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Targeting a major HIV-1 vulnerability region: the gp41 Membrane Proximal External Region. Balance between neutralizing and non-neutralizing antibodies and implications for vaccine design

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hacen constar

que el trabajo experimental y la redacción de la memoria de la Tesis Doctoral titulada "*Targeting a major HIV-1 vulnerability region: the gp41 Membrane Proximal External Region. Balance between neutralizing and non-neutralizing antibodies and implications for vaccine design*" han sido realizados bajo su dirección por
LUIS MANUEL MOLINOS ALBERT y

consideran

que la memoria resultante es apta para optar al grado de Doctor en Inmunología por la Universitat Autònoma de Barcelona.

Y para que quede constancia, firman el presente documento en Badalona,
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Dr. Jorge Carrillo Molina

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A mis padres, por la paciencia

Me lo contaron y lo olvidé,

lo vi y lo entendí,

lo hice y lo aprendí.

-Confucio-

CONTENTS

Abbreviations

ADCC	Antibody-Dependent Cellular Cytotoxicity
AID	Activation-Induced cytidine Deaminase
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cell
AZT	Zidovudine
bNAb	Brodaly Neutralizing Antibody
CCR5	Chemokine Receptor 5 (CD195)
CDC	Centers for Disease Control
CDR H3	Heavy Chain Complementary Determinant Region 3
CHOL	Cholesterol
CRAC	Cholesterol Rich Aminoacid Region
CRF	Circulating Recombinant Form
CT	Gp41 Cytoplasmic Tail
CXCR4	CX Chemokine Receptor 4 (CD184)
DC	Dendritic Cell
DNA	Desoxirribonucleic Acid
DSL	Gp41 Disulfide Loop
ELISA	Enzyme-Linked Immunosorbent Assay
Env	HIV-1 Envelope Glycoprotein
Fab	Fragment Antigen-Binding
FDA	Food and Drug Administration
FP	Gp41 Fusion Peptide
FWR	Framework Region
GALT	Gut Associated Lymphoid Tissue
GFP	Green Fluorescent Protein
GM3	Monosialodihexosyl Ganglioside
Gp120	Glycoprotein 120
Gp160	Glycoprotein 160
Gp41	Glycoprotein 41
HA	Hemagglutinin
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus Type 1
HIV-2	Human Immunodeficiency Virus Type 2
HR1	Gp41 N-terminal Heptad Repeat
HR2	Gp41 C-terminal Heptad Repeat
HTLV	Human T-Cell Lymphotropic Viruses
IC50	Inhibitory Concentration 50
IgG	Immunoglobulin G

KYNU	Kynureninase
LAV	Lymphadenopathy-Associated Virus
LTR	Long Terminal Repeat
LUV	Large Unilamellar Vesicle
Mab	Monoclonal Antibody
MPER	Gp41 Membrane Proximal External Region
MPLA	Monophosphoryl Lipid A
mRNA	Messenger RNA
Nab	Neutralizing Antibody
NHP	Non-Human Primate
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
POPC	1-Palmitoyl-2-Oleoylphosphatidylcholine
PS	Phosphatidylserine
RNA	Ribonucleic Acid
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electroforesis
SF3B3	Splicing Factor 3b Subunit 3
SHIV	Simian-Human Immunodeficiency Virus
SHM	Somatic Hypermutation
SIV	Simian Immunodeficiency Virus
SM	Sphingomyelin
TLR	Toll-Like Receptor
TM	Gp41 Transmembrane Domain
TT	Tetanus Toxoid
UCA	Unmutated Common Ancestor
VHH	Variable Domain Heavy Chain Antibody
VL	Viral Load

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The elicitation of broadly neutralizing antibodies (bNAbs) against HIV-1 constitutes the major goal of an effective vaccine able to control the epidemic caused by this virus. The HIV-1 envelope glycoprotein is the only viral antigen exposed on the surface of the virus and the main target of protective humoral responses. The identification within this protein of functional regions recognized by bNAbs has delineated an HIV-1 vulnerability map that has guided efforts for rational immunogen design. In this regard, the gp41 Membrane Proximal External Region (MPER) is a major HIV-1 vulnerability site because is highly conserved, plays a major role in viral infectivity and is targeted by protective bNAbs. However, its localization, next to the viral membrane, results in a hardly accessible, transiently exposed and hydrophobic domain, strongly influenced by membrane lipids. Therefore, the immunogenicity of the MPER offers a high level of complexity that needs to be further explored.

In this thesis we provide new knowledge on the MPER immunogenicity in both natural HIV-1 infection and immunization in animal models. We generated gp41-based miniproteins which properly exposed the MPER region that have been used 1) as novel platforms for MPER antibody detection and 2) as immunogen candidates presented in different proteoliposome compositions. In humans, the results revealed a strong immunogenicity of the MPER that correlated with a global response against the envelope glycoprotein suggesting no special constraints for the immune system to target this region. However, the antibodies elicited showed heterogeneous functionality in terms of neutralizing capacity and epitope competition, regardless MPER specificity. Interestingly, these results were reproduced by immunization in animal models. High antibody titers were achieved that were specially enhanced by the addition of lipid mixtures mimicking the viral membrane. Interestingly, a non-neutralizing immunodominant response against an epitope that overlapped the 2F5 neutralizing antibody binding motif was identified. Overall, the anti-MPER response in both humans and animal model settings was not correlated with the neutralizing capacity and antibodies detected or induced by immunization were preferentially non-neutralizing.

Our results suggest that the balance between neutralizing and non neutralizing responses may represent an important issue in the global response against MPER. Therefore, further redesign of immunogens able to skip non-neutralizing determinants will benefit from the knowledge derived from new anti-MPER antibodies that reflect the functional heterogeneous profile observed in our studies.

La generación de anticuerpos ampliamente neutralizantes contra el VIH-1 es el principal objetivo de una vacuna que sea capaz de controlar la epidemia causada por el virus. La glicoproteína de la envuelta del VIH-1 es el único antígeno viral expuesto en la membrana del virus y la principal diana de respuestas humorales protectoras. Dentro de esta proteína se han identificado regiones funcionales que son reconocidas por anticuerpos ampliamente neutralizantes, lo cual ha permitido establecer puntos de vulnerabilidad del virus hacia los que una vacuna debería dirigirse. En este contexto, la región externa próxima a la membrana (MPER) de gp41 es uno de los sitios de vulnerabilidad del virus más representativos, ya que está altamente conservado, juega un papel crucial en la infectividad del virus y es reconocido por anticuerpos protectores ampliamente neutralizantes. Sin embargo, su localización, contigua a la membrana, hace del MPER un dominio poco accesible, expuesto de forma transitoria, hidrofóbico y altamente influenciado por lípidos de membrana. En consecuencia, su inmunogenicidad presenta una alta complejidad que debe ser explorada en mayor profundidad.

En esta tesis, aportamos nuevo conocimiento sobre la inmunogenicidad del MPER a nivel de la infección natural y en modelos animales de inmunización. Hemos evaluado miniproteínas basadas en gp41 que sobreexponen el MPER, para ser utilizadas como 1) nuevas plataformas para la detección de anticuerpos anti-MPER y 2) prototipos de inmunógenos presentados en proteoliposomas de diversa composición. Los resultados han revelado una alta inmunogenicidad del MPER en humanos, que correlaciona con la respuesta global contra la proteína de la envuelta. Esto sugiere que el sistema inmune no tiene una especial restricción para la generación de este tipo de anticuerpos. Sin embargo, los anticuerpos detectados mostraron una funcionalidad heterogénea, en términos de capacidad neutralizante y competición por diferentes epítopos, independientemente de la especificidad por el MPER. Además, los resultados fueron reproducidos en animales inmunizados. En estos últimos, conseguimos generar un alto título de anticuerpos específicos, potenciados por la incorporación en los proteoliposomas de mezclas lipídicas complejas que mimetizaban las de la partícula viral. Sorprendentemente, se generó una respuesta inmunodominante no neutralizante que solapaba con un epítopo reconocido por el anticuerpo neutralizante 2F5. En resumen, la repuesta humoral natural contra el MPER en pacientes infectados por el VIH-1 y la generada en modelos animales mediante inmunización comparten ciertas características como son la especificidad y la escasa capacidad neutralizante.

Estos resultados sugieren que el balance entre la respuesta neutralizante y no neutralizante podría tener una importante relevancia en la respuesta global contra el MPER. De este modo, es necesario un mayor refinamiento de inmunógenos que sean capaces de sortear respuestas no neutralizantes. Este rediseño se verá altamente beneficiado del conocimiento generado a partir de nuevos anticuerpos monoclonales contra el MPER que reflejen la heterogeneidad funcional observada en nuestros estudios.

La generació d'anticossos àmpliament neutralitzants contra el VIH-1 es el principal objectiu d'una vacuna que sigui capaç de controlar l'epidèmia causada per aquest virus. La glicoproteïna de l'embolcall del VIH-1 es l'únic antigen viral exposat a la membrana del virus y la principal diana de respostes humorals protectores. Dins d'aquesta proteïna han sigut identificades regions funcionals reconegudes per anticossos àmpliament neutralitzants, fent possible la definició de punts de vulnerabilitat del virus, cap als que una vacuna hauria de dirigir-se. En aquest context, la regió externa pròxima a la membrana (MPER) de la gp41 es un dels llocs de vulnerabilitat més representatius del virus, ja que està altament conservat, té un paper crucial per a la infectivitat del virus i es reconegut per anticossos protectors àmpliament neutralitzants. Tot i així, la seva situació, prop a la membrana, fa que el MPER sigui un domini poc accessible, exposat de manera transitòria, hidrofòbic i altament influenciat pels lípids de la membrana. Per tant, la seva immunogenicitat presenta una alta complexitat que ha de ser explorada en major profunditat.

En aquesta tesi, aportem nou coneixement sobre l'immunogenicitat del MPER durant l´infecció natural i en models animals d'immunització. S'han avaluat miniproteïnes derivades de la gp41 que sobreexposen el MPER, per ser utilitzades com 1) nous plataformes per a la detecció d'anticossos anti-MPER i 2) prototipus d'immunògens presentats en proteoliposomes de diversa composició. Els resultats van revelar una alta immunogenicitat del MPER en humans, que correlaciona amb la resposta global contra la proteïna de l'embolcall, suggerint que el sistema immune no té cap restricció per a la generació d'aquest tipus d'anticossos. Tot i així, els anticossos detectats van mostrar una funcionalitat heterogènia, tant pel que fa a la capacitat neutralitzant com a la competició per diferents epítops, independentment de l'especificitat per MPER. A més, aquest resultats van ser reproduïts en animals immunitzats. En aquest últims, s'ha aconseguit generar un alt títol d'anticossos específics, que van ser potenciats per la incorporació als proteoliposomes de lípids complexes que mimetitzaven als de la partícula viral. Sorprenentment, es va generar una resposta immunodominant no neutralitzant contra un epítop solapant amb el de l'anticòs neutralitzant 2F5. En resum, la resposta humoral contra el MPER a humans i animals immunitzats no va correlacionar amb la capacitat neutralizant d'aquests i els anticossos generats són principalment no neutralitzants.

Aquests resultats suggereixen que el balanç de la resposta neutralitzant i no neutralitzant té una important rellevància en la resposta global contra el MPER. Conseqüentment, sembla necessari un major refinament d'immunògens capaços d'evitar respostes no neutralitzants. Aquest redisseny es veurà altament beneficiat pel coneixement generat a partir de nous anticossos monoclonals contra el MPER que recullin l'heterogeneïtat funcional observada als nostres estudis.

CHAPTER 1

Introduction

1. The virus, the pandemic and of the quest for a vaccine

1.1. **Changing the paradigm.** From the empiricism to the rational vaccine design

"Chance favors the prepared mind". This aphorism, attributed to Louis Pasteur, illustrates how eventually certain observations by some intuitive minds resulted in discoveries that have determined the progress of humanity. The origins of vaccination can be traced back in the Ancient Chinese for smallpox prevention. The observation that prior smallpox disease protected from a subsequent exposure led to the practice of insufflation of variola scabs for preventive purposes. In the 18th century, the awareness of those ancient practices conducted to the first vaccine investigations. In 1796, Edward Jenner observed that milkmaids previously exposed to cowpox rarely were sickened of smallpox disease. This observation led him to perform the first vaccine clinical trial. Jenner demonstrated scientifically that inoculation of cowpox scabs in healthy individuals could in fact protect against smallpox infection upon challenge with virulent virus (1). 183 years later, Jenner´s discoveries led to the eradication of smallpox in 1979 by a global vaccination strategy (2). This historic milestone represented the triumph of humanity against an infectious agent, initially based on a simple observation.

During the last quarter of the 19th century, subsequent discoveries by Louis Pasteur, Robert Koch, Paul Ehrlich or Emil von Behring contributed enormously to our knowledge of microbiology, immunology and vaccinology disciplines, expanding

enormously the rudimentary first steps advanced by Jenner, who was not aware of the microbial etiology of infections. Consequently, vaccines against different pathogens were developed thereafter. Besides clean water, the implementation of vaccination in healthcare systems was the most cost-effective intervention in human health history for controlling many fatal infectious diseases.(3).

Classic vaccines were developed following empirical approaches by direct mimicry of the organism or toxin causing the disease, usually as attenuated or inactivated form (3). Starting in late 1970, new technologies were developed, bypassing the classic conception of vaccinology and contributing to the development of novel vaccines. Those included glycoconjugate vaccines (*H influenzae b)*, recombinant DNA technology (HBV); genome-based reverse vaccinology (*N. meningitides* type B); or the use of molecular adjuvants as linkages of adaptive and innate immune responses (4). In spite of the great achievements in vaccinology during the 20th century, there are still some devastating diseases such as malaria, tuberculosis or HIV-1 infections for which attempts for generating effective vaccines remain elusive. These pathogens display an unprecedented complexity and classic vaccine approaches have not been successful in providing protection. In these cases, further development of rational-based vaccine concepts offers a great promise for advancing towards improved vaccines.

1.2. **The Human Immunodeficiency Virus**. A worldwide health issue

1.2.1. HIV and AIDS

Only two years after smallpox was officially eradicated, an emerging epidemic would determine the life of millions of people throughout the world for the next years. In 1981, the Centers for Disease Control (CDC) identified the first cases of the Acquired Immunodeficiency Syndrome (AIDS) in individuals with unusual opportunistic infections such as *Pneumocystis carinii* (now *Pneumocystis jirovecii*). Shortly after, those cases were found to be associated with a low CD4 T-cell count (5–7). In 1983, at the Pasteur Institute, one retrovirus was isolated from a patient lymph node. The virus was initially related with the Human T-cell lymphotropic viruses (HTLV-1 and HTLV-2). However, further investigations revealed that this new virus had distinct properties such as CD4 T-cell killing and high growth in CD4 T-cells (8). Within the same year, those findings were published in Science magazine and the

causative agent of AIDS was described as a lentivirus and designed as Lymphadenopathy-Associated Virus (LAV) (9). Further studies in North America confirmed that LAV was indeed the causative agent of AIDS, and lately was renamed as Human Immunodeficiency Virus (HIV) (10).

1.2.2. The pandemic

HIV originates from multiple zoonosis of the simian immunodeficiency virus (SIV) in non-human primates (NHP) into humans in West and Central Africa (11). This cross-species transmission was probably due to primate manipulation during haunting, butchering and trading processes (11). Although it was identified in early 80s, the earliest evidence of HIV infection in humans was found in stored serum and lymph node samples in 1959 and 1960 respectively in Kinsasha, Democratic Republic of Congo (12). These samples have been useful to estimate the most recent common ancestors and to trace the evolutionary rates of different HIV lineages (13, 14).

The AIDS pandemic constitutes a dramatic public health issue, especially in developing countries where over 95% people infected with HIV live. Since the identification of the causative agent of AIDS, more than 60 million people have become infected with HIV, with 30 millions of deaths (15). Life expectancy has become considerably reduced in certain areas of the world especially in sub-saharan Africa, where the epidemic reached dramatic levels (16). According with the UNAIDS report, at the end of 2014 over 36.9 millions of people were living with HIV infection and 22 million need to be reached with ART; 1.2 million people died of AIDS-related diseases and there were 2 millions of new infections. Just 15 countries accounted for the 75% of these new infections (15).

After the approval by the FDA of zidovudine (AZT) as the first anti-HIV drug in 1987, significant advances have been made in the development of antiretroviral treatment (ART) and more than 25 different antiretroviral drugs have been approved (17). Starting in 1995, the combination of drugs blocking different steps of the viral cycle, often refereed as highly active antiretroviral therapy (HAART or ART), was a major breakthrough in the HIV history (17). It changed the perception of the disease from being a rapid deadly infection to a chronic disease. Global efforts for universal HAART coverage have been implemented. In 2000, less than 1% of people living in developing countries had access to HAART. In 2014, the global HAART coverage was

40% (15). This is a crucial intervention for controlling the pandemic, not only to improve life expectancy but also to avoid future transmissions.

1.2.3. HIV diversity

Since the discovery of HIV as the causative agent of AIDS, two related but divergent types of virus have been identified: HIV-1 and HIV-2, being the former the one isolated in 1983 and the responsible for the pandemic. HIV-2, although can also cause AIDS, is a less easily transmitted and a less pathogenic virus mainly located in West Africa (14). Phylogenetically, HIV-1 can be divided in groups M (main), N (new) and O (outliers). The M group is the most prevalent and comprises the majority of circulating HIV-1 isolates. The HIV-1 M group can be divided in 9 genetically different subtypes or clades (A, B, C, D, F, G, H, J, K) presenting a genetic variation of 15-20% within subtype and 25-35% between subtypes. Whereas subtype B is the most prevalent in Europe and America, subtype C accounts for the great majority of infections worldwide, as it is present in Africa and India. Moreover, interclade combinations result in a great number of circulating recombinant forms (CRFs) mainly present in Southeast Asia and Central Africa (14, 18) **(Figure 1).** Additionally, in 2009 a gorilla-related Simian Immunodeficiency Virus (SIV) strain was discovered in Cameroon, and was designated group P (Pending) (19).

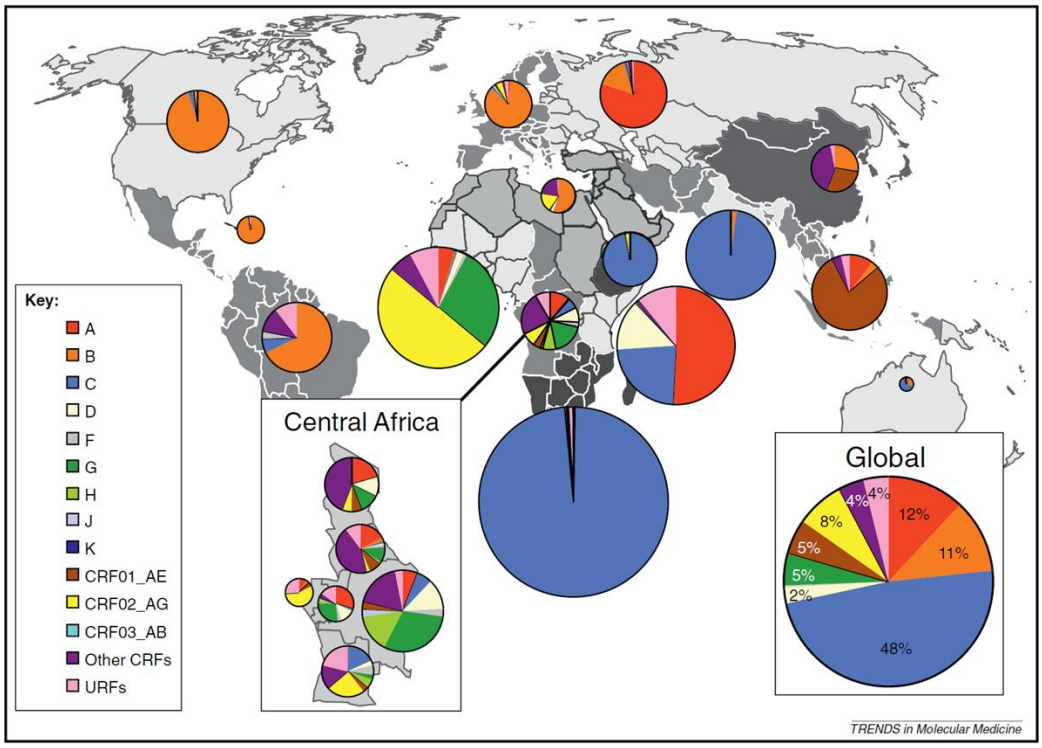

Figure 1 *(Legend on next page)*

(Figure on previous page) **Figure 1. Prevalence and global distribution of the different HIV-1 subtypes and Circulating Recombinant Forms (CRFs).** Pie charts of the main figure indicate the geographical distribution of HIV-1 subtypes and CRFs from 2004-2007. Pie chart size is proportional to the prevalence of people living with HIV-1 in the region. HIV-1 subtype distribution around the world and Central Africa are indicated in the inset figures. Color key identifying HIV-1 subtype or CRFs is indicated in the legend on the left-hand side. *Reproduced from Hemelaar, J., 2012* (14)

1.2.4. The HIV-1 life cycle

The HIV-1 genome comprises nine genes encoding for 15 proteins. Includes the genes *env* (envelope glycoprotein); *gag* (structural nucleocapsid) and *pol* (functional enzymes retrotranscriptase (RT), protease (P) and integrase (I)), common to all retroviruses. Additionally the virus presents a set of genes encoding for different accessory proteins (20) **(Figure 2).**

The HIV-1 lifecycle is a complex interplay between viral and host proteins that begins when the gp120 subunit of the envelope glycoprotein attaches to the CD4 receptor and CCR5 or CXCR4 correceptor, on the surface of target cells, triggering the exposure of the internal gp41 subunit that ultimately mediates membrane fusion (21–26). A detailed description of the HIV-1 fusion process is described in **Section 2.1**. Once the viral content is released into the cytoplasm, single stranded RNA is retrotranscribed to double stranded DNA by the RT enzyme. Subsequently, viral integrase catalyzes the integration of viral DNA into the cell genome by the long terminal repeats (LTR) regions (27). The early transcripts encode for the accessory proteins nef, rev and tat.

Translation of partially spliced mRNA produces the polypeptides Env, Gag and Pol. The final steps of the viral replication process occur in the viral membrane. After cleaved by cellular proteases, envelope glycoproteins remain associated and anchored into the cellular membrane. Gag forms the nucleocapsids and recruits viral RNA and cellular proteins required for viral budding and newly assembled virions are released (20). Then, the viral protease facilitates the immature to mature step of the virion by processing polypeptides resulting in the viral nucleocapsid and the enzymes retrotranscriptase, protease and integrase (20)

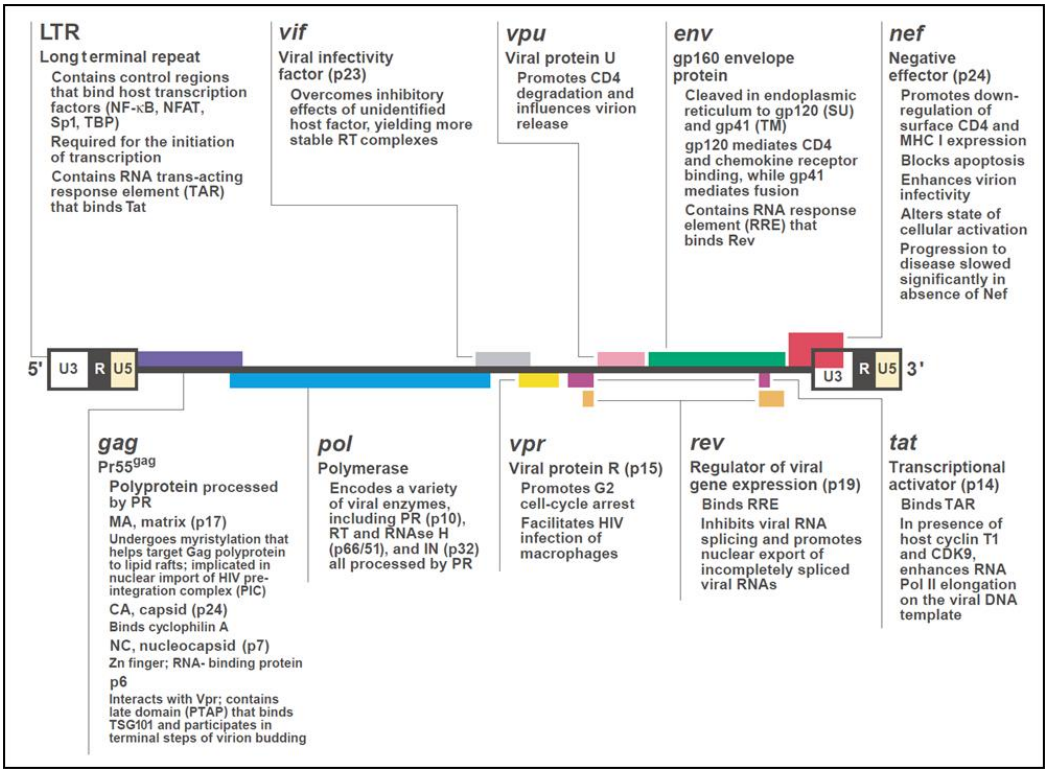

Figure 2. **The HIV-1 genome.** Schematic representation of the genome of the HIV-1 provirus and description of the nine genes encoding for viral proteins. *Reproduced from Greene, W. C., 2002* (20)

However, the infectious process *in vivo* is much more complex. HIV-1 can be acquired by blood contact, vertical transmission or through mucosal barriers by microabrasions or transcytosis, reaching quickly dendritic cells and CD4+ T-cells in the lamina propia (28). After local expansion, the virus reaches the draining lymph nodes and then the bloodstream to disseminate towards secondary lymphoid organs **(Figure 3)**. During this initial step, there is a preference for the CD4+ T-cells located in the gut associated lymphoid tissue (GALT), which is massively colonized and destroyed (29). During the process of viral dissemination, the impact of cell-associated virus transmission (cell-to-cell transmission) was estimated to be 100-1000 times more efficient in establishing new infections comparing with cell-free virions. Accordingly, this mechanism has been proposed to be the main contributor to viral spread (30).

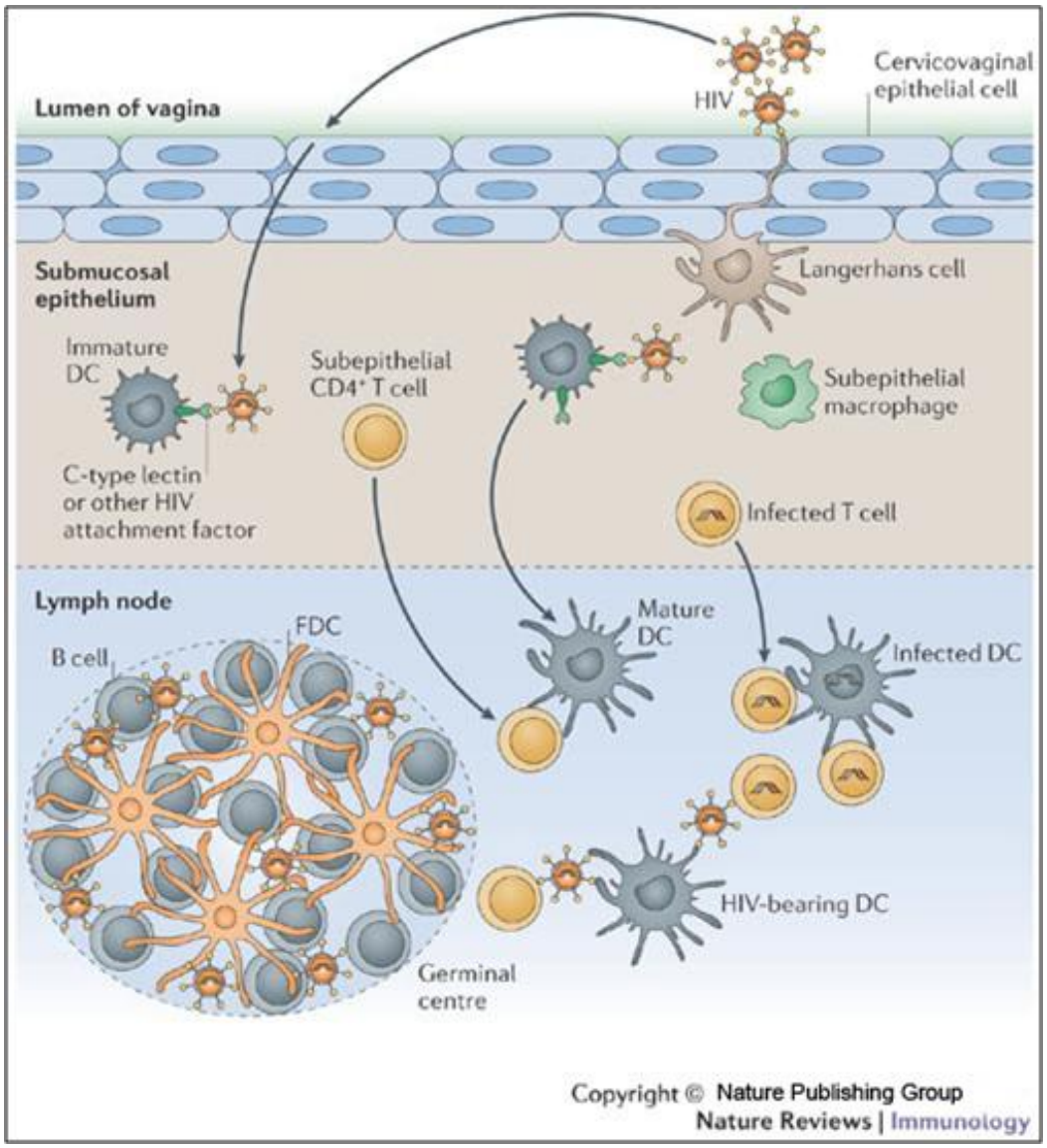

Figure 3. Initial steps of HIV-1 transmission through mucosal barriers. At mucosal surfaces, dendritic cells (DCs) are probably the main target to interact with the virus. DCs capture the virus by c-type lectin attachment and migrate to lymphoid tissue. Here, HIV-1 *trans*-infection to activated CD4+ T-cells occurs, facilitating viral dissemination. *Reproduced from Wu&Kewalramani, 2006* (31)

1.3. Lessons learned from HIV-1 vaccine clinical trials

A remarkable effort has been made to slow down the HIV-1 spread by different prevention strategies over the years. Those include educational programs that promote effective condom use, pre-exposure prophylaxis interventions (PrEP), tenofovir-based vaginal microbicides, prevention of mother-to-child transmission, harm reduction in injecting drug users and improving accessibility to antiretroviral treatment (32–34). A preventative HIV-1 vaccine will provide a cost-effective tool to such prevention initiatives.

To date, four vaccine concepts have been explored in six phase IIb or III efficacy trials (35) **(**detailed in **Figure 4)**. The AIDSVAX® B/B or B/E was a bivalent gp120-based candidate that was tested in the Vax004 and Vax003 trials, as a certain protection was observed in immunized chimpanzees against intravenous HIV-1 challenge. However, this candidate did not prevent HIV-1 acquisition (36, 37). Due to the difficulties found for eliciting protective humoral responses, research on HIV-1 vaccines was turned toward the cellular immunity. Accordingly, the Step (HTVN 502) and Phambili (HTVN 503) trials performed in the USA and South Africa, respectively (38) explored the induction of cellular responses as correlate of protection. The vaccine candidate evaluated was the Merck recombinant Ad5 vector expressing HIV-1 clade B antigens (MRCKAd5 HIV-1 *gag/pol/nef* B). The Step trial and, short after, the Phambili trial were stopped prematurely for futility (38). In both trials an increase of the infection rate in vaccinees was reported. This effect was associated with a preexisting immunity to the adenovirus vector and thus a major immune activation in vaccine recipients. The outcomes of the Step trial were recapitulated in Indian rhesus macaque models (39).

The RV144 vaccine trial (40) performed in Thailand, was a second big study, enrolling more than 15000 heterosexual individuals, at low risk of infection. This trial aimed to generate an improved CD4+ T-cell response that would enhance host defenses, by combining a B-cell immunogen AIDSVAX B/E envelope gp120 and the CD4+T cell-stimulating ALVAC canarypox vaccine. The sponsoring of the U.S government for this trial was questioned by a group of investigators arguing that the evidence supporting the vaccination strategy proposed for the trial was extremely weak (41). The RV144 trial was performed finally in 2004.

The HIV-1 incidence in the placebo group was 0.28 infections per 100 persons-year, ten-fold lower than previous efficacy trials. The estimated vaccine efficacy at 42

months, by using the modified intention to treat analyses was 31.2% of protection (40). This trial represented an opportunity to define immune correlates of protection in humans. The major findings encountered included production of specific CD4+T cells and antibody-dependent cell-mediated cytotoxicity (ADCC). Neutralizing antibodies detected were only effective against sensitive HIV-1 strains (42). Furthermore, robust assays identified two strong correlates with infection risk: i) IgG against the V1/V2 loop region of gp120 envelope domain was inversely correlated with infection risk and ii) high plasma concentrations of IgA antibody to HIV-1 envelope glycoprotein were directly correlated with acquisition of infection (43). Neutralizing responses against sensitive virus were inversely correlated with infection risk only in the setting of low level of Env-specific IgA (42). The outcomes of the RV144 trial pointed the importance of non-neutralizing antibody effector mechanisms for protection like antibody-dependent cell-mediated cytotoxicity or antibody-mediated phagocytosis (44)

Study	Vaccines	Phase	Volunteers' risk	HIV incidence per 100 person-years	Location	Result
Vax004	AIDSVAX® B/B gp120 in alum	III	MSM and women at high risk	2.6%	United States, Europe	No efficacy
Vax003	AIDSVAX® B/E gp120 in alum	III	Injecting drug users	3.4%	Thailand	No efficacy
HVTN 502 Step trial	MRKAd5 HIV-1 gag/pol/nef B	IIb	MSM and heterosexual women and men	3.0%	United States	No efficacy; transient increased infection rate in vaccinees
RV144	ALVAC-HIV (vCP1521) and AIDSVAX® B/E rgp120 in alum	III	Community-risk population	0.28%	Thailand	31.2% efficacy at 42 months, 60% at 12 months against HIV acquisition. No effect on plasma viral load and CD4 count
HVTN 503 Phambili trial	MRKAd5 HIV-1 gag/pol/nef B	IIb	Heterosexual men and women	3.7% Placebos 4.54% Vaccinees	Republic of South Africa	No efficacy; increased HIV infection rate in vaccinees
HVTN 505	DNA and rAd5 (A, B, and C)	IIb	MSM with Ad5-specific antibody titers <1:18 (negative)	0.018%	United States	Stopped for futility; no efficacy on HIV acquisition, plasma viral load and CD4 count

Figure 4. Vaccine efficacy trials. Overview of the main human preventive HIV-1 vaccine human trials. *Reproduced from Kim J.H., 2014* (44)

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2. Targeting HIV-1 vulnerability regions. The Envelope Glycoprotein (Env) as target of Broadly Neutralizing Antibodies

2.1. **The target.** Structural features and function of the HIV-1 Env

The HIV-1 envelope glycoprotein (Env) is the sole viral antigen displayed on the viral surface and mediates viral entry. It is, therefore, the major target of humoral neutralizing responses and it is presumed that antibodies targeting certain functional regions of Env could block viral entry and potentially protect upon infection (45–47). Env is constituted by the non-covalent association of 3 gp120 (surface) with 3 gp41 (transmembrane) subunits, resulting in a heavily glycosylated trimer of heterodimers (48–52) **(Figure 5A-C).** Initially, translation of *env* mRNA in the endoplasmic reticulum generates an uncleaved gp160 polypeptide precursor which is transported through the Golgi apparatus where glycosylation by high mannose sugars takes place. After cleaved by furin proteases, gp120 and gp41 glycoproteins are generated (47, 53). Fully cleaved Env proteins are displayed on the cell membrane and then incorporated during the budding process to virions that are released as new infectious viral particles (20).

The HIV-1 envelope glycoprotein trimer exhibits a similar architecture to that of influenza hemagglutinin (HA) protein, which is a prototypic type I membrane-fusion protein (54, 55). The highly conserved HIV-1 Env fusion machinery corresponds to

gp41 which is anchored to the membrane, whereas the surface head gp120 is more variable, distal to the membrane and contains the receptor (CD4) and coreceptor (CCR5 or CXCR5) binding sites (56, 57). According with sequence analysis, gp120 is divided in five variable regions (hypervariable loops, V1-V5) and five conserved regions (C1-C5) (58, 59). Gp120 shows a higher level of variability than gp41, probably because gp41 houses the hydrophobic fusion machinery that is less exposed to antibody pressure. The gp41 ectodomain is composed by an N-terminal hydrophobic fusion peptide (FP), two alpha-helical repeat domains (HR1 and HR2) separated by an immunodominant disulfide loop (DSL). Gp41 is connected to the membrane by the tryptophan-rich membrane proximal external region (MPER) which connects the HR2 with the transmembrane domain (TM), which is followed by an exceptionally long cytoplasmic tail (CT), in comparison with other retroviruses, that modulates the conformation of external domains of Env and its fusogenicity (25, 60, 61). As the main target of study of this thesis, the MPER region is further reviewed in **Section 3**.

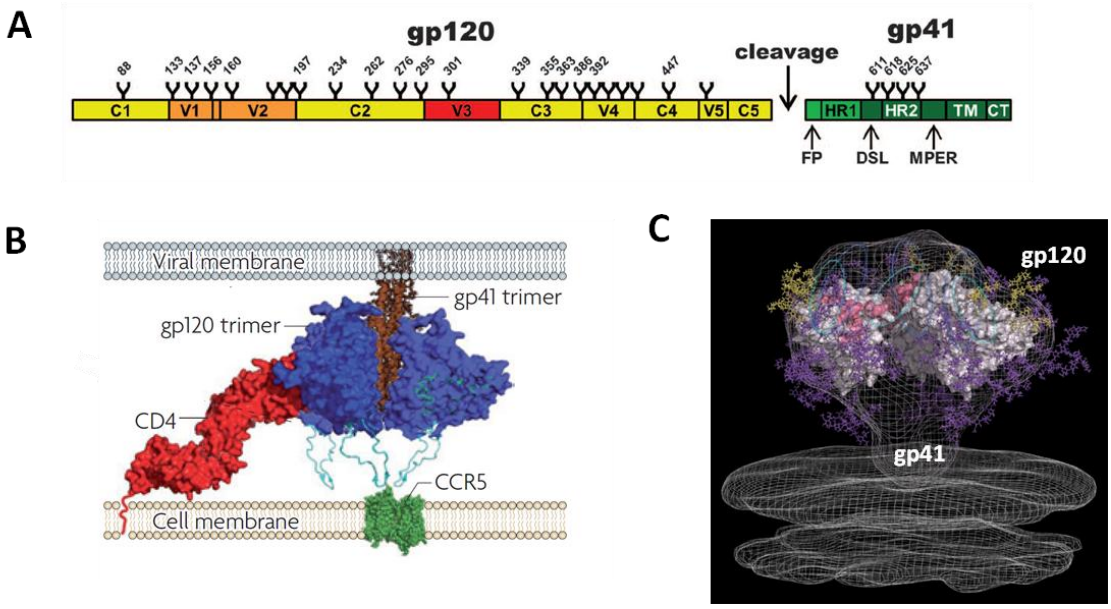

Figure 5. The HIV-1 Envelope glycoprotein. **A.** Schematic representation of the HIV-1 Env gp120 and gp41 subunits after gp160 cleavage. Gp120 (constant, C1-C5, and variable, V1-V2, loops) and gp41 (FP, HR1 and HR2 helices, DSL, MPER, TM domain and CT*)* regions are indicated. N-linked glycosilation sites are indicated and numbered. *Reproduced from Julien J.P., et al 2013* (56). **B**. Structure-based model of the HIV-1 Env in complex with the CD4 and CCR5 receptor and coreceptor, respectively. Gp120 and Gp41 are depicted. *Adapted from Karlsson G., 2008.*(62) **C.** Structural diagram of Env surrounded by the glycan shield. *Adapted from Burton&Weiss, 2010* (63)

The immediate process before HIV-1 entry into the target cell starts with the fusion of the viral and host cell membranes, mediated by Env (64) **(Figure 6)**. The process initiates with the high affinity interplay between gp120 and the CD4 molecule on the surface of target cells. This interaction promotes a series of conformational changes that transiently exposes the gp120 coreceptor binding site (V3 loop), allowing the attachment to CCR5 or CXCR4 chemokine receptors (21, 65, 66). Coreceptor ligation triggers structural rearrangements in gp41 that permit the initiation of viral fusion. In a first step, the gp41 fusion peptide inserts into the target cell membrane accounting for a short-life prehairpin fusion intermediate in which both cellular and viral membranes are connected by an extended conformation of gp41. Next, alpha-helical domains HR1 and HR2 of each gp41 monomer are folded back together to generate a 6-helix bundle conformation that brings both target cell and viral membranes closer to finally produce the membrane merge (67, 68), where the FP and the MPER play a relevant role in membrane destabilization (69) **(Figure 6.A).** Subsequent virus-cell or cell-to-cell membrane fusion encompasses a number of steps including lipid mixing, the opening of a small pore and a final expansion of the fusion pore that enables the transfer of the viral nucleocapsid into the cytoplasm **(Figure 6.B)**. When a CD4+ infected T-cell contacts a non-infected one, the outer leaflets of their lipid bilayers may fuse (hemifusion) without progression to the fusion pore formation. If the complete fusion process is not accomplished, it forces the target cell to undergo apoptosis (70, 71)

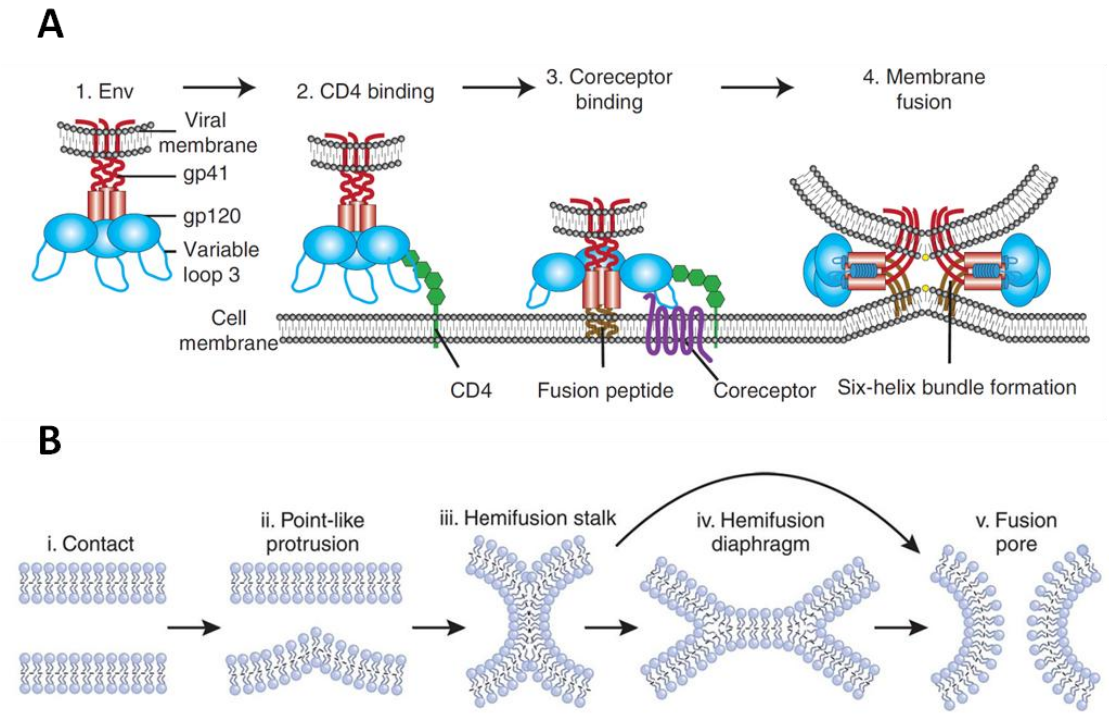

Figure 6. HIV-1 entry to the target cell . A. Overview of the HIV-1 entry into the target cell, mediated by Env [1]. Gp120 attaches to the CD4 receptor [2], inducing conformational changes that permit coreceptor binding, which triggers the initiation of the fusion process by gp41. In a prehairpin intermediate conformation of gp41, the fusion peptide anchors into the target cell [3] and membrane fusion is completed after the 6-helix bundle formation brings both membranes together [4]. *Reproduced from Wilen C.B., et al., 2012* (72) **B.** Lipid rearrangements during the fusion process**.** First, both membranes establish a contact site (i). A point-like protrusion contributes to diminish the energy required to minimize the hydration repulsion, coming into immediate contact (ii). Transient displacement of polar head groups leads to the formation of a hydrophobic patch. These hydrophobic patches attract each other resulting in a hemifusion stalk (iii), which expands and yields the hemifusion diaphragm (iv). The fusion pore (v), forms either in (iii) or (iv). *Reproduced from Chernomordik L.V., 2008* (73)

2.2. **A continuous race.** Antigenic diversity and other immune evasion strategies of HIV-1 Env

To avoid protective humoral responses, HIV-1 presents a unique set of strategies, which compromise viral control by the immune system and challenge the generation of successful immunogens. Accordingly, a tremendous research effort has been focused in understanding the mechanisms of viral immune evasion. In this context, the plasticity of the envelope glycoprotein plays a major role. Env is the only viral antigen displayed on the surface of HIV-1 particles and thus, it is continuously exposed to the humoral immune pressure. This pressure has brought the virus to develop a series of features in Env to facilitate antibody evasion. Those features are mainly derived from

the development of rapid escape mutations, structural constraints within the trimer architecture and masking of neutralizing determinants (74) **(Figure 7).**

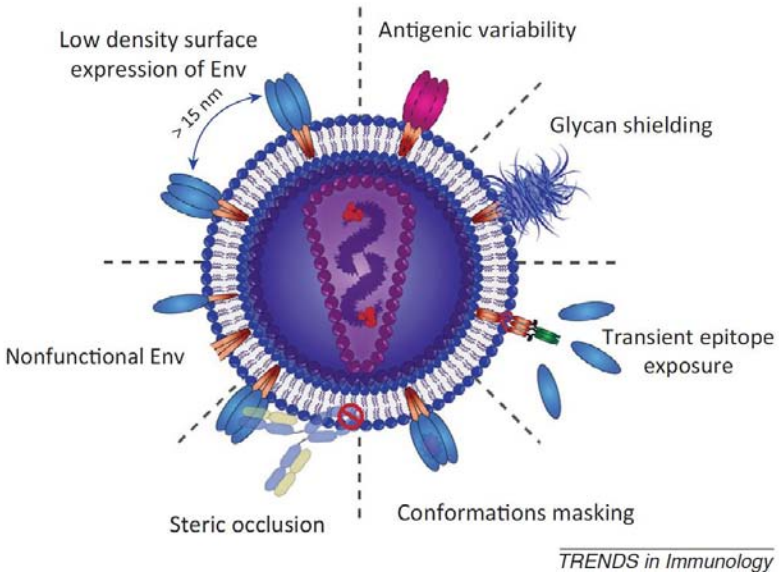

Figure 7. Antibody evasion mechanisms of HIV-1 Env. The continuous immune pressure to which Env is exposed has driven a series of features, besides antigen variation, such as architectural and structural constraints that allows the virus to overcome antibody recognition. *Reproduced from Mouquet H., 2014* (75)

2.2.1. Env diversity

The particularly high mutation and replication rate of HIV-1 results in an extremely high sequence variability, which represents a major hurdle for the generation of a universal HIV-1 vaccine (76, 77). Like other retroviruses, HIV-1 reverse transcriptase is error-prone and lacks the proof reading mechanisms of DNA polymerases (78). As a result, mutations are mainly introduced when viral RNA is reverse transcribed into DNA. The mutation rate has been estimated to occur between 10^{-4}-10^{-5} errors per base pair, which means approximately one nucleotide mutation per replication cycle (79, 80). This high mutation rate is amplified by the production of about 10^{10} virions per day (81). Consequently, a highly diverse viral swarm is generated within an infected individual (82, 83). This viral diversification impacts on the inability of the immune system to control the progression of the infection (84).

During natural HIV-1 infection, initial antibody response is mainly mounted against gp41 and is not neutralizing. This occurs about 8 days after detection of plasma viremia (85, 86). After 4-14 weeks from HIV-1 acquisition an autologous neutralizing response against gp120 is developed, starting the selective pressure which accounts for

the emergence of new Env variants (87–89). The extraordinary high rate of mutation of the virus is fast enough to generate a highly diverse viral population within the host able to escape from the contemporary neutralizing response. As a result, a continuous race between the developing humoral response and rapidly evolving viruses takes place (87). Years after infection, a small percentage of HIV-1 infected individuals develop an antibody response able to neutralize a wide range of viral strains **(Section 2.3)**. However, this broadly neutralizing response seems to be not effective to control viral dissemination (84).

2.2.2. Concealment of neutralizing determinants

A successful HIV-1 vaccine should elicit neutralizing antibodies targeting Env conserved functional epitopes in order to overcome the high antigenic diversity. Such kinds of antibodies are designed as broadly neutralizing antibodies (bNAbs) (**Section 2.3**). However, the structural architecture of the Env spike has evolved to restrict the access of those conserved epitopes to antibodies. Conformational masking and transient exposure of key neutralizing determinants has a major impact in antibody escape. For example, the coreceptor binding site is only exposed following CD4 engagement (48). In a similar manner, the MPER is mainly transiently exposed during the fusion process when gp41 acquires the prehairpin fusion intermediate conformation (90). Additionally, the CD4 binding site resides in a pocket to which antibody access is restricted (48, 91). Moreover, N-linked glycosylation by host-derived carbohydrates has a major role in masking functional epitopes. The HIV-1 Env gp120 subunit is highly glycosylated (about 25 N-linked glycosylation sites) and, to a less extent, gp41 (92, 93). Given the molecular weight of these structures, the Env trimer is decorated by a glycan shield accounting for about 50% of its total mass, preventing the virus from antibody recognition(48, 62). Since glycans are originated from the host cell, they are indistinguishable for the host immune system, which impedes the generation of a glycan-specific antibody response. Finally, the large size of immunoglobulines may eventually result in the steric occlusion of nearby neutralizing epitopes (94).

2.2.3. What is different with HIV-1 spikes?

In addition to the challenges presented by conformational masking and glycan shield, the HIV-1 antibody response needs to overcome the relatively low number of Env spikes displayed on the viral surface. While other pathogens, like influenza or papillomavirus, present a far great number of surface antigens, it has been estimated that

HIV-1 displays about 14 Env spikes per virion, randomly distributed throughout the membrane (51, 95, 96) **(Figure 8.A).** Consequently, the average distance between two adjacent spikes exceeds the maximun distance that two arms of an antibody can cope with. As a result, the bivalent binding by anti-HIV-1 antibodies is usually rare and therefore avidity is greatly compromised (96). It has been suggested that due to this low chance of bivalent binding, HIV-1 bNAbs need to present polyreactivity at some degree, as a manner to ensure a tight binding to the virion (95–97) (**Section 2.4**).

HIV-1 Env spikes are prone to disassembly, resulting in a heterogeneous population of Env versions within viral particles and infected cells. These forms include not only functional Env trimers but also uncleaved gp160, gp41 stumps after gp120 shedding, alternative trimer isoforms or gp120/gp41 monomers (98, 99) **(Figure 8.B)**. All these non-functional Env forms have been proposed to divert the humoral response towards non neutralizing epitopes. If we assume that antibody binding to functional forms of the Env trimer would predict neutralization, then the existence of non-functional forms of Env may compromise such neutralizing responses (100). This may explain the lack of early neutralizing responses during natural infection. Therefore, the generation of this alternative forms of Env has been proposed as an additional immune evasion mechanism (100). However, non-neutralizing antibodies that presumably are elicited against these forms are able to mediate viral capture (99) and thus they might present antiviral activity through complement or by binding to Fc receptors (FcγR) on the surface of immune cells. Therefore, the generation of non-neutralizing antibodies by non-functional Env forms could be also relevant, as they could mediate ADCC, an immune correlate of protection reported in the RV144 trial (101)

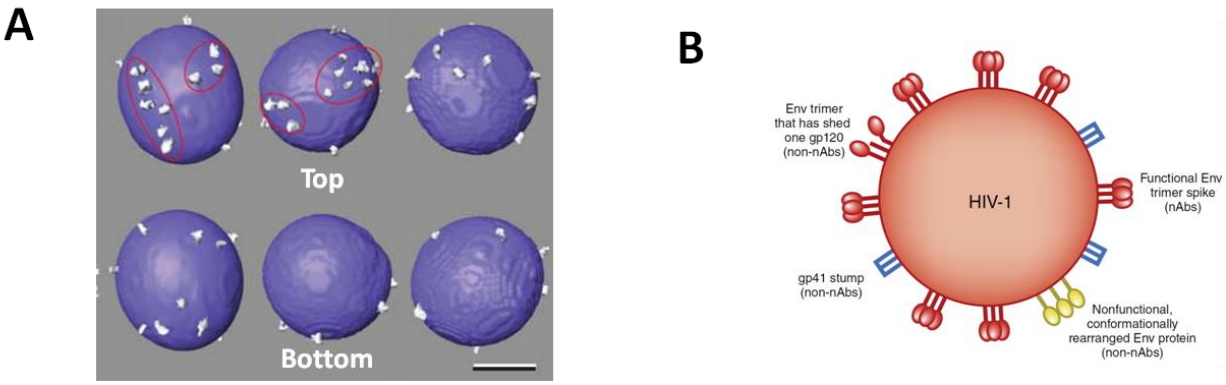

Figure 8. What is different with HIV-1 spikes? A. The HIV-1 viral particle displays very few Env spikes on the surface. Surface-rendered model of three HIV-1 virions (Top and bottom views) with highlighted Env spikes. Scale bar 50nm. *Adapted from Zhu P., et al., 2006* (51). **B.** Non-functional Env forms may divert neutralizing responses. Schematic representation of some Env forms that may be displayed on the surface of virions and elicit antibody responses.. *Reproduced from Burton&Mascola, 2015* (100)

2.3. **Identifying vulnerability regions.** Broadly Neutralizing antibodies (bNAbs)

The notion that the human immune system is capable of mounting a broadly neutralizing response against HIV-1 has been established by two pieces of evidence: i) the identification of sera from HIV-1 infected individuals displaying such broadly neutralizing activity and ii) the isolation of bNAbs from these individuals, capable to cross-react against a wide range of viral isolates (74, 102). The identification of different regions within the HIV-1 Env targeted by bNAbs allowed researchers to delineate an HIV-1 Env vulnerability map. Accordingly, the study of anti-HIV-1 bNAbs and the epitopes targeted by them has been guiding the rational design of immunogen candidates during the last years (74, 103)

2.3.1. The beginning

Some years after the identification of HIV as the causative agent of AIDS, investigators demonstrated, by using a cell-to-cell transmission assay, that sera from HIV-1 infected donors contained antibodies capable of blocking infection *in vitro*. These antibodies were directed against the Env gp120 subunit (45, 104, 105). These results led to some optimism that a protective vaccine could be achieved within few years. However, although initial vaccine candidates including recombinant gp160 and

gp120 proteins demonstrated the elicitation of specific antibodies, to the surprise of most researchers, they were unable to neutralize primary viral isolates (106, 107).

Our early knowledge of the precise neutralizing determinants within the HIV-1 Env came from the isolation of the firsts neutralizing antibodies (NAbs), reported between 1993 and 1994. These antibodies identified three Env regions including the CD4 binding site (NAb b12); a surface glycan patch on gp120 (NAb 2G12) and the Membrane Proximal External Region (MPER) within the gp41 ectodomain (NAbs 2F5, 4E10) (108–113). These mAbs were able to neutralize laboratory-adapted and primary viral isolates with moderate potency at different levels. While antibodies b12, 2G12 and 2F5 exhibited limited breadth and/or potency against non-clade B isolates, 4E10 displayed a greater breadth (>90% of isolates from different clades) but relatively weak potency (114). Thus, the principle that a neutralizing response could be achieved during HIV-1 infection was considered feasible. Remarkably, passive infusion of these mAbs to non-human primates (NHP) demonstrated protection against simian-human immunodeficiency virus (SHIV) challenge, supporting that the elicitation of this kind of antibodies by vaccination would confer protection against infection (46, 115–118). Additionally, clinical studies of high-dose cocktails of 2F5, 4E10 and 2G12 in HIV-1 infected individuals showed a delay in viral rebound (119). Therefore, this first generation of NAbs provided the starting point for rational immunogen design. However, it became clear that none of these antibodies could neutralize the majority of circulating HIV-1 strains (114).

2.3.2. Identification of bNAbs during HIV-1 infection

Until 2007, our knowledge about the prevalence of cross-reactive neutralizing antibodies during natural infection was limited. One of the main difficulties that researchers had encountered when analyzing serum-mediating HIV-1 neutralization was the use of peripheral blood mononuclear cells (PBMCs) or CD4+ T cells-based assays, where the infectivity was quantified after several rounds of viral replication. In consequence, the results usually were neither accurate enough nor reproducible. However, in 2005, high-throughput neutralization assays were developed by the generation of recombinant Env pseudotyped viruses that mediated a single-round of infection (120, 121). The use of these pseudotyped viruses, allowed the generation of large and genetically defined viral libraries better representing the global viral diversity of HIV-1. These newly developed assays permitted the screening of large panels of

HIV-1 infected individuals for the detection of neutralizing responses. Accordingly, several cohorts of HIV-1 infected individuals with different clades were analyzed to systematically identify individuals that developed bNAbs with remarkable neutralization breadth and potency (122–131). The observation that some HIV-1-infected individuals were able to mount cross-reactive neutralizing responses with greater potency than those reported by the current immunogen candidates changed the previous negative perception about the potential of the immune system to generate HIV-1 bNAbs (132).

These studies reported that, depending of the cohort of study, between 10-25% of HIV-1-infected individuals could generate a relatively potent cross-neutralizing response (74, 123, 132–135). The development of potent heterologous neutralizing responses was observed to take about 2-3 years after infection and correlated with a high antigenic viral load, highlighting the importance of a prolonged exposure to Env antigen. It was also reported that the viral diversity developed in individuals with cross-reactive neutralizing activity was higher compared to those individuals without broadly neutralizing activity (133, 136, 137). Furthermore, in a cohort of 1798 mostly non-clade B HIV-1 infected individuals, 1% of sera were found to display an unusually potent neutralizing activity against the majority of the viral isolates tested (126). These individuals, designed as "elite neutralizers", were considered the starting material for the isolation of new anti-HIV-1 monoclonal bNAbs in an attempt to understand the nature of this immune response (126).

2.3.3. Neutralizing determinants. The HIV-1 Env vulnerability map

The specificities mediating cross-neutralizing responses observed in sera of HIV-1 infected individuals were determined by a variety of methodologies including selective removal of specific antibodies by antigen-coated beads and neutralization of chimeric viruses engrafted with specific Env neutralizing epitopes. The main neutralizing determinants observed were the CD4 and coreceptor binding sites in gp120 and, in a minority of cases, the MPER of gp41 (122, 123, 133–135, 138). However, a substantial fraction of sera was found to target unidentified epitopes. The possibility of the existence of antibodies recognizing quaternary epitopes within the native trimer or carbohydrate molecules covering large Env areas was considered. Therefore, it was clear that the broad and potent neutralizing activity observed could not be only explained by the handful of known NAbs discovered more than ten years back (132).

Consequently, starting in 2009, a substantial effort was focused in the isolation of new bNAbs in order to delineate a complete map of the neutralizing determinants of HIV-1 Env. The development of high-throughput analysis of single memory B cells and the use of fluorescently labeled Env protein probes to isolate antigen specific B cells (135, 139, 140) contributed enormously to the discovery of new HIV-1 neutralizing antibodies. Large-scale cultures of single memory B cells were then performed under conditions in which enough supernatant could be recovered for neutralization screening (141–143). Once identified, the specific heavy and light chains of individual B cells were recovered by PCR amplification and cloned into IgG expression vectors (144, 145). These methodological advances led to the isolation of a new generation of HIV-1 bNAbs, some of them with a remarkable breadth and potency. Their characterization has redefined our understanding of the HIV-1 envelope vulnerability sites and a new HIV-1 "Achilles Heel" map has been traced including the major epitopes defined by bNAbs (100, 102) **(Figure 9)**. At present, the major neutralizing clusters within HIV-1 Env include the CD4 binding site (109, 114, 139, 142, 146); two glycan-dependent epitopes defined by residues N332 and N160 in the V3 and V1/V2 loops respectively (141, 142, 147); the MPER of gp41**(Section 3)** (110, 113, 143, 148); and recently discovered epitopes located at the gp120-gp41 interface (bNAbs 8ANC195 (149) and 35O22 (150)); a glycan-dependent epitope at the gp41 prefusion state (bNAb PGT151 (151)); and the fusion peptide of gp41, by the identification of the bNAb VRC34.01(152).

The HIV-1 envelope vulnerability map highlights the potential of the human immune system to mount potent cross-neutralizing responses against HIV-1. Although these discoveries raised some optimism into the HIV-1 vaccine field, no immunogen candidates have been able to mimic such potent responses (74, 153). The study of bNAbs offers important clues for the rational design of novel immunogens. However further study of bNAbs revealed certain features that may hamper the elicitation of similar antibodies by immunization. These unusual features are discussed in the next section.

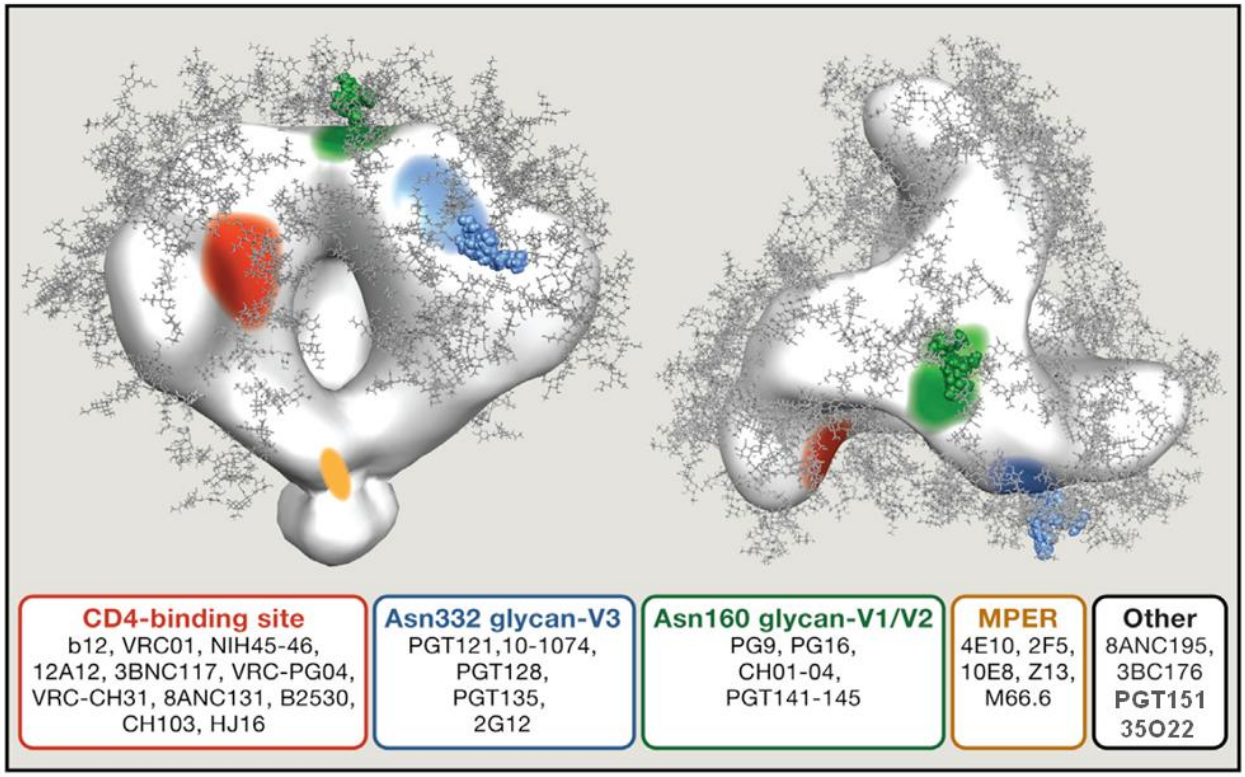

Figure 9. The HIV-1 Env vulnerability map. Structure of the Env spike. N-linked glycans are shown. The approximate surface location of the four main bNAb targets are highlighted in colors: CD4 binding site (red); glycan patch at V3 (blue) and V1/V2 (green); and MPER (yellow). BNAbs from different families are indicated in the color boxes. Recently described bNAbs, targeting distinct epitopes are group in "other" box (see text). *Adapted from West A., et al., 2014* (102)

2.4. **Why are bNAbs so difficult to make?** Unusual traits

The landmark for a successful preventative HIV-1 vaccine is that it should elicit bNAbs targeting different functional conserved sites within Env (154). However, after more than 20 years of extensive experimentation in animal models and several HIV-1 vaccine trials, the elicitation of bNAbs by vaccine candidates has not been achieved (153). Thus, a relevant question in the field is why these antibodies are so difficult to make. Identifying the roadblocks responsible for impeding bNAbs induction by current immunization protocols and designing strategies to overcome them is crucial for a successful HIV-1 vaccine development. Molecular characterization of over more than 100 bNAbs identified to date revealed some predictive traits of negative selection during B-cell development (74, 102) Further study of these features are providing some clues to our understanding of the nature of these unconventional antibodies

2.4.1. Polyreactivity

Polyreactive antibodies are promiscuous binders able to interact with numerous antigens with relatively low affinities. It has been reported that HIV-1 bNAbs are much more polyreactive than HIV-1 nNAbs (155). A percentage of polyreactive antibodies are expressed by immature B-cells, which subsequently are removed at different tolerance checkpoints (144). The exception of HIV-1 bNAbs has been attributed to the low density of Env spikes in the viral surface which prevent bivalent binding and polyreactive antibodies are selected to provide a second binding interaction (96, 97). The particular polyreactivity issues of anti-MPER bNAbs have focused a wide interest and are discussed in **Section 3.3**.

2.4.2. Long CDR H3 loops

Many bNAbs present unusually long CDR H3 loops, between 20-34 residues. This is in contrast with the human antibody average of 16 residues (156, 157). Long CDR H3 loops are probably a physic requirement for epitope accessibility rather than an obligate characteristic for all bNAbs. For example, anti-CD4 binding site antibodies present short CDR H3, and some of them are within the broadest and most potent anti-HIV-1 antibodies described (158). However long CDR H3 loops seems to be relevant for antibodies targeting the V1/V2 loops, the V3 glycan supersites, the MPER or the gp120/gp41 bridging region. A long CDR H3 is important for the penetration into the glycan shield in the case of glycan-binding bNAbs (159, 160), whereas anti-MPER

bNAbs need to extend its CDR H3 hydrophobic apex to reach residues embedded within the membrane (161) **(Section 3.3)**. The low frequency of human B cells encoding for long CDR H3 and its relation with self-reactivity, are considered as a potential hurdle for bNAbs induction in humans. However, it is important to highlight that human IgGs with long CDR H3 loops are indeed generated and neither all of these antibodies are autoreactive nor all autoreactive antibodies present a long CDR H3 (157). Therefore, the requirement of long CDR H3 for some bNAbs can be achievable by the human immune system.

2.4.3. Somatic Hypermutation (SHM)

Whereas not all HIV-1 bNAbs present long CDR H3 and/or polyreactivity, all HIV-1 bNAbs isolated to date, irrespectively of their specificities, present an unusual degree of somatic hypermutation (SHM) (140). Whereas mature human V_H domains usually carry between 15-20 nucleotide mutations (162), HIV-1 bNAbs are far more mutated carrying 40-100 gene mutations (139, 141, 163–165). Particularly, anti-CD4 binding site bNAbs present extremely high levels of SHM (139). Somatic mutations are introduced by the enzyme activation-induced cytidine deaminase (AID) which preferentially targets hot spots mainly located in the complementary determinant regions (CDR), but also in the framework regions (FWR) (166). Somatic mutations of HIV-1 bNAbs are essential for antibody binding and neutralization as reversion to their germline results in a complete loss of neutralizing capacity (146, 163, 165). The high degree of SHM reported suggests that HIV-1 bNAbs are the result of a long process of antigen selection in the germinal centers. In order to delineate the maturation pathways of HIV-1 bNAbs, the study of concomitant evolution of virus and antibody since transmission have centered a great deal of interest and evolution of different bNAb lineages have been reported (146, 167, 168). Immunization strategies by using immunogens targeting unmutated common ancestors (UCA) to trigger potential neutralizing antibody lineages in combination with Env proteins are being explored as future vaccine approaches (74). Finally, the possibility that neutralizing activity could be achieved without high level of SHM may be now considered, as a bNAb isolated recently from an HIV-1 infected infant showed low level of SHM (6.8% at the nucleotide level) (169). Future study of this antibody lineage will focus a great interest.

3. The Membrane Proximal External Region (MPER) of gp41

3.1. **The MPER.** An apparently easy vaccine target

The MPER region is a major neutralizing determinant of gp41. This highly conserved and unusual tryptophan-rich motif is located adjacent to the viral membrane, covering the last 24 C-terminal residues of the gp41 ectodomain (aa 660-683, HXB2 numbering) and connecting the extracellular portion of Env with the TM domain (170, 171) **(Figure 10.A)**. The importance of the MPER on Env functionality was highlighted by several studies using viral mutants containing deletions, insertions or substitutions within this region (171–173). Substitution of the five conserved tryptophan residues (W1-5) dramatically compromises the incorporation of gp41 into virions and, thus, abolishes viral entry (171). Moreover, simple deletion of the sequence spanning the residues W_{666}-I_{682} completely abolishes syncytium formation (174). These observations concluded that the MPER plays a major role in the HIV-1 Env-mediated fusion and viral infectivity, which explains the high level of sequence conservation (170). Moreover, the MPER has a role in HIV-1 CD4-independent viral transcytosis at the epithelial barrier (175), where the conserved $_{662}$ELDKWA$_{667}$ gp41 sequence interacts with galactosyl ceramide receptors (176). It was demonstrated that secretory IgA in cervicovaginal secretions from HIV-1 infected individuals were capable of blocking viral transcytosis via $_{662}$ELDKWA$_{667}$ sequence binding (177). Furthermore, induction of specific vaginal IgA antibodies with transcytosis-blocking properties by vaccination

with gp41-containing virosomes induced protection against viral challenge in macaque model (178).

The functional implications in viral infectivity, the high level of conservation and the lack of N-linked glycosylated residues, together with the discovery of highly potent and/or broadly neutralizing antibodies targeting linear MPER sequences (2F5, 4E10, 10E8), all able to protect against viral challenge in animal models (115, 116, 179), makes the elicitation of MPER-specific neutralizing responses highly desirable by immunogen candidates. Therefore, the MPER is a major HIV-1 vulnerability site and a potential vaccine target (148, 180, 181).

However, the presentation of presumably simple linear epitopes targeted by MPER-specific bNAbs does not seem to facilitate the elicitation of this kind of antibodies by immunogen candidates (148, 181). Moreover, anti-MPER neutralizing response arises in a lower proportion of HIV-1 infected individuals comparing with other specificities **(Section 3.2)**. The immunogenicity of the MPER presents physical and immunological hurdles for immunogen design that need to be overcome. Physicochemical properties include high degree of hydrophobicity that promotes the MPER to be partially embedded within the viral membrane **(Figure 10B-C)**, compromising antibody accessibility (161). Structurally, the native conformation of the MPER within the Env trimer is not fully understood, adding the challenge of developing an immunogen against a structurally ambiguous epitope. Finally, MPER-specific bNAbs show reactivity against self-antigens and host tolerance mechanisms have been suggested to influence the elicitation of anti-MPER bNAbs **(Section 3.3)**.

In this section, the particular features of the MPER will be reviewed in order to put in context the scope of this thesis.

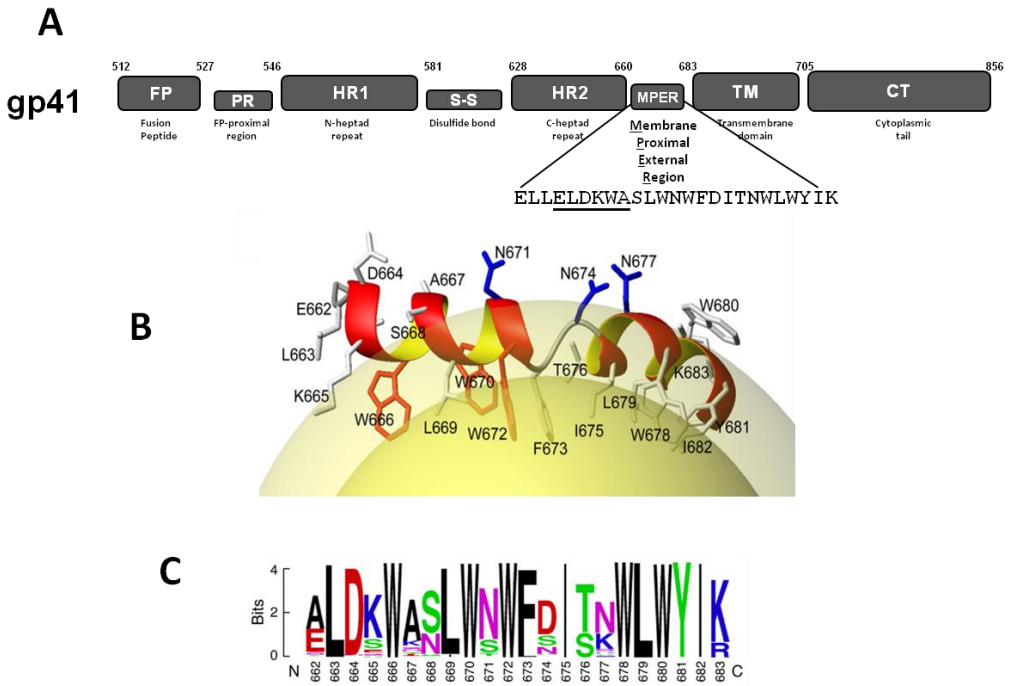

Figure 10. The Membrane Proximal External region of HIV-1 gp41. **A.** The different domains of gp41 are shown using the HXB2 numbering scheme. The MPER is depicted (aa 660-683). The galactosyl ceramide binding sequence $_{662}$ELDKWA$_{667}$ is underlined. **B.** Amphiphilic behaviour of the MPER within membranes. Hydrophobic residues are embedded into the membrane whereas the more polar ones are solvent exposed. The three conserved tryptophan residues important for fusion are highlighted in red. The most variable asparagine residues are depicted in blue. **C.** MPER sequence conservation. Representation of MPER aminoacid patterns within sequence alignment. The most variable residues are those solvent-exposed. *Images **B** and **C** reproduced from Sun Z.J., et al., 2008* (161)

3.2. **Natural responses against the MPER.** bNAbs discovery

Within the native viral spike, gp41 is occluded by gp120, compromising antibody accessibility to the MPER region, which is transiently exposed during the fusion process (172). In spite of this, a strong antibody response against the whole gp41 subunit during natural infection is generated, probably due to gp120 spontaneous shedding, non-functional forms of Env or transient epitope exposure during viral entry (182). The humoral response against gp41 is typically non-neutralizing and mainly focused against immunogenic regions (especially the immunodominant disulfide loop), different from the MPER (86, 183). Notwithstanding, the MPER has been reported to be relatively immunogenic. Since early 90s, a number of studies demonstrated the presence of anti-MPER antibodies at variable levels albeit without addressing the question if

those responses were neutralizing (reviewed in (148)).

3.2.1. MPER-specific neutralizing responses during natural infection

In order to delineate the specific contribution of MPER-specific antibodies to neutralizing activity of human plasma samples, chimeric SIV and HIV-2 viruses engrafted with the HIV-1 sequences targeted by 2F5 and 4E10 MPER antibodies were constructed (184). Chimeric SIV viruses were validated and initially used to characterize 96 plasma samples identifying only one sample showing 4E10-like neutralizing activity, without showing epitope competition (184). Similarly, another study used both SIV and HIV-2 chimeric viruses to define the specificities of samples from three asymptomatic HIV-1 infected individuals without finding MPER-specific neutralizing activity (122). Subsequent analysis of different cohorts including subtypes A, B and C concluded that, when compared with other neutralizing specificities, MPER-specific neutralizing responses are usually raised in less proportion during natural infection (89, 124, 127–129, 131, 134, 136) (see **Table 1** for details). Interestingly, in a South African cohort of 156 HIV-1 infected individuals, three samples were found to present high titers of anti-MPER antibodies (124). Depletion of these antibodies by MPER-coated beads resulted in loss of the neutralization breadth in the three samples but the antibody specificities were found to be targeting a distinct epitope from those recognized by previously identified NAbs 4E10 or 2F5, highlighting the existence of additional neutralizing epitopes within the MPER (124).

Table 1. Summary of human studies detecting MPER-specific neutralizing responses

Year published	Number of Participants	Main findings	Reference
2006	96	One individual with 4E10-like neutralizing activity. No epitope competition	(184)
2007	3	No MPER-specific neutralizing activity	(122)
2007	14	4 individuals with MPER-specific neutralizing activity. 2 of them within the 6 months after seroconversion. No correlation with breadth.	(89)
2009	156	3 individuals high MPER titer, associated with breadth. Distinct epitope from 4E10, 2F5 or z13	(124)
2009	70	MPER titer correlated with breadth. 4E10-like. Anti-cardiolipin antibodies correlated with breadth and MPER titer	(127)
2009	32	MPER-specific neutralization in 4 individuals	(134)
2010	19	Modest MPER-specific neutralization in 6 individuals	(128)
2011	308	4 out of 9 breadth neutralizers displayed MPER-specific neutralization (17-30% contribution)	(129)
2011	40	7 individuals >40% breadth MPER cross-neutralizing antibodies	(136)
2012	78	21 MPER-specific neutralizing activity. 8 out of 21 displayed 10E8 neutralization pattern	(143)
2016	439	One individual with potent MPER-specific neutralizing activity	(131)

3.2.2. Isolation of bNAbs targeting the MPER

In spite of the low frequency of MPER-specific neutralizing responses reported during natural infection, some neutralizing antibodies have been isolated, designed as 2F5, 4E10, 10E8, z13, m66.6 and CH12, and three of them (2F5, 4E10 and 10E8) display a broadly neutralizing activity (110, 111, 113, 116, 143, 185–188) (**Figure 11**) and (**Table 2**).

Table 2. *bNAbs targeting the MPER (source: BNAber www.bnaber.org)*

	2F5	**4E10**	**10E8**
Neutralization breadth at IC50 < 50 μg/ml (%)	67	98	98
Neutralization breadth at IC80 < 50 μg/ml (%)	43	36	NA*
Neutralization potency IC50 (μg/ml)	1.44	3.41	0.25
CDR H3 length (Kabat aa)	22	18	20
VH mutation (% aa)	15	20	26
Reference	(108) (114)	(108) (114)	(143)

** NA,data not available*

As mentioned in **Section 2.3.1,** 2F5 and 4E10 were some of the first HIV-1 bNAbs discovered and were generated by electrofusion of Peripheral Blood Mononuclear Cells (PBMC) mixtures from different HIV-1 infected individuals (108). 2F5 targets the linear sequence $_{662}$ELDKWA$_{667}$ (110) within the N-terminal moiety of the MPER, where the central core $_{664}$DKW$_{666}$ is essential for neutralization, as demonstrated by alanine-scanning mutagenesis assays (189). 2F5 has a relatively high potency and was found to neutralize 67% of the viral panel tested (114). However, subtype C viruses are usually 2F5-resistant due to a mutation in the central core epitope (DSW instead of DKW) (114, 190, 191). 4E10 targets the conserved tryptophan rich moiety located C-terminal to the 2F5 epitope which includes the sequence $_{671}$NWFDIT$_{676}$ and extended toward C-terminal residues, where W672, F673, I675, T676, L679 and W680 have the most significant contacts with the antibody (113).

Although less potent than 2F5, 4E10 displays a remarkable breadth against 98-100% of the viral isolates, depending of the panel tested (114, 141). Moreover, both 2F5 and 4E10 were shown to protect against viral challenge in macaques (115, 116).

As many other bNAbs, both 2F5 and 4E10 antibodies present particular features. They are highly mutated and present an unusually long and hydrophobic CDR H3 loops (**Table 2)**. In fact, these antibodies share a common neutralization mechanism by which the interaction of the hydrophobic CDR H3 apex with the membrane seems to be essential (192, 193) **(Section 3.4)**. Depending of the bound antibody, the MPER can acquire different structures. Crystal structures of 4E10 and 2F5 in complex with their epitope-containing antigens have been determined, providing a snapshot of the MPER structures bound by specific neutralizing antibodies. 2F5 in complex with an MPER peptide showed that the core motif DKW forms a type 1 β-turn structure (194) and the MPER in complex with 4E10 was found to form a α-helical conformation from D674 to K683 (195, 196) **(Figure 11.B)**.

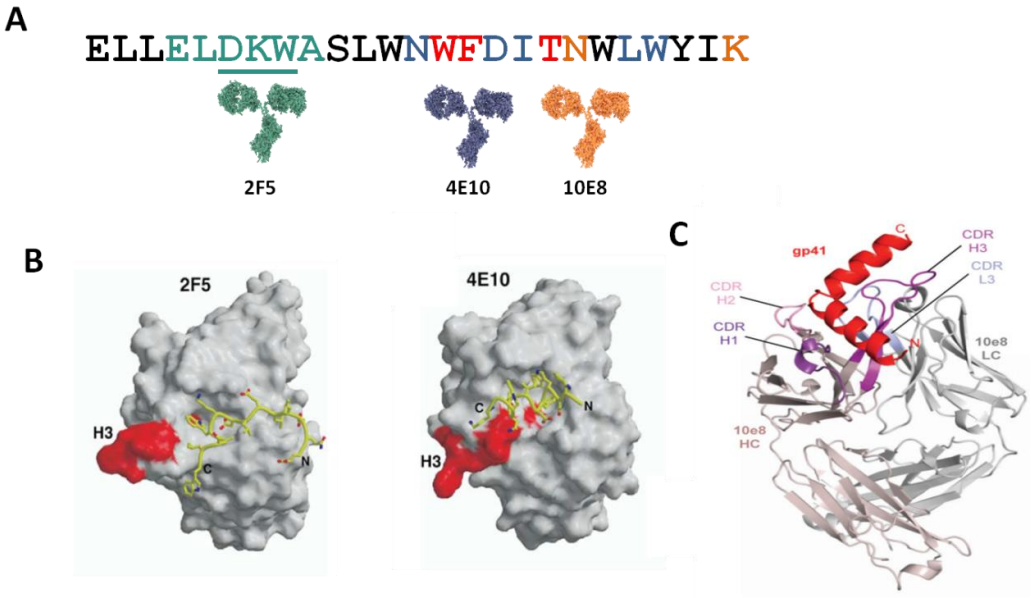

Figure 11. Binding determinants of MPER-specific bNAbs. A. MPER residues for neutralizing antibody binding. 2F5 (green) binds to the N-terminal MPER moiety. The 2F5 neutralizing core DKW is underlined. 4E10 (blue) and 10E8 (orange) bind to overlapping sequences at the C-terminal region. Residues recognized by both are highlighted in red **B.** MPER structure (yellow) differs between 2F5 (extended β-turn) or 4E10 (α-helix) binding. *Reproduced from Cardoso R.M.F. et al., 2005* (195) **C.** Crystal structure of 10E8 Fab in complex with its MPER epitope. *Reproduced from Huang J., et al., 2012* (143)

Monoclonal antibody 10E8 was isolated in 2012 by Huang and colleagues and was found to present a remarkable potency and breadth. With an IC50 below 50ug/mL,

10E8 neutralized 98% of a panel of 181 pseudovirus whereas 72% of the virus were neutralized with an IC50 below 1ug/mL (143). Therefore, 10E8 neutralizes with a 10-fold greater potency than previously discovered anti-MPER bNAbs 2F5 and 4E10 and this potency is comparable with some of the most potent HIV-1 bNAbs like VRC01 or PG9/PG16 (102). 10E8 presents a long CDR H3 and is highly mutated, showing a 21% and 14% of mutations within the variable genes of the heavy and kappa chains, respectively (143). Initially, 10E8 was reported to be non-poly/autoreactive although some studies have suggested that 10E8 needs to bind membrane lipids, especially cholesterol, to mediate neutralization (143, 197). Encouragingly, the frequency of 10E8-like antibodies in infected individuals seems to be superior comparing with 2F5 or 4E10 specificities (143). 10E8 has also been reported to protect against viral challenge *in vivo* (179), supporting the role of neutralizing antibodies with these specificity in protection (179, 198).

3.3. **Physical and Immunological hurdles.** Lipid membranes and polyreactivity

3.3.1. MPER and Lipids

In 2000, Suarez and colleagues showed that a peptide corresponding to the MPER sequence was able to partition completely into membranes of unilamellar vesicles that were analogous to the viral membrane (199). Therefore, the MPER was able to destabilize lipid membranes. This was attributed to the high tryptophan content (200). Structurally, biophysical models suggest that the MPER acquires an alpha-helical conformation partially embedded into the viral membrane, constituted by two independent domains separated by a flexible hinge (161, 201). These two segments have been shown to present different membrane-interacting properties. The C-terminal domain remains embedded into the membrane (161, 201–203), whereas the N-terminal domain is more exposed. Given the amphiphilic properties of the MPER, hydrophobic residues remain buried into the membrane whereas the most polar are solvent-exposed (161) **(Figure 10.B)**. The MPER topology also depends on the membrane context where it is presented (204, 205). Furthermore, membrane lipids such as cholesterol and sphingomyelin can modulate the capacity of the MPER to destabilize membranes (204, 205). MPER and cholesterol interactions are further supported by the existence of the sequence 679-LWYIK-683 located at the C-terminus which was identified as a

cholesterol recognition aminoacid consensus (CRAC) motif (206). This motif seems to play an important role during the incorporation of Env into the virion, stabilizing the trimer complex (181).

3.3.2. **Neutralization mechanisms.** The role of membrane interactions

The inhibition of the MPER membrane perturbing properties involved in viral fusion was attributed to the neutralization mechanism of antibodies 2F5 and 4E10. In this regard, the interaction with membranes has an important role. It has been suggested that the MPER undergoes a conformational change upon binding by the 4E10 antibody, which extracts the membrane-embedded epitope (161). Moreover, the lipid components involved in 4E10 binding as well as its lipid binding site have been recently determined by crystallographic studies (207) **(Figure 12.A)**, resolving the full 4E10 epitope. Therefore, affinity by the peptide sequence is not the only determinant for MPER neutralization but also other physicochemical properties may be involved. As an example, z13e1 or 13H11 mAbs overlap the sequences bound by 4E10 and 2F5 respectively with the same affinity but displaying considerably lower neutralization potency (186, 208)

It is established that anti-MPER bNAbs share a common mechanism by which interactions of the long CDR H3 hydrophobic loops with membrane lipids seems to be essential for neutralization but not for peptide recognition (192, 193, 209). Accordingly, binding studies demonstrated that whereas anti-MPER bNAbs bind to a peptide sequence following a Langmuir curve model, binding against peptide-membrane complexes follow a two steps (encounter-docking) model. First, the antibody attaches to the lipid membrane through its long hydrophobic CDR H3 and concentrates within the proximity of the MPER epitope to subsequently bind to the prehairpin intermediate of gp41, once the conformational change takes place (192, 193). This mechanism facilitates the accessibility of the antibody to its epitope, overcoming the poor exposure of the MPER and takes advantage of its close proximity to the viral membrane. Interestingly, neutralizing activity of an anti-MPER single-chain bivalent llama antibody induced by immunization was also dependent of the hydrophobic CDR H3 apex which was not involved in antigen recognition (210) **(Figure 12.B)**.

Given the considerations exposed in **3.3.1** and **3.3.2**, it has been proposed that membrane lipids could be the equivalent to a natural scaffold that shapes the MPER structure resulting in a conformation recognized by currently described bNAbs.

Therefore, it is widely accepted that the generation of a robust anti-MPER response should require its presentation within a membrane environment to properly present neutralizing determinants and to implement lipid cross-reactivity. The role of membrane lipids over MPER immunogenicity is a relevant question addressed in this thesis

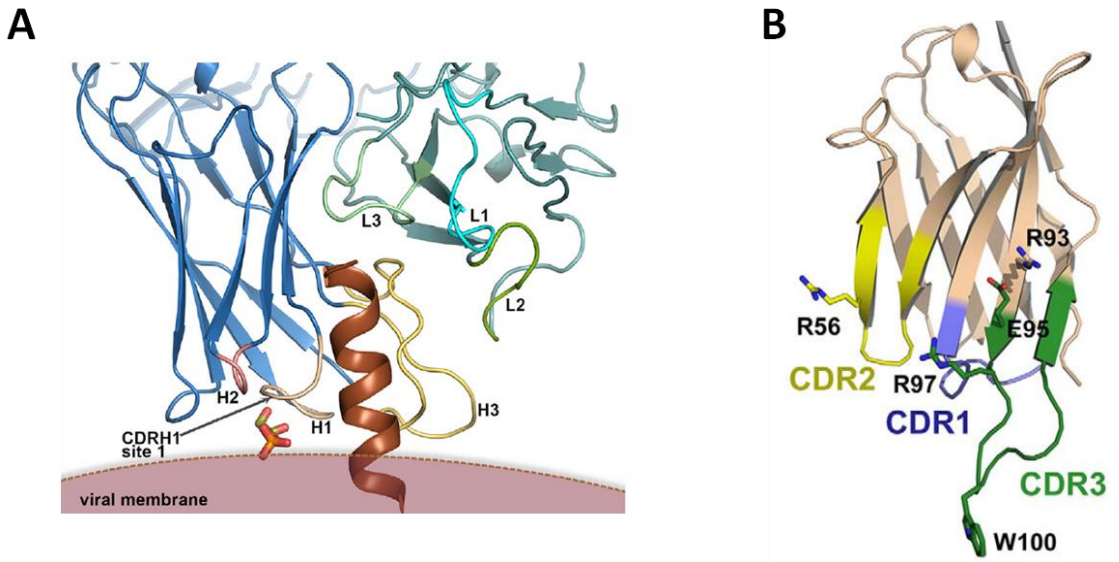

Figure 12. Interaction with viral membrane by MPER bNAbs. A. Crystal structure of 4E10 Fab bound to gp41 MPER peptide. Lipid binding site of the 4E10 CDR H1 revealed by binding to sn-glycerol-3-PO4 (sticks). Reproduced from *Irmia A., et al., 2016* (207). **B.** Ribbon representation of the single chain llama antibody 2H10. MPER binding residues are highlighted with sticks. The tryptophan residue at the CDR3 apex (W100) does not participate in antigen binding but is required for neutralization. Reproduced from *Lutje Hulsik D., et al., 2013*(210)

3.3.3. Particular polyreactivity issues

Reactivity with self-antigens has been suggested to explain the low frequency of MPER-specific bNAbs induced during immunization regimens or natural responses. In 2005, polyspecific binding to cardiolipin and other anionic phospholipids by 4E10 and 2F5 mAbs was reported (211). This finding led to the hypothesis that immunological tolerance might be playing a role in HIV-1 evasion of immune responses and autoreactive B-cells that cross-react with MPER sequences are deleted from the naïve repertoire (212). In consequence, the diversity of primary antibody repertoire could be limited. This hypothesis was demonstrated by monitoring B cell development in knock-in mice models carrying the original V(D)J rearrangements of mature bNAbs 2F5 and 4E10. These models showed a normal early B cell development but exhibited a blockade in the transition of pre-B to immature IgM+ B cells, which is defined by the first tolerance checkpoint (213–216). This behavior is characteristic of antibodies

involved in autoimmune diseases (217). Furthermore, conserved vertebrate host antigens bound by 2F5 and 4E10 have been reported. 2F5 binds to kinureninase (KYNU), which contains the identical sequence (ELDKWA) as the 2F5 epitope, and is highly conserved between different mammal species. 4E10 binds to splicing factor-3b subunit-3 (SF3B3) and type I inositol triphosphate (IP_3R1). The authors concluded that HIV-1 host mimicry could be an immune evasion strategy that limits the repertoire of naïve B-cells available to be triggered by immunogens aiming to generate MPER-specific neutralizing responses (155, 218). The role of immune tolerance accounting for weak anti-MPER neutralizing responses was challenged by the discovery of the 10E8 antibody, which was initially reported to be non-polyreactive (143). However, some authors have reported that 10E8 binds cholesterol, while a protein microarray confirmed that was non-polyreactive but exhibited a strong binding against a human protein expressed in different organs (155, 197). The physiological relevance of 10E8 self-reactivity needs to be further evaluated.

3.4. **Eliciting anti-MPER antibodies by immunization.** From linear sequences to structurally relevant epitopes in a membrane environment

Given its crucial role on Env-mediated membrane fusion and its high grade of conservation, a great effort has been focused in generating a neutralizing antibodies against the MPER by immunogen candidates (148). The poor immunogenicity reported comparing with other immunodominant gp41 domains and the close proximity to the membrane that results in its partial occlusion constitute important hurdles for producing an effective immunogen targeting the MPER. There are two major questions that a candidate MPER-based immunogen should address: i) what are the relevant structures that most likely mimic the native-bound form of MPER bNAbs and ii) which is the role of the membrane over the MPER immunogenicity, in terms of magnitude and quality, to induce neutralization. From a rational-based perspective, the isolation of bNAbs targeting different sequences within the MPER presumably informed about the functional epitopes that an immunogen should target. Since these antibodies bind to linear sequences within gp41, initial approaches to induce 2F5 or 4E10-like antibodies attempted to introduce its corresponding binding sequences in foreign proteins by generating chimeric viruses or fusion proteins (reviewed in (148)). Although this epitope-targeting approaches were able to induce high titers of MPER-specific

antibodies, neutralizing responses were not achieved and further progress has not been made by using those platforms. As mentioned above, it was assumed that other variables beyond the recognition of certain sequences within the MPER should be incorporated into the rational MPER-based immunogen design. Conformational states bound by anti-MPER bNAbs have been explored as potential targets. The MPER is transiently exposed during the fusion process and the gp41 prehairpin fusion intermediate state was suggested to be the main target of 2F5 and 4E10 bNAbs (219).

Accordingly, different approaches aiming to mimic the fusion intermediate conformation and/or the precise structures recognized by 2F5 and 4E10 antibodies have been explored. Computational methods were used to design scaffolds consisting in unrelated protein structures selected from database to accommodate the neutralizing 2F5 binding sequence in a conformation close to the peptide-bound crystal structure. These scaffolds were able to induce polyclonal responses mimicking a 2F5-like binding profile. Crystallographic studies confirmed that monoclonal antibodies isolated induced by these scaffolds presented the same conformation of 2F5 in a flexible gp41 peptide, which was bound with high affinity to the same sequence and similar angle of epitope approach (220, 221). However, the authors argued that other 2F5 features such as membrane binding were not addressed in the design of these platforms and neutralizing activity was difficult to achieve. The same approach was used to target the response against the 4E10 (222) and z13e1 (223) binding motifs, with similar results.

Non-neutralizing MPER-specific antibodies directed specifically against the 2F5 DKW tripeptide core were obtained by a prime/boost regimen combining a gp140 oligomer with liposomes containing an MPER peptide in macaques. Antibodies from immunized animals recognized preferentially the prehairpin fusion intermediate rather than the recombinant gp41 construct (224). In spite of the use of liposomes, neutralization was not achieved. However, the authors concluded that the preferential binding to the fusion intermediate of immunized sera was due to structural modifications induced by liposomes where the antigen was presented (224). The results derived from these studies suggested that recreating the epitope-bound conformation, addressing the response towards neutralizing sequences or binding to the fusion intermediate are relevant but insufficient to achieve the full properties of MPER-specific bNAbs (224). Probably, the additional membrane-binding component suggested to be responsible of a substantial portion of the free energy of 2F5, 4E10 and

10E8 binding (192, 209, 225) was missed. As a proof of concept, the generation of CDR H3 mutants, abrogating lipid binding but maintaining fusion intermediate recognition, resulted in loss of neutralizing activity (193, 224, 226).

To address this question, different membrane-mimicking platforms have been explored for presenting the MPER in a membrane context such as viral-like particles (VLP) (227, 228) or liposomes (229, 230). Liposomes are synthetic nanocarriers of relevant interest for immunogen formulation because they are safe, well tolerated and show self-adjuvant properties that enhance antigen uptake by antigen presenting cells (APCs) and activation of B-cells by presenting surface-conjugated antigens in a repetitive manner (231, 232). As mentioned before, lipids can also modulate the MPER structure generating a native-like conformation. Therefore, besides their intrinsic adjuvant effect, liposomes may play an important role on shaping relevant MPER structures that may also result in improved immunogenicity. Thus, the potential role of lipids on inducing MPER-specific neutralizing responses may be further explored by these platforms.

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Given the physicochemical features of the MPER region and the role of membrane interaction on antibody functionality, structural formulations like proteoliposomes are immunogen platforms worth exploring. Particularly, the versatility of proteoliposomes offers the opportunity to systematically explore the effect of different lipid components over the immunogenicity of a given antigen such as the MPER. The improvement of MPER immunogenicity by these platforms may result in optimal immunogen candidates aiming to induce neutralizing responses.

The work performed in this thesis analyzes the immunogenicity of the MPER in depth. We aimed to delineate the specificity and functionality of naturally-induced anti-MPER antibodies and those elicited by immunogen candidates overexposing the MPER in a membrane environment. The knowledge extracted from the results will contribute to evaluate and consider the MPER as a feasible vaccine target.

CHAPTER 2

Hypotheses & Objectives

The generation of protective antibodies is one of the objectives of a preventative HIV-1 vaccine. In this context, to delineate the neutralizing determinants that an immunogen should be focused is essential. The gp41 Membrane Proximal External Region (MPER) constitutes one of the main vulnerability sites of the HIV-1 envelope glycoprotein because is highly conserved and plays an essential role in viral infectivity. However, targeting MPER neutralizing determinants by immunogens has been hampered by its particular localization at the base of the envelope trimer, together with its high hydrophobicity, resulting in a hardly accessible domain. Therefore, we hypothesize that novel membrane-based MPER presentations could represent a new tool to: i) identify anti-MPER antibodies in HIV-1 infected individuals and ii) to generate anti-MPER antibodies in immunized animals. In addition, we hypothesized that membrane lipids could have a strong influence over the MPER exposure, conformation and immunogenicity. Thus, the incorporation of the MPER into a virion-like lipid mixture would improve its presentation and enhance the magnitude and the quality of the anti-MPER humoral response.

These hypotheses were addressed by the following specific aims:

To design new tools to quantify anti-MPER humoral responses

1. To identify the minimal fragment of gp41 that better exposes MPER neutralizing determinants.
2. To evaluate gp41-based miniproteins as potential tools for the detection of MPER-specific neutralizing antibodies in plasma from HIV-1 infected individuals.

To characterize the immunogenicity of the MPER region during chronic HIV-1 infection

3. To determine the prevalence, stability and specificity of MPER-specific antibodies.
4. To determine immune correlates of viral control with MPER-specific antibodies.
5. To delineate the MPER-specific neutralizing and non-neutralizing profiles.

To evaluate the selected gp41-based miniprotein that overexposes the MPER region, as a potential immunogen candidate in mouse models

6. To generate a recombinant protein of the gp41-based construction, identified in **objective 1**, and evaluate its immunogenicity.
7. To evaluate the effect of different lipid mixtures over the MPER immunogenicity and the potential to induce specific neutralizing antibodies.
8. To determine the MPER binding determinants induced by immunization and to establish a relation with a neutralizing profile.
9. To perform molecular dynamics of the interactions of the MPER-specific responses elicited.

CHAPTER 3

Results

Results I

Anti-MPER antibodies with heterogeneous neutralization capacity are detectable in most untreated HIV-1 infected individuals

This chapter corresponds to the manuscript:

Luis M. Molinos-Albert[#], Jorge Carrillo[#], Marta Curriu, Maria L. Rodriguez de la Concepción, Silvia Marfil, Elisabet García, Bonaventura Clotet and Julià Blanco**.** ***Anti-MPER antibodies with heterogeneous neutralization capacity are detectable in most untreated HIV-1 infected individuals****. Retrovirology 2014, 11:44*

[#] Equal contribution

1. Presentation

The localization of the MPER, close to the membrane and mostly occluded, may compromise antibody accessibility. In order to evaluate potential restrictions on MPER immunogenicity, we generate gp41-based miniproteins able to overexpose this region and were used to characterize the humoral response of 35 HIV-1 infected individuals. Evaluation of these proteins as novel tools for antibody detection; mapping of the humoral response as well as delineation of the MPER-specific neutralizing profile of this patient cohort are presented here.

2. Introduction

The highly conserved Membrane Proximal External Region (MPER) of the gp41 HIV-1 glycoprotein contains linear epitopes targeted by the broadly neutralizing antibodies (bNAbs) 2F5, 4E10 and 10E8; all isolated from HIV-1 infected subjects (110, 113, 143, 185). The ability of the human immune system to mount a neutralizing response against this region and their protective activity in animal models (116) made the MPER a promising target for vaccine design aiming to develop a protective neutralizing response against HIV-1 (148, 153, 233). However, the elicitation of such neutralizing responses against the MPER is challenging likely because of its poor immunogenicity due to topological constraints or to the existence of immunodominant not neutralizing regions within gp41 (148, 203, 234). Furthermore, some of the features presented by both 2F5 and 4E10 antibodies including lipid recognition and autoreactivity, represents a considerable immunological barrier when designing immunogens aiming to mimic anti-MPER responses(192, 211, 235). The development of the B-cell cloning technology led to the recent isolation of the monoclonal antibody 10E8 (143), which is among the broadest and most potent neutralizing antibodies identified to date. Although it was shown initially to lack the limiting features presented by the previous anti-MPER bNAbs (143, 236), it has been shown that 10E8 does bind membrane lipids by two hydrophobic residues in the CDR H3 loop, suggesting that anti-MPER bNAbs could mediate neutralization by similar mechanisms where the binding to the viral membrane plays a role (197). Despite this controversy, it seems that the presentation of MPER epitopes in a lipid environment or in a soluble form may modify its recognition by anti-MPER antibodies (161, 181).

The efforts to characterize bNAbs against the MPER have abridged the full characterization of other anti-MPER humoral responses, which also include several antibodies with low or null neutralizing capacity (113, 208). The characterization of these non-neutralizing anti-MPER antibodies may provide further insights in the mechanisms and molecular determinants of neutralization. For this reason, we aimed to characterize the diverse MPER responses in HIV-1 infected individuals. To this end, we developed small gp41-derived proteins that properly exposed the MPER epitopes recognized by 2F5 and 4E10 bNAbs on the surface of HEK-293 cells. By using cell lines stably transfected with these proteins, we characterized plasma samples from untreated HIV-1 infected individuals. We could detect anti-MPER antibodies in most of these individuals. Furthermore, we found that MPER-specific responses were elicited in the context of a global response against the envelope, which suggest that there is no specific constraint in the elicitation of anti-MPER antibodies. Further characterization of the MPER-specific neutralizing activity showed that anti-MPER responses were highly heterogeneous in terms of neutralization and specific epitope recognition.

3. Materials and methods

3.1. Samples

We selected plasma samples from untreated HIV-1 infected individuals according to the following criteria: HAART naïve individuals with VL > 50 copies/ml, with at least two plasma samples available separated by one year. A total of 35 individuals fulfilled selection criteria and were classified in three groups according to the VL in the first time-point analyzed: Group 1: VL > 50000, Group 2: 50000 > VL > 5000 and Group 3: VL < 5000 copies/mL. Blood from 10 uninfected healthy donors were collected by venipuncture. Plasma was prepared by blood centrifugation for 10 minutes at 3000 x g.

3.2. Construction of Gp41 derivatives

The pHXB2 plasmid containing a full HIV-1 sequence and the peGFP plasmid containing an eGFP coding sequence were used to amplify gp41 and GFP sequences. The primers were designed according to published sequences, and were as follows (5´-3´): i) for the fusion peptide region gp41-EC-f **CACC**ATGGCAGTGGGAATAGGAGCTATG and FP-r

*TACCGTCAGC*GTCATTGAGGCTG; ii) for the HR1 region GP41-2 L-f **CACC**ATGCAGGCCAGACAATTATTGTCTG; iii) for the HR2 region GP41-MAX-f **CACC**ATGATTTGGAATAACATGACCTGG and HR-2 f-2 *GCTGACGGTA*ATTTGGAATCACACGACCTGG; iv) for the transmembrane region GP41-r *GCCACCGCCACC*TAGCTCTATTCACTATAGA and v) for the intracellular region GP41-MAX-r *GCCACCGCCACC*TAGCAAAATCCTTTCCA. The bold text indicates overhang sequences for directional cloning. The italic text indicates overlapping sequences for the generation of fused proteins. All amplification reactions were performed using Amplitaq DNA polymerase (Applied Biosystems) in a 2720 Thermal cycler (Applied Biosystems). For each construct, an additional plasmid containing the green fluorescent protein (GFP) coding sequence fused to gp41 in C terminal was constructed. The final PCR products were cloned into a pcDNA vector using the pcDNA 3.1 Directional TOPO® Expression Kit (Invitrogen). The positive clones were selected in ampicillin plates, recovered, screened by PCR, sequenced and then tested for functional expression. A previously reported pcDNA3.1 plasmid coding for the NL4.3 envelope gene was used to express the full-length envelope (237).

3.3. Transient and stable expression in 293 T cells

Human embryonic kidney HEK-293 T cells (ATCC Accession No. CRL-11268) were cultured in DMEM medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen). One day before transfection, the cells were detached using versene (Invitrogen), washed in supplemented DMEM and split in six well plates at a density of 400000 cells/well. For transfection, each well was transfected with 2 μg of one of the plasmids coding for gp41constructs described herein using the CalPhos Mammalian Transfection Kit (Clontech). Transient expression was assayed 24–48 hours after transfection.

Stable expression of gp41 proteins was assayed by flow cytometry after culturing transfected cells in supplemented DMEM containing 1 mg/ml of the selection antibiotic G418 (Invitrogen). Transient and stable expressions were assayed by determining cell surface expression and total levels of gp41 proteins in cell extracts. In addition, molecular weight, western blot analysis, MPER integrity, antibody recognition and residual fusogenic activity assays were also performed.

293 T cells were stained with the anti-gp41 2F5 or 4E10 monoclonal antibodies (Polymun Scientific) at a concentration of 4 μg/ml for 30 minutes at room temperature. After washing, bound antibodies were revealed using a PE-labeled Goat-anti-human IgG (Jackson ImmunoResearch) and analyzed in a LSR II flow cytometer (Becton Dickinson). The level of expression was determined as the % of positive cells or the Mean Intensity of Fluorescence (MFI). Mock transfected HEK-293 T cells were used as negative controls. The ratio of MFI observed for samples and negative controls was measured as a surrogate parameter of MPER exposure.

3.4. Peptides and proteins

HIV-1 IIIB C34 and T20 peptides were obtained through the NIH AIDS Reagent Program (Division of AIDS, NIAID). A 28-mer MPER peptide (EQELLELDKWASLWNWFNITNWLWYIKL) was ordered to ThermoFisher Scientific. The OLP#19 peptide covering the C-terminal part of MPER was kindly provided by C. Brander (IrsiCaixa, Spain). Gp41-Min sequence was cloned in a pET-21d(+) expression vector (Novagen) and produced by *E. coli* BL21 DE3 strain (Invitrogen). Inclusion bodies were prepared from 1 L of bacterial culture and solubilised using 8 M urea. Highly pure protein was obtained through niquel-based Immobilized Metal Affinity Chromatography (GE Healthcare) and gel filtration using a Sephacryl S-100 HR column (GE Healthcare).

3.5. Enzyme Linked Immunosorbent Assays (ELISA)

Peptides C34, T20, OLP#19, MPER and recombinant gp41-Min protein were coated in 96-well Maxisorp Nunc-immuno plates (Fisher Scientific). After blocking, plates were incubated with 100ul of previously diluted plasma samples overnight at 4ºC. Plates were then washed and 100ul of a Horseradish Peroxidase (HRP)-conjugated F(ab)2 Goat antihuman IgG (Fc specific) (Jackson Immunoresearch) were dispensed for one hour at room temperature. Plates were developed with 100ul of O-Phenylenediamine dihydrochloride (OPD) substrate (Sigma-Aldrich) and stopped with 100 ul of 4 N H_2SO_4. Optical density was measured at 492 nm for specific signal and at 620 nm for background.

3.6. 2F5 competition assay

The 2F5 antibody was labeled with the DyLight 649 Microscale Antibody Labeling Kit (Pierce) and titrated in 293 T cells expressing gp41-Min protein. Competition of plasma samples with labeled 2F5 was performed by preincubating 293-Min cells with 1/10 dilutions of plasma for 15 minutes at room temperature, and then with 0.5 ug/ml of 2F5 for 30 min. Cells were washed in PBS, fixed in FA 1% in PBS and analyzed by flow cytometry.

3.7. Viruses and neutralization assays

HIV-2 chimeras were made in the context of the full-length p7312A HIV-2 molecular clone (GenBank accession number L36874). Expression vectors for the wild type HIV-2 (p7312A) and HIV-2 chimeras containing the HIV-1 gp41 Membrane Proximal External Region (p7312A-C1), the 2F5 (p7312A-C3) or 4E10 epitopes (p7312A-C4), were kindly provided by G.M Shawn (University of Pennsylvania) (122). Pseudoviruses were generated by transfection of plasmids in 293 T cells. After 24 hours post-transfection, supernatants were harvested, filtered at 0.45 micron and viral stocks frozen at -80ºC.

HIV-1 isolates NL4.3, BaL, AC10 and SVP16 were generated as pseudoviruses using Env expression plasmids and the pSG3 vector as described (238). Cell-free virus neutralization by plasma samples was tested by a standard TZM-bl based assay (121). Briefly, in a 96-well culture plate, 100ul of previously diluted plasma samples were preincubated with 50ul of pseudovirus stock, using 200 TCID50, at 37ºC, one hour. Then, 100ul containing 10,000 TZM-bl luciferase-reporter target cells per well were added. Plates were cultured at 37ºC and 5% CO2 for 48 hours. 2F5, 4E10 and IgGb12 (Polymun Scientific), and anti-CD4 clone SK3 (BD Biociences) were used as controls. Plasma samples were inactivated (56ºC, 30 minutes) prior to the assay and threefold serial dilutions were tested, from 1/60 to 1/4960. TZM-bl reporter cells were treated with dextran (Sigma Aldrich) to enhance infectivity. Luciferase substrate, Britelite Plus (Perkin-Elmer) was used for the read out.

3.8. Statistical analysis

Variables were expressed as the median (interquartile range) and compared using Mann–Whitney test. Spearman's correlation coefficient was calculated to assess the association between variables. Non-linear fit of neutralization data were calculated

using normalized values fitted to an one-site inhibition curve with fixed Hill slope (239). All statistical analyses and non-linear fitting were performed using the GraphPad Prism v5.0 software. Positivity cutoffs for ELISA and flow cytometry assays were calculated using the MEAN + 2xSD of values obtained using HIV-1 seronegative individuals. Bonferroni correction has been calculated for multiple comparisons.

4. Results

4.1. Generation and characterization of gp41-derived proteins

We designed a series of proteins containing the MPER of gp41 by generating deletion mutants of gp41 **(Figure 13.A).** Starting from a complete gp41 sequence devoid of the cytoplasmic tail (gp41-EC), we sequentially removed the fusion peptide to generate the gp41-2 L (2 helicoidal regions and loop) protein, the HR1 and the loop region to generate the gp41-Min protein. Finally, we fused the fusion peptide to the gp41-Min protein to limit HR2 flexibility and to putatively increase the association of the protein to the membrane (Gp41-Staple construct, **Figure 13.A**). All proteins were cloned in pcDNA3.1 expression vectors fused with a GFP sequence at the C-terminal end and transiently transfected in 293 T cells to assess MPER exposure on the surface of transfected cells. As shown in **Figure 13. B**, all proteins were similarly expressed as assessed by the intensity of GFP expression, although the proper exposure of MPER epitopes on the cell surface differed among constructs. The binding of two different anti-MPER antibodies (4E10 and 2F5) to the gp41-EC protein was hardly detectable, and the removal of the fusion peptide had little effect on cell surface MPER exposure, that remained only detectable at low level using the 2F5 antibody. Conversely, removal of the loop and the HR1 region greatly increased MPER exposure that become readily detectable by 4E10 and 2F5 in Gp41-Min transfected cells. Addition of the gp41 fusion peptide at the N-terminal end failed to increase cell surface expression of MPER, rather a decrease was observed for the binding of the 4E10 antibody **(Figure 13.B).**

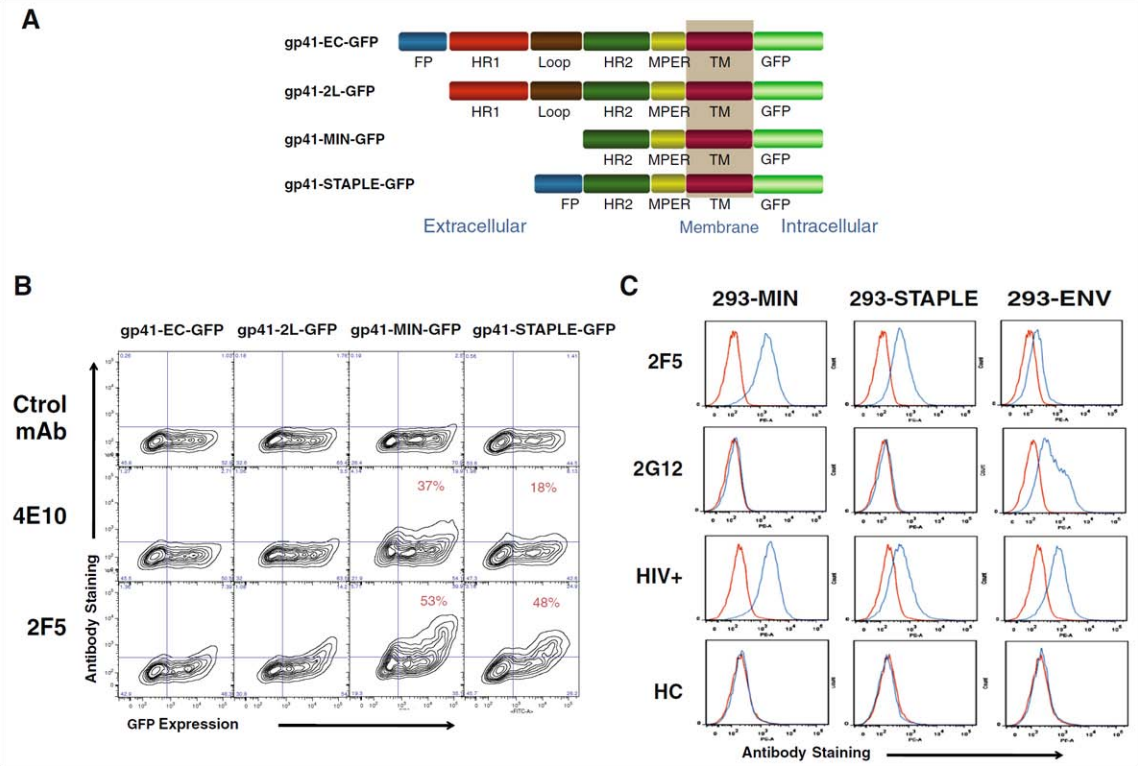


Figure 13. Characterization of gp41-derived proteins. **A**. Different gp41-derived proteins used in this study are depicted. The different regions of gp41 are depicted in blue (fusion peptide), red (helicoidal region 1, HR1), brown (disulfide loop), green (HR2), yellow (membrane proximal external region, MPER) and purple (Transmembrane region, TM). The GFP fused to the C-terminal sequence is also depicted in light green. **B**. Flow cytometry analysis of MPER exposure on the surface of transfected cells. 293T cells transiently transfected with the constructions shown in panel A were analyzed for cell surface MPER exposure. Plots of GFP expression and binding of control, 4E10 and 2F5 antibodies are shown. **C**. 293T cells stably expressing the gp41-Min (left panels) or gp41-Staple (middle panels) constructions were selected and the binding profile of different antibodies was compared with a 293T cell line stably expressing a full-length HIV-1 envelope construct (right panels). Antibodies tested were the anti-MPER mAb 2F5, the anti-gp120 glycan shield mAb 2G12 and plasma samples from HIV-1 infected or uninfected individuals.

We selected gp41-Min and gp41-Staple constructs to determine the level of anti-MPER antibodies in HIV-1 infected individuals, and generated 293 T cell lines stably expressing these proteins. For comparative purposes, a 293 T cell line stably expressing the full-length HIV-1 envelope (gp160 protein, isolate NL4-3) was also selected. 293 T cells expressing gp41-Min and gp41-Staple showed higher level of cell-surface MPER exposure than cells expressing full-length Env as assessed by 2F5 staining. The low 2F5 signal in the latter cell line was not due to low full-length Env expression, since a strong positive signal was obtained after staining with the 2G12 anti-gp120 antibody **(Figure 13.C).** Plasma from an HIV-1 infected individual showed reactivity against all cells,

while background levels of antibody binding were detected when plasma from an uninfected individual was used **(Figure 13.C).**

4.2. Analysis of anti-gp41 responses in HIV-1 infected individuals

Stably transfected cell lines characterized in **Figure 13.C** were used to analyze the binding of 35 plasma samples obtained from viremic untreated HIV-1 infected individuals. Recognition of gp41-Min, gp41-Staple and full-length Env was assessed by calculation of the ratio of MFI from each plasma obtained in the different cell lines and the MFI obtained using a control untransfected 293 T cell line **(Figure 14.A).** Background staining was defined as MEAN + 2xSD of values obtained using 10 plasma samples from uninfected individuals and showed that 94% of samples yield positive signals against gp41-Min protein, 85% against gp41-Staple and 97% against the full-length Env. No major differences among HIV-1 infected individuals were found when samples were classified according to VL, only a lower global anti-Env response was noticed in the group of patient showing VL < 5000 copies/ml **(Figure 14. A).** The stability of humoral responses was assessed using longitudinal samples separated at least one year **(Figure 14. B)** that showed a general conservation of specific responses; only a significant increase was observed for anti-gp41-Min antibodies in the lowest VL group **(Figure 14. B),** probably associated to a significant increase in VL (data not shown).

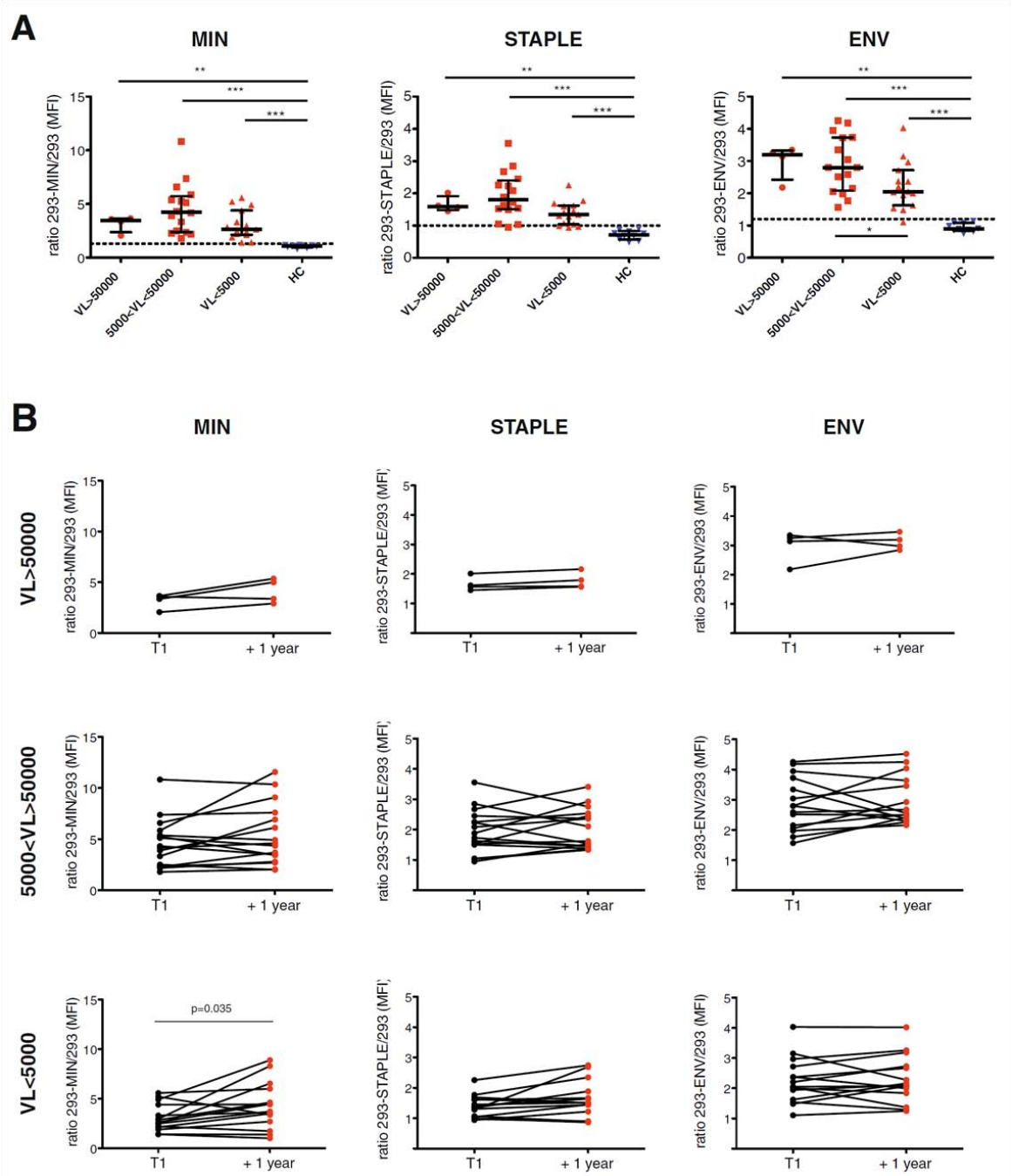


Figure 14. Identification of anti-MPER antibodies in HIV-1 infected individuals. **A**. The presence of antibodies recognizing the gp41-Min, gp41-Staple or full-length HIV-1 envelope was tested using the 293T cells stably expressing these proteins. The upper plots show the ratio of mean fluorescence intensity (MFI) of plasma IgG bound to 293–Min, 293-Staple or 293-ENV (full-length) and control 293 cells. Plasma samples from HIV-1 infected individuals were classified according to plasma viral load (VL > 50000, 50000 < VL < 5000 and VL < 5000). Plasma samples from uninfected individuals (HC) were tested as control. Dotted lines show the positivity cutoff calculated as Mean + 2xSD of uninfected plasma samples. **B**. The longitudinal evolution of gp41-Min, gp41-Staple and full-length HIV-1 envelope recognition by plasma samples from HIV-1 infected individuals is shown for the different VL groups defined in A. Time points are separated at least one year. All figures show the ratio of MFI between cells stably expressing gp41-Min (left) gp41-Staple (middle) or full-length envelope (ENV, right) and 293T control cells. Significant p values are shown.

Furthermore, a strong correlation was observed between the recognition of gp41-Min and gp41-Staple when all samples were analyzed **(Figure 15)** emphasizing the similarities between both proteins tested. Interestingly, the amount of antibodies bound to both gp41-derived proteins strongly correlated with the total anti-Env response **(Figure 15)**, suggesting that anti-gp41 responses are generated in the context of a potent general anti-Env response.

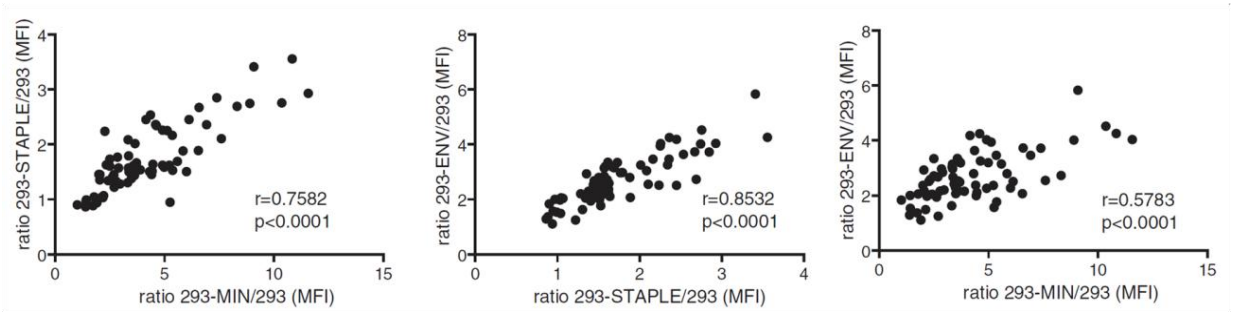


Figure 15. Antibodies against Gp41-Min and Gp41-Staple are elicited in the context of a wide anti-Env response. Spearman´s correlation analysis of the signals obtained between 293-Min and 293-Staple cell lines (left panel) and between both cell lines and 293-ENV cell line (middle and right panels, respectively). Figures show the correlation coefficient (r) and p values (p).

4.3. Mapping anti-gp41 responses

To determine the peptidic regions recognized by plasma samples and to evaluate the potential differences in recognition of soluble and membrane bound forms of our proteins we performed a series of ELISA assays using a purified full-length gp41-Min protein, the C34, T-20, MPER and OLP#19 peptides covering respectively the 628–661, 638–673, 659–683 and 671–684 residues of gp160 (HXB2 numbering, **Figure 16.A**). All plasma samples from HIV-1 infected individuals recognized the soluble form of gp41-Min protein with titers above the cutoff defined by uninfected individuals (Figure 16B). However, the recognition of the MPER peptide yielded positive titers for 66% of plasma samples **(Figure 16.B).** A similar percentage of samples recognized the T-20 peptide, which contains the 2F5 core epitope but lacks the 4E10 binding motif **(Figure 16.B)**, while a lower percentage of samples (55%) yielded positive titers for C34 peptide binding **(Figure 16.B)** and only 17% of samples recognized the OLP#19 peptide encompassing the 4E10 epitope. Furthermore, a strong positive correlation was found between anti-MPER responses and both the recognition of cell surface expressed gp41-Min protein and anti-T-20 titers (Figure 16.C), while a poor correlation was observed between anti-MPER titers and either anti-C34 antibodies or anti-4E10 epitope

antibodies **(Figure 16.C).** Altogether these data suggest that a robust response against the 2F5 epitope is generated in HIV-1 infected individuals.

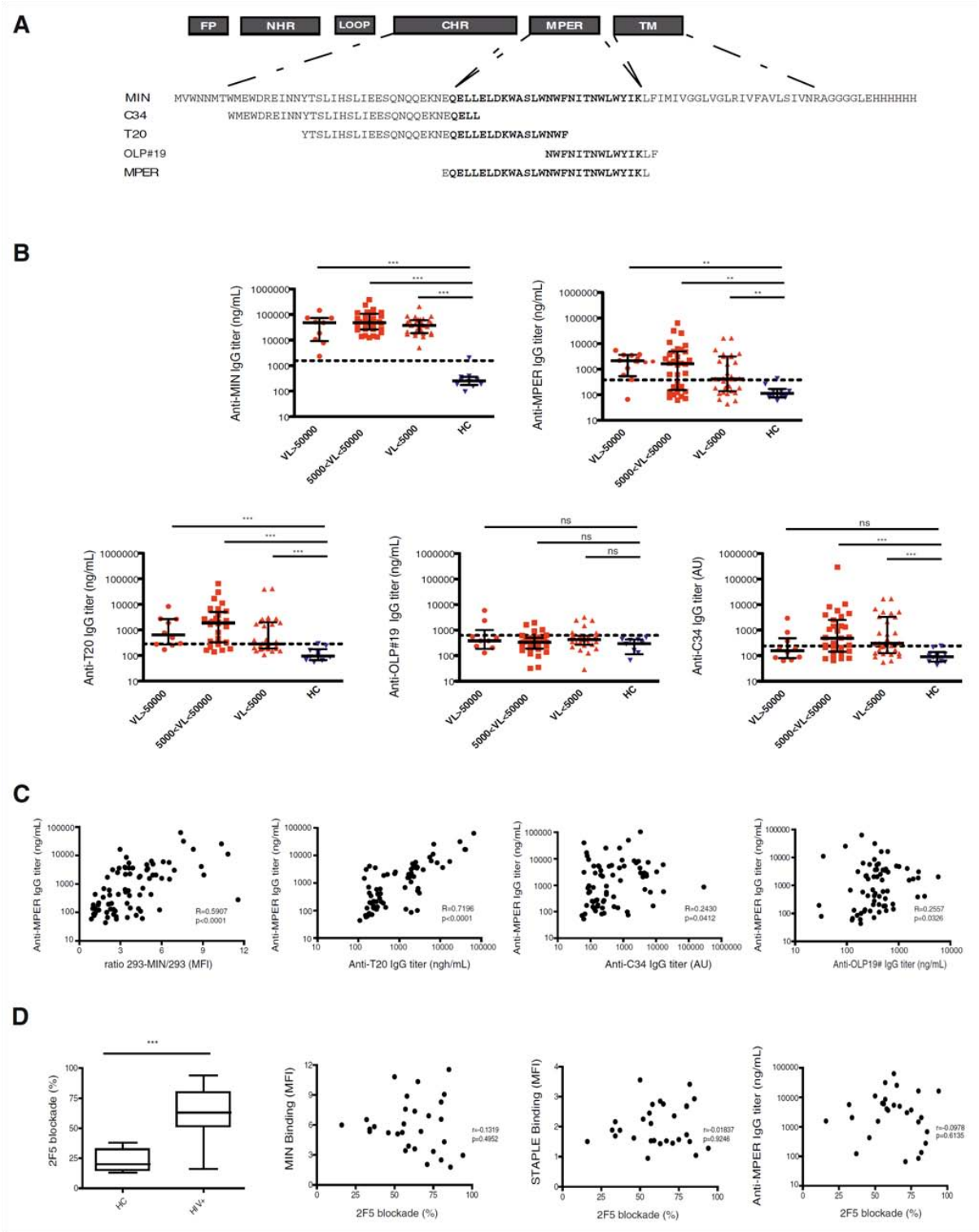


Figure 16 *(legend on next page)*

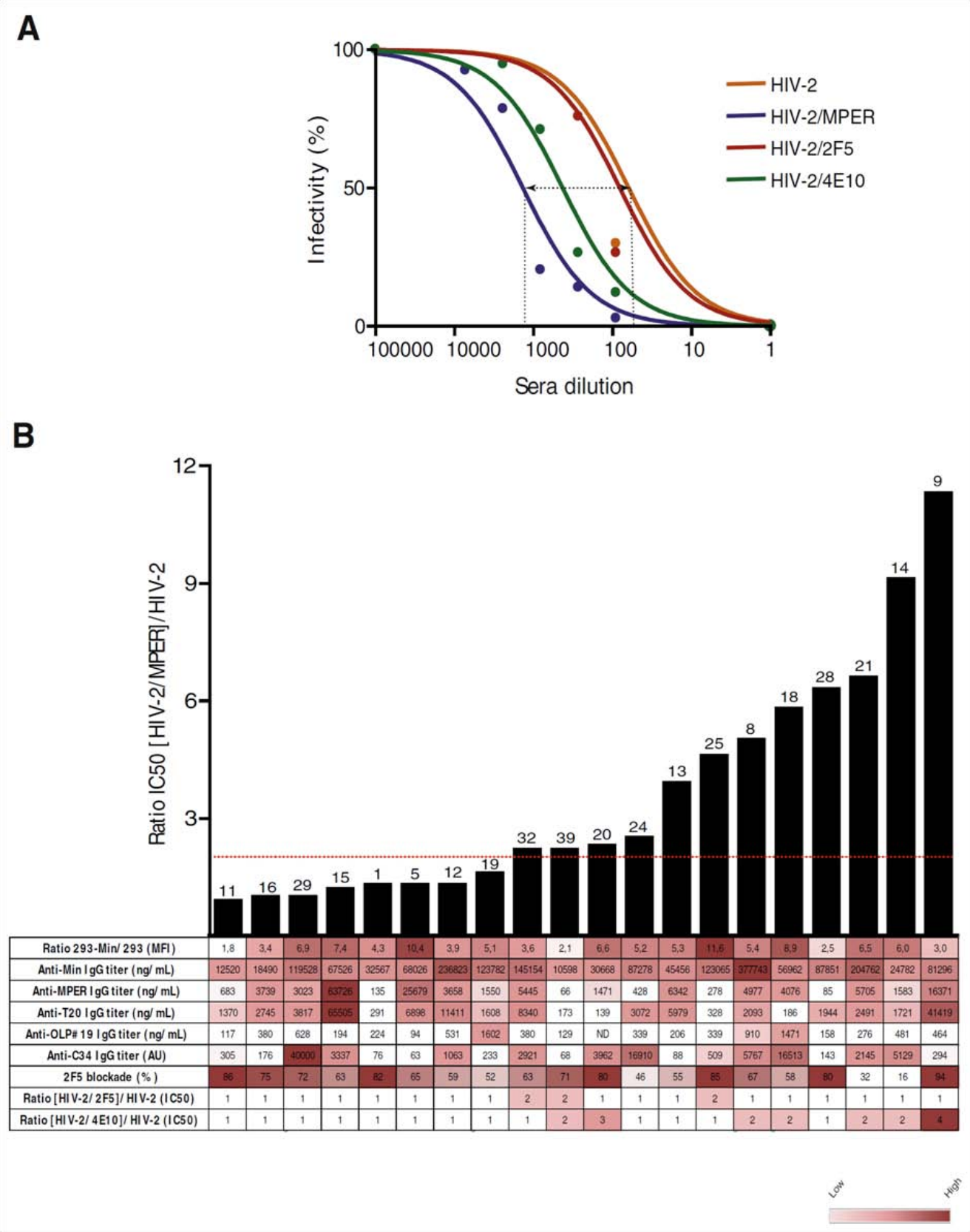
(Figure on previous page) **Figure 16. Mapping anti-gp41 responses**. **A**. Schematic representation of the antigens used for our fine mapping of anti-gp41 responses. Amino acid sequences of a recombinant full-length gp41-Min protein and peptides C34 (gp41 aa 628–661), T20 (gp41 aa 638–673), OLP#19 (gp41 aa 671–684) and MPER (gp41 aa 659–683) are displayed. **B**. Specific IgG titers for the recognition of gp41-Min, MPER, T20, OLP#19 and C34 peptides by plasma samples. Titers are indicated in equivalents of 2F5 in ng/mL for gp41-Min, T-20 and MPER or 4E10 equivalents in ng/ml for OLP#19. For C34 Arbitrary Units (AU) relative to one highly positive plasma sample used as standard are indicated. **C**. Spearman´s correlation between standard anti-MPER ELISA assay and specific IgG signal displayed by 293-Min cell line stained by plasma samples from HIV-1 infected individuals, anti-T20 ELISA titers, anti-C34 ELISA titers and anti OLP#19 ELISA titers. **D**. Plasma samples from HIV-1 infected individuals and healthy controls (HC) were tested in a competition assay by using the 293-Min cell line and a fluorescently-labeled 2F5 antibody. The percentage of blockade of 2F5 binding is shown for both groups of samples. Correlations between 2F5 blockade and specific recognition of 293-Min, 293-Staple and anti-MPER ELISA titers by plasma samples are shown. 2F5 competition assays and fine gp41-peptide mapping confirms the presence of anti-MPER antibodies in plasma from HIV-1 infected individuals. In B and D, ***denotes $p < 0,001$. In panels C and D the correlation coefficient (r) and p values (p) are shown.

Therefore, we assayed the functional binding of plasma antibodies to MPER in a competition assay using 293T cells expressing the gp41-Min protein and labeled 2F5 antibody. As expected, plasma from HIV-1 infected individuals induced a significant blockade of the 2F5 epitope compared to background levels induced by control uninfected samples **(Figure 16.D).** However, the extent of inhibition was not correlated to the titers of anti-MPER antibodies measured by different methods: direct binding to gp41-Min or gp41-Staple proteins or MPER peptide ELISA **(Figure 16.D)**, suggesting heterogeneity in functional binding to the targeted epitopes.

4.4. Neutralization capacity of plasma samples

The potency of neutralization of different HIV-1 isolates (NL4.3, BaL, AC10 and SVPB16) was evaluated for plasma samples in TZM-bl cells. A positive correlation was found between the levels of gp41-Min recognition and the neutralization titers for all isolates (data not shown). However, this observation is probably related to the strong correlation between anti-Min antibodies and global anti-Env responses that may mediate neutralization. Therefore to ascertain the specific neutralizing capacity of anti-MPER antibodies detected in plasma samples, we tested plasma samples against a collection of chimeric HIV-2 viruses engrafted with different MPER sequences (122). IC-50 values were calculated for the wild type (wt) HIV-2, HIV-2 containing the full MPER sequence (aa 661–684), the 2F5 epitope (aa 661–670) or the 4E10 epitope (aa 671–684). The increase in IC-50 between the wt and the different engrafted viruses was assumed to be the specific contribution of MPER or specific regions to neutralization

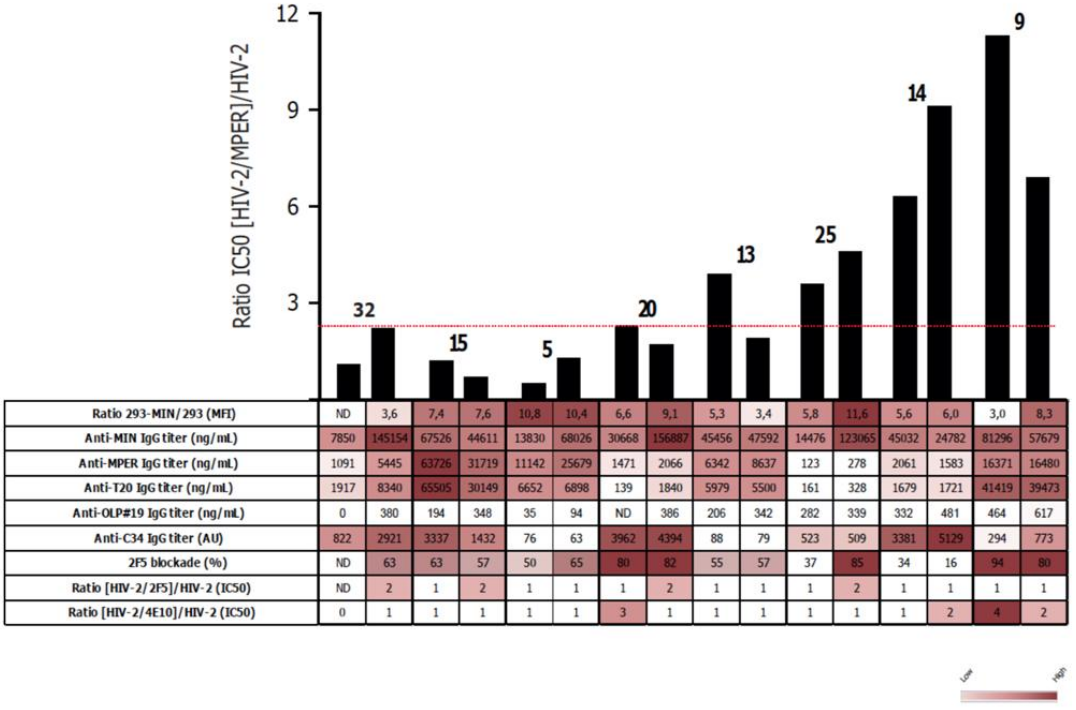
(Figure 17.A). Using this approach, only a percentage of plasma samples showed specific neutralization against the full MPER sequence. Neutralizing activity was hardly detected when using HIV-2 viruses engrafted with shorter epitopes of either 2F5 or 4E10 antibodies. A direct comparison of neutralizing plasma with those lacking neutralization capacity showed an unexpected similarity in ELISA, or flow cytometry parameters evaluated to quantify MPER recognition **(Figure 17.B)**. Indeed, several plasma samples exhibited high titers of anti-MPER antibodies in the absence of measurable neutralization capacity (see samples 15, 5 and 12 in **Figure 17.B**), while several plasma samples showed an inverse behavior, with neutralizing capacity in the absence of high anti-MPER titers (samples 25 or 28). Furthermore, the longitudinal analysis of several plasma samples confirmed a robust reproducibility in the different parameters measured, indicating that the very diverse profiles in gp41 humoral responses are stable overtime **(Figure 18).** In summary, these data suggest that both neutralizing and non-neutralizing responses are generated against the MPER epitope and that standard or new epitope binding measurements hardly identify neutralizing activity of polyclonal plasma samples



Ratio 293-Min/ 293 (MFI)	1,8	3,4	6,9	7,4	4,3	10,4	3,9	5,1	3,6	2,1	6,6	5,2	5,3	11,6	5,4	8,9	2,5	6,5	6,0	3,0
Anti-Min IgG titer (ng/ mL)	12520	18490	119528	67526	32567	68026	236823	123782	145154	10598	30668	87278	45456	123065	377743	56962	87851	204762	24782	81296
Anti-MPER IgG titer (ng/ mL)	683	3739	3023	63726	135	25679	3658	1550	5445	66	1471	428	6342	278	4977	4076	85	5705	1583	16371
Anti-T20 IgG titer (ng/ mL)	1370	2745	3817	65505	291	6898	11411	1608	8340	173	139	3072	5979	328	2093	186	1944	2491	1721	41419
Anti-OLP# 19 IgG titer (ng/ mL)	117	380	628	194	224	94	531	1602	380	129	ND	339	206	339	910	1471	158	276	481	464
Anti-C34 IgG titer (AU)	305	176	40000	3337	76	63	1063	233	2921	68	3962	16910	88	509	5767	16513	143	2145	5129	294
2F5 blockade (%)	86	75	72	63	82	65	59	52	63	71	80	46	55	85	67	58	80	32	16	94
Ratio [HIV-2/ 2F5]/ HIV-2 (IC50)	1	1	1	1	1	1	1	1	2	2	1	1	1	2	1	1	1	1	1	1
Ratio [HIV-2/ 4E10]/ HIV-2 (IC50)	1	1	1	1	1	1	1	1	1	2	3	1	1	1	2	2	1	2	2	4

Figure 17 *(legend on next page)*

(figure on previous page) **Figure 17. MPER-like neutralization capacity of selected plasma samples shows diverse antibody specificities**. Panel A. Example of the neutralization profile of one plasma sample against a collection of chimeric HIV-2 viruses engrafted with the whole MPER region or the 2F5/4E10 epitopes. Specific neutralization capacity was calculated as the ratio of IC50 between engrafted viruses and wild type HIV-2, corresponding with the curve shift relative to that for wild type HIV-2. Panel B. Bar graph shows the level of specific MPER-like neutralization, expressed as described in panel A. Numbers on the top of bars indicate patient code. The table displays the values of the different parameters evaluated in the study for each tested plasma sample. Color code is indicated in the lower right corner.



Ratio 293-MIN/293 (MFI)	ND	3,6	7,4	7,6	10,8	10,4	6,6	9,1	5,3	3,4	5,8	11,6	5,6	6,0	3,0	8,3
Anti-MIN IgG titer (ng/mL)	7850	145154	67526	44611	13830	68026	30668	156887	45456	47592	14476	123065	45032	24782	81296	57679
Anti-MPER IgG titer (ng/mL)	1091	5445	63726	31719	11142	25679	1471	2066	6342	8637	123	278	2061	1583	16371	16480
Anti-T20 IgG titer (ng/mL)	1917	8340	65505	30149	6652	6898	139	1840	5979	5500	161	328	1679	1721	41419	39473
Anti-OLP#19 IgG titer (ng/mL)	0	380	194	348	35	94	ND	386	206	342	282	339	332	481	464	617
Anti-C34 IgG titer (AU)	822	2921	3337	1432	76	63	3962	4394	88	79	523	509	3381	5129	294	773
2F5 blockade (%)	ND	63	63	57	50	65	80	82	55	57	37	85	34	16	94	80
Ratio [HIV-2/2F5]/HIV-2 (IC50)	ND	2	1	2	1	1	1	2	1	1	1	2	1	1	1	1
Ratio [HIV-2/4E10]/HIV-2 (IC50)	0	1	1	1	1	1	3	1	1	1	1	1	1	2	4	2

Figure 18. Longitudinal analysis of MPER-like neutralization capacity of selected plasma samples. Bar graph show the level of specific MPER-like neutralization, expressed as described in Figure 17. for longitudinal analysis of plasma samples. Numbers on the top of paired bars indicate patient code. The table displays the values of the different parameters evaluated in the study for each plasma tested. Color code indicated in the lower right corner and corresponds to that used in Figure 17...

5. Discussion

The MPER of gp41 is an attractive vaccine candidate that exposes linear peptides as target of broadly neutralizing antibodies. However, the particular localization of the MPER in the HIV-1 envelope glycoprotein trimer may represent a limiting factor for neutralizing activity. Indeed, this sequence is partly inserted in the viral or cellular membrane due to its amphiphilic properties (148), and is located in the base of the inverted pyramid formed by the envelope trimer (240). However, monoclonal antibodies against the MPER have been shown to exert similar protective effects than anti-gp120 antibodies in non human primate models (116). Therefore the elicitation of neutralizing anti-MPER responses by several candidate immunogens is still a major issue in HIV-1 vaccine research, although it has been unsuccessful to date (210, 241–243).

All these failed attempts may be explained by the requirement of hydrophobic residues in the CDR H3 loop of anti-MPER antibodies to allow them to access the hydrophobic environment of the targeted sequence (210). This is the case of the different bNAbs isolated (148, 197). Furthermore, the hydrophobic CDR H3 regions recognize lipids (148, 197) and at least 2F5 and 4E10 bNAbs are also cross reactive with human proteins, thus suggesting that tolerance may limit anti-MPER responses (214, 244). All these limitations seem to favor the diversion of humoral immune responses towards other gp41 regions, in particular the external loop, which has been described as an immunodominant non-neutralizing region (245).

To evaluate the impact of the latter limitations in the generation of anti-MPER antibodies, we analyzed the responses against the MPER elicited by natural infection. Responses were quantified by using miniproteins devoid of the immunodominant regions of gp41 but containing the HR2 sequence adjacent to the MPER. Maintaining this region was necessary since removal of the HR2 sequence reduced the binding of 2F5 to the cell surface expressed miniproteins (data not shown, Carrillo et al. in preparation). Unexpectedly, most HIV-1 infected individuals showed specific recognition of two different miniproteins displaying the HR2 and the MPER regions of gp41. In fact, both proteins only differ on the flexibility of the HR2 sequence, being this region free in the gp41-Min protein, while conformationally constrained by the potential interaction of the fusion peptide with the membrane in the gp41-Staple protein. Thus, the strong association between the recognition of these two proteins suggested that anti-

MPER antibodies were responsible for binding. This observation was further confirmed by classical ELISA assays using full length gp41-Min protein, and C34, T-20 and MPER peptides; the latter peptides share the N-terminal MPER sequence and showed strong positive correlation in ELISA data. Consistently, a peptide spanning the C-terminal moiety of MPER, OLP#19, showed much lower reactivity. These data reinforced the notion that HIV-1 infected individuals develop anti-MPER antibodies, mainly against the 2F5 epitope, that are stable overtime. Furthermore, another relevant observation is that strong anti-MPER responses occurs in patients with strong anti-Envelope responses, suggesting that, no specific requirements are needed for such a responses, and that the general immunocompetence, defined by the suboptimal function of CD4 and B cell compartments (246), may be the main limiting factor in the elicitation of strong humoral responses in natural HIV-1 infection. Finally, our analysis also searched for immune correlates of viral control; however, no correlation of viral load was observed with any measured parameter, either related to MPER-specific or general anti-Env responses. This is consistent with previous data showing a lack of correlation between neutralizing activity and virological control and progression to AIDS (84, 247).

While the wide presence of anti-MPER responses could be good news for the development of MPER based immunogens, our data points to a more negative aspect, the strong functional heterogeneity of the anti-MPER antibodies detected in HIV-1 infected individuals. We show that plasma samples show divergent neutralizing capacity or ability to block 2F5 binding and that both activities are unrelated to the level of anti-MPER antibodies measured by flow cytometry or ELISA. Several reasons may explain these paradoxical results. The existence of non-neutralizing anti-MPER antibodies, that may compete for the 2F5 binding epitope, has been reported in mice and rhesus macaques immunized with MPER-containing proteins (208, 248). These antibodies seem to be also generated in the context of natural HIV-1 infection and may provide positive results in flow/ELISA assays that detect MPER binding, while failing to induce detectable neutralization. Consistently, a wide analysis of gp41 responses showed that all monoclonal antibodies elicited in humans against the cluster II of gp41 lacked neutralizing activity (249). Similarly, llama immunization with liposomal formulations of gp41 miniproteins is able to induce neutralizing antibodies despite the absence of neutralizing activity observed in plasma samples (210), suggesting that non-neutralizing antibodies may not only divert neutralizing immune responses but also

block the binding of neutralizing antibodies elicited, as shown for gp120 C1 antibodies (250). However, the ability of non-neutralizing antibodies to bind MPER may allow them to interfere with HIV-1 replication by alternate mechanisms, such as Antibody Dependent Cellular Cytotoxicity (ADCC) that seems to be a protective factor in the RV144 study (101) and has been described for neutralizing anti-MPER antibodies (251). Our data also provide evidence for a completely opposite setting, the presence of neutralizing antibodies that poorly bind peptidic sequences (this is the case of samples 25 and 28 in **Figure 17.B** and **18**). In these cases, flow cytometry approaches yield higher positivity, suggesting the involvement of lipid membranes in binding to the target epitopes and therefore a benefit of flow cytometry methods to detect these antibodies.

Given the great complexity of the immunogenicity of the MPER, it seems necessary to better understand the natural humoral response to this region. For instance, further characterization of new monoclonal antibodies isolated from HIV-1 infected individuals displaying high titers of anti-MPER antibodies with neutralizing and non-neutralizing activity will be beneficial for the definition of the mechanisms and the structural requirements involved in the elicitation of broadly neutralizing antibodies. In this regard, the role of HR2, that seems to be necessary for immunogen conformation but also responsible for the elicitation of non-neutralizing antibodies, should be defined. These data will provide an improved framework for the design of novel MPER-based immunogens that directs the humoral response towards neutralizing activities.

Results II

Proteoliposomal formulations of an HIV-1 gp41-based miniprotein elicit a lipid-dependent immunodominant response overlapping the 2F5 binding motif

This chapter corresponds to the manuscript:

Luis M Molinos-Albert, Eneritz Bilbao, Luis Agulló, Silvia Marfil, Elisabet García, Maria L. Rodríguez de la Concepción, Nuria Izquierdo-Useros, Cristina Vilaplana, F.-Xabier Contreras, Martin Floor, Pere J Cardona, Javier Martinez-Picado, Bonaventura Clotet, Jordi Villà-Freixa, Maier Lorizate[*], Jorge Carrillo[#], Julià Blanco[*#]. ***Proteoliposomal formulations of an HIV-1 gp41-based miniprotein elicit a lipid-dependent immunodominant response overlapping the 2F5 binding motif.*** *Submitted for publication.*

* Corresponding authors
# Equal contribution

1. Presentation

The complexity of the MPER immunogenicity observed in the previous chapter made us to explore how anti-MPER antibodies are generated. Accordingly, we formulated one of the gp41-based miniproteins that better exposed the MPER region into proteoliposomes of diverse lipid mixtures, for mice model immunization. A systematic analysis of the influence of lipid components over the MPER immunogenicity, epitope immunodominance mapping and molecular dynamics of the residues involved are presented in this chapter.

2. Introduction

The HIV-1 envelope glycoprotein (Env) is a trimer of heterodimers composed by the non-covalent association of gp120 and gp41 subunits (252). It is the sole viral protein exposed on the viral surface and, thus, is the main target of neutralizing antibodies. In spite of more than 30 years of research, an immunogen capable of inducing a broadly neutralizing antibody response against Env has not been achieved yet. Due to the high variation rate and immune evasion, a successful preventive vaccine should target conserved functional epitopes within the envelope. The identification of a small percentage of broadly neutralizing humoral responses within different cohorts of HIV-1 infected individuals highlighted the uncommonness but also the feasibility of the human immune system to develop this kind of responses (114, 123, 126, 128, 131). Furthermore, the isolation of broadly neutralizing antibodies (bNAbs) from some of these individuals identified several antigenic vulnerability sites within Env including the CD4 binding site (109, 114, 139, 142, 146); glycan-dependent residues located in the V1/V2 (N332) and V3 (N160) gp120 loops (141, 142, 147); the gp41 Membrane Proximal External Region (MPER) (110, 113, 143, 148); and recently discovered extended regions including residues from both gp120 and gp41 (149–151) and the gp41 fusion peptide (152). The study of these regions has guided efforts in HIV-1 vaccine development during the last years (74, 103).

The MPER is a highly conserved tryptophan-rich region that has crucial roles in both viral fusion (170, 171) and CD4-independent transcytosis across the epithelial cell barriers (176). Moreover, the MPER includes linear, transiently exposed epitopes targeted by bNAbs such as 2F5, 4E10 and 10E8. All of them show a wide in vitro activity and are able to protect animals upon viral challenge *in vivo* (115, 116, 143, 179). Particularly 10E8 is one of the broadest and most potent bNAbs isolated to date

(143). Therefore, the MPER is considered as a potential HIV-1 vaccine target. However, several barriers to generate a neutralizing response against this region make MPER vaccine design considerably challenging (148). Structurally, MPER peptide conformation is highly influenced by lipids and seems to be embedded into the viral membrane (204, 205), which is unusually rich in cholesterol (CHOL) and sphingomyelin (SM) (253). Accordingly, anti-MPER bNAbs show cross-reactivity against lipids that seem to be essential for their neutralizing capacity (193, 197, 254, 255).

In order to dissect how lipids may modulate the immunogenicity of the MPER region, we generated a collection of proteoliposomes containing a gp41-based miniprotein, previously shown to overexpose the MPER region [described in **results I** and (256)]. We included several structural lipids overrepresented in the viral membrane (CHOL and SM) (253) and lipids that may influence immune responses by promoting selective capture by antigen presenting cells (APC). In this regard, we tested phosphatidylserine (PS), that binds to different receptors on the surface of phagocytic cells, and the ganglioside GM3, which binds to CD169 (SIGLEC-1) on the membrane of subcapsular sinus macrophages (257–260), a highly specialized antigen presenting cells population that play a pivotal role during the induction of the humoral response (261). Moreover, monophosphoryl lipid A (MPLA), a ligand of TLR4, was included as molecular adjuvant (262).

By using our proteoliposome collection, we systematically evaluated the impact of lipid composition over MPER immunogenicity. We also mapped the responses elicited within the sequence of our immunogen. Remarkably, we found that regardless of the lipid composition used, most of the humoral response was focused on an epitope overlapping the 2F5-binding motif, revealing the existence of a gp41 immunodominant but non-neutralizing region that cannot be bypassed by any lipid mixture.

3. Materials and methods

3.1. Immunogen construction

Protein gp41-Min has been previously described[**results I** and (256)]. A tetanus toxoid (TT) promiscuous T-helper epitope $_{830}$QYIKANSKFIGITEL$_{844}$ (263) was added in frame at the end of the transmembrane domain of the gp41-Min. A linker of four glycines was introduced between them. The construct was generated by three sequential

round of PCR amplification using the gp41-Min plasmid as template and the following primers: sense primer: Nco I-Min S 5´ TTTGGCCATGGTTTGGAATAACAT 3´ and the following three antisense overlapping primers: Min-TT as1: 5´ GTTCGCTTTAATATACTGGCCACCGCCACCAGCTC-3´, Min-TT as2 5´ ATGCCAATAAATTTGCTGTTCGCTTTAATATACTG-3´and Min-TT as3 5´ TTTAACTCGAGCAGTTCGGTAATGCCAATAAATTTGCTG-3. The final construct was cloned into a pET21d (+) plasmid (Novagen), in frame with the C-terminal 6xHis tag, using the NcoI and XhoI restriction enzymes (Fermentas).

3.2. Recombinant gp41-MinTT production

Gp41-MinTT was produced in *E. coli* BL21 DE3 strain (Invitrogen). Cells were transformed with the plasmid pET-21-d+Min-TT and cultured in LB medium supplemented with ampicillin. Cells were harvested by centrifugation and lysed with 50 mM Tris-HCl, 100 mM NaCl, (pH=8) buffer supplemented with 2mg/mL of lysozyme. Crude extract was then treated with 0.5% of Triton X-100 and inclusion bodies were collected after centrifugation. Inclusion bodies were solubilized using an urea-containing buffer (8M urea, 20mM Tris-HCl, 500mM NaCl, 30mM imidazole, pH=8). Gp41-MinTT protein was purified by immobilized-metal affinity chromatography using sepharose-Ni^{2+} affinity chromatography (GE Healthcare). An additional gel filtration purification step (Hiprep 16/60 Sephacryl S200 HR, GE Healthcare) was performed in the presence of 1% sodium dodecyl sulfate (SDS) buffer (pH=7). Purified gp41-MinTT protein was dialyzed against PBS and concentrated with a 3kDa Amicon Ultra-15 device (Milipore) to reduce SDS concentration to <0.1%. Purity was assessed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and G-250 Coomassie staining (Biorad).

3.3. Proteoliposome production and characterization

Large unilamellar vesicles (LUV) were prepared following the extrusion method described before (264). Briefly, lipids from Avanti Polar Lipids were mixed in chloroform:methanol (2:1) ratio (v/v) and dried under a nitrogen stream. Traces of organic solvent were removed by vacuum pumping for 1–2 h. Subsequently, the dried lipid film was dispersed in 50mM MES, 50mM Tris, 1mM EDTA (pH 7.3) and subjected to 10 freeze-thaw cycles prior to extruding 10 times through two stacked polycarbonate membranes with a 100 nm pore size (Nucleopore) using the Thermo-

barrel extruder (Lipex extruder, Northern Lipids, Inc.). To make proteoliposome fraction visible, 1% of Rho-DHPE (Molecular Probes) was added in the organic phase during LUV preparation. Highly pure Gp41-MinTT and Liposomes were mixed in a 1:250 mole ratio (1:10 (w/w)). Octylglucoside (OG) (Sigma-Aldrich) was the detergent used for proteoliposome preparation. OG was removed by extensive dialysis using 14 kDa membranes (Sigma-Aldrich) in 50mM MES, 50mM Tris, 1mM EDTA (pH 7.3) buffer, changing the buffer solution every 4-8 hours for two days. The sample to buffer ratio used during dialysis was 1:2000 ratio (v/v). Newly formed proteoliposomes were subsequently purified by floatation experiment using ultracentrifugation for 210 min through a sucrose gradient (0-25-30% of sucrose) in a TLA 120.2 rotor at 100,000 rpm. Each sample was in 30% sucrose. After ultracentrifugation six fractions were collected following the next volumes from 1 to 6: 300-100-200-200-200 and 100 µl. The proteoliposomes were layered in the first two fractions as proved by the rhodamine fluorescence and the silver staining of each fraction. Once the proteoliposomes were purified, they were immediately freeze-dried in a FreeZone lyophilizer (Labconco). After proteoliposome characterization only those that fulfill the required characteristics were used for immunization. Proteoliposomes were characterized based on their vesicle size distribution using a Malvern Zeta-Sizer instrument (Malvern Instruments, Malvern, UK), and their lipid/protein quantification. For lipid quantification membrane labeled rhodamine fluorescence was measured in a BioTek Synergy HT plate reader. In all cases, Triton X-100 was added to avoid fluorophore quenching effect induced by the order degree of different lipid compositions. Protein quantification was achieved by a quantitative western blotting using anti-Histidine (Invitrogen) antibody. Detection was carried out on a LI-COR Odyssey system (LI-COR Biosciences) following manufacturer recommendations. All proteoliposomal preparations were aliquoted, freeze-dried and stored at –30ºC, and reconstituted shortly before use.

3.4. Experimental Animal modeling and Immunization regimens

Seven-week old female C57 BL/6 mice were purchased from Harlan Interfauna Iberica (Spain). The animals were shipped under suitable conditions, with the corresponding certificate of health and origin. Upon arrival, mice were kept under controlled conditions in a P3 high security facility with sterile food and water "ad libitum". All animal procedures were approved and supervised by the Animal Care Committee of the Germans Trias i Pujol University Hospital and by the Department of

Environment of the Catalan Government. Mice (five per experimental group) were subcutaneously immunized four times at weeks 0, 3, 6 and 9 with proteoliposome preparations (2 µg gp41-MinTT protein: 20µg lipids/inoculum), liposomes (20µg/inoculum) or recombinant gp41-MinTT (20ug protein/*inoculum*), reconstituted in PBS buffer. Mice were examined daily following a protocol that monitored weight loss, apparent good health (bristle hair and wounded skin) and behavior (signs of aggressiveness or isolation). Blood samples were collected from facial vein before each immunization point as well as at week 10. Mice were euthanized at week 12 with isoflurane (inhalation excess) in order to avoid any suffering, and total blood and spleen were collected. Serum was obtained by blood centrifugation at 5000xg for 10 minutes.

3.5. Peptide and protein antigens

15-mer overlapping peptides covering the gp41 HR2 and MPER regions were obtained from the NIH AIDS reagent program. Alanine mutants of #162 peptide (QEKNEQELLELDKWA) were purchased from Covalab. Recombinant gp41-Min protein was produced as described previously (256).

3.6. Western blot and ELISA assays

Samples were loaded onto a NuPAGE® Novex® 4-12% Bis-Tris Gel (Life Technologies) and run at 180V, 110 mAmp for 50 minutes. Membrane transfer was performed in an iBlot Gel Transfer Device (Life Technologies). After blocking (5% w/v skimmed milk PBS buffer + 0.05% v/v Tween 20), membrane was incubated at 4ºC overnight with anti-Histidine (Life technologies), D50 (NIH AIDS Reagent Program) or 2F5 (Polymun) antibodies. Membrane was then washed in PBS twice and incubated with Horseradish Peroxidase (HRP)-conjugated $F(ab)_2$ Goat anti-mouse IgG Fc specific or (HRP)-conjugated-$F(ab)_2$ Goat anti-human IgG Fc specific (Jackson Immunoresearch). Membrane was developed using an Enhanced Chemioluminiscent HRP substrate (Fisher Scientific). For ELISA, recombinant gp41-Min protein and peptides were prepared at 1 µg/mL and 10 µg/mL in PBS or carbonate/bicarbonate buffer (pH=9.6) respectively to coat 96-well Maxisorp Nunc-immuno plates (Fisher Scientific, 50 µl/well). After blocking with 1% bovine serum albumin (Sigma-Aldrich), plates were incubated with 100 ul of previously diluted serum samples overnight at 4ºC. Plates were then washed and 100 ul of a (HRP)-conjugated $F(ab)_2$ Goat anti-mouse IgG (Fc specific, Jackson Immunoresearch) were dispensed for one hour at room

temperature. Plates were developed with 100ul of O-Phenylenediamine dihydrochloride (OPD) substrate (Sigma-Aldrich) and stopped with 100 ul of 4N H_2SO_4. Optical density was measured at 492 nm for specific signal and at 620nm for background.

3.7. Viruses and neutralization assays

Pseudotyped HIV-1 were generated by cotransfection of Env expression plasmids and the pSG3 vector as described elsewhere (238). Cell-free virus neutralization by sera samples was tested by a standard TZM-bl based assay. Briefly, in a 96-well culture plate, 100ul of previously diluted plasma samples were preincubated with 50ul of pseudovirus stock, using 200 $TCID_{50}$ at 37ºC for one hour. Then, 100ul containing 10,000 TZM-bl luciferase-reporter target cells per well were added. Plates were cultured at 37ºC and 5% CO_2 for 48 hours. 2F5, 4E10 and IgGb12 (Polymun Scientific), and anti-CD4 clone SK3 (BD Biociences) were used as controls. Serum samples were inactivated (56ºC, 30 minutes) prior to the assay. Before addition to the assay, TZM-bl reporter cells were treated with dextran (10µg/mL) (Sigma Aldrich), to enhance infectivity. Luciferase substrate (Britelite Plus, Perkin-Elmer) was used for the read out.

3.8. Structural modeling

An *in silico* structural model of the extracellular and transmembrane segments of gp41-Min (including a few residues of the intracellular segment, 707 to 714, to stabilize the transmembrane domain) was constructed using Modeller 9v14 (265). The RCSB Protein Data Bank (PDB) structures 3VGX and 3H01 were used as templates for HR2 and MPER regions, while 2MG1 structure was used as template for the transmembrane domain. Alignment was done using 3D-Coffee (266) The best of six models obtained from Modeller, as assessed by the DOPE (Discrete Optimized Protein Energy) method, was embedded in a POPC bilayer using the VMD (267) membrane plugin, solvated with TIP3P water molecules (15 Å as minimal distance to the edge of the box). Additionally, Cl^- and Na^+ ions were added up to a final concentration of 0.15 M. The final structure was minimized, thermalized, and then submitted to about 310 ns of molecular dynamics at 310 K, using the CHARMM36 force field, with a timestep of 2 fs, a cut-off distance for non-bonded interactions of 10 Å and Particle-Mesh-Ewald for long-range electrostatic interactions. Protein RMSD throughout the dynamics is shown on supplemental file 3. The same protein structure but in the absence of the lipid bilayer was also submitted to a shorter molecular dynamics (150 ns) for comparative analysis.

Dynamics were performed with NAMD (268) at BSC supercomputing facilities (Barcelona). VMD was used for protein dynamics analysis.

4. Results

4.1. Antigen design, production and characterization

From our collection of gp41-based miniproteins [**results I** and (256)], we selected a minimal gp41-based construction that greatly increased the MPER exposure, designed as gp41-Min. This protein contains the HR2, the MPER and the transmembrane (TM) domains of gp41. To increase immunogenicity, a tetanus toxoid (TT) promiscuous T-helper epitope $_{830}$QYIKANSKFIGITEL$_{844}$ (263) was fused in frame with the TM domain. A six-histidine tag was added at the C-terminal end to allow protein purification. This construct, designed as gp41-MinTT (**Figure 19. A**), was cloned into a prokaryotic expression vector and the protein was produced in *E. coli*. After metal affinity and gel filtration chromatography, a highly pure 15kDa gp41-MinTT recombinant protein peak was achieved (**Figure 19. B**). The integrity of the protein was confirmed by SDS-PAGE and coomassie staining, Western Blott and ELISA using the specific antibodies D50 (HR2) and 2F5 (MPER) (**Figure 19 C-D).**

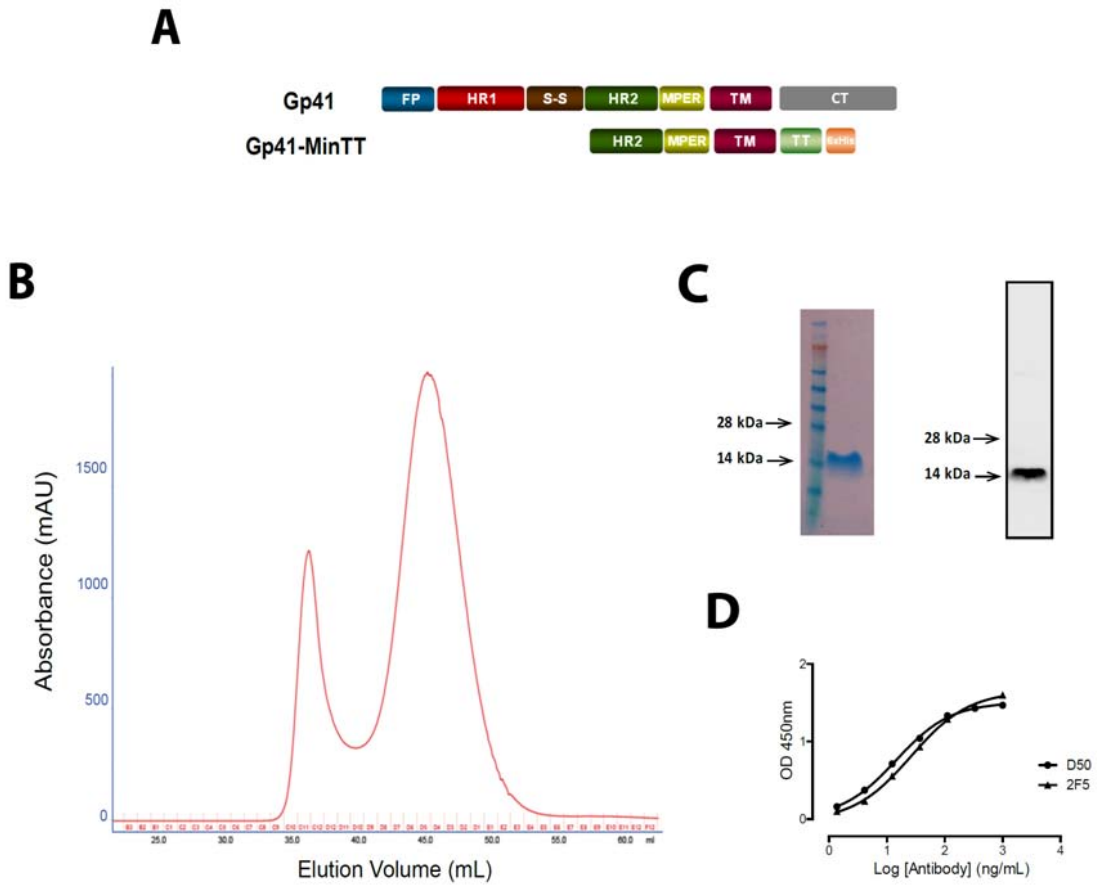


Figure 19. Gp41-MinTT expression and purification. **A**, schematic representation of gp41 and gp41-MinTT proteins is shown. FP, fusion peptide (blue); HR1, N-terminal heptad repeat (red); S-S, disulfide loop (brown); HR2, C-terminal heptad repeat (green); MPER, membrane proximal external region (yellow); TM, transmembrane domain (purple); CT, cytoplasmic tail (gray); TT, tetanus toxoid epitope (light green); 6xHis, 6-histidine tag (orange). **B**, gp41-MinTT recombinant protein was purified by IMAC (not shown) and gel filtration chromatography. Elution profile of the latter step is shown. **C**, a highly pure 15KDa protein was recovered and concentrated from central fractions of the largest peak shown in panel B (44-49 mL fractions), as confirmed by SDS-PAGE and comassie staining (left) and by Western blot using the 2F5 antibody (right). Molecular markers are indicated. D, antigenicity of purified gp41-MinTT protein determined by ELISA using serial dilutions of D50 (anti-HR2) and 2F5 (anti-MPER) antibodies.

4.2. Proteoliposome production and characterization

Since membrane environment influences the conformation of the MPER (204), we generated gp41-MinTT-based proteoliposomes using POPC and lipids overrepresented in the viral membrane-like environment, such as CHOL or SM (253, 269, 270). The molar ratios chosen mimic viral membrane composition and rigidity (270). The proteoliposomes used in this study were classified as complex or simple according to the presence or absence of CHOL and SM, respectively. In addition, we included lipids

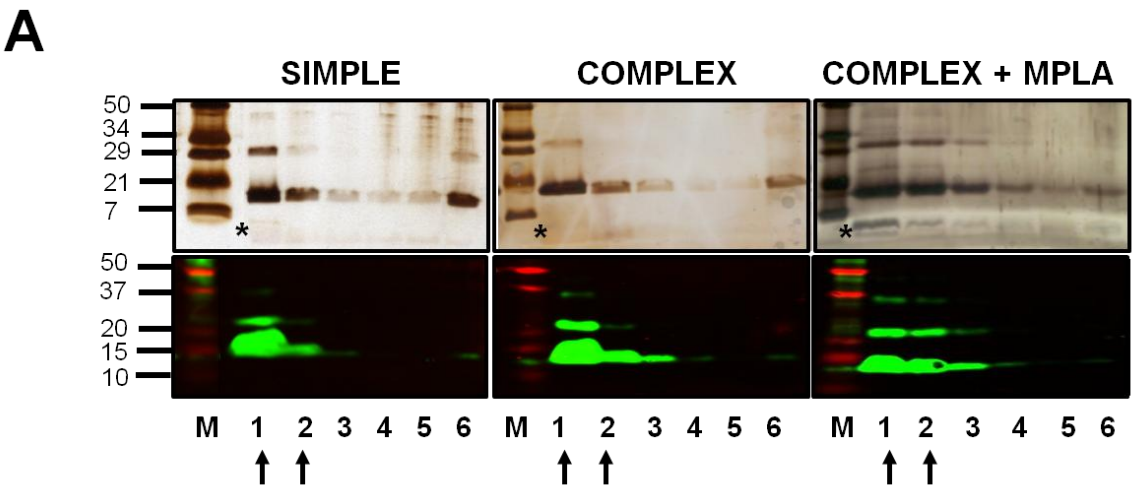
that bind to several receptors on the surface of antigen presenting cells (APC), such as the GM3 ganglioside (259) and PS (271), to seek whether the route of presentation may influence the immunogenicity of gp41-MinTT. We selected a GM3 molar ratio that induces optimal capture by dendritic cells (272). The same amount of PS was used. Finally, we evaluated the effect of the TLR4 agonist MPLA over the effect of complex proteoliposomes. A summary of the composition of the proteoliposomes used in this study is specified in **Table 3**.

Proteoliposomes were purified by ultracentrifugation on a sucrose gradient. Since proteoliposomes have a lower density than the protein alone, they float up and are recovered from the first two fractions **(Figure 20. A).** Protein signal was detected by silver staining and quantified by western blot analysis. Proteoliposomes were labeled with rhodamine, which was incorporated as DHPE-Rho into lipid mixtures. Fluorescence signal was used to quantify the recovered lipid amount, which was measured by plate reader (data not shown) and it is also visible in silver stained gels **(Figure 20. A).** All proteoliposomes incorporated more than 85 % of gp41-MinTT protein and showed an overall size distribution between 100-200 nm **(Figure. 20. B).** The lipid amount ranged between 220-240 µM. For protein characterization, we performed quantitative western blot. For this purpose, labeled secondary antibodies were used in a LiCoR system, which allows measuring the signal of each sample in the linear range. From quantitative experiments, a protein to lipid mole ratio obtained was always close to 1:250 **(Figure 20. B).** Western blot of collected fractions, showed similar protein amounts in all the samples tested. Interestingly, in the absence of lipids, gp41-MinTT protein showed a single band corresponding to a protein monomeric form whereas in the presence of lipids, SDS and mercaptoethanol resistant oligomeric bands appears **(Figure 20. B).** Consequently, all the proteoliposomes prepared were similar in size and lipid to protein mole ratio and therefore, suitable for comparable immunization.

FORMULATION	LIPID COMPOSITION (mole ratio)	PROTEIN	DOSE/INOCULUM
Recombinant protein		MinTT	20 µg
Simple proteoliposomes	POPC (100)	MinTT	2 µg
	POPC:GM3 (95:5)	MinTT	2 µg
	POPC:PS (95:5)	MinTT	2 µg
	POPC:GM3:PS (90:5:5)	MinTT	2 µg
Complex proteoliposomes	POPC:SM:CHOL (30:25:45)	MinTT	2 µg
	POPC:SM:CHOL:GM3 (30:20:45:5)	MinTT	2 µg
	POPC:SM:CHOL:PS (25:25:45:5)	MinTT	n.d*
	POPC:SM:CHOL:GM3:PS (25:20:45:5:5)	MinTT	2 µg
Complex proteoliposomes+MPLA	POPC:SM:CHOL:MPLA (29:25:45:1)	MinTT	2 µg
	POPC:SM:CHOL:GM3:MPLA (29:20:45:5:1)	MinTT	2 µg
	POPC:SM:CHOL:PS:MPLA (24:25:45:5:1)	MinTT	2 µg
	POPC:SM:CHOL:GM3:PS:MPLA (24:20:45:5:5:1)	MinTT	2 µg

**n.d.: not determined*

Table 3. Gp41-MinTT-containing immunogens used in this study. Immunogens were categorized in recombinant protein (gp41-MinTT), simple (POPC), complex (POPC CHOL SM) and complex+MPLA (POPC CHOL SM MPLA) proteoliposomes. GM3, PS or both were added to each category. Percentages are referred to lipid components. Lipid mole ratios were used. POPC: 1-Palmitoyl-2-oleoylphosphatidylcholine; CHOL, cholesterol; SM, sphingomyelin; GM3, monosialodihexosylganglioside; PS, phosphatidylserine; MPLA, monophosphoryl lipid A.



B

	SIMPLE	COMPLEX	COMPLEX + MPLA
Size (nm)	175 ± 44.4	168 ± 20.3	123 ± 15.6
[MinTT] (μM)	0.95 ± 0.031	0.95 ± 0.017	0.87 ± 0.1
[Lipid] (μM)	233 ± 5.7	234 ± 4.1	221 ± 11.6
MinTT/Lipid	1/235-1/258	1/244-1/252	1/235 -1/278

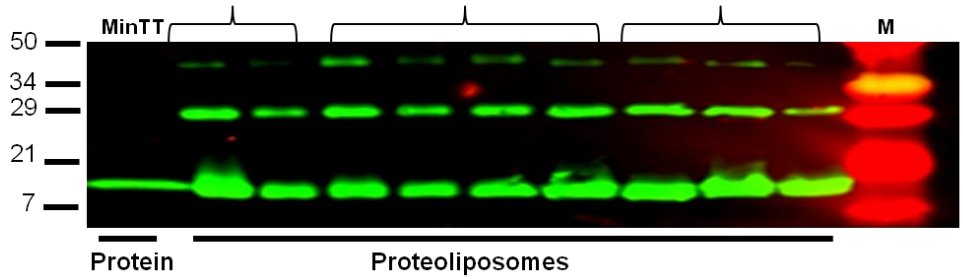


Figure 20. **Proteoliposomes characterization**. **A**, silver stained gels (top) and Western blots (bottom) of each sucrose gradient fractions corresponding to representative proteoliposomes of different composition (simple, complex and complex+MPLA). After proteoliposomes floatation, a sample of each recovered fraction was used for silver staining and Western blot analysis. Proteoliposomes could be detected in the first two fractions (arrows) by the signals of the protein and lipids (asterisks). M: Molecular Weight Marker and numbering 1 to 6 corresponds to the collected fractions from the top to the bottom of the ultracentrifuge tube. **B,** Mean values and standard deviations of vesicle size, quantified protein and lipid amounts of each proteoliposome group are indicated. Range of protein/lipid ratios are also shown. Western blot analysis of the selected proteoliposome fractions for immunization is shown below. Purified gp41-MinTT protein was used as reference (left lane).

4.3. Gp41-MinTT immunogenicity can be modulated by modifying the lipid composition

C57 BL/6 mice were immunized four times with 2μg of gp41-MinTT-containing proteoliposomes or with 20 μg of soluble recombinant protein in a 12-weeks immunization regimen. Three weeks after the last inoculum, anti-gp41 IgG titers were determined by ELISA using the gp41-Min protein, lacking the TT epitope (256). As expected, and despite the lower inoculum dose used, all proteoliposome-immunized

animals showed higher titers than those immunized with soluble protein **(Figure 21. A).** A global comparison between animals immunized with simple and complex proteoliposome formulations showed at least 1-log anti-gp41 titer increase in the complex group, indicating the enhancing effect of CHOL-SM addition **(Figure 21. A)**. When we evaluated the adjuvant activity of MPLA, we found an additional 1-log titer increase, highlighting the potential benefits of this TLR agonist on anti-gp41 responses **(Figure 21. A).** Moreover, analysis of antibody titer of immunized mice against a recombinant gp41-MinTT protein and those determined against the original gp41-Min protein showed a positive correlation ($r=0.8373$, $p<0.0001$), indicating that the humoral response generated by proteoliposomal formulations were directed mostly against the gp41 domain. However, mice immunized with recombinant gp41-MinTT protein showed gp41-MinTT binding in the absence of gp41-Min titers, indicating that the response was exclusively focused against the more immunogenic TT epitope **(Figure. 21. B)**. These findings demonstrate that the humoral response induced by gp41-MinTT immunogen can be dramatically modulated by the addition of lipids, which not only increase the magnitude of antibody titers but also guide the response towards the gp41 domain.

We next evaluated the effect of individual lipid components over gp41-MinTT immunogenicity **(Figure. 21. C-D).** Although a slight but not significant effect was observed in terms of immunogenicity when GM3 or PS were individually incorporated into simple compositions **(Figure 21. C),** we detected faster kinetics of anti-gp41 antibodies in GM3-containing liposomes **(Figure 21. D).** In spite of this, the individual contribution of GM3 or PS to the global response was not statistically significant in any of the conditions tested **(Figure 22).** However, a great increase in antibody titer was detected when both components were formulated together in the context of simple POPC formulations **(Figure 21. C).** This synergistic effect was not additive to the enhanced effect caused by either CHOL-SM or MPLA, since it was lost in complex formulations **(Figure 21. C).** Remarkably, we observed that kinetics of the antibody response were faster in those animals immunized with complex proteoliposomes as well as for those receiving GM3 and PS in combination **(Figure 21. D).**

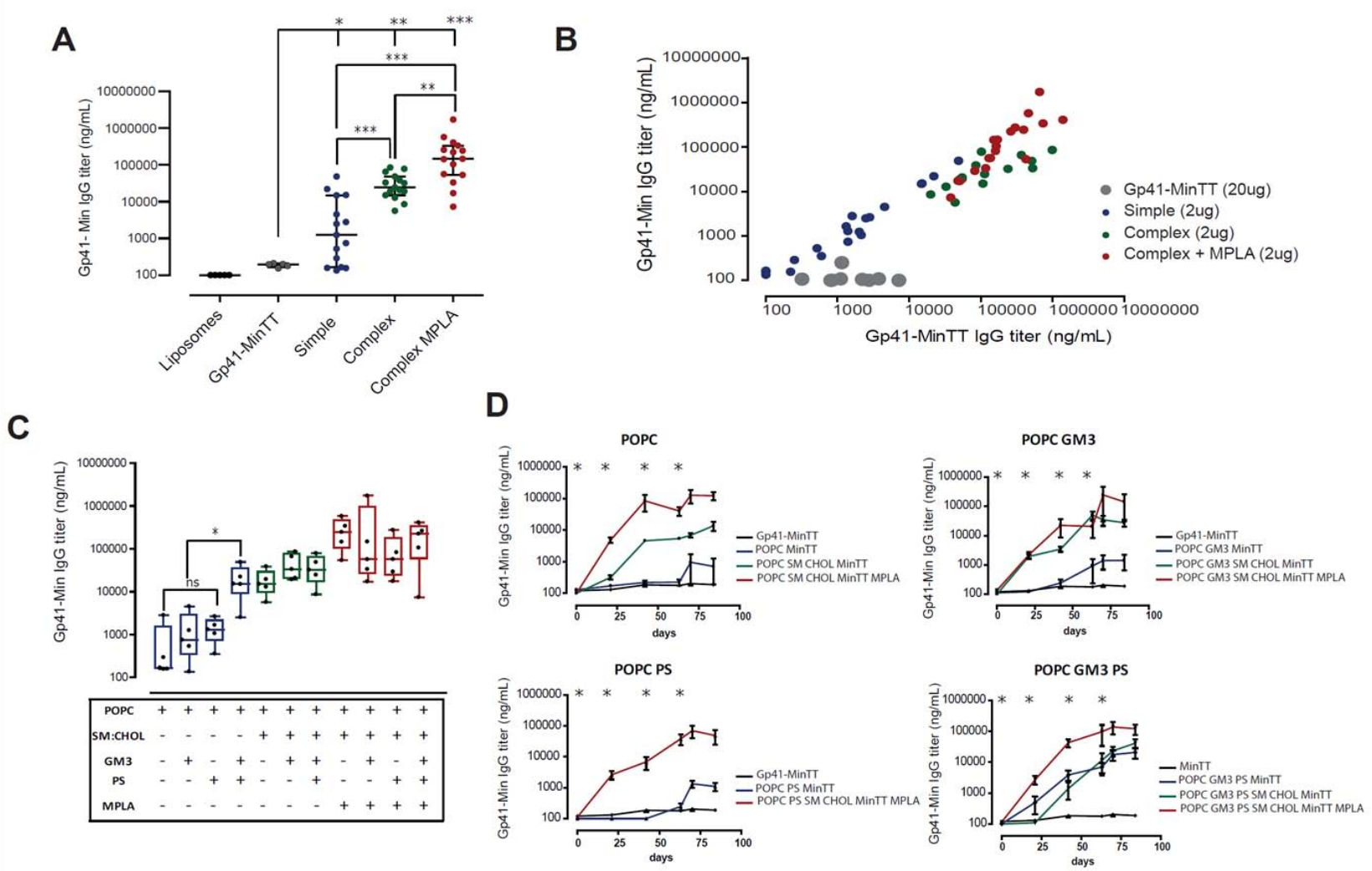


Figure 21. **Effect of lipid composition on anti-gp41 IgG response**. **A**, summary of anti-gp41-Min specific IgG titers, at sacrifice day, of C57 BL/6 mice immunized with gp41-MinTT proteoliposomes of simple (blue), complex (green) and complex incorporating MPLA (red) compositions. Data from animals immunized with control liposomes (black) and soluble gp41-MinTT protein (grey) are included. **B**, correlation of IgG titers, at sacrifice, against gp41-MinTT and gp41-Min antigens of mice immunized with gp41-MinTT as recombinant protein or formulated in proteoliposomes. **C**, Influence of lipid mixtures over gp41-MinTT immunogenicity. Gp41-IgG titer at sacrifice day is shown. **D**, Evolution of the gp41-Min IgG response of animals immunized with gp41-MinTT proteoliposomes based on POPC (upper left); POPC and GM3 (upper right); POPC and PS (down left); POPC combined with GM3 and PS (down right) in a simple, complex or complex with MPLA composition (blue, green and red lines respectively). Asterisks indicate immunization time points. In all panels, IgG titer is displayed as ng/mL referred to the D50 antibody, which was used as standard. Data show the median and interquartile range of at least two independent determinations. In panels A and C, ***, ** and * denote $p<0.001$ and $p<0.01$ respectively. *ns*, not significant.

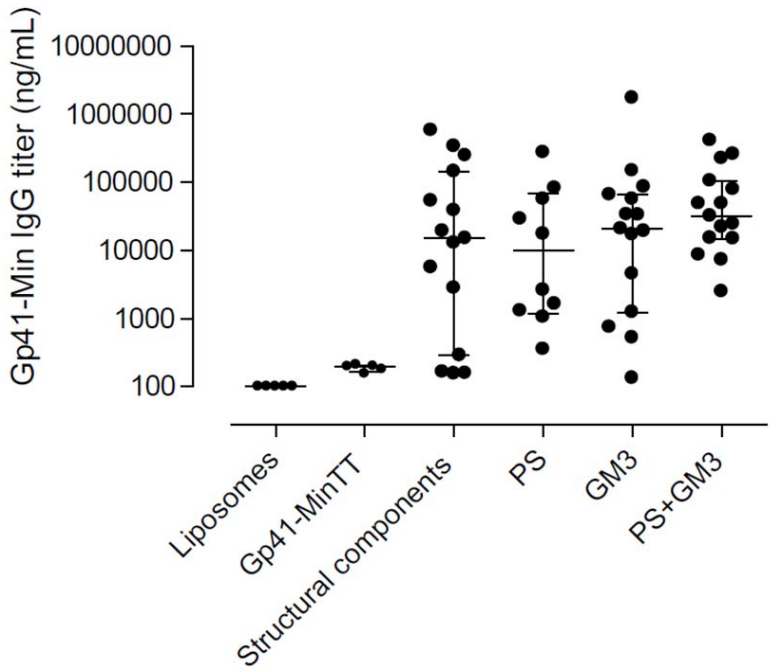


Figure 22. Individual contribution of PS and GM3 to gp41-MinTT immunogenicity. Gp41Min specific IgG titers of immunized animals with liposomes containing only structural components (POPC with or without CHOL and SM) or adding GM3, PS or both are displayed.

4.4. Immunodominant response is focused on the N-terminal MPER region

In order to explore the gp41 regions targeted in immunized animals, we assayed sera against a collection of 15-mer overlapping peptides covering the HR2 and the MPER of gp41 **(Figure 23. A).** The results, summarized in **Figure 23. B**, showed that the global response was mostly directed against a peptide, designed as #162 (residues 653-667), that overlaps between HR2 and MPER and includes the 2F5 binding domain **(Figure 23. C).** This major response correlated with the global anti-gp41-Min titer **(Figure 23. D),** indicating that humoral responses against this region are especially favored by gp41-MinTT immunogen formulated within a membrane environment. Furthermore, addition of MPLA to complex formulations increased the titer **(Figure 21. A)** of antibody but did not modify the specificity of the response since it was focused almost exclusively in the #162-covering region **(Figure 23. B).** We found hence that immunization with gp41-MinTT-based proteoliposomes generated an immunodominant response overlapping the N-terminal part of the MPER region, which contains the 2F5 binding domain.

To gain insights into the features of elicited antibodies, we analyzed the relevant residues for #162 binding. We selected 29 serum samples that displayed the highest #162 binding signals, and we assayed those sera against a collection of peptides in which each position of the #162 sequence was substituted by an alanine residue **(Figure 24. A)**. On average, we found that 6 residues (N656, E657, L660, L661, K665 and W666) were especially relevant for #162 binding **(Figure 24. B).** Importantly, four out of six of these residues are located within the MPER region and two of them (K665 and W666) correspond to the 2F5 neutralizing core ($_{664}$DKW$_{666}$) (189). In contrast, only a reduced number of sera recognized the D664 residue within the 2F5 epitope. Despite the binding of mice sera to a 2F5-overlapping region, no clear neutralization activity was observed in any animal at 1/100 dilution against a panel of four subtype B viruses including NL4-3, BaL, AC10 and SVPB16. (**Figure 25** and data not shown).

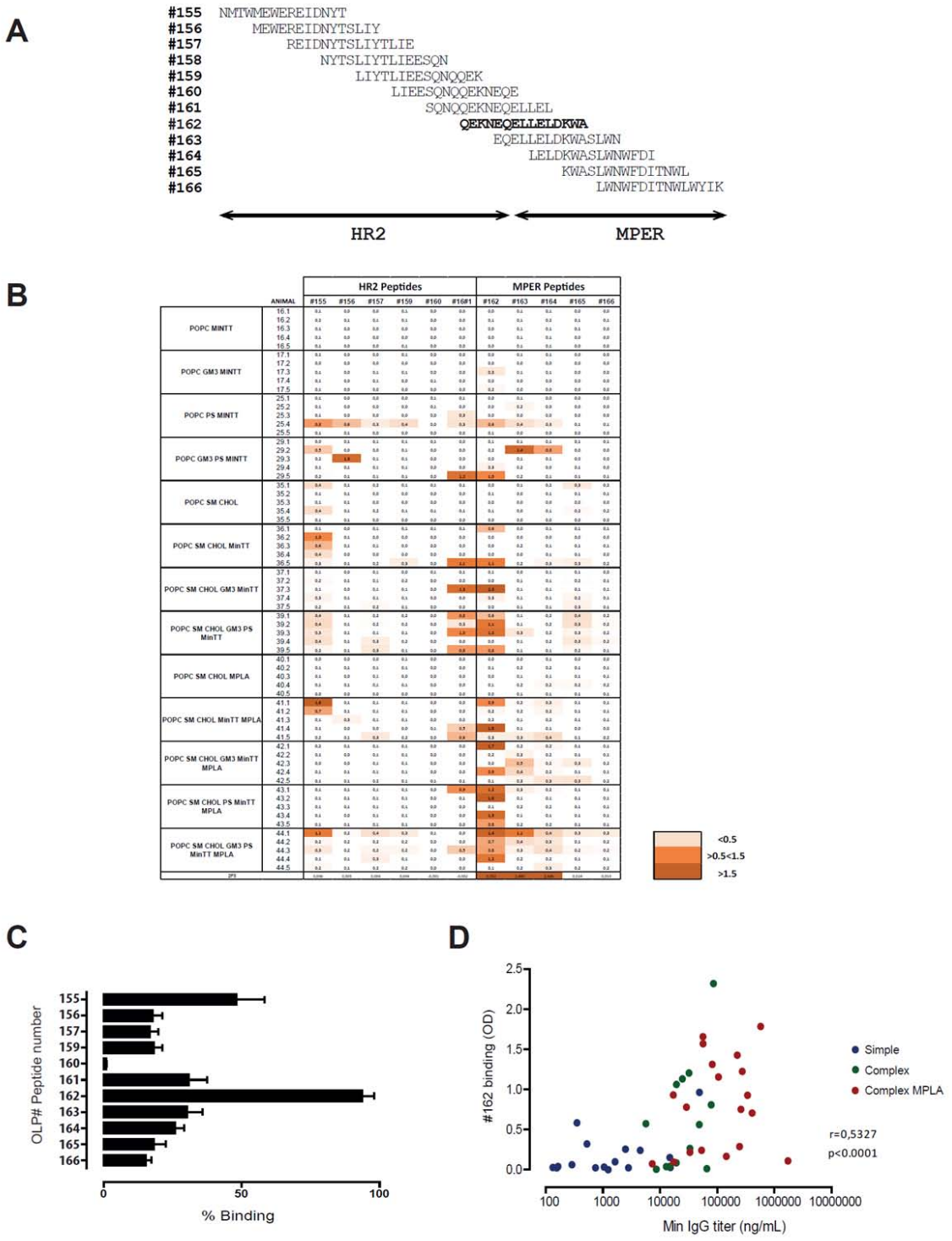


Figure 23. **Mapping of humoral response against gp41-overlapping peptides**. **A**, 15-mer overlapping peptide collection covering the HR2 (OLP peptides #155-161) and the MPER (OLP peptides #162-166) used for mapping. **B**, OD values of the indicated immunized mice sera (60-fold dilution) against OLP peptides in ELISA. 2F5 binding is also indicated. Color code is shown. **C**, for each animal the peptide yielding the strongest signal was assigned 100%. Bar diagram shows the percentage of signal of each peptide in all animals (mean +/- SD), highlighting the immunodominance of the #162 peptide sequence. **D**, Spearman´s correlation of #162 binding signals and gp41-Min titer of immunized mice sera. Data are representative of two independent experiments. Spearman´s correlation coefficient and p value are indicated.

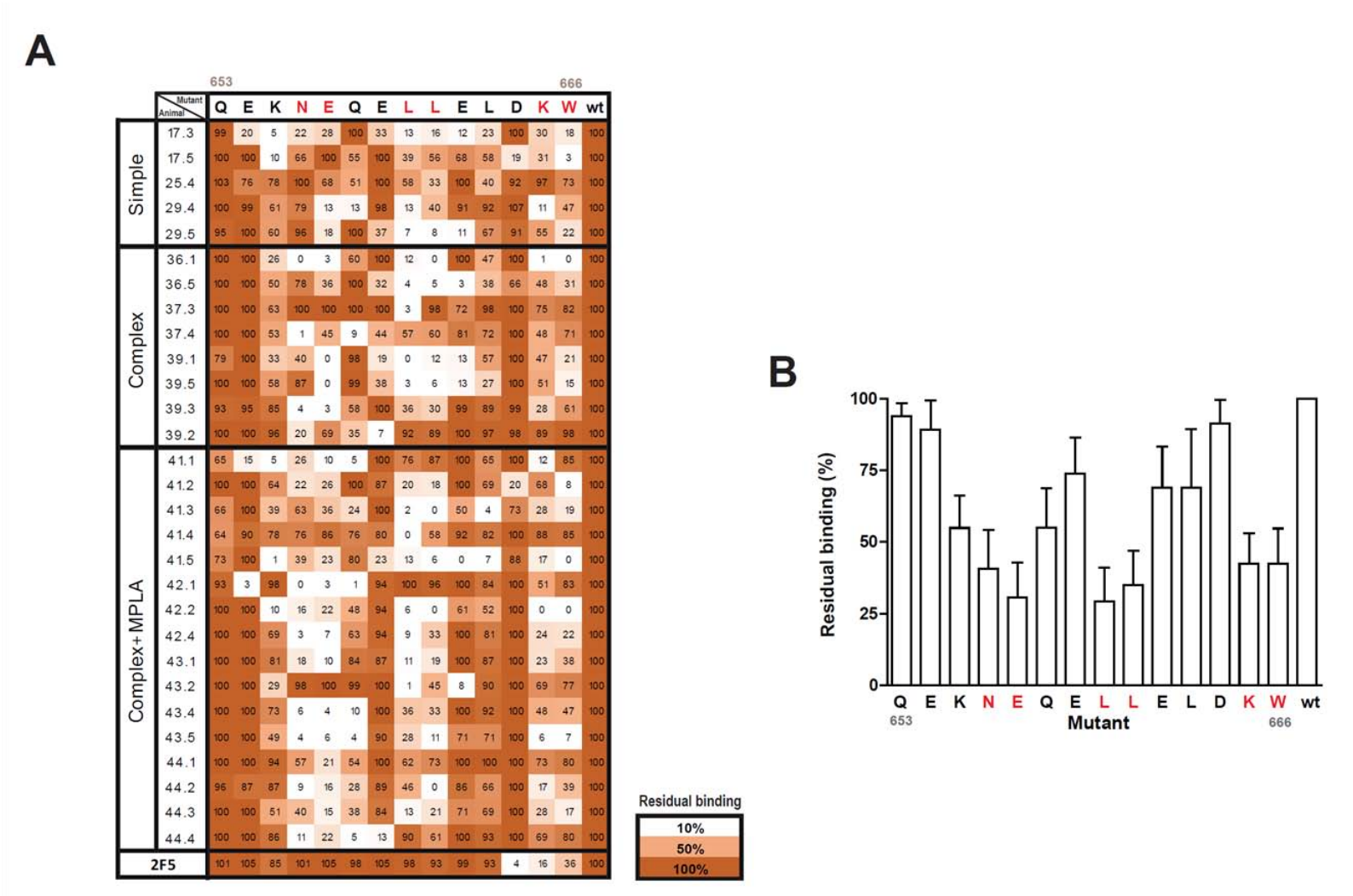


Figure 24. **Alanine-scanning analysis of the immunodominance against #OLP-162**. **A**, 29 mice sera displaying the highest signals against OLP#162 peptide were tested for binding against a collection of OLP#162-alanine mutants. Values indicate the binding percentage relative to the wild type peptide signal. 2F5 profile is also included. Color code is indicated. **B**, percentage of residual binding (mean+/-SD) for each alanine mutant peptide is shown. Immunodominant residues (<50% residual binding) are highlighted in red in both panels. HXB2 numbering of Q_{653} and W_{666} residues is indicated in gray.

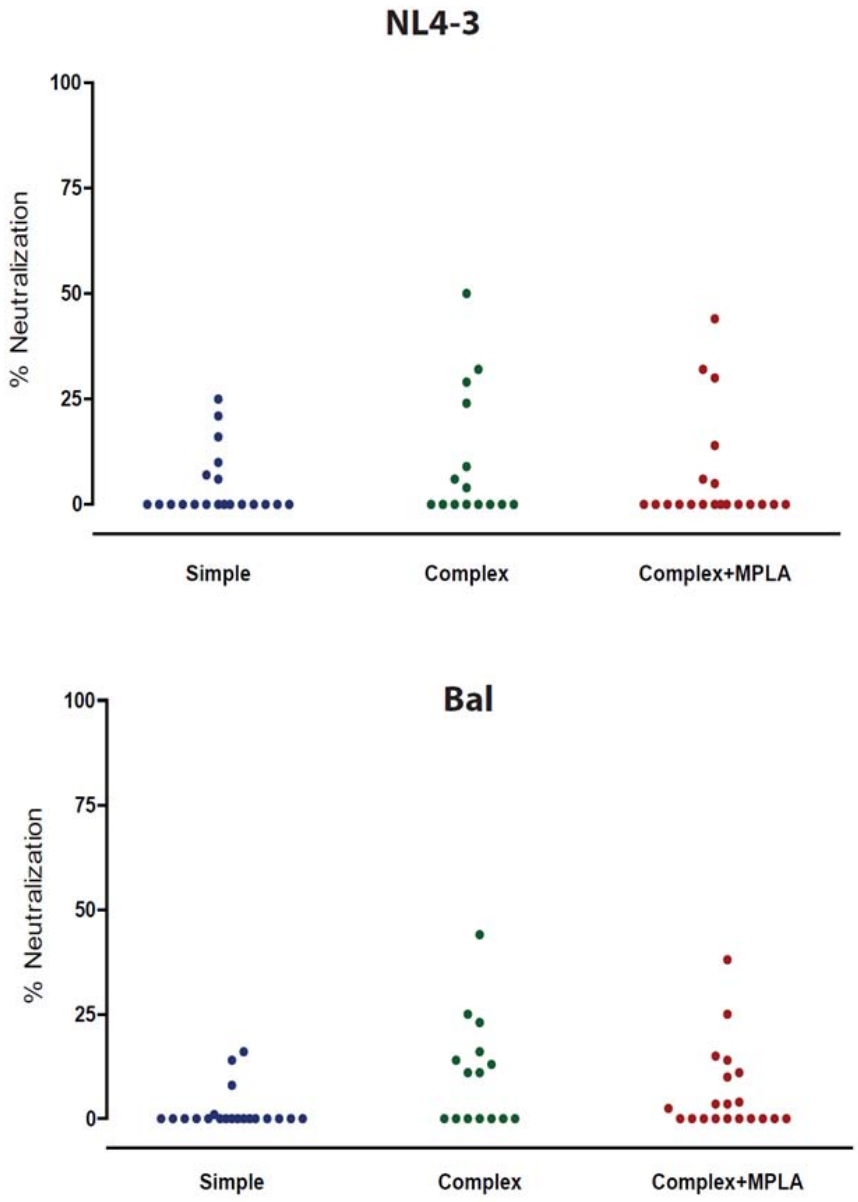


Figure 25. Neutralizing activity of sera from immunized animals. The neutralizing activity of sera obtained at sacrifice was assessed in TZM-bl assays against the HIV-1 isolates NL4-3 and BaL at a single 1/100 dilution. Neutralizing activity is shown for the different groups of immunized animals.

4.5. Structural analysis of gp41-Min protein

Molecular dynamics of an *in silico* model of the extracellular and TM sequences of gp41-Min protein embedded in a POPC bilayer showed that the 6 relevant residues for #162 binding belong to two different turns of the MPER helix that are located on the membrane interface **(Figure 26. A-B and Figure 27).** The turn containing W666 strongly interacts with the lipid bilayer throughout the dynamics (W666 with the hydrophobic environment of the bilayer and K665 with phospholipid polar heads; total non-bond interaction energy of -55.2 kcal/mol), similarly to the following two turns (residues 667 to 670: -38.6 kcal/mol; residues 671 to 674: -38.2 kcal/mol). On the contrary, the previous turn, containing N656, E657, L660 and L661, shows a much weaker interaction with the viral membrane (+5.4 kcal/mol).

The turn containing W666 also contains K665 and D664. In spite of the proximity between these three residues, only W666 and K665 were shown to be relevant for antibody response to #162 (**Figure 24. A-B**). Polar residues are extremely solvatable in water solution, making less strong interactions with other polar solvents than when they are in a water excluded patch (273). Thus, a simple way to identify hot spots for strong protein-protein interactions is to analyze the hydration patterns for the residues and regions of interest. To analyze the hydration pattern of W666, K665 and D664 we compared the water shell around each residue for the membrane embedded protein model and the water-only solvated model. We observed that W666 and K665 showed a lower level of hydration in a lipidic bilayer when compared to soluble forms **(Figure 26. C)**, while hydration of D664 was extremely similar in both models, suggesting that extraction of W666 from the membrane will modify the hydration shell for the first two residues in a much larger extent than D664. In addition, K665 is much less hydrated than D664, indicating that the W666 prevents water molecules to fully solvate the lysine residue, thus, offering a much favorable propensity to create strong charge-charge interactions with other proteins. This may explain the selectivity of antibody binding to K665 when the turn is exposed to the solvent.

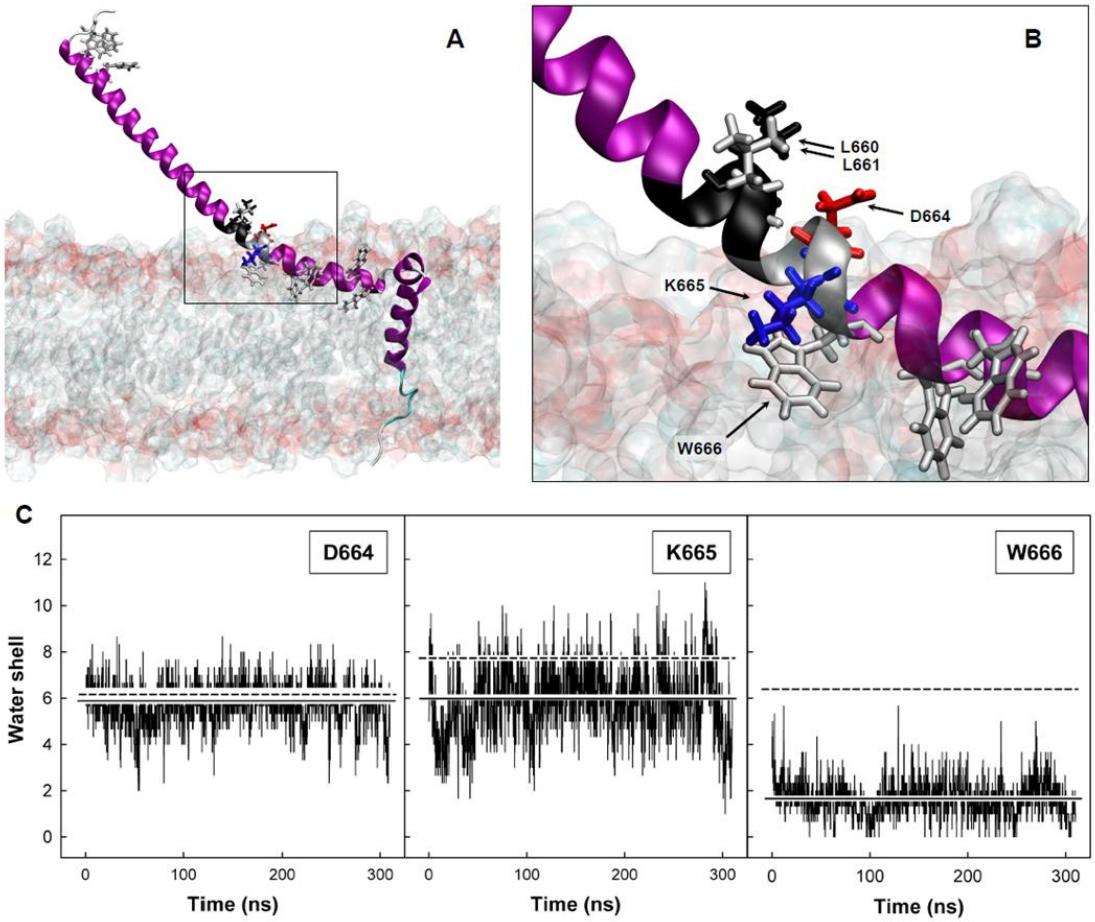


Figure 26. ***In silico*** **structural model of gp41-Min. A**, general view of the structural model embedded in a POPC bilayer. **B**, detail of the helix turns containing relevant residues for #162 binding. Protein is shown in a ribbon representation. The helix turn containing L660 and L661 is shown in black and the turn containing W666 is shown in grey. In addition, all tryptophans present in gp41-Min and some residues specifically named in the text are labeled with arrows and represented as sticks. POPC bilayer is represented as solvent accessible surface, truncated along the main protein axis to allow its visualization. **C**, number of water molecules within a sphere of 3 Å around lateral chains of residues D664 (left), K665 (middle) and W666 (right) in the gp41-Min model embedded in a POPC bilayer throughout the molecular dynamics. Mean values are represented by a continuous horizontal line. The respective mean values obtained from the molecular dynamics performed in the absence of the lipid bilayer are also shown for comparison (discontinuous line).

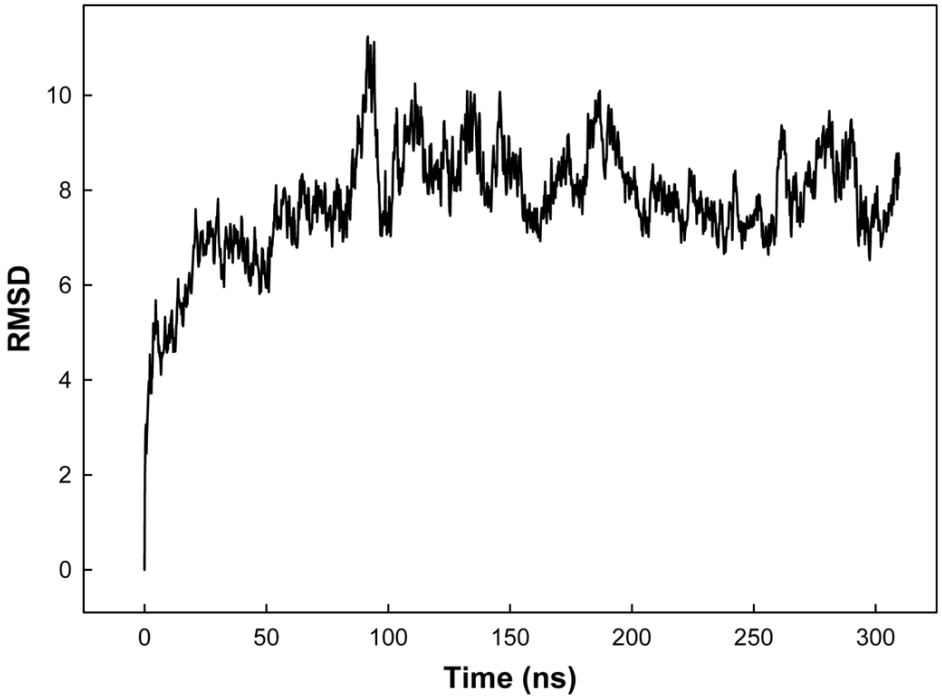


Figure 27. Molecular dynamics of gp41-Min embedded in membrane. Root-mean-square deviation of atomic positions (RMSD) during molecular dynamics of the gp41-Min protein embedded in a POPC bilayer**.**

5. Discussion

Anti-MPER bNAbs share a common neutralization mechanism in which interactions of CDR H3 hydrophobic residues with membrane lipids seems to be essential (197, 207, 210, 226, 274). Additionally, MPER structure is influenced by membrane lipid composition (204, 205). Therefore, it is widely assumed that the generation of a robust anti-MPER response should require its presentation within a membrane environment to properly present neutralizing determinants and to implement lipid cross-reactivity (210, 224, 229, 230, 275).

In this study, we evaluated to what extend lipids may modulate the MPER immunogenicity. Accordingly, we selected an immunogen containing the HR2, the MPER and the transmembrane domain of gp41 resulting in an increased MPER exposure compared with the whole gp41 protein (256). Additionally, we incorporated a promiscuous T-cell Tetanus Toxoid (TT) epitope to harness the MHC-II antigen presentation pathway that has been shown as a major limiting factor in MPER responses in murine models (276). Likewise, in a cohort of untreated HIV-1 infected viremic controllers it has been recently shown an association between gp41-specific CD4 T cell responses with the magnitude and breadth of anti-Env neutralizing antibodies (277). We incorporated gp41-MinTT protein into liposomes of diverse composition. Starting from simple (POPC-based) liposomes, we increased complexity by adding i) CHOL and SM overrepresented in the viral membrane (253); ii) GM3 and PS, whose receptors are present on the surface of phagocytic cells, and may trigger specific delivery to specialized APCs (such as the CD169+ subcapsular sinus macrophages) (231, 261, 271, 278, 279) and iii) the monophosphoryl Lipid A, a TLR4 agonist approved for human use, as a molecular adjuvant (262).

The results showed that animals immunized with the recombinant protein gp41-MinTT developed a weaker humoral response than those immunized with 10-fold lower inoculum dose of the same protein formulated as proteoliposomes. Interestingly, humoral response elicited by soluble protein was focused mainly on the TT peptide, while proteoliposomes guided the humoral response towards the gp41 extra-cellular portion of the recombinant protein. Besides differences in oligomerization status in soluble and proteoliposomal preparations, these results can be explained by the presence of lipids. In proteoliposomes, the gp41 region is surrounded by specific lipids that results in surface exposure of the extracellular portion of the protein possibly mimicking

a native conformation and enhancing immunogenicity. Furthermore, we observed modest contributions of individual GM3 and PS incorporation into POPC proteoliposomes, although synergistic activity was noticed in this context, probably due to multivalent presentation of both ligands through different pathways. In contrast, incorporation of CHOL and SM significantly increased the gp41-MinTT immunogenicity compared to the simplest POPC proteoliposomes. This immunogenicity boost supports the notion that certain lipids may modulate the MPER structure and/or accessibility by modifying its membrane insertion index (181, 204) or membrane fluidity, accounting for a higher antibody response. (203, 232) The membrane of HIV-1 virions shows an unusual high content of sphingolipids and CHOL (253) which seems to be essential for MPER-induced membrane fusion (280) and whose depletion strongly affected to HIV-1 infectivity (281). Furthermore, the C-terminal end of the MPER contains the LWYIK "Cholesterol Recognition Amino acid Consensus" (CRAC) motif by which MPER-CHOL interactions would be supported (206). Accordingly, the use of a virion-like membrane, in which the MPER region is properly anchored, might mimic the MPER native context accounting for higher immunogenicity (181). In addition, we cannot exclude that distinct liposome composition might modulate other membrane features (such as membrane structure) that may impact on the stability of membrane-dependent structures, antigen uptake by draining lymph nodes and the interaction with antigen presenting cells (271). Finally, we have confirmed that addition of the TLR4 agonist MPLA, as a molecular adjuvant, considerably enhanced the immunogenicity of the immunogen. Therefore, the use of CHOL and SM-containing liposomes in combination with MPLA constitutes an excellent carrier system for MPER-based immunogens.

A deeper analysis showed that the response of immunized animals was focused on peptide #162, which overlapped the HR2 and the MPER regions including the 2F5 binding domain. This immunodominance did not depend on the proteoliposome composition since it was detected regardless of the immunization regimen used. It included two of the 2F5 neutralizing core residues, the K665 and W666, the latter one being essential for viral fusion (170, 171). Despite this close specificity, sera from immunized animals showed a poor neutralizing capacity. Similarly, an study in rhesus macaques by Dennison et al. showed that antibodies that recognized the 2F5 neutralizing core epitope ($_{664}$DKW$_{666}$), with a preferential binding for the gp41 fusion intermediate, lacked neutralizing activity (224). Moreover, llama immunized with a

gp41 mini protein generated a polyclonal humoral response that failed to neutralize HIV-1. However, the later authors isolated a single chain antibody (VHH) that binds partially to the 2F5 epitope, but lacked binding to W666 (210). This antibody became neutralizing when was made bivalent and displayed a hydrophobic CDR H3 tip which was essential for neutralization, a common feature of many other anti-MPER bNAbs (210, 226, 274). In our case, the lack of neutralizing activity in immunized animals, could be also related to the observed reactivity against the HR2 region of gp41, that may limit the accessibility of antibodies to the target epitope either in the native envelope trimer or in the fusion intermediate structures, which are thought to be the main target of MPER bNAbs (197, 219). Our data support the notion that antibodies elicited by proteoliposome immunization extract W666 from the membrane, as it has been proposed for other anti-MPER antibodies (161); although the response observed here lacked D664 reactivity. Structural information of the system suggest that hydration shells of the different residues may determine the strength of charge-charge interactions and therefore the folding structures, the exposure and the reactivity of these key residues. A more detailed structural analysis may provide insights for fine tuning of the hydrophobic/solvent interactions of these key residues that might impact the specificity of antibody responses.

Overall, the results presented here show that lipid modulation of membrane-dependent antigens impacts immunogenicity and elicit high antibody titers. We propose that CHOL and SM-containing liposomes in combination with molecular adjuvants like MPLA constitute excellent vaccine platforms for the MPER immunogens. The identification of non-neutralizing immunodominant residues and its comparison to anti-MPER bNAbs will provide helpful information to redesign immunogens to specifically eliciting MPER-neutralizing responses.

CHAPTER 4

Discussion & Future Directions

Plagues of humanity such as smallpox, measles or polio have been successfully controlled by vaccination, highlighting this intervention as the most important advance in human medicine. However, advances in the vaccinology field have not been yet translated into the generation of a preventive vaccine able to control the HIV-1 pandemic, which continuous to be devastating. The high mortality rate in the absence of a cure, the lack of worldwide HAART availability or a natural mechanism to clear infection makes the generation of a preventive vaccine a global health priority. In this regard, the detailed study of bNAbs and the conserved functional regions of HIV-1 Env targeted by them represent the "Holy Grail" for the rational design of HIV-1 immunogen candidates for a vaccine.

In our quest for neutralizing responses we focused on one of the major HIV-1 Env vulnerability sites: the MPER. This region presents a high level of conservation, according to its crucial role in viral fusion and infectivity; and is targeted by bNAbs. Two out of three MPER-specific bNAbs isolated to date show a neutralization breadth close to 100% against viruses from different clades, and all of them confer protection against infection upon viral challenge *in vivo*. Additionally, further functionalities such as ADCC (251), inhibition of viral transcytosis at the mucosal barrier (178) or blockade of cell-to-cell viral spread (282) have been attributed to MPER-specific bNAbs. Therefore, a successful MPER-based immunogen would induce potent neutralizing antibodies against a wide range of viral isolates together with complementary humoral effector functions able to control viral spread. Given these considerations, the inclusion of MPER-based immunogens in future HIV-1 vaccine settings is highly desirable.

The work presented in this thesis aimed to shed new light into the development, specificity and functionality of antibodies targeting the MPER region and generate knowledge for improved immunogen platforms. We designed a series of tools to perform a deep analysis of the MPER immunogenicity in two different settings: i) HAART-naïve HIV-1 infected individuals; and ii) mouse model immunized with a membrane-embedded MPER antigen.

The gp41-based miniproteins described here constitute novel platforms for analyzing MPER-specific humoral responses in HIV-1 infected individuals. We were able to identify anti-MPER antibodies in a high percentage of HIV-1 infected patients analyzed and their titer correlated with the total anti-Env humoral response, indicating that the MPER is much more immunogenic than previously thought. Therefore, our

results suggest that there are no specific constraints to generate a humoral response against this region. However, only few of these patients showed MPER-specific neutralizing activity, highlighting that anti-MPER antibodies are functionally heterogeneous.

Due to its proximity to the viral membrane, the MPER structure is highly influenced by lipids and the cross-reactivity against them seems to be one of the features of MPER bNAbs. Thus, we explored whether the lipid environment might modulate the humoral response against the MPER. With this aim, we made proteoliposomes combining a recombinant protein that overexposed the MPER region and several lipids overrepresented in the viral membrane and/or with strong immunomodulatory properties.

Consistent with previous studies (229), we confirmed the importance of liposomes on modulating humoral responses against MPER and determined optimal lipid mixtures that achieved higher titers of MPER-specific antibodies. Our systematic analysis revealed the potential of certain components such as cholesterol and sphingomyelin in combination with molecular adjuvants such as MPLA to induce the highest antibody responses. Therefore, lipids overrepresented on the viral membrane induce a strong effect over the MPER immunogenicity, supporting their inclusion on MPER-based immunogen platforms. The reason for this could be related with changes on the structure and/or exposure of the sequence; although the influence of lipids in antigen stability in germinal centers or other effects in antigen processing cannot be ruled out.

Remarkably, the humoral response observed in human samples was reproduced in immunized animals in terms of titer magnitude, specificity and heterogeneous functionality. In fact, there was a clear preference for the N-terminal moiety of the MPER in both, human and immunized mice settings. Ongoing analysis of MPER-binding profiles of our cohort seems to reveal a similar pattern to that raised by proteoliposomes in immunized mice.

The strong MPER immunogenicity observed in HIV-1 infected individuals and immunized mice settings contrasts with the functional heterogeneity displayed by specific antibodies. In this context, it is important to highlight that non-neutralizing responses were preferentially raised. Previous studies have reported a limited prevalence of MPER-specific neutralizing responses during natural infection (128, 131),

indicating that these neutralizing responses are less favored comparing with other Env specificities. This has been attributed to poorly immunogenic epitopes, accessibility to native conformation and/or immunological constraints due to self-reactivity issues (283). It has been suggested that antibodies targeting the 2F5 binding moiety are under host tolerance control because it binds human proteins (155, 284). Our results showed that anti-MPER antibodies detected in HIV-1 infected individuals were mostly directed against the N-terminal moiety of MPER, where the 2F5 binding motif is present. Confirmation of the precise residues involved in antibody binding of our cohort will confirm the feasibility of generating anti-MPER antibodies mimicking the 2F5 neutralizing epitope. Interestingly, as mentioned above, we could reproduce this behavior in immunized animals, where the response induced by gp41-MinTT formulated in different lipid mixtures was always focused against an epitope overlapping the 2F5 binding motif. The results suggest that presentation of the MPER within a certain lipid environment modulates the antigen conformation, stability or processing and presentation resulting in greater antibody titers; however, this would not necessarily correlate with the induction of neutralizing responses. In this regard, it was surprising that, although the gp41-MinTT-spanning region included the HR2, MPER and the TM domain, the response was mainly focused on a unique epitope. Molecular dynamics of the antigen presented in a POPC environment revealed that most of the MPER was embedded within the membrane, whereas the immunodominant residues where located within two turns at the membrane interface, where the D664 residue could be less favored to establish antibody interactions, explaining its lack of reactivity.

Therefore, whereas our data provided valuable information about optimal liposome carriers to present MPER-based immunogens, further refinement of gp41-MinTT antigen may be required in order to focus the response not only to the 2F5 overlapping region but also additional MPER epitopes including the 4E10, which is the most conserved one. In this regard, the influence of the TM should be also evaluated, as this domain was reported to shift the response from the C-terminal to the N-terminal MPER helix, probably related to changes in the immersion depth of the 4E10 binding epitope making it a more buried epitope and less accessible (203). On the contrary, the maintenance of the TM domain may be relevant as it anchors the MPER into the liposome surface avoiding early dissociation of the antigen, accounting for higher immunogenicity.

Although we reported that the MPER region seems to be sufficiently immunogenic to induce specific antibodies, the balance between non-neutralizing and neutralizing antibodies targeting similar or overlapping epitopes seems to be a relevant issue with important implications in vaccine design. This balance could be explained by competition between non-neutralizing B-cell clones and those encoding for bNAbs during the germinal center reaction. Easier accessibility to non-neutralizing determinants or host tolerance control mechanisms that may limit the frequency of B-cell clones encoding for bNAbs lineages (285) may be involved in this competition. On the other hand, bNAbs are the result of a long SHM process in which, after sequential accumulation of mutations, some B-cell lineages acquire neutralization breadth (146, 168). Therefore other variables such us longer immunization protocols or prolonged permanence in germinal centers should be further evaluated. Thus, a future goal in the field will be to further explore both neutralizing and non-neutralizing responses in order to provide better clues for the redesign of immunogens focused exclusively on neutralizing determinants. The generation of an immunogen able to skip non-neutralizing immunological distractions but keeping the recognition by NAbs will be of great interest. This could be the case of BG505 SOSIP.664 native-like trimers, which are almost exclusively recognized by bNAbs and achieved high, although autologous, neutralization levels when used to immunize rabbits (286, 287). However, these trimers did not include the MPER region on their structure and we still lack sufficient number of MPER-specific mAbs to systematically approach this issue.

In fact, our knowledge of MPER-specific neutralizing responses is limited to the low number of specific mAbs available. From the different bNAbs families encompassing more than one hundred antibodies, only three of them target the MPER region (2F5, 4E10, 10E8) (114, 143), although this small collection can be enlarged with additional neutralizing antibodies with limited breadth and potency (z13, m66.6 and CH12) (113, 188, 288). A non-neutralizing 2F5 cross-reactive murine antibody (13H11) resulted to be non-polyreactive, did not bind to peptide-liposomes and, most importantly, it bound to a well defined six helix MPER post-fusion conformation, different from the conformation recognized by bNAbs (208, 289). The common features, highlighted by NAbs (and not presented by 13H11), particularly membrane cross-reactivity and recognition of the gp41 prehairpin intermediate (193, 219), supported the rational, also followed by us, that similar antibodies would be generated

by presenting MPER antigens in a membrane-like environment. Whereas the implementation of these strategies achieved MPER-specific antibodies, only weak neutralizing titers have been reported by a few studies. For example, the use of an HA/gp41 fusion protein in viral like particles achieved modest 4E10-like neutralization against 4 viral strains (243). W. Weissenhorn´s group designed a gp41-based immunogen that mimicked the fusion intermediate presented in proteoliposomes. In this case, superior neutralizing responses were gained, although the epitope specificity was not delineated (230). A third study evaluating liposome-peptide platforms in combination with MPLA molecular adjuvant led to the isolation of two MPER-specific IgM antibodies showing lipid cross-reactivity but very limited neutralizing capacity against one virus tested (275). These few examples suggest that lipid reactivity or binding to fusion-mediating gp41 conformations by MPER-specific antibodies induced by immunization may be achievable but does not guarantee a potent and broad neutralizing activity. Most importantly, it does not exclude the elicitation of non-neutralizing antibodies whose B-cell precursors may compete for the antigen presented. Therefore, the knowledge extracted from the few MPER bNAbs isolated seems to be not sufficient for optimal immunogen design as non-neutralizing responses have resulted to be favored by the different immunization strategies followed to date.

In conclusion, there are still some gaps in our knowledge regarding the specific features that an immunogen must have to generate an MPER-specific neutralizing response. The limited number of MPER-specific mAbs isolated constitutes a bottleneck for a feasible comparison between neutralizing and non-neutralizing antibodies and thus, to establish solid conclusions regarding their specific features. Accordingly, future characterization of new MPER-specific antibodies reflecting the heterogeneous responses observed in natural infection and immunization settings in our studies will provide helpful information for the redesign of immunogens able to skip non-neutralizing determinants.

CHAPTER 5

Conclusions

To design new tools to quantify anti-MPER humoral responses

1. To identify the minimal fragment of gp41 that better exposes MPER neutralizing determinants.
2. To evaluate gp41-based miniproteins as potential tools for the detection of MPER-specific neutralizing antibodies in plasma from HIV-1 infected individuals.

1. The MPER accessibility is topologically compromised by the presence of gp41 immunodominant domains. Removal of the HR1 and disulfide loop from gp41 results in an improved exposure of the MPER region.
2. Gp41-based miniproteins with improved exposure of the MPER are useful tools for the detection of MPER-specific antibodies. However those miniproteins are not capable of distinguish between neutralizing and non-neutralizing antibodies.

To characterize the immunogenicity of the MPER region during chronic HIV-1 infection

6. To determine the prevalence, stability and specificity of MPER-specific antibodies.
7. To determine immune correlates of viral control with MPER-specific antibodies.
8. To delineate the MPER-specific neutralizing and non-neutralizing profiles.

3. The MPER region is immunogenic. Long-lasting MPER-specific antibodies, mainly directed against the 2F5 binding motif, are generated during natural HIV-1 infection.
4. The magnitude of anti-MPER antibodies correlated with total anti-Env humoral responses. None of these parameters correlated with viral load.
5. MPER-specific antibodies are functionally heterogeneous, in terms of neutralizing capacity or competition with neutralizing antibodies.

To evaluate the selected gp41-based miniprotein that overexposes the MPER region, as a potential immunogen candidate in mouse models

6. To generate a recombinant protein of the gp41-based construction, identified in **objective 1**, and evaluate its immunogenicity.
7. To evaluate the effect of different lipid mixtures over the MPER immunogenicity and the potential to induce specific neutralizing antibodies.
8. To determine the MPER binding determinants induced by immunization and to establish a relation with a neutralizing profile.
9. To perform molecular dynamics of the interactions of the MPER-specific responses elicited.

6. Recombinant Gp41-MinTT can be successfully produced in prokaryotic cells and purified to homogeneity. Soluble forms are poorly immunogenic in the absence of adjuvants.

7. The magnitude of immune responses to proteoliposomal formulations of gp41-MinTT is modulated by the lipid composition. The highest titers are obtained using cholesterol and sphingomyelin and MPLA containing proteoliposomes.

8. Immunization with gp41-MinTT formulated into lipid mixtures promotes an immunodominant IgG response overlapping the HR2 and the 2F5 binding motif o. This immunodominance includes the residues N656, E657, L660, L661, K665 and W666 of gp41 and did not correlate with neutralizing activity.

9. W666 prevents water molecules to fully solvate the K665, offering a much favorable propensity to create strong charge-charge interactions with other proteins, explaining the selective antibody binding to K665.

CHAPTER 6

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CHAPTER 7

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