HT594 (primary isolate), SL162, JRCS envelope proteins. The aMLV was the negative control virus, and HIV positive plasma Z23 was the positive serum sample.

Z23 plasma

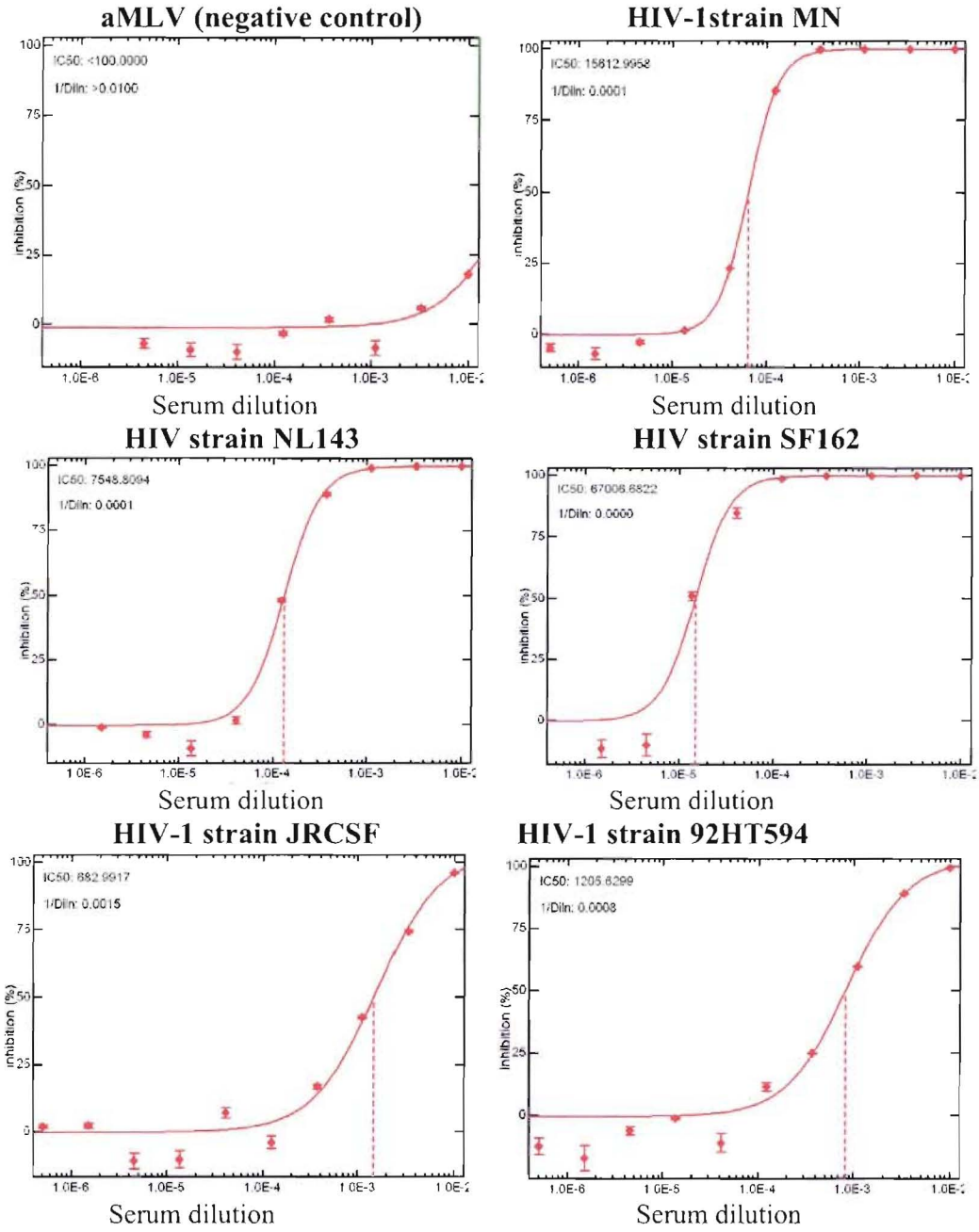


Figure 3-27. Neutralization data for the HIV Nt plasma Z23. HIV-1 Neutralization activity from serum the HIV-1 positive Nt plasma Z23 was accessed. The assay was done with pseudoviruses bearing the MN (TCLA), and 92HT594 (primary isolate), SL162, JRCS envelope proteins. Data courtesy of T. Wrin from Monogram Biosciences.

R1 PurAb

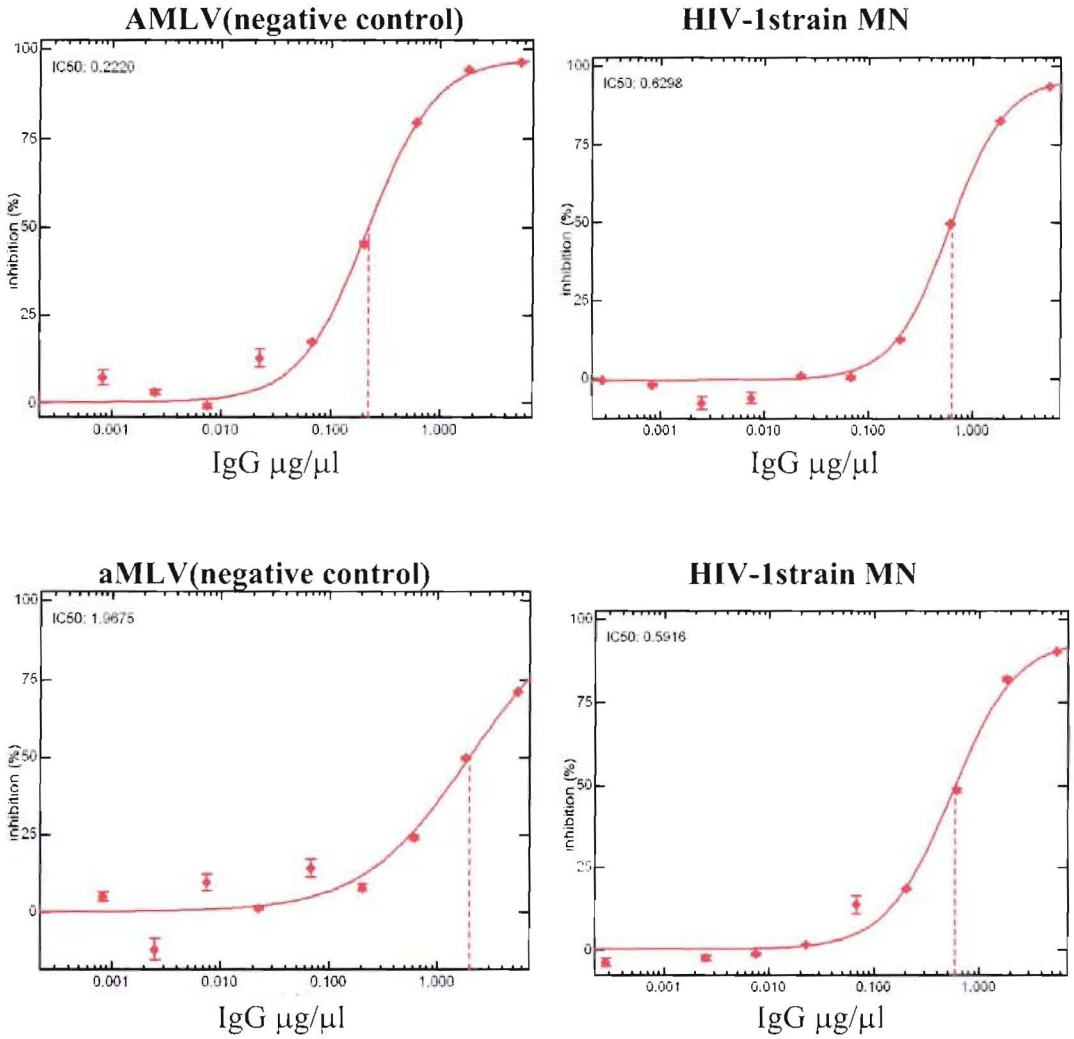


Figure 3-28. Neutralization data for the R1 purified Ab. HIV-1. The assay was done with pseudoviruses bearing the MN (TCLA), and 92HT594 (primary isolate), SL162, JRCS envelope proteins.

R3 PurAb

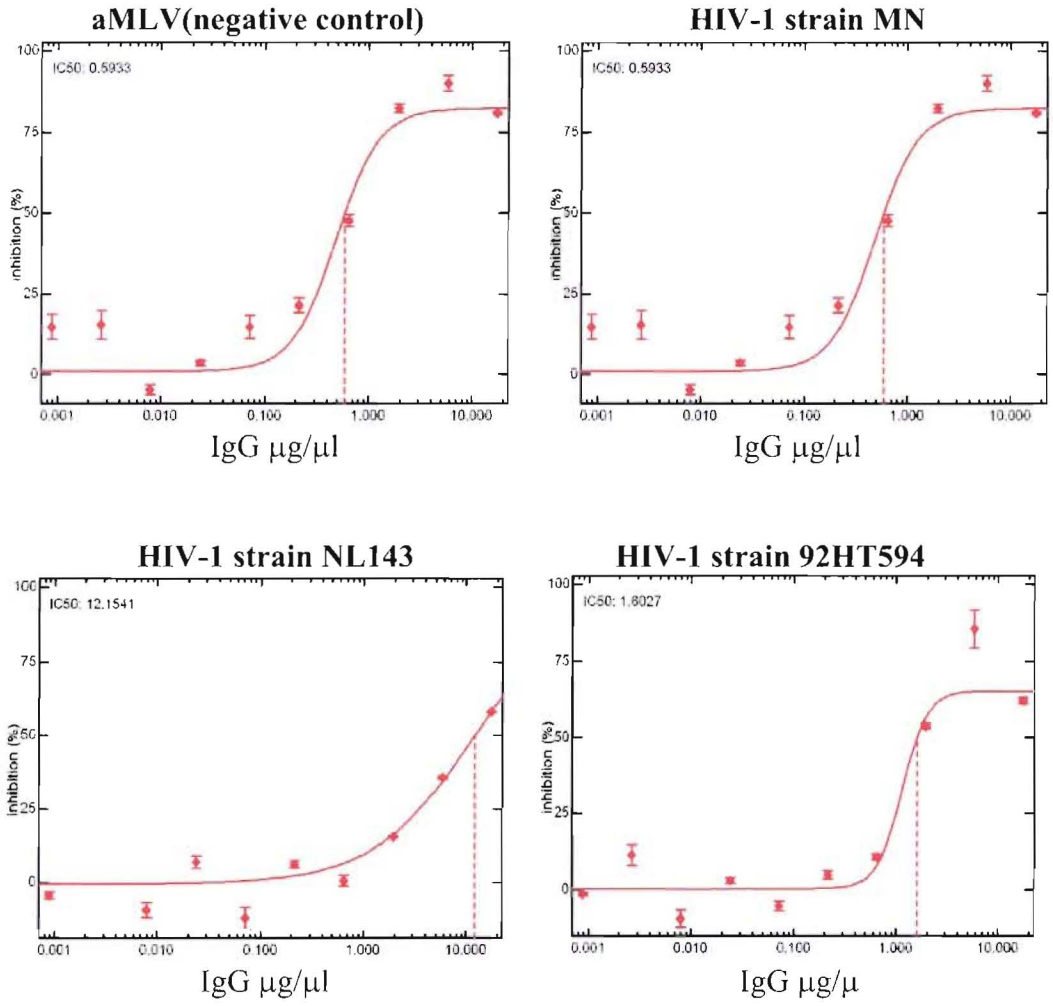


Figure 3-29. Neutralization data for the R3 purified Ab.

R4 PurAb

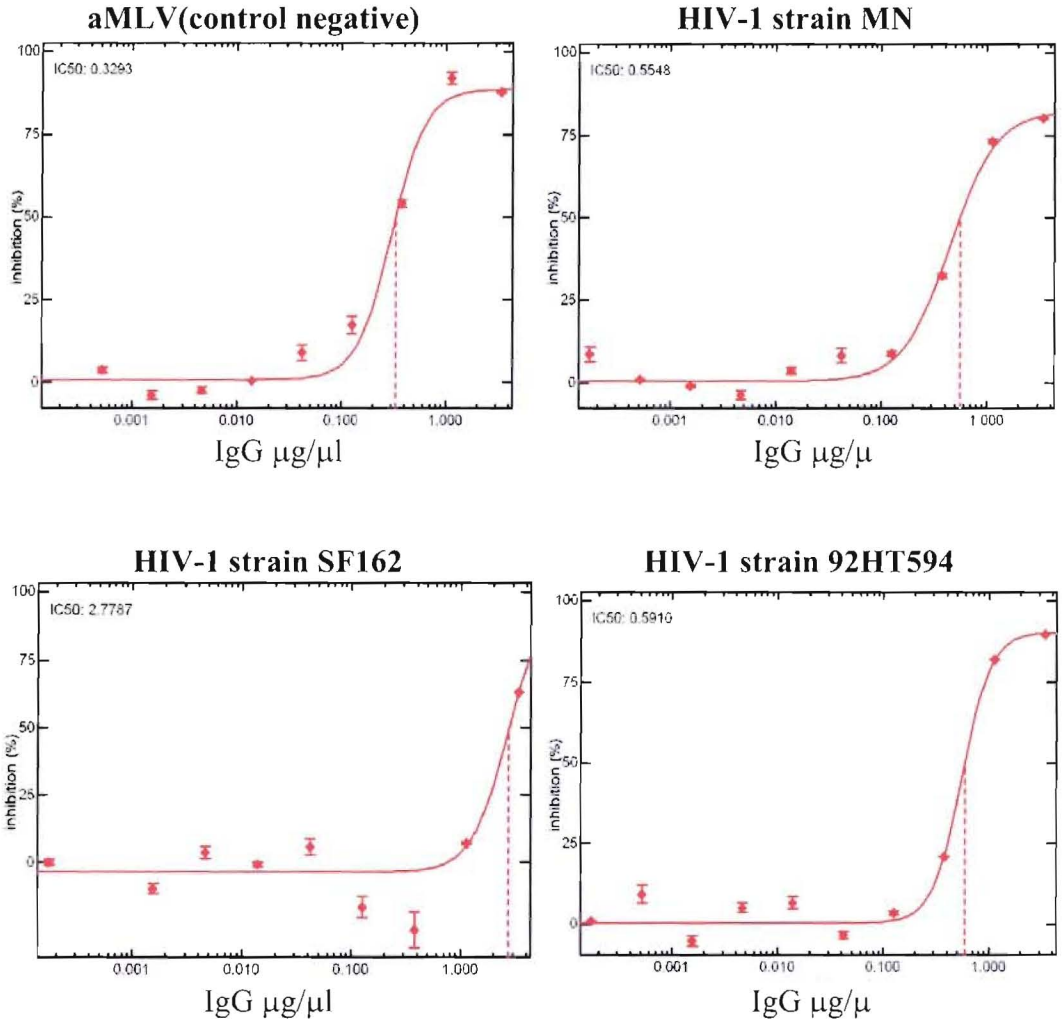


Figure 3-30. Neutralization data for the R4 purified Abs. The assay was done with pseudoviruses bearing the MN (TCLA), and 92HT594 (primary isolate), SL162, JRCS envelope proteins.

R6 PurAb

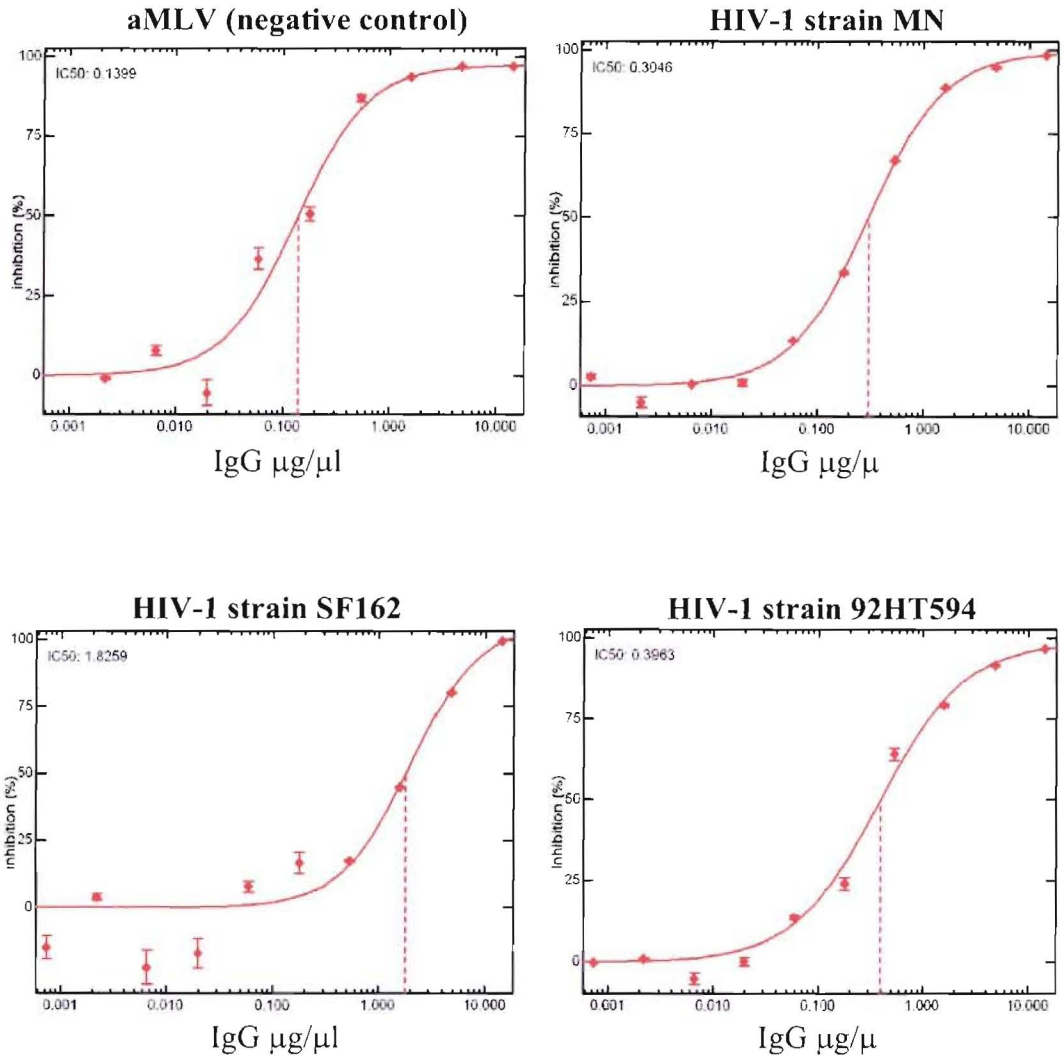


Figure 3-31. Neutralization data for the R6 purified Ab. The assay was done with pseudoviruses bearing the MN (TCLA), and 92HT594 (primary isolate), SL162, JRCS envelope proteins.

CHAPTER 4. Discussion

The MPER is a distinctive domain with a high degree of sequence conservation between HIV-1 isolates (174, 197, 210, 290). About half of the residues are hydrophobic with five being tryptophan. Mutation studies suggest a critical role of the MPER in membrane fusion (194, 210), which explains why it is such a sensitive target for Ab-mediated viral neutralization. However, as for many other regions of HIV-1 Env proteins, in spite of being immunogenic (23, 26, 35, 84, 111, 170, 246) most of the Ab response against the MPER seems irrelevant to virus neutralization (2, 89). The reasons for this are unclear but can be related to the structural complexity of gp41 and the dynamic nature of its biological functions. The 3D structure of gp41 undergoes major changes through the steps of viral attachment, fusion, pore formation and entry. Thus, it is possible that the adequate structure to elicit Nt Abs is only transiently available, while all others are the main targets of the Abs response. Most of the gp41 structures (including those of the MPER) have not been adequately resolved, and it is remains unclear which of the potential structures are permissive to Ab-mediated and neutralization. An already complicated scenario has become more so after several lines of evidence have suggested that the MPER may interact with the viral membrane (37, 98, 169, 183, 219, 232, 233, 251); thus, lipid membrane can be part of, or somehow influence the epitopes recognized by the bNt MAbs 2F5 and 4E10. Of particular interest is the finding that the affinity of 2F5 and 4E10 for the MPER increases when gp41 is presented in association with lipid (98, 183), suggesting that the epitopes recognized by 2F5 and 4E10 on the MPER may depend on membrane.

To study the influence of membrane and other regions of gp41on the antigenicity and immunogenicity of the MPER, we generated 29 DNA constructs encoding for various fragments of gp41, tethered either to the PDGF-TM region or the gp41 TM region **Table 2-4**, and expressed the recombinant proteins in the surface of COS-7 cells (**Figures 2-6 and 2-13**). Expression of the MPER was influenced by several factors; it was very low encoded by the viral DNA sequence on the pDisplay vector, whereas transfer of the genes to the pJW4303 increased protein expression significantly. The pDisplay and pJW4303 vectors carry the CMV early promoter, but pJW4303 also carries the CMV/intron A combination, which has proven to be more efficient for protein expression (257). The two vectors also have different secretion signal sequences; pDisplay carries the murine Ig κ signal sequence whereas the pJW4303 carries the highly efficient human tissue plasminogen (tPA).

The HIV-1 gene codon usage is very different from that of most mammalian genes (130, 229), and therefore a different pool of tRNAs is required to translate HIV-1 genes. Several examples have shown increased immunogenicity of DNA vaccines if the viral coding sequence has been optimized for mammalian codon usage while maintaining other vector elements constant (6, 100, 126, 286). We optimized the coding sequence of gp41 from the HIV-$1_{JR\text{-}CSF}$ isolate, which was used as template for the MPER constructs generated in pJW4303. However, no obvious increase in protein expression with respect to the constructs previously developed in pJW4303 using viral wild-type Env gene (**Figure 2-4, Figure 2-9 and Figure 2-11**). A recent study from Wang *et al.* addressed the influence of codon usage, promoter efficiency and leader sequence to the antigen expression and immunogenicity of a gp120 DNA vaccine (257). The study showed the

superiority of the tPA signal peptide over the Env signal peptide, and that its combination with the CMV IE/Intron A, had a positive contribution to protein expression. These two combined elements marked in synergy to increase the expression even in the case of protein expressed from the HIV-1 wild type gene. Our results are in general agreement with these finding, although in our system codon usage did not seem to be a limiting factor.

Fusion of gp41 fragments to proteins expressed from constructs bearing the gp41 transmembrane region (TM1) and the PDGFR-TM localized to the cell surface, as demonstrated by Ab reactivity with impermeabilized, transfected cells (**Figures 2-6 and 2-14**). To our knowledge, this is the time that the MPER has been expressed separated from the rest of gp41 on the cell surface. Our results validate the concept that the MPER (and therefore gp41) may be engineered to expose regions of immunological interest on the cell surface, and in the context of biological membranes. Interestingly, the cytoplasm of intact cells expressing gp41 fragment containing the full CT were labelled by 2F5 (*i.e.,* the Ab penetrated the cell), indicating that their plasma membrane was permeable to Ab even though no permeabilazing agent was used in preparing the cells (**Figures 2-19 and 2-20**). This suggests that the CT directly affected cell membrane permeability. Three regions with membrane-perturbing activity, and known as lentiviruses lytic peptides (LLPs) have been described in the CT region of gp41, LLP-2 (aa 768-788, LLP-3 (aa 788-814) and LLP-1 (aa 828-855) (69, 101, 102, 163). Synthetic peptides from these regions also disturb membrane (8, 40, 48). In a virus-infected cell, the CT interacts with the matrix protein (p17) and nucleates the viral assembly. However, in a situation like

ours, where no matrix protein is available, the CT region is free to interact with other cell elements and perhaps exert its membrane-disrupting activity.

The proteins expressed in the cell surface were recognized by HIV-1 bNt Ab 2F5 and 4E10, as well as Abs in the sera of HIV positive donors. We observed differences in antigenicity depending on the protein size or the TM region used. When the MPER was tethered to the membrane by the PDGFR-TM (**Figure 2-8**) 2F5 IgG showed better binding than 4E10, in all the expressed proteins; however, the gp41 TM constructs were equally antigenic for both Abs. This suggests that the PDGFR-TM causes the occlusion of the 4E10 epitope or forces it into a less antigenic structure, and that the antigenicity of the 4E10 epitope is influenced by the TM region. A recent study by Brunel *et al.* suggests the importance of the orientation of the MPER helix on 4E10 binding to its epitope, thus it is possible that the correct orientation of the helix is supported by the gp41 TM region but not by the PDGFR-TM. The expression of 2F5 and 4E10 epitopes in heterologous viral scaffolds have been reported before, but to our knowledge, this is the first time that the antigenicity of the 4E10 epitope has been linked to the gp41 TM region. The MPER has been previously inserted into the Env of other retroviruses including SIV and HIV-2, with the objective of using the chimeric viruses for detecting neutralizing Abs to the MPER in sera from infected donors, in a scenario where the presence of other Nt Abs can be disregarded (56, 60, 281). In these studies, no differences were observed in the interaction of 4E10 with HIV-1 and the chimeric HIV-2 and SIV, but the TM regions of these viruses share approximately 80 % homology. We speculate that the homology between the TM of these three related viruses supports a similar 4E10-MPER interaction and it is the reason that the importance of the gp41 TM

region for 4E10 binding was not appreciated. In contrast, the TM regions of gp41 (HIV-1_{JR-CSF}) and PDGFR show only a 20 % homology. In our study, we do not see differences in Z13e1 reactivity associated with the TM region. In a recent study, Nelson *et al.* determined the CBRs for a Z13e1 Ab (optimized version of Z13); Z13e1 CBRs localize to the face of the helix opposite from the CBRs for 4E10. They also suggested that although 4E10 and Z13 epitopes share the same region on the MPER, divergent MPER residues affect their reactivity, residues important for Z13 are located N-terminal from the core epitope, while 4E10 important residues and a separate CBR Trp_{680} are located C-terminal from the core epitope (177). Thus the TM region does not necessarily influence the binding of all Abs against the MPER.

Han *et al.* generated 3 GST-fusion proteins, which consisted of the last C-terminal 30, 64, or 100 amino acids of the gp41 ectodomain from an M group consensus sequence (MCON6). The purified proteins were tested in ELISA with 2F5 and 4E10, and they found that the three recombinant proteins were equally recognized by 2F5, but 4E10 recognized GST-gp41-64 and -100 better than GST-gp41-30, suggesting that the 4E10 epitope is influenced by upstream sequences (CROI, 2007 meeting, poster 472).

The epitope of 2F5 was equally antigenic with the two different TM regions, indicating that it is less sensitive to modifications on that part of the protein. However, we observed differences in the reactivity of 2F5 with proteins of different sizes, whereas such an effect was not evident for 4E10. 2F5 interacts better with the MPER expressed by the shortest fusion proteins (pJW-MPER and pMPER-TM1). A possible explanation is that a larger region of the ectodomain expressed on the cell surface can mask parts of the MPER and affect the 2F5 epitope exposure. Alternatively, a larger ectodomain may

prevent the access of the Ab by steric hyndrance, however, this is not supported by studies that showed no increase in the Nt activity of the 2F5 and 4E10 Fab compared with the IgG, in which a decrease in the neutralizing activity was observed for both Abs when the Fab was used in neutralization assays (183). Since no gross difference in expression between the proteins tested was observed, our results with 2F5 reactivity might also indicate differences in structures adopted by MPER among the constructs, which in turn allow differences in structures adopted by the 2F5 epitope. It is possible that these differences are due to membrane presentation, since no difference in 2F5 reactivity was observed in the study of Han *et al.*, using recombinant proteins.

It is possible that a better recognition of the 4E10 epitope due to membrane presentation might influence its immunogenic properties; however, we are aware that is not possible to truly predict the immunogenic properties of individual MPER epitopes based only on their antigenic properties (109, 131, 135, 159). Different from antigenicity, which only depends on two molecular partners (Ab and epitope (Ag)), immunogenicity is a more complex parameter. The immunogenic character depends on a whole variety of complex interaction involving several components of the immune system, including host immunoglobulin gene repertoire, cytokines, and different cell types (B-cells, T-cells, APC), thus, it is hard to predict how immunogenic a protein can be.

The expressed gp41 fragments are immunogenic in rabbits as DNA vaccines (**Table 3-1, Figure 2-3**). All the constructs tested elicited Abs against the MPER, and the sera also cross-reacted with recombinant gp41. The highest gp41-reactive Ab titers were obtained with the vaccination with pJW-NHR, while pJW-MPER and pJW-CHR

produced lower titers. The pJW-NHR produces a larger protein compared with the other constructs; thus, its higher Ab response could be attributed to an increase in the immunogenic property of the protein as a function of the molecular weight (*i.e* epitope availability). Also, the larger protein has more T-cell epitopes ("gp160 T-Helper/CD4+ Epitope Map," (http://www.hiv.lanl.gov/content/immunology/maps/helper/gp160.html), which would help to elicit a stronger response. However, these higher titers of Ab do not necessarily indicate that pJW-NHR is our best candidate for targeting the MPER and/or elicitation of 2F5 and 4E10-like Nt MAbs; the quality (specificity) of the Ab response is also very important. We also showed the elicitation of a MPER specific Ab response by all the DNA constructs tested, using cell lysate ELISA, however this type of assay can not define the immunogenic epitopes within the MPER responsible for this Ab reactivity. Thus, to further characterize the nature of the Ab response produced by the different DNA constructs, we performed a serum epitope mapping. A 2F5 epitope crossreactive Abs were elicited for almost all the animals, indicating the immunogenic nature of the epitope using this approach.

We observed a correlation between the higher antigenicity for 2F5 observed with pJW-MPER, as compared to the other constructs, and its elicitation of 2F5 cross reactive Abs. It will be interesting to confirm whether the correlation between antigenicity and immunogenicty is reproducible, using a larger number of animals.

No Ab cross-reactivity with the 4E10 epitope peptide was observed using this mapping procedure. However, it is known that peptides can adopt different conformations, which can influence their interaction with Abs (248). In our experimental conditions, in which peptides are adsorbed to microwell plates, a significant portion of

their surface is unavailable for interaction with the Ab and thus, their antigenicity may be diminished. On the other hand, the majority of the Abs elicited in the polyclonal response after immunization are usually directed against discontinuous epitopes on proteins (13, 117), which are difficult to mimic or recapitulate by small linear peptides. Small peptides can either recreate continuous linear epitopes or linear fragments of a complex discontinuous epitope. The possible negative influence on the 4E10 peptide structure or residues availability determined by the peptide capturing procedure, was assessed by performing the serum mapping ELISA using biotinylated 4E10 epitope peptide captured on streptavidin (SA) coated plates, but no reactivity with the peptide was detected, suggesting that indeed, Ab against the 4E10 peptide were not produced at detectable levels.

The X-ray structure of the 4E10 Fab with a synthetic peptide (37) indicates that only one face of the peptide helix interacts with the Ab. Based on this structure, Brunel *et al.* designed peptides to optimize the interaction with the 4E10 Ab (27). They proposed that immunization with immunogens in which the non Nt face of the helix is occluded by a non-immunogenic bulk will allow the amplification of Abs more specific for the Nt epitope, however no immunization studies has been reported with such an Ag. Recently, in an effort to increase the antigenic characteristic of the 4E10 epitope Law *et al.* grafted the epitope into the V1/V2 loops of gp120 protein. Sequential deletion of amino acids (LWN) at the N-terminal of the epitope allowed the rotation of the helix, and the creation of an Ag with an increased affinity for the 4E10 Ab. Nevertheless, immunization studies in mice and rabbits with a plasmid which produced such an optimized antigen, followed by a protein boost, did not elicited any 4E10 peptide cross reactive Abs. The Ab

response elicited was directed against the gp120 protein, mainly the immunodominant epitope V3. Thus, an increase in the antigenicity of the 4E10 epitope did not correlated with elicitation of 4E10-like Ab. The structural complexity of the 4E10 epitope and its low immunogenicity are the probable determinants of the failure to elicit a cross-reactive Ab response to the epitope. Since gp41 protein goes through several conformational changes during the infection process, it is likely that the epitope for the 4E10 Ab is much more complex that the linear region known so far. It is also possible that 4E10 binding is extremely dependent on transitional structures of the MPER involving other regions of gp41, which are transiently exposed during infection.

The cluster I is a very conserved region among lentiviruses, and it is generally accepted that more than 98 % of the sera from HIV infected individuals have Ab reactivity with this region (42, 85, 86, 111, 176). MAbs with epitopes in the cluster I region has been isolated in several studies (16, 38, 51, 203). A high Ab response to the cluster I region has been reported after immunization of healthy humans volunteers with rgp160 (90, 249), and Ab to this region are prevalent in HIV-1^{+} sera (254). Surprisingly, in our study a much lower Ab response than expected was mapped to the cluster I epitope for the animals immunized with pJW-NHR. Although the native structure of gp41 in the viral spike is not known, it has been suggested that the interaction with gp120 imposes some structural constraints to gp41, so that the FP is not exposed and gp41 remains in a extended prefusogenic state. However, in the absence of gp120 and the FP (such as in cells expressing the NHR-MPER protein, the gp41 ectodomain could adopt the 6HB conformation and/or form oligomers, and in so doing expose the immunodominant region in a structure that is not the most significant for the induction of Ab response.

Conversely pJW-MPER and pJW-CHR elicit Abs crossreactive with the 2F5-binding site (peptide #6373) as seen in **Figures 3-5 and 3-6.**

The serum peptide mapping also revealed reactivity with a region of the MPER, peptide #6378 (aa 677-694 TNWLWYIKIFIMIVG), which is located C-terminal to the 4E10 core epitope. Intriguingly, an Ab response against this peptide was also observed in the highly-neutralizing FDA-2 serum (**Figure 3-11**), indicating the presence of an immunogenic epitope very close to the gp41 TM region. The third amino acid of this peptide is Trp_{680}, a residue that was recently reported as a new CBR for the recognition of the MPER and virus neutralization by the 4E10 Ab (27, 288). The relevance of an Ab response to this region could be interesting to explore.

An interesting finding of this study is the difference in the prevalence of the Ab response to the MPER depending on the antigen used. The presence of Ab to a specific epitopes is usually determined using synthetic peptides, in spite of the well-known limitations of this approach. We found that although a minor fraction of the Ab generated by our DNA constructs were cross-reactive with peptides corresponding to the 2F5 epitope peptide, the reactivity of the sera with the cell lysate expressing the MPER was higher (**Figure 3-2**). For these experiments we used the lysate from cells transfected with the pMPER-TM1 DNA construct. The binding of sera to the protein elicited by these constructs can only be due to its MPER reactivity and not other epitopes from gp41 (**see Figure 2-8**). This result indicates that peptide mapping alone can not truly reflect the prevalence of Ab response to the MPER. Therefore, the use of cell lysates expressing MPER could be a valuable tool for the analysis of MPER Ab response after immunization or even in natural infection. The cell lysate ELISA also revealed that the

Ab response to the MPER can be boosted by means of DNA immunization. In this study an increased response to the MPER was noticed after the final boost with the pJW-MPER DNA constructs.

The sera from immunized rabbits were tested for HIV-1 neutralization after the fourth and fifth DNA immunization (neutralization studies with bleed II, were not shown). No Nt activity was found in any of the sera tested (**Figures 3-21 to 3-27**, Appendix 2). Ab affinity-purified on 2F5 peptide, from rabbit sera were evaluated for Nt activity. However, we cannot rule out that the low level of Ab elicited is limiting the neutralization and that an increase of the Ab level by a protein boost increase the Nt specific response. Although presentation of the MPER on membrane has been considered important for elicitation of 2F5 and 4E10 MAbs, the immunization study of Grudner *et al.* where trimeric gp160 protein formulated in liposomes was used for immunization did not elicited any gp41 specific Nt Ab. Similarly, Lenz *et al.*, using trimeric gp41 embedded in liposomes did not elicit Nt immune response (133). These results suggest the membrane presentation is not the only requirement for the elicitation of 2F5- and 4E10-like Nt Abs, more likely the complete Nt epitopes have not been recreated by any of these studies. Thus, definition of the complete epitopes (*i.e.* structural features) will have a profound impact in immunogenicty results. Interestingly, a recent study from Ye *et al.* where a DNA immunization approach was also used for evaluating the Ab response to the MPER indicates that the structure adopted by gp41 is probably the determining factor in the production of a Nt Ab response. Ye and coworkers substituted the gp120 protein by the influenza virus HA domain with the rationality that a smaller protein can make the MPER accessible and at the same time keep its structure in a

conformation more similar to the natural one. Conversely, their immunogenicity study in mice elicited a Nt Ab response to the TCLA adapted isolate SF162 at very low dilution (278). In this study, low Nt titers were achieved using only DNA immunization. It will be interesting to see if similar or better results can be obtained in other animal models or whether Nt Ab to primary isolates can also be induced by this type of approach in a more efficient immunization strategy using a DNA prime/protein boost combination.

Our immunization study used a gp41 gene that was non-codon optimized for mammals, however an Ab response to the 2F5 epitope was mounted with the three different plasmids expressing the gp41 MPER. These results suggest the possibility that the codon usage optimized version of the gp41 gene may improve the expression *in vivo* and positively influence the immune response generated to the MPER. DNA immunization constitutes a very important emerging strategy for vaccine development (for review see (99)). The use of DNA as a vaccine has some advantages over direct protein immunization, since they are easy to produce and Abs elicited after DNA vaccination show broader neutralization activity as it has been shown in recent vaccination studies (131, 256). However, Ab responses generated after DNA immunizations are usually low and require a protein boost to increase the Ab levels.

Using DNA immunization, we show that an Ab to the MPER in the context of membrane can be mounted, and the elicited Abs crossreact with gp41 recombinant protein, 2F5 epitope peptide as well as the MPER derived proteins presented in the membrane context. However, the sera from immunized animals did not show Nt activity. Thus, the results presented here suggest the validity of this line of research and emphasizes the requirement of more effort directed to increase the Abs specificity and the

magnitude of the response. The use of molecular adjuvant together with the appropriate protein boost could be important tools to complement and enhance the Ab response to these MPER DNA constructs. We are currently developing these tools to be used in our next immunization study with the codon usage optimized gp41 constructs. The present situation of the global HIV-1 infection rates together with the available Nt studies with bNt Abs 2F5 and 4E10 highlight not only the necessity but also the great significance of the continuous effort in targeting the MPER for vaccine development.

Future studies

DNA vaccines have proven to behave different in different species. Usually more success has been obtained in small animal models, and only low immunogenicty has been reported for larger animals (9). Thus, new approaches are required to increase the immunogenicity of DNA vaccines. First a combination of DNA with a protein boost has been shown to significantly amplify Ab production (175, 202). This is an approach that can be explored in future studies. However, the use of protein boost like recombinant soluble protein can divert the original Ab response to a different one, since in this case, the MPER epitopes can be in a different conformation. An ideal protein boost for our study will be with gp41 fragment tethered to the plasma membrane *via* the gp41 TM region. Virus like particles (VLPs) may be the best choice for presenting this Ag. To make such VLPs the gene encoding the HIV-1 Gag and Env are produced from the cells in the absent of other viral proteins. In this case non-replicative particles are assembled that can be purified from culture supernatants (63, 279). The Scott laboratory already has an ongoing collaboration with Dr. T. Ross (University of Pittsburgh), who has made

VLPs using one of our DNA constructs (pMPER-CT). We are planning to do immunization experiments with a DNA prime/VLPs boost regimen.

Another approach used to increase the DNA vaccine immunogenicity is the addition of molecular adjuvants including cytokines, chemokines, co-stimulatory molecules (218), complement protein C3d, TLR agonist CpG (TLR9) and flagellin (TLR5). We are planning to use two different molecular adjuvants, the complement derived protein C3d and the bacterial protein flagellin. C3d is part of the complement cascade and it is the ligand for the B-cell co-receptor molecule CD21/CR2. C3d-mediate B-cell activation occurs as a consequence of simultaneous engagement of the Ag with the surface-specific immunoglobulin (B-cell receptor) and CD21 by antigen/C3d complexes. The adjuvant activity of C3d was originally reported for the HEL model (58). Dempsey and colleagues showed that inclusion of one, two or even three copies of the C3d increased the Ab response. C3d has increased the Ab response for several DNA vaccines when fused to pathogen proteins, including HIV-1, influenza, meales, bovine viral diarrhea virus (96, 204, 205, 255). It is believed that C3d increased the Ab response to the Ag by reducing the threshold required for activation of the B cell receptor (166). Flagellin protein is the TLR-5 ligand, which was previously reported to increase the Ab response to Ova when plasmids expressing the two proteins were used together in immunization studies (7). It is believed that flagellin can activate the immune system by targeting the Ag to the APCs, which carry the TLR-5. We constructed plasmids with the codon-optimized region of human and murine C3d, and have cloned the two coding regions into the pDisplay vector, fused to the PDGFRR-TM region. Similarly, we also amplified by PCR the coding region for the *fliC* gene of *Salmonella thyphimurium*

(SL4413) and cloned it into the pJW4303, fused to the gp41 TM1 region. Expression of the desired proteins after transient transfection of COS-7 cells with the three DNA constructs was detected by ELISA. Confocal microscopy studies have indicated that the proteins are displayed on the cell surface. Our goal is to incorporate C3d and flagellin proteins into VLPs. We also designed immunization protocols to test the constructs expressing flagellin and the murine C3d. Currently, we are performing DNA immunization studies in Xenomouse ™ animals (mice transgenic for the human Ab genes). Thus, it will be defined which one of these molecular adjuvants (C3d or flagellin) or their combination is more suitable for increasing the MPER antigenicty using DNA immunization or a DNA-priming/protein boosting combination.

Conclusions

In this study, DNA constructs expressions of the MPER tethered to cells membrane were produced. We showed that our constructs display the MPER on the cell surface and also that the elicited proteins were antigenic for the bNt MAbs 2F5 and 4E10. Differences were observed in the interaction of the MAbs with their epitopes, and probably were influenced by the presence of other domains besides the MPER in the recombinant proteins expressed on mammalian cells. The 2F5 MAb showed better reactivity to the proteins carrying only the MPER, and 4E10 binding to the MPER was affected by the presence of a heterologous TM region, that affected the membrane display of the MPER. In contrast, the gp41 TM was important for the interaction of the 4E10 Ab with the MPER in the context of the cell. This has not been described before, and it may be critical for presenting for presenting the 4E10 epitope in its native form. Finally we

used DNA immunization approach to evaluate the ability of three constructs to elicit Nt Abs against MPER. Our study indicates that higher Ab titers are required in order to reach Nt capacity, or that the desired Abs were not elicited with the evaluated constructs. Ultimately, our ability to induce Abs with the viral Nt activity of 2F5 and 4E10 will depend in the understanding of the structural and functional elements required for full recapitulation of the neutralization-sensitive MPER structure.

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