Universidade de Lisboa

Faculdade de Farmácia

Development and pre-clinical evaluation of a new HIV-1 vaccine concept

Rita Diogo de Almeida Calado

Orientador: Prof. Doutor Nuno Eduardo Moura dos Santos da Costa Taveira

Co-orientador: Prof. Doutor José António Frazão Moniz Pereira

Tese especialmente elaborada para obtenção do grau de Doutor em Farmácia, especialidade Microbiologia

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“The important thing is to never stop questioning”

-Albert Einstein

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Preface

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1. Bartolo I*, **Calado R***, Borrego P, Leitner T, Taveira N. Rare HIV-1 Subtype J Genomes and a New H/U/CRF02_AG Recombinant Genome Suggests an Ancient Origin of HIV-1 in Angola. AIDS Res Hum Retroviruses. 2016 Aug;32(8):822-8. * These authors have contributed equally to the work.

2. **Calado R**, Duarte J, Borrego P, Marcelino J.M, Wilton J, Bártolo I, Martin F, Figueiredo IB, Almeida SCP, Vítor J, Graça L, Taveira N. A prime-boost immunization strategy with Vaccinia virus expressing novel envelope gp120 glycoproteins from non-B subtypes induces cross-clade tier 2 HIV-1 neutralizing antibodies in mice (Manuscript submitted).

3. **Calado R**, Martin F, Figueiredo IB, Vítor J, Carrapiço B, Dias I, Taveira N. Induction of tier 2 cross-clade neutralizing antibodies in rabbits by a prime-boost immunization strategy with Vaccinia virus expressing novel HIV-1 gp120 glycoproteins from clade CRF02_AG (Manuscript in preparation).

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Resumo

A SIDA continua a ser uma prioridade em saúde pública com o número de pessoas infetadas com o HIV-1 a aumentar. Apesar dos progressos feitos na área da prevenção, o controlo da infeção por HIV-1 dependerá do desenvolvimento e aplicação de uma vacina segura e eficaz contra este vírus. Os anticorpos neutralizantes de largo espetro (bNAbs) são considerados um elemento crucial numa vacina preventiva para o HIV sendo capazes de se ligar ao vírus bloqueando a sua entrada na célula hospedeira. Assim, o desenvolvimento de imunogénios que induzam a produção de bNAbs contra as diversas estirpes de HIV-1 constitui a maior prioridade na área das vacinas. A maior parte dos regimes vacinais usados atualmente para a indução de bNAbs consiste na administração combinada e sequencial de diferentes imunogénios. O uso de vetores virais recombinantes como primeira imunização (designada "*priming*") seguido de reforços ("*boosts*") com proteínas do invólucro do HIV constitui uma das abordagens mais usadas uma vez que induzem no hospedeiro um largo espetro de respostas imunes incluindo anticorpos e células T. Baseado numa estratégia semelhante, o ensaio clínico RV144 foi até à data o único em que se observou algum grau de proteção, embora modesto, contra a infeção por HIV reforçando a ideia de que o desenvolvimento de uma vacina preventiva contra o HIV-1 é uma tarefa concretizável. Contudo, com a exceção dos recentes resultados observados em vacas imunizadas com o trímero BG505 SOSIP, nenhum candidato a vacina foi capaz de induzir de forma consistente bNAbs contra vírus *tier* 2 heterólogos de diferentes subtipos [1].

Num estudo recente, a imunização de ratinhos BALB/c com vírus da vacina recombinante a expressar a glicoproteína de superfície do invólucro juntamente com um reforço com polipéptidos recombinantes constituídos pela *loop* V3 e pelas regiões circundantes C2 e C3 do invólucro de um isolado HIV-2 com tropismo R5 induziu a produção de bNAbs contra diversos isolados de HIV-2 com tropismo R5 [2]. Este trabalho demonstrou pela primeira vez que, com uma estratégia de vacinação relativamente simples e os antigénios adequados, é possível obter uma resposta neutralizante de largo espetro. Assim, o principal objetivo deste trabalho foi investigar se uma estratégia vacinal semelhante induzia a produção de bNAbs contra o HIV-1 em ratinhos BALB/c e em coelhos. Para aumentar as hipóteses de sucesso no contexto da enorme diversidade do HIV-1, decidiu-

se utilizar glicoproteínas do invólucro representativas dos genótipos mais comuns a nível mundial e provenientes de vírus característicos de uma epidemia antiga e estável como é o caso de Angola. Neste contexto, o primeiro objetivo específico desta tese foi caracterizar a nível genómico diferentes isolados de HIV-1 provenientes de Angola. Outros objetivos específicos foram expressar genes *env* dos isolados virais em vírus da vacina, produzir gp120 solúvel e polipéptidos recombinantes contendo as regiões C2, V3 e C3 dos diferentes isolados, caracterizar a antigenicidade e imunogenicidade, em particular a resposta em anticorpos neutralizantes, dos novos antigénios em ratinhos BALB/c e coelhos com diferentes estratégias vacinais e, finalmente, caracterizar as respostas celulares envolvidas na neutralização, nomeadamente, células T foliculares auxiliares (Tfh) e reguladoras (Tfr).

Angola tem uma epidemia antiga de HIV-1 que data da primeira metade do século 20 e terá desempenhado um papel crucial na disseminação inicial do HIV-1 a nível regional e mundial. Para melhor compreender a origem e dinâmica de transmissão dos subtipos e formas recombinantes do HIV-1 presentes neste país, sequenciou-se e analisou-se filogeneticamente os genomas completos de três vírus isolados em 1993 provenientes de três indivíduos originários de Cabinda (capítulo 2). Descobriu-se que um dos isolados era do subtipo J, outro era maioritariamente do subtipo J mas tinha uma pequena região de classificação incerta no local *gag/pol* e o último era um recombinante H/U/CRF02_AG nunca anteriormente descrito. Os resultados sugeriram ainda que o subtipo J de Angola poderá estar na origem mundial do subtipo J. Estes resultados confirmam que a epidemia de HIV-1 é antiga em Angola e contribuem para a expansão das bases de dados mundiais de sequências do HIV-1 dos subtipos mais raros (H e J).

O objetivo do capítulo 3 foi o de investigar no modelo murino se a estratégia semelhante à usada previamente contra o HIV-2 induzia a produção de anticorpos neutralizantes contra o HIV-1. Um imunogénio vacinal ideal contra o HIV-1 deverá induzir no hospedeiro uma resposta em anticorpos neutralizantes de largo espetro, ou seja, anticorpos que neutralizem vírus contemporâneos de todos os genótipos. A estratégia adotada para gerar uma vacina que fosse de largo espetro foi recorrer aos vírus em circulação nas epidemias Angolana (vírus não-B) e Portuguesa (subtipo B) entre 1993 e 2008. A hipótese que se colocou foi a de que os invólucros dos vírus ancestrais presentes nestas duas epidemias exporiam epítopos conformacionais conservados ao longo da evolução devido a restrições de natureza funcional e induziriam de forma eficaz a

produção de anticorpos capazes de neutralizar todo o tipo de vírus contemporâneos. Assim, os genes *env* de isolados dos genótipos B, C, CRF02_AG, G, H e J foram amplificados, sequenciados e clonados em vetores de expressão do vírus da vacina. Todos os isolados possuíam tropismo para o co-receptor CCR5 indicando que seriam aptos para vacinação uma vez que a maioria dos vírus transmitidos usa este co-receptor. Foram produzidos com sucesso vírus da vacina recombinantes a expressar glicoproteínas gp120 dos subtipos B, C, CRF02_AG e J. Adicionalmente produziram-se polipéptidos recombinantes contendo as regiões C2V3C3 e a proteína gp120 solúvel de cada um dos subtipos. Todas as novas proteínas reagiram positivamente em ELISA e *Western Blot* com anticorpos presentes no soro de indivíduos infetados com HIV-1 indicando que apresentavam uma conformação apropriada. Por outro lado, o anticorpo monoclonal humano 447-52D, direcionado contra o motivo GPGR da região V3, reagiu fortemente em ELISA e *Western Blot* com a proteína gp120 do subtipo CRF02_AG, enquanto o anticorpo monoclonal HJ16, específico para a região de ligação ao CD4, reagiu contra todas as proteínas embora apenas em ELISA. Estes resultados demonstram que os novos imunogénios apresentam adequadamente o epítopo neutralizante presente no *loop* da V3 e, ainda, algum grau de exposição dos epítopos presentes no local de ligação ao CD4.

Ratos BALB/c foram imunizados com três combinações vacinais incluindo vírus da vacina a expressar as glicoproteínas gp120 dos subtipos B, C, CRF02_AG e J, polipéptidos recombinantes contendo as regiões C2V3C3 e a proteína gp120 solúvel de cada um dos subtipos. Em geral todos os ratos imunizados desenvolveram anticorpos de ligação contra as proteínas gp120 autólogas e heterólogas sendo que apenas alguns desenvolveram anticorpos de ligação contra a C2V3C3. Os animais inoculados com imunogénios derivados do genótipo CRF02_AG produziram uma boa resposta neutralizante (>50%) contra seis pseudovírus heterólogos de *tier* 2 e três isolados primários de diferentes subtipos de HIV-1. A produção de bNAbs foi observada apenas nos grupos de animais infetados inicialmente com vírus da vacina recombinantes suportando, por um lado, a importância do uso de vetores vacinais replicativos como componente de uma vacina para o HIV-1 e, por outro, o potencial do uso de monómeros da gp120 quando usados em regimes de combinação.

O alvo dos anticorpos neutralizantes não foi identificado formalmente. No entanto, uma vez que a gp120 do isolado vacinal CRF02_AG se ligou a um anticorpo monoclonal neutralizante que têm por alvo a região V3, juntamente com o facto do isolado

neutralizado por todos os grupos imunizados com este imunogénio ter o motivo GPGR na V3 (PX2278) e os não neutralizados terem um motivo diferente (GPGQ), sugere que os anticorpos neutralizantes induzidos nos ratos têm sobretudo por alvo o motivo GPGR da região V3. No entanto, a ligação, embora fraca, do anticorpo monoclonal HJ16 a todos os imunogénios gp120 em ELISA juntamente com a indução de bNAbs contra pseudovírus e isolados primários de subtipos B e não B (que não possuem o motivo (GPGR) indica que outros epítopos possam estar envolvidos. Assim, embora os novos imunogénios utilizados neste estudo não pareçam originar epítopos conformacionais, os resultados demonstraram que as respostas induzidas contra os epítopos na V3 foram suficientes para neutralizar vários isolados *tier* 2 de HIV-1 suportando a importância da inclusão da região V3 numa vacina contra o HIV-1.

Ao contrário do que tinha sido observado previamente para o HIV-2, verificou-se que os polipéptidos recombinantes C2V3C3 não induzem uma resposta neutralizante contra o HIV-1 provavelmente porque os epítopos neutralizantes da V3 não são apresentados de forma correta por estes polipéptidos. Nos ratos imunizados com vírus da Vacina recombinante a expressar gp120 truncada dos subtipos C, CRF02_AG e J seguidos de um reforço com polipéptidos recombinantes C2V3C3 e a proteína gp120 solúvel dos mesmos isolados (ensaio piloto) o número de células Tfh correlacionou-se de forma positiva com o número e níveis de anticorpos de ligação contra os imunogénios gp120 e a frequência de células Tfr correlacionou-se negativamente com as respostas neutralizantes. Estes resultados constituem um suporte adicional para o uso de vírus da vacina replicativos como componente de uma vacina para o HIV uma vez que estes são capazes de induzir respostas celulares específicas envolvidas na indução de anticorpos anti-HIV.

No capítulo 4 efetuou-se um novo estudo vacinal em coelhos com o objetivo de investigar se o nosso protótipo de vacina baseado em imunogénios de genótipo CRF02_AG, também induzia bNAbs num modelo animal distinto e mais próximo dos primatas. Os coelhos foram imunizados com vírus da vacina recombinante a expressar a proteína gp120 truncada derivada do subtipo CRF02_AG seguido de reforço com a proteína gp120 truncada do mesmo isolado. Todos os coelhos imunizados desenvolveram elevados títulos de anticorpos de ligação contra proteínas do invólucro autólogas e heterólogas tendo um dos animais neutralizado a mais de 50% a maioria (13/16) dos vírus testados. Estes resultados confirmaram os observados previamente em ratos (capítulo 3), reforçando o potencial desta estratégia vacinal.

Em conclusão, o trabalho desenvolvido nesta tese demonstrou que uma estratégia vacinal baseada no uso de um vetor viral replicativo a expressar uma proteína do invólucro derivada de um isolado ancestral de subtipo não-B juntamente com um reforço com a proteína gp120 autóloga induziu a produção de bNAbs contra diversos subtipos de HIV-1 em dois modelos animais distintos sendo este o tipo de resposta pretendido com uma vacina preventiva contra este vírus.

Palavras-chave: diversidade genética do HIV, vacinas contra HIV/SIDA, imunogénios do invólucro, anticorpos neutralizantes, células T foliculares

Abstract

New immunogens that elicit the production of broadly neutralizing antibodies (bNAbs) are needed to prevent and control HIV-1 epidemic. However, their induction by vaccination is still a difficult task. Prime-boost immunization strategies combining poxvirus with envelope glycoproteins constitutes a promising approach for an HIV-1 preventive vaccine as they provide strong immune responses. Recently, bNAbs against HIV-2 were elicited in mice using a Vaccinia vector-prime C2V3C3 polypeptide boost vaccination strategy. Thus, the main goal of this thesis was to determine if a similar strategy would elicit the production of bNAbs against HIV-1. The general aims of this thesis were: obtain and examine HIV-1 samples derived from Angolan isolates as a paradigm of the ancestral viruses we intended to use in a new type of vaccine, express envelope genes from Angolan and Portuguese isolates in Vaccinia virus and produce the autologous C2V3C3 recombinant polypeptides, investigate the immunogenicity of these immunogens in mice and rabbits using different regimens and quantify the respective cellular immune responses. In chapter 2, three full-length genomes from Angolan patients were sequenced and analyzed in order to better understand the origin and dynamics of HIV-1 in Angola. A pure subtype J, a subtype J with a small uncertain region and the first H/U/CRF02_AG recombinant were identified. Overall, these results supported the extraordinary genetic diversity of HIV-1 and confirm the ancestral presence of this subtypes in Angola. In chapters 3, gp120 glycoproteins expressed in Vaccinia virus, soluble gp120 and C2V3C3 polypeptides derived from several HIV-1 isolates from Angola and Portugal (clades B, C, CRF02_AG and J) were produced and used as immunogens in mice and rabbits (chapter 4). CRF02_AG based immunogens were able to elicit bNAbs against several heterologous HIV-1 tier 2 viruses and V3 region was found to be one of the main target of this immunogen. Antibody responses were associated with adequate Tfh and Tfr responses indicating that this strategy targeted the cellular subsets required for the induction of an effective NAb response. In conclusion, the results obtained suggest that the novel CRF02_AG based immunogens and prime-boost immunization strategy may be able to induce the type of response intended in a preventive HIV-1 vaccine.

Keywords: HIV genetic diversity, HIV/AIDS vaccines, Envelope based immunogens, neutralizing antibodies, follicular T cells

Abbreviations

Ab	Antibody
Ad	Adenovirus
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADCVI	Antibody-dependent cell-mediated virus inhibition
aLRT	Approximate likelihood-ratio test
AIDS	Acquired Immunodeficiency Syndrom**e**
APC	Antigen presenting cells
ART	Antiretroviral therapy
BI	Branching index
BCG	Bacille Calmette-Guerin
BSA	Bovine serum albumin
BSL-2	Biosafety level 2
bNAb	Broadly neutralizing antibody
CA	Conic shaped viral capsid
CD4	Cluster of differentiation
CD4bs	CD4 binding site
CCR5	C-C chemokine receptor type 5
CDC	Center for disease control and prevention
CDR H3	Third heavy chain complementary determining regions
CLR	C-type lectin receptors
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CRF	Circulating recombinant form

CTL	T cytotoxic lymphocytes
DC	Dendritic cell
DMEM	Dulbecco´s minimal essential medium
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
DRC	Democratic Republic of Congo
ELISA	Enzyme-Linked Immunosorbent Assay
Fab	Antigen-binding fragment
FBS	Fetal bovine serum
Fc	Fragment crystallizable region
FcRs	Fc receptors
FP	Fusion peptide
GALT	Gut associated lymphoid tissue
GC	Germinal Centers
GPCR	G-protein-coupled receptor
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
HLA	Human leukocyte antigen
HR1	Heptad Region 1
HR2	Heptad region 2
HTLV	Human T-cell leukaemia viruses
Hu-BLT	Humanized mice
HuMAbs	Human Monoclonal antibodies
ICOS	Inducible T cell co-stimulator
IDUs	Injecting Drug users

IFN	Type I interferon
IgG	Immunoglobulin
IN	Integrase
IQR	Interquartile range
jpHMM	Jumping profile Hidden Markov Model
KIR	Killer immunoglobulin receptor
LCMV	Lymphocytic choriomeningitis virus
LTR	Long terminal repeat
MA	Matrix protein
MALT	Mucosa-associated lymphoid tissue
mDC	Myeloid dendritic cell
ML	Maximum likelihood
MPER	Membrane proximal external region
mRNA	Messenger RNA
MRCA	Most recent common ancestor
MSM	Men who have sex with men
MVA	Modified Vaccinia virus Ankara
NAb	Neutralizing antibody
NC	Nucleocapsid protein
NHPs	Non-human primates
NK	Natural killer cells
NYVAC	Vaccinia virus derived from the Copenhagen Vaccinia strain
OD	Optical density
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PEP	Post-exposure prophylaxis
PIC	Pre-integration complex
PNGS	N-linked glycosylation sites
PrEP	Pre-exposure prophylaxis
PRRs	Pathogen-recognition receptors
PR	Protease
RC	Republic of Congo
RIP	Recombinant Identification Program
RLU	Relative light units
RNA	Ribonucleic acid
RRE	Rev Response element
RT	Reverse transcriptase
RTC	Reverse transcriptase complexes
SD	Standard deviation
Sgp120	Supernatant containing gp120 protein
SHM	Somatic Hypermutation
SHIV	Simian-Human Immunodeficiency Virus
SIV	Simian immunodeficiency virus
SLPC	Short-lived plasma cells
SPF	Specific pathogen free
SU	Surface glycoprotein
TAR	Trans-acting response

TB	Tuberculosis
TD	Transmembrane domain
Tfh	T Follicular helper cells
Tfr	T Follicular regulatory cells
TI	Thymus-independent antigens
TLR	Toll-like receptor
TM	Transmembrane glycoprotein
TNF- α	Tumor necrosis factor α
URF	Unique recombinant form
USA	United States of America
VACV	Vaccinia virus
VLPs	Virus-like particles
VSV	Vesicular stomatitis virus
VV	Vaccinia virus
VV_{WR}	Western Reserve strain of Vaccinia virus
WHO	World Health Organization

Units

ºC	Celsius degrees
kb	Kilobase
kDa	kilodalton
ml	milliliter
nm	nanometers
µg	micrograms
µl	microliter

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Chapter 1

Introduction

1. General introduction

1.1. Discovery of HIV

The discovery of the Human Immunodeficiency virus type 1 (HIV-1) as the causative agent of the Acquired Immunodeficiency Syndrome (AIDS) constitutes one of the major scientific discoveries in recent history. AIDS was first described in 1981, when several cases of *Pneumocystis carinii pneumonia* and Kaposi´s Sarcoma were observed in healthy men who have sex with men (MSM) in Los Angeles, New York and California[3, 4]. After one year, there were 593 reported cases of AIDS of which 243 resulted in death (41%)[5]. Between 1982 and 1983 the disease was also reported in patients from Haiti, Africa and Europe [6-11] and in other risk groups like injection drug users, hemophiliacs, heterosexual women and infants (vertical transmission) [5, 12-14]. In 1983, Luc Montaigner and Françoise Barré-Sinoussi from the Pasteur Institute in France isolated a new retrovirus from an AIDS patient belonging to the family of the human T-cell leukemia viruses (HTLV) that could be the causative agent of AIDS[15]. In 1984, a group of American investigators announced the discovery of a similar retrovirus (HTVL-III) which later in 1986 was officially called HIV and considered to be the causative agent of AIDS [16-20]. Soon after this discovery, a new retrovirus distinct from HIV-1 that caused clinical symptoms similar to AIDS was isolated in patients from Guinea-Bissau and Cape Verde Islands that were admitted in a hospital in Lisbon, Portugal. Later, this new virus was classified as Human Immunodeficiency virus type 2 (HIV-2) [21, 22].

1.2 Origin and Genetic diversity of HIV-1

HIV-1 and HIV-2 are of zoonotic origin and result from multiple transmission events from SIVs (Simian immunodeficiency virus) of non-human primates (NHPs) to humans[23]. Although it is not known how humans acquired the virus it is believed that transmission must have occurred as a result of the frequent contact with primates in many parts of Africa due to primate hunting and meat consumption[24, 25]. SIV infection have been documented in more than 40 different primate species from Africa with a

seroprevalence that can be close to 50% in some species[26]. The great majority of primate species harbor a single strain of SIV[27].

HIV-1 was originated from SIVcpz that infects West Central African chimpanzees (*Pan troglodytes troglodytes*) and from SIVgor that infects Western lowland gorillas *(Gorilla gorilla gorilla)* [28-30] whereas HIV-2 descend from SIVsmm which is found in sooty mangabey monkeys (*Cercocebus atys*)[31-34]. The four groups (M, N, O, P) that constitutes HIV-1 are the result of four independent cross-species transmissions from chimpanzees and gorillas. HIV-1 groups M and N descend from SIVcpzPtt that was transmitted from chimpanzees to humans in southern Cameroon whereas HIV-1 groups O and P descend from SIVs (SIVgor) infecting western lowland gorillas [29, 35-38]. Phylogenetic analysis of divergent HIV-2 strains demonstrated that at least nine cross-species transmissions have been described, resulting in nine HIV-2 groups (A-I)[39].

Transmission from simians to humans is estimated to have occurred between the end of the 19th century in Kinshasa (now Democratic Republic of Congo, DRC) and the beginning of the 20th century [40-46]. In fact, HIV-1 strains were identified in a blood sample from DRC and in a biopsy in 1960 and they already presented a high genetic diversity [40, 42]. Molecular epidemiological studies demonstrated that HIV-1 group M started to spread in humans around 1908 (1884-1924) and that the epicenter was the western part of the DRC where ancestors have been identified [37, 46-49]. For HIV-1 group O radiation is estimated to be around 1920 (1890-1940) [43, 45, 50] and for group P the estimated date is between 1845 and 1989[51]. Group N presents the lower intragroup genetic diversity suggesting a more recent introduction in human population (1948-1977)[45].

The extreme genetic diversity of HIV-1 is the result of several mechanisms as the lack of proofreading activity of the reverse transcriptase, host immune pressure and high recombination rate [35, 52-54]. HIV-1 group M is responsible for the pandemic being the only HIV-1 group that has spread across Africa and all the globe [35, 49, 55-57]. The remaining groups are restricted to West Africa and are much less prevalent [58-61]. HIV-1 M group is divided in nine different subtypes or clades (A, B, C, D, F, G, H, J, K) which have arisen from just one cross-species transmission event[34]. Subtypes E and I have been described but further analysis revealed that they have a mosaic structure [62, 63].

Within group M the intersubtype genetic variability is 15% for the *gag* gene and 25% for the *env* gene[64]. Within a subtype it is still possible to identify several subsubtypes which are phylogenetically more related to each other than to other subtypes. For instance, clades A and F can be separated in subsubtypes A1-A5 and F1-F2, respectively[63].

HIV-1 group M also includes more than 70 circulating recombinant forms (CRFs) and many unique recombinant forms (URFs)[62, 65, 66]. Of note, a recombinant form is named CRF if it is documented in at least three individuals that are not epidemiological related and URF if it is restricted to a limited number of individuals. When three or more subtypes are involved the term cpx is used to refer a complex mosaic structure. CRFs can recombine and in that case the viruses are called second or even third generation CRFs[35]. On a global perspective, the most prevalent subtypes are C (50%), A (12%), B (11%), followed by CRF02_AG (8%), G (5%), CRF01_AE (5%) and D (2%). The remaining subtypes and recombinant strains represent less than 1% of all HIV-1 infections (Figure 1)[35]. In fact, there are less than nine full-length genomes of subtypes J and H available in the Los Alamos sequence database[65]. Geographic distribution of HIV-1 subtypes is illustrated in figure 1.

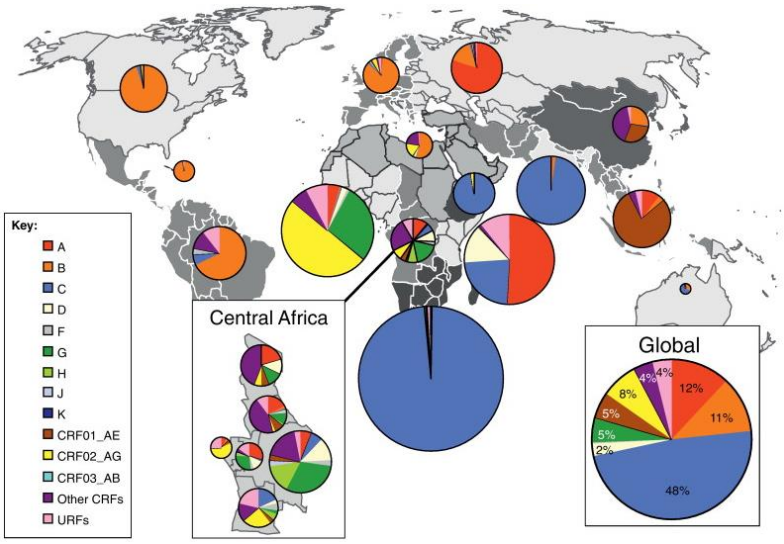

Figure 1- Global distribution and prevalence of HIV-1 (adapted from[64]) .

Because HIV-1 pandemic originated in West Central Africa, the highest genetic diversity is observed in DRC and surrounding countries. For instance, in Angola, a country surrounded by Namibia, Zambia, DRC and Republic of Congo (RC) all subtypes except B and many CRFs and URFs have been detected[66-68]. In Portugal, the most prevalent subtypes are B, G and CRF14_BG [69-71].

1.3 HIV-1 Genome and structure

HIV-1 and HIV-2 belong to the *Retroviridae* family, *Orthoretrovirinae* subfamily and *Lentivirus* genus[72]. HIV is a spherical enveloped retrovirus with 120nm in diameter[73]. HIV particles are surrounded by an envelope that is composed of a lipid bilayer where a transmembrane glycoprotein (TM) with approximately 41kDa is inserted.

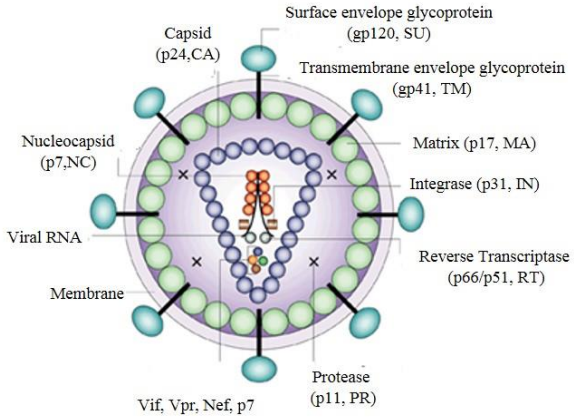

Figure 2- Schematic structure of HIV-1 particle (adapted from[74]).

Anchored to the TM there is an outer surface glycoprotein (SU) with approximately 120kDa[75]. In the mature virion, glycoproteins TM and SU are associated as trimers in the cell surface by non-covalent interactions [76]. The envelope is surrounded internally by matrix proteins (MA) and within the matrix there is a conical shape viral capsid (CA) which includes two identical copies of a positive sense single stranded ribonucleic acid (RNA) associated with nucleocapsid proteins (NC)[75]. HIV-1 capsid is formed by approximately 1500 CA subunits and provides structural stability to the virion[77]. Inside the particle there are the viral enzymes reverse transcriptase (RT), integrase (IN), protease (PR) and the accessory proteins Nef, Vif, Vpr and Vpu[75].

Each RNA molecule that constitutes HIV-1 genome consists of approximately 9800 nucleotides long and is flanked by long terminal repeats (LTR) at both ends (5´-3´) (Figure 3). HIV genome contains nine genes that encode major structural, regulatory and accessory proteins in the mature virion[75]. Structural proteins and viral enzymes are essential components for the retroviral particle whereas regulatory proteins modulate transcriptional and posttranscriptional steps of gene expression being also essential for

virus propagation. Although not necessary for viral propagation in tissue culture, accessory proteins play an important role *in vivo*[78].

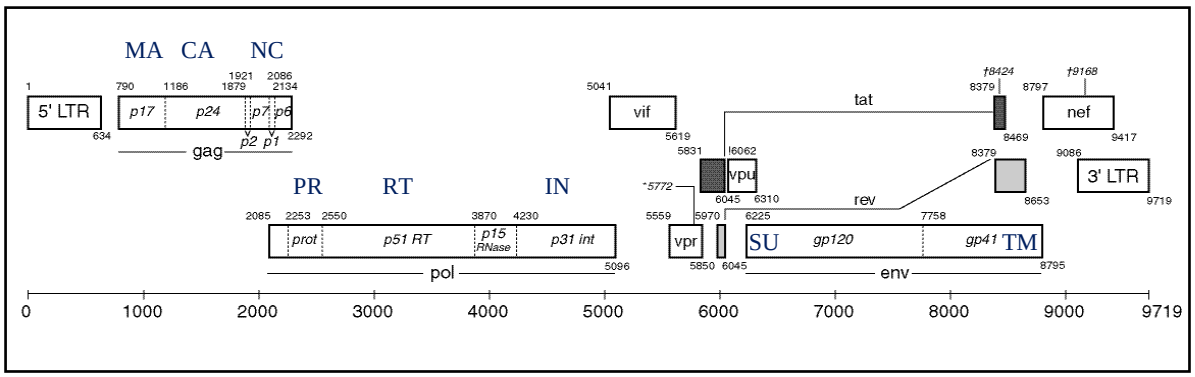

Figure 3- Genomic organization of HIV-1. MA- matrix proteins); CA- capsid; NC-nucleocapsid proteins; PR-protease; RT- reverse transcriptase; IN-integrase; SU- surface glycoprotein; TM-transmembrane glycoprotein (adapted from https://www.hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html).

Genes *gag*, *pol* and *env* encode for structural or enzymatic proteins, *tat* and *rev* for regulatory proteins and finally *nef*, *vif*, *vpr* and *vpu* for accessory proteins[75, 79, 80]. The *gag* encodes the polyprotein precursor $P55^{Gag}$ that is posteriorly cleaved by the viral protease into the MA (p17), CA (p24), NC (p7), p6 proteins and two additionally spacer peptides (p1 and p2)[75]. The *pol* gene encodes the viral enzymes PR (p15), IN (p31) and RT (p66 and p51 subunits). These enzymes are produced as a Gag-Pol precursor polyprotein ($Pr160^{GagPol}$) that is processed by PR. The *env* gene encodes for a polyprotein precursor $Pr160^{Env}$ which is processed by furin into the SU (gp120) and TM (gp41) glycoproteins [75, 81].

1.4 HIV-1 life cycle

The initial step of the HIV life cycle consists of viral entry into the host cells in order to initiate infection. Host cells (*e.g.* T-helper cells, monocytes, macrophages and dendritic cells) which expresses the CD4 (cluster of differentiation 4) glycoprotein receptor on its surface are the main target of HIV[78]. Binding of HIV Env (SU, gp120) to CD4 receptor present in the host cell surface induces conformational changes in the SU and a second receptor (co-receptor) belonging to the chemokine receptor family, CXCR4 and CCR5 becomes exposed [82, 83]. Binding of gp120 both to CD4 and co-receptor triggers new

conformational changes in TM glycoprotein that result in the insertion of gp41 fusion peptide into the host cellular membrane leading to the fusion of viral and cellular membranes and the release of the viral core into the cytoplasm[75, 84]. After uncoating of the virus in the cytoplasm, the viral RNA is reverse transcribed by the viral RT enzyme generating a linear double-stranded DNA molecule (dsDNA)[84]. Uncoating of the viral core leads to the establishment of the reverse transcriptase complexes (RTCs) and pre-integration complexes (PICs)[75, 78, 84]. PIC, which includes dsDNA, MA, NC, IN, RT and Vpr, is then conducted to the nucleus using the cytoplasmatic microtubules network and this process is mediated by IN and Vpr [75, 78]. Still outside the nucleus, IN binds to each end of the newly formed cDNA and removes 2 nucleotides at each 3´end of both DNA strands originating two recessive ends[75, 78]. Later in the nucleus, PIC-dsDNA is inserted into an open region of the host chromosomal genome. Finally, the unpaired regions between HIV and host dsDNA are repaired through cellular cofactors and a provirus is generated[78]. In productively infected cells, integrated provirus serves as a template for the transcription of both viral mRNA and genomic RNA. Transcription of proviral DNA is mediated by the promotor region within the 5´LTR. However, when 5´LTR is defective, 3´LTR activation can occur [85]. Successful transcription leads to the generation of HIV viral transcripts which are derived from a single full-length transcript by alternative splicing, generating messenger RNA (mRNA) with common 5´and 3´ends [86]. HIV-1 transcripts can further be grouped into three different classes: **completely spliced mRNA** or early transcripts (encoding early regulatory proteins as Tat, Nef and Rev**), incompletely spliced mRNA** or late transcripts (encoding Env, Vif, Vpr and Vpu) and **unspliced and complete mRNA** that encode for the polyprotein precursors Gag and Gag-Pol[75, 86]. Unspliced mRNAs are later incorporated in the viral particles as genomic RNA. To complete the expression of the later transcripts proteins from the early transcripts (Tat and Rev) are necessary. Tat binds to a secondary structure located in the R region of the 5' LTR, named the trans-acting response (TAR) element resulting in an increased processivity of RNA polymerase. Rev is responsible for the transport of unspliced and incompletely spliced mRNA outside the nucleus to the cytoplasm to be translated. This process is mediated by the binding of Rev to the Rev response element (RRE), a 240 base region of complex RNA secondary structure [75, 87].

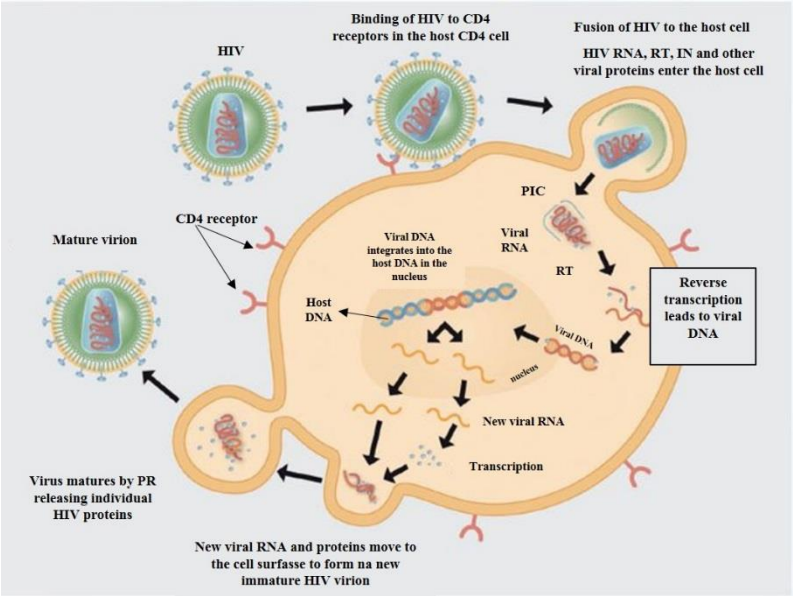

Figure 4- Life cycle of HIV-1 (adapted from www.niaid.nih.gov and [88])

The Env precursor polyproteins are glycosylated in the Golgi apparatus before they oligomerize in trimers. Then, polyproteins are cleaved into the SU and TM glycoproteins and conducted to the cytoplasmatic membrane in order to initiate assembly process. The assembling virion includes all of the components necessary for infectivity, *i.e.*, two copies of viral RNA, cellular tRNA, molecules to prime cDNA synthesis, Env, Gag, PR, RT and IN. HIV-1 Gag and Gag-Pol precursor polyproteins mediate the virion assembly including the generation of spherical particles and genomic RNA packaging[80]. Immature viral particles bud from the cell by gemulation of the cytoplasmatic membrane and acquire the lipid envelope containing the TM/SU trimers. The last step of HIV-1 life cycle consists in the release of new HIV mature particles and cleavage of the Gag and Gag-Pol precursors into the functional proteins by PR [75, 80].

1.5 HIV-1 transmission

In 2015, about 2.1 million (1.8 million-2.4 million) new HIV cases were reported resulting in a total of 70 million infected people since the beginning of the HIV epidemic [89]. Although this represent a reduction in new infections (2.6 million new infections in 2009), the reduction has not been uniform across different regions and group risks. Thus, HIV infection continues to be a major global public health issue [90, 91].

HIV-1 transmission mainly results from sexual contact across mucosal surfaces, maternal-infant exposure (during pregnancy, delivery and breastfeeding) and percutaneous inoculation (through contaminated blood or blood products)[92] . Once a person is infected, HIV is present in semen, vaginal fluids, breast milk, blood and rectal secretions. Sexual contact with an infected person constitutes the most frequent mode of transmission of HIV and the majority of HIV transmissions worldwide occur through heterosexual contacts [93]. The risk of transmission of HIV-1 depends largely on the susceptibility of the uninfected host but also on the viral features. HIV viral load (*i.e*. amount of HIV in a body fluid) in the transmitting partner seems to affect the efficiency of the infection and varies according to the stage of disease [94]. In fact, HIV-1 transmission is likely to occur with more probability during the acute stage of HIV infection (earliest days) and in the latest stage of the disease (AIDS) where intense viral replication is observed [90, 94-96]. Moreover, taking antiretroviral therapy (ART) can reduce the risk of an HIV-infected person transmitting the infection to another by as much as 96%[97] . HIV transmission is also influenced by the presence of co-infection with other STDs (Sexual Transmitted Diseases) such as syphilis and herpes simplex-2 (HSV-2) [90, 98, 99]. Also, genital ulcers and inflammation can contribute to enhance HIV-1 sexual transmission with ulcerative STDs presenting an additional entry point for HIV [90, 99].

Despite the high diversity of HIV variants observed in infected patients, HIV transmission involves a limited number of variants (bottleneck effect) that are not necessarily the dominant variant in the donor [90, 100]. Thus, while chronically infected patients present a set of viral quasispecies that are genetically diverse, acutely infected patients frequently present a more "homogenous" set of viral variants that result from the transmission of one or few closely related viruses [90, 100, 101]. This is observed both in sexual and percutaneous routes of infection albeit at different levels [100, 101]. Transmission of multiple and more heterogeneous variants can be observed in injecting drug users (IDUs) probably due to the lack of a mucosal barrier and the associated protective cells (*e.g*. Langerhans cells) and innate immune response (*e.g.* production of IFNɣ by local macrophages) that play an important role in reducing HIV-1 transmission [101, 102]. Because the majority of HIV variants that are transmitted present a strong preference for CCR5 co-receptor, the capacity of HIV to establish an efficient infection also depends on the availability of target CD4 cells expressing CCR5 co-receptors [101, 103]. In fact,

the defective expression of CCR5 in humans (Δ32 mutation) is known to confer protection from HIV infection[104].

1.6 HIV-1 prevention

In the absence of a cure, the control of HIV/AIDS epidemic requires the implementation of several prevention measures. There are several options to reduce the risk of acquisition and transmission of HIV. This includes behavioral interventions (*e.g*. counseling and sex education of susceptible populations, use of condoms, needle and syringe programs) and biomedical interventions (*e.g*. male circumcision, HIV testing, antiretroviral drugs, pre and post-exposure prophylaxis[105].

Because globally only 54% of individuals infected with HIV-1 are aware of their status, HIV testing should be considered one of the first prevention methods. Awareness of HIV status is crucial to identify HIV infected individuals in order to establish the appropriate measures to get HIV treatment and also to prevent HIV transmission to others [92]. In fact, the WHO established with the 90-90-90 objectives that by 2020, 90% of all people living with HIV will know their status, 90% of all people with diagnosed HIV infection will receive sustained antiretroviral therapy and 90% of all people receiving ART will have viral suppression[106].

Besides their role in the improvement of health quality and survival rate in HIV-1 infected individuals, antiretroviral therapy is also crucial in preventing HIV transmission and infection. In fact, ART contributes crucially to lower viral load to undetectable levels in blood and genital secretions in HIV-1 infected individuals thereby reducing HIV transmission to negligible levels (< 1%) and can also be used to prevent infection very efficiently in uninfected (PreP, Pre-Exposure Prophylaxis) or recently infected individuals (PEP, Post-Exposure Prophylaxis) [97, 107-110]. Currently, oral PrEP consists of a combination of tenofovir disoproxil fumarate and emtricitabine and is able to reduce the risk of getting HIV by more than 90% if taken consistently everyday [111-114]. More recently, topical microbicides have been proposed to prevent HIV sexual transmission by directly inactivate or prevent HIV entry or replication in susceptible target cells present in the vagina and/or rectum. Topical microbicides can be delivered

over a prolonged period of time (*e.g.* intravaginal ring) or applied on a daily basis (*e.g.* gel)[115]. Recent results from the ASPIRE study conducted in Malawi, South Africa, Uganda and Zimbabwe demonstrated that the use of a vaginal ring continuously releasing dapivirine provided a modest degree of protection against HIV particularly in subgroups with evidence of increased adherence[116]. Currently, there is still no safe and effective microbicide available to the public.

Despite the important role of ART and PrEP in lowering and prevent HIV infection, in 2015 less than 50% of HIV infected adults and children were accessing treatment and 2.1 million people became newly infected with HIV[117]. Therefore, control and ultimately eradication of HIV will depend on the development of a safe and effective HIV vaccine accessible to all. Despite several years of investigation and important advances in vaccine field there is still no effective vaccine available for HIV. Currently, vaccine trial HVTN 702, based in the optimization of the RV144 trial[118], is being conducted among South African adults in order to test if an experimental vaccine regimen safely prevents HIV infection[119]. Vaccination including HIV vaccines will be explained in more detail in chapter 3.

Other biological interventions like male circumcision also contributes to lower HIV infection. In fact, this method reduces the risk of infection up to 60% by eliminating many cellular targets of HIV (*e.g.*Langerhans' cells) that are present in the penile foreskin [120, 121]. Nonetheless, behavioral interventions (*e.g.* sexual abstinence, reduced number of sexual partners, use of condoms, implementation of needle and syringe cleaning practices among IDUs) and better access to healthcare services are also important strategies to reduce the risk of HIV infection[105, 122]. Because no single intervention measure has been found to be completely effective it is believed that the combination of different prevention approaches that integrate behavioral and biomedical strategies will lead to an important reduction in new HIV infections [123].

1.7 HIV-1 pathogenesis

The first stage of HIV infection (acute phase or primary infection) is characterized by an intense viral replication that, in the absence of antiretroviral therapy, declines after three to four weeks after initial exposure (Figure 5) [103]. The first symptoms occur two to

four weeks after transmission and includes flu-like clinical manifestations, fatigue, myalgia, fever and lymphadenopathy[124]. During several months plasma viremia continues to gradually decline before reaching a steady state (viral set point)[103]. Viral set point constitute an important determinant on the rate of disease progression because it points out the beginning of the chronic stage of HIV infection[103].

CD4 T cells are the first targets of the virus. Along with the high replication of HIV in the mucosa, submucosa and draining lymphoreticular tissues there is a dramatic loss of $CD4^+$T cells in the peripheral blood that is associated with the depletion of $CCR5^+$ memory $CD4^+$ T cells in the gut associated lymphoid tissue (GALT)[103, 125]. In fact, GALT harbors the majority of lymphoid tissue being an important site for viral replication and interactions with the host immune system during HIV infection[126]. This condition is followed by the dissemination of the virus to peripheral lymphoid tissue, particularly lymph nodes, and the establishment of persistent lymphoid tissue viral reservoirs[103]. The decline of $CD4^+$ T cells is closely related with the direct effect of the viral infection of these cells together with the host cellular responses (*e.g.* host cytotoxic responses and natural killer cells)[127].

Seroconversion, with detection of HIV specific antibodies, occurs normally after three to 12 weeks after HIV exposure [103, 128]. The first antibodies detected are those directed against p24 antigen followed by antibodies directed to gp120 and gp41 (envelope)[128]. Approximately 12 weeks after transmission neutralizing antibodies (NAbs) start to arise and evolve but this response seems not to be enough to clear the virus. The first NAbs are directed against the autologous virus and are not able to neutralize more divergent virus (heterologous viruses) [128-132].

The chronic stage of HIV-1 infection (second stage) is asymptomatic (or latent) and lasts between eight to ten years (Figure 5). It is characterized by low but persistent levels of viral replication in the lymph nodes and constant antigen stimulation by the host immune system [103, 127, 133]. During this period, the immune system becomes activated by several factors that include viral proteins, microbial products that are translocated from the GALT and host responses. As cells are activated, they produce a set of proinflammatory cytokines which in addition with viral replication will ultimately lead to chronic immune activation[103]. This persistent immune activation is seen by an increased T cells turnover, monocytes and natural killer cells (NK), high levels of CD4

and CD8 T cell apoptosis and polyclonal B cell activation that can lead to hypergammaglobulinemia [103, 127, 134-136].

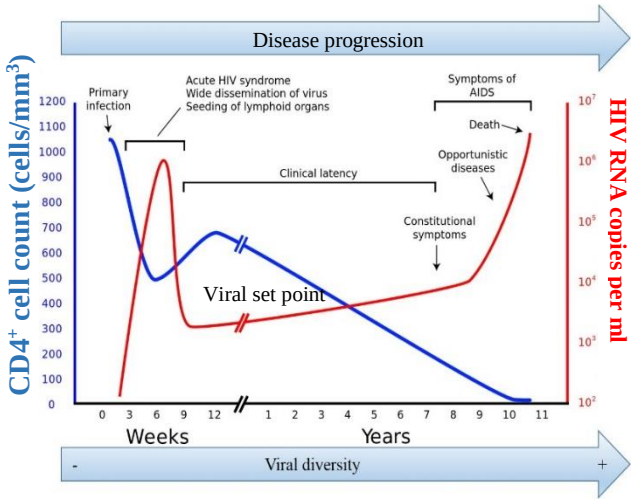

Figure 5- Natural course of untreated HIV-1 infection. Representation of the relation between HIV viral load (red) and CD4 counts (blue) during the course of HIV infection (adapted from [137] and [138].

Together, these changes within each cell population greatly affect the overall immunologic competence leading to the exhaustion of the immune system. Moreover, in the absence of ART the majority of HIV infected patients become susceptible to the occurrence or reactivation of opportunistic infections (*e.g.* candidiasis, pneumonia, tuberculosis) as well as the development of virus induced tumors (*e.g.* Epstein-Barr virus related lymphomas, Kaposi´s sarcoma and cervical cancer caused by Human Papillomavirus [103, 134]). These symptoms define the early symptomatic stage of HIV chronic phase. In untreated patients, the progressive loss of $CD4^+$ T cells can lead to an increased state of immunodeficiency that mark the onset of the last stage of HIV-1 infection: AIDS. According to Center for disease control and prevention (CDC), CD4 counts less than 200 cells/ul associated with opportunistic infections is a criterion that defines the AIDS stage [139]. Most untreated HIV-1 infected patients develop AIDS and eventually die [140]. A small minority (<5%) of individuals termed long-term nonprogressors remains healthy for several years in the absence of treatment [140, 141].

2. HIV-1 envelope

2.1 Molecular and structural organization

HIV-1 entry to host cells is mediated by interactions between the virus envelope (SU and TM glycoproteins) and the host cell receptors. The HIV-1 envelope glycoprotein (Env) is a trimer composed of three copies of non-covalently associated heterodimers of gp120 (SU) and gp41 (TM) that represent several spikes in the viral surface. These glycoproteins result from a gp160 precursor glycoprotein that is cleaved by cellular proteases and remain non-covalently associated on the cell or viral surface [142, 143]. Whereas gp120 interacts with cellular receptors and co-receptors, gp41 mediates fusion between viral and cellular membranes [142]. In this context, the main exposed surface of HIV-1 envelope is composed of gp120 while gp41 is mostly shielded. HIV-1 gp120 envelope include five conserved regions (C1-C5) and five variable regions (V1-V5) (Figure 6)[144]. Whereas the five conserved regions compose the structural core of gp120, the five variable regions are highly glycosylated and protect the core from neutralizing antibodies [143, 145]. Four of these hypervariable regions (V1-V4) tend to form loops through disulfide bonds that are exposed on the outer surface of the viral Env [146, 147].

The V1/V2 loop is involved in Tat binding and Tat-mediated viral entry, a previously unknown mechanism[148], whereas V3 loop plays an essencial role in coreceptor binding and viral entry[83]. In addition, both regions represent a target for antibodies, including neutralizing antibodies, when accessible on the surface of the virion [149, 150].

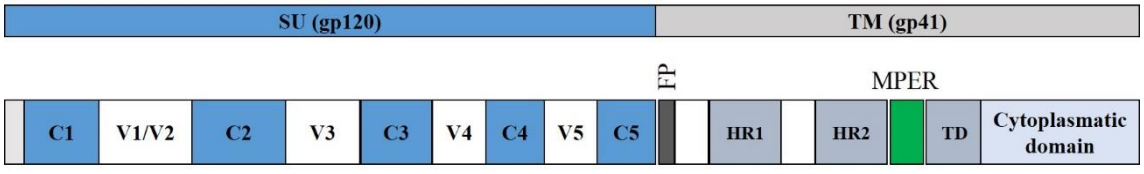

Figure 6- S linear representation of the surface and transmembrane envelope glycoproteins. Surface glycoprotein (SU, gp120) includes five conserved (C1-C5) and five variable regions (V1-V5). Transmembrane glycoprotein (TM, gp41) contains the fusion peptide (FP), two heptad regions (HR1 and HR2), the membrane proximal external region (MPER), one transmembrane domain (TD) and a cytoplasmatic domain (adapted from [75] and [151]).

In its native trimeric conformation, SU has one inner and outer domain (Figure 7). The outer domain is highly glycosylated and is involved in the interaction between SU and

the cellular receptors and co-receptors. The outer domain is the region more exposed to the host immune responses and has most of the antigenic determinants in Env including, for example, the potent neutralizing antibody epitopes in V1/V2 and V3 loop [150, 152-155]. The inner domain is hydrophobic and essential for the association between SU and TM. Between the inner and outer domain there is another domain named bridging sheet (Figure 7). This bridging sheet is formed between V1/V2 stem and β sheets 20 and 21 of C4 as the result of conformational changes that occur after gp120-CD4 binding [75, 146, 156, 157]. After binding of the viral receptor and co-receptor by gp120 the fusion machinery in the gp41 subunit is readily activated.

HIV gp41 glycoprotein is divided in three major domains: one extracellular domain or ectodomain (512-683 in HXB2), one transmembrane domain (TD) that inserts in the host membrane cell (683-707 in HXB2) and one cytoplasmatic domain (708-856 in HXB2).

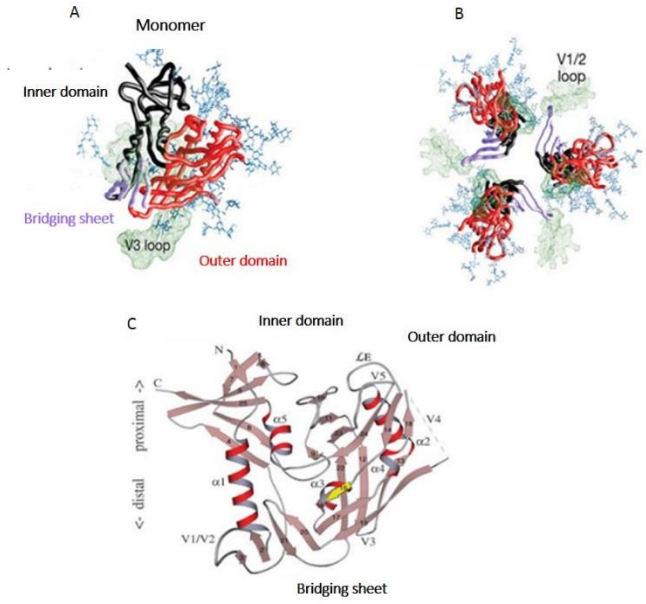

Figure 7- HIV-1 gp120 structure (adapted from [158, 159]). Monomeric (A) and trimeric (B) gp120 core with inner (black) and outer domain (red) and the bridging sheet (violet). Secondary structure of gp120 (C);

The extracellular domain, which mediates the major functions of TM, can be subdivided in five functional regions: a fusion peptide (FP, 512-534 in HXB2) followed by two α-helices containing leucine-zipper motifs designated heptad repeat 1 (HR1, 542-591 in HXB2) and heptad repeat 2 (HR2, 623-661 in HXB2), the loop region (593-622 in HXB2) that separate the HR1 and HR2 and finally the membrane proximal external region

(MPER, 662-683 in HXB2)[160]. Both fusion peptide and HR1/HR2 have a crucial role on the fusion of the virus to the host cell. The cytoplasmatic domain of TM binds to the matrix protein during the assembly of new viral particles [75, 160, 161].

2.2 HIV-1 entry and interaction between the Env protein and the cell

HIV-1 entry into host cells represents the first step in the viral infection cycle and is mediated by the Env glycoprotein. This process involves three major steps: 1) adhesion of the virus to the host cell and binding of the SU glycoprotein to the CD4 receptor, 2) binding of the SU glycoprotein to the cell co-receptor (CCR5 and/or CXCR4) and 3) fusion of the viral envelope with the cell membrane (Figure 8) [83].

Adhesion of the virus to the host cell brings Env glycoprotein into close proximity with the CD4 receptor. CD4 is a 60 kDa membrane glycoprotein belonging to the immunoglobulin superfamily and is expressed in functionally mature T cells, macrophages, dendritic cells, and monocytes [83, 162]. After binding to CD4 in the host cell, SU suffers major conformational changes that include rearrangements and exposure of V1/V2, V3 and C4 regions and formation of a bridging sheet as described previously. These conformational changes result in the approximation between the viral envelope and the cellular membranes which consequently leads to the interaction of the SU (namely V3 region) with the host cell co-receptors [83]. CCR5 and CXCR4 are considered the major HIV-1 co-receptors *in vivo* [75, 83, 163]. The chemokine receptor CCR5 is predominantly expressed on memory $CD4^+$ T lymphocytes, activated T lymphocytes (mainly Th1 $CD4^+$T cells) and macrophages whereas CXCR4 is mainly found on $CD4^+$ and $CD8^+$ T lymphocytes, monocytes, dendritic cells and B lymphocytes[104, 164, 165].

Viruses that infect preferentially macrophages typically use the CCR5 coreceptor (R5 viruses) mainly during the initial and asymptomatic stage of infection while viruses that infect mainly lymphocytic cell lines use the CXCR4 coreceptor (X4 viruses) preferentially later in the infection (AIDS stage)[104, 166].There are also viruses (the R5/X4 viruses) that are able to use indifferently the both co-receptors producing an effective infection [104, 165, 167, 168]. Moreover, R5 viruses seems to replicate more efficiently in $CD4^+$ T cells compared with X4 viruses contributing to the R5 viral

dominance[169, 170]. As described above, the interaction of gp120 with CD4 results in the exposure of V3 loop of gp120 that subsequently binds to the co-receptors (Figure 8). V3 loop is the major determinant of cellular tropism and co-receptor specificity being responsible for the molecular recognition of the chemokine co-receptor in the cell membrane[171]. Specifically, the V3 tip and V3 base are the main regions involved in the co-receptor binding [172-174].

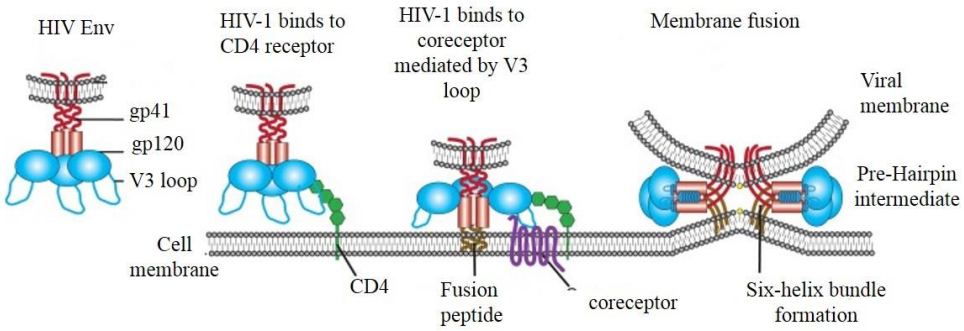

Figure 8- HIV entry mechanism. HIV-1 Env (gp120 and gp41) attaches to the host cell and binds to CD4 receptor. This leads to conformational changes in SU (gp120) and to the interaction of V3 loop of Env with the cell co-receptor. Finally, membrane fusion begins with the insertion of the fusion peptide of gp41 (TM) in the target membrane (adapted from [83]).

Coreceptor binding causes the exposure of the gp41 fusion peptide which is inserted into the cytoplasmatic host membrane creating a prehairpin intermediate configuration of TM (Figure 8). After this, HR1 and HR2 fold in an antiparallel form originating a six-helix bundle which brings the viral envelope and the cellular membrane in close association resulting in the formation of the fusion pore and consequently in the entry of the viral capsid into the cell[83, 175].

3. Immune responses against HIV-1

The immune system protects the host from a large range of pathogens (*e.g.* viruses, bacteria) and toxic or allergenic substances by controlling and/or eliminating them. Any immune response involves the recognition of a pathogen or other organism plus a reaction to eliminate it. In a general way, the immune response can be classified as innate or adaptive. Innate immune response constitutes the first line of defense against pathogens and is characterized for being non-specific and fast allowing a rapid response to invasion.

In contrast, the adaptive immune response is highly specific against a certain pathogen or substance and normally starts after an innate response. While the innate response remains unchanged after pathogen exposition, adaptive response can generate immunological memory that allows a more specific and efficient response in each subsequent exposition to the same pathogen. Although innate and adaptive responses behave in a totally different way they work together in order to achieve the same goal: an effective immune response capable of protecting the body from "invaders" [176, 177].

In HIV infection, both innate and adaptive immune responses are raised but they are inefficient to eliminate the virus.

3.1 Innate immune responses

The innate immune system includes external barriers as the skin, mucous membranes, cilia, secretions and tissue fluids containing antimicrobial agents. Soluble proteins and small molecules present in biological fluids (*e.g*. complement proteins, defensins and ficolins) or released from cells (*e.g*. cytokines, chemokines, lipid mediators of inflammation, reactive free radical species and enzymes) are also part of the innate response as well as membrane receptors and cytoplasmatic proteins that bind to molecular patterns expressed in the pathogen surface [176]. Innate immune system is composed of an array of cell subsets derived from the bone marrow that include phagocytes (monocytes, macrophages, neutrophils), cytolytic cells (natural killer cells and neutrophils) and professional antigen-presenting cells (dendritic cells)[177-179]. Phagocytes, a particular group of cells that engulf pathogens via phagocytosis, bind to the foreign pathogen using receptors that recognize Pathogen-associated molecular patterns (PAMPs) which includes microbial nucleic acids (*e.g*. DNA and RNA), lipoproteins, surface glycoproteins and membrane components (*e.g*. peptidoglycans and lipopolysaccharide) [180, 181]. PAMPs are normally present in the pathogen surface and are recognized by innate immune receptors on certain leucocytes providing exogenous signals that alert the immune system to the presence of the pathogen [176, 177, 182]. This process can lead to the phagocytosis of the pathogen highlighting the interaction between the innate and adaptive immune responses.

Recognition of PAMPs is performed by pathogen-recognition receptors (PRRs) expressed by cells of the innate immune system. An example of PRR is the Toll-like receptor (TLR). Human TLRs can be expressed on the cell plasma membrane (*e.g*. TLRs 1,2, 4, 5 and 6) or in endosomes (*e.g.* TLRs 3,7,8 and 9)[183]. TLRs are expressed at mucosal surfaces in several cell types including neutrophils, macrophages, DCs, T cells and epithelial cells [184, 185]. TLR function in epithelial tissues is particularly important since these are the main sites for microorganism exposure[186]. Besides the activation of phagocytes, killing pathogens and release of pro-inflammatory cytokines and anti-microbial peptides, the activation of DCs by TLRs lead to the stimulation of the adaptive immune responses[182, 187].

HIV-1 can be recognized by the innate immune system through PAMPs present in the viral particle or generated during different stages of HIV life cycle. For instance, the viral envelope glycoproteins may be recognized by TLRs and CLRs (C-type lectin receptors) as described for other retroviruses[188]. Also, genomic RNA, mRNA and viral structural and regulatory proteins may represent potential ligands for PRRs. For instance, HIV (ssRNA) is recognized by TLR7 and TLR8, which are present in DCs and monocytes, leading to the potent activation of these cells and the release of type I interferons (IFNs) and tumor necrosis factor α (TNF- α)[189]. This contributes to the inhibition of viral replication (antiviral activity) in infected cells and activation of the immune response by recruiting other immune cells to the sites of infection [190, 191]. Interestingly, TRL7/8 expression is reduced in male and female lower genital tract [192, 193] and absent in the gut[186], two major sites for HIV exposure. However, several studies have shown that TLRs 7-9 are constitutively expressed in the upper female reproductive tract (reviewed in [194]. In addition, responsiveness to TLR9 is defective in HIV-1 infection. Direct interaction of gp120 with pDCs seems to inhibit TLR9-mediated IFN-α secretion leading to a reduced capacity to induce cytotoxic activity of NK cells and therefore to a decreased ability to initiate host immune responses [195, 196].

Among the innate immune cells involved in the early control of HIV-1 infection, NK cells play a major role. These cells are a subset of granular lymphocytes that do not express an antigen-specific receptor but instead, express a set of inhibitory and activating receptors on their surface [178, 191, 197]. NK cells recognize and kill virus-infected cells and are regulated by the balance between incoming inhibitory and activating signals with the inhibitory signals (received from Human leukocyte antigen class I molecules) being

usually predominant maintaining NK cells in a resting state [179, 198]. NK activation can be achieved when the magnitude of the activating signal overwhelms the dominant inhibitory signal (reviewed in [179]). In acute HIV-1 infection, NK cells expand rapidly specially in the seronegative window [191]. Co-expression of NK-cell receptors (named KIR-Killer immunoglobulin receptor) in association with their ligands is related with a slower HIV disease progression and early control of viremia supporting an important role for these cells in the first stage of the infection [199, 200]. Despite the important role of NK cells in controlling early infection, HIV-1 can impair NK cells and escape from recognition by several mechanisms, including the expression of counteracting proteins (*e.g.* Nef), leading ultimately to persistent infection[179]. Nef is able to down-regulate HLA class I molecules on the surface of the infected cells by decreasing HLA-A and HLA-B and maintaining HLA-C and HLA-E unchanged. This selective HLA-I downregulation protects HIV-1 infected cells from lysis mediated by NK cells expressing inhibitory receptors that are specific for HLA-C or HLA-E (reviewed in [198]).

Finally, DCs, one of the earliest targets of HIV, are the main antigen-presenting cells and induce a primary immune response in resting naïve T cells[201]. Immature DCs develop into mature effector DCs upon activation by microorganisms or inflammatory signals and migrate to the draining lymph nodes where they stimulate naïve Th cells. Specifically, HIV-1 is captured by DCs in the mucosa and is delivered to the lymph node where it replicates [202]. Based on differences in function and expression of surface markers, DCs are divided in myeloid DCs (mDCs) and plasmacytoid DC (pDCs). mDCs include Langerhans cells, dermal DCs and interstitial DCs and are found in the blood, skin and mucosal tissues and act by capturing antigens, processing them and presenting them on the cell surface linking the innate and adaptive systems. pDCs are located in blood and secondary lymphoid organs (but they can be recruited to inflammation sites) and play an important role in innate immune responses through the production of type I IFN, as described above. In HIV-1 infected individuals the number of mDCs and pDCs are substantially reduced. Interestingly, while mDCs can enhance HIV infection through capture and subsequent transmission of the virus, pDCs are capable to inhibit HIV replication due to the antiviral activity of IFN-α[202].

3.2 Adaptive immune responses

Adaptive immune response is characterized by the ability to recognize a wide range of antigens, for its high specificity and for a long immunological memory. There are two types of adaptive immune responses: cellular responses, mediated by T lymphocytes (or T cells) and humoral responses, mediated by antibodies produced by B lymphocytes (or B cells).

3.2.1 Cellular responses

There are several types of T lymphocytes according to its function, namely, helper T cells ($CD4^+$), cytotoxic T cells ($CD8^+$) and regulatory T cells ($CD4^+FOXP3^+$). Helper T cells include several main subsets of cells: Th1, Th2, Th9, Th17 and more recently, T follicular helper cells (Tfh). Th1 cells, which produce IFN-γ and IL-2, are involved in an efficient antiviral response and in the pathogenesis of autoimmunity, whereas Th2 cells, which secrete IL-4, IL-5, IL-10 and IL-13 cytokines, are involved in allergic responses and clearance of extracellular pathogens[203-205]. Th17 cells are one of the predominant proinflammatory cell types and through IL-17 production attract other innate immune cells such as macrophages and neutrophils to further aggravate chronic inflammation. Th17 are predominantly present in the gut to maintain homeostasis and are depleted to some degree in HIV infected individuals contributing to the pathogenesis of HIV infection [205, 206]. T-cytotoxic lymphocytes (CTL), which have cytotoxic activity, are responsible for the recognition and destruction of host cells infected by pathogens [177, 207]. Follicular T cells will be described in detail further below.

After infection, naïve T cells circulating through the lymphoid organs encounter specific antigens presented at the surface of antigen presenting cells (*e.g.* DCs). Due to this interaction, the antigen-specific T cell undergoes a process of extensive division and differentiation and becomes an activated effector T cell that migrates to tissues in order to fight the infection. This will lead to the production of a pool of memory T cells that are responsible for the rapid response to subsequent encounters with the same pathogen. By the end of puberty, memory T cells are predominantly present in lymphoid tissues, mucosal sites and skin and persist throughout adult life representing the most abundant

lymphocyte population in the body[208]. Although both $CD4^+$ and $CD8^+$ T cells differentiate simultaneously, $CD8^+$ T cells divide sooner and more rapidly and therefore develop into effector cells quicker than $CD4^+$ T cells. $CD4^+$ T cells are important for optimal generation of memory $CD8^+$ T cells following acute infections and for sustained $CD8^+$ T cell responses during chronic infections[207, 209, 210].

Most cellular responses against HIV are mediated by $CD8^+$ T cells. These cells have the capacity to kill HIV-1 infected cells and secrete IFN-γ being detected early in infection shortly before the peak of viremia, declining after 1-2 weeks when viremia also declines[211]. The rapid decline of $CD8^+$ T cells responses observed during early HIV-1 infection can be explained by several mechanisms that include a decrease in antigen load as viremia is brought under control, T cell exhaustion (due to persistent antigen exposure) and subsequent deletion of the early responding T cell populations and selection of viral escape mutations that interfere with $CD8^+$ T cells recognition [211-214].

$CD8^+$ T cells have an important antiviral activity against HIV-1 as they are associated with a rapid and drastic decline of the initial viral replication and also with the establishment and maintenance of the viral set point and thus the rate of disease progression [215, 216]. The antiviral effect of $CD8^+$ T cells is particularly evident in HIV controllers, *i.e.* HIV-1 positive individuals who maintain plasma viral loads below the limit of detection without antiretroviral therapy. In contrast with HIV progressors, in HIV controllers there are persistent and highly strong and specific $CD8^+$ T cells which are associated with the control of viral replication[217].

In contrast with $CD8^+$ T cells, which recognize viral determinants at the surface of infected cells through antigen presentation by HLA class I inducing apoptosis, $CD4^+$ T cells recognize viral determinants through antigen presentation by HLA class II and act by proliferating and releasing cytokines[177, 218, 219]. IL-2 is a cytokine that regulates the proliferation, differentiation and survival of T cells [220-222]. It is secreted by activated $CD4^+$T cells and its expression is regulated by a complex network involving transcription factors, chromatin remodeling and CD28 costimulation signaling [223]. $CD4^+$ T cells help in maturation of B cells and antibody class switching and also in the generation of cytotoxic $CD8^+$ T cell responses and memory development [224, 225].

$CD4^+$ T cells are particularly susceptible to HIV infection representing the main target of HIV-1[103, 226]. HIV infection is characterized by an acute stage of intense viral

replication and a decrease in $CD4^+$ T cell counts, followed by a partial control of viremia and entry in a chronic state of infection (figure 5). Through the course of infection $CD4^+$ T cells are progressively depleted and functionally impaired leading eventually to AIDS and death in untreated individuals [133, 140, 211, 227].

In early HIV-1 infection, memory $CD4^+$T cells are depleted from the lymphoid system, mainly in the gut. This depletion is observed in all memory $CD4^+$T cells populations although those specific for HIV are preferentially infected and destroyed. However, only a few percent of these cells are infected suggesting that the majority of HIV-specific $CD4^+$T cells escape [228]. The causes of $CD4^+$ T cell depletion in the course of HIV infection have not been fully elucidated. However, apoptosis caused by a variety of HIV proteins (*e.g.* gp120, Tat, nef, Vpr) and the chronically activated and hyper inflammatory immune status characteristic of HIV infection have been associated to HIV-1 induced $CD4^+$ T cell death [229-231]. DNA-dependent protein kinase during viral integration is also associated with cell death during HIV infection [232]. More recently, $CD4^+$ T cell death during HIV infection has been linked to a mechanism of caspase-1-mediated pyroptosis triggered by abortive viral infection[233]. In addition, HIV-1 infection of $CD4^+$ T cells leads to an abnormal expression of the IL-2 gene disturbing the anti-viral immune responses mediated by IL-2 secretion (reviewed in [224]). This gradual loss of IL-2 secretion and proliferation during the course of infection is strongly associated with T cell exhaustion[225]. Despite $CD4^+$ T cell responses to HIV proteins are still unclear, HIV-1 Gag protein seems to constitute an important epitope for these cells in chronically infected patients. Streeck and colleagues have demonstrated that lower levels of viremia associated with an effective immune control were correlated with a high frequency and magnitude of Gag responses and low proportion of Env responses. Specifically, targeting of Gag was exhibited by elite controllers while Env was targeted by HIV progressors[234].

The recently characterized T follicular helper cells, which reside in follicles of secondary lymphoid organs assisting B cells in the generation of antibodies are a key component of the adaptive immune system (Figure 9) [218, 235-238]. Tfh cell differentiation process starts at initial dendritic cell priming of a naïve $CD4^+$ that subsequently undergoes cell division. If a CXCR5 chemokine receptor is expressed, the early Tfh cell migrate to the border of the B cell follicle leading to further Tfh cell differentiation. In contrast, if the cell receives Th1, Th2 or Th17 cell signals, $CD4^+$ T cell will follow a Th1, Th2 or Th17

cell differentiation program that includes the upregulation of chemokine receptors for inflammatory chemokines which in turn will drive the effector cell to the site of infection or inflammation [239]. These cells are primarily found in germinal centers (GCs) and play a significant role supporting B cell activation, somatic hypermutation (SHM) and antibody class switching, following natural infection or vaccination [235, 240-242].

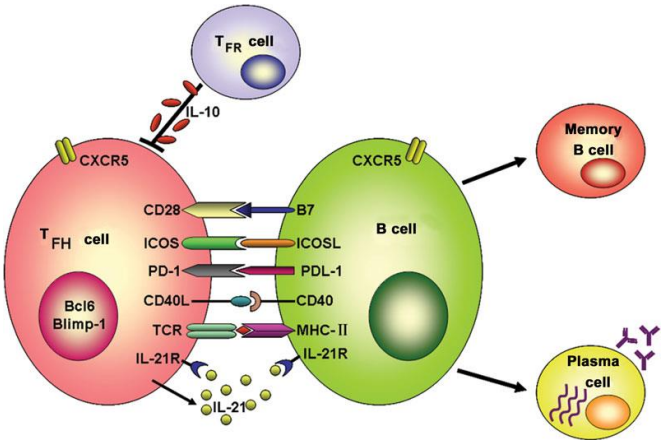

Figure 9- T follicular helper cell and B cell interaction in the lymph node (extracted from [243]). Activated Tfh (red) upregulate CXCR5 and migrate toward B cell follicles to form GC. Tfh in GC interact with antigen-specific B cells (green) through several molecules as ICOS-ICOSL, PD-1-PDL-1, CD40-CD40L and IL-21R-IL-21 leading to memory B cells and plasma cells production. Plasma cells secrete long-lived antibodies in order to combat infectious agents. Tfr (purple) can regulate Tfh and GC responses to limit autoantibody production.

Tfh cells are defined by the expression of CXCR5 chemokine receptor, program death-1 (PD-1), ICOS (Inducible T cell co-stimulator), and secretion of IL-21 and their differentiation is regulated by B-cell lymphoma 6 (Bcl6) [235, 244, 245]. IL-21, primarily produced by CD4 T cells, is a key cytokine involved in the promotion of GC formation and maintenance, Tfh and B cell proliferation, and memory B cells/plasma cells differentiation. It is also critical to the generation of antigen-specific IgG antibodies and expansion of class-switched B cells and plasma cells *in vivo*, an important process during the production of NAbs[242, 246, 247].

The interplay between HIV infection and Tfh is still not fully understood. Because Tfh cells have a central memory phenotype and express CCR5 coreceptor they are potentially susceptible to HIV infection[248]. In fact, Perreau *et al.*, have demonstrated that Tfh cells are a major reservoir for HIV infection, replication and production [249]. In the earlier stages of the disease, Tfh levels are increased promoting HIV replication and disease progression [133, 236]. In fact, because Tfh are primarily found in follicles and GCs of

secondary lymphoid organs they are more susceptible to be infected compared to a CD4 T cell outside the follicle[249, 250]. Despite being highly permissive to HIV, Tfh cells expand in early and chronic stage of the disease and part of this expansion is driven by antigen stimulation[251].

Increased evidences have linked many of HIV-infection-induced immune deficits (*e.g.* B cell dysfunction, decreased GC B cell and plasma cell numbers, hypergammaglobulinemia, loss of T-dependent responses) to impaired Tfh function [237, 249, 252]. Recently, Haddak and colleagues [253] have demonstrated that, despite Tfh expansion during HIV infection, these cells were functionally impaired being unable to provide adequate B cell help. This was due to an increased PD-L1 expression by B cells from the GC leading to PD-1 triggering on Tfh cells which in turn affected Tfh cell proliferation, activation, ICOS expression and IL-21 secretion. Impairments in Tfh contribute to the ability of HIV to persist and evade humoral immunity interfering with the effective production of NAbs[237]. It was recently demonstrated that impairments in the capacity of Tfh to stimulate HIV specific antibody production by B cells occur soon after infection. In addition, Tfh from lymph nodes of untreated and treated HIV infected patients seems to be much more sensitive to Gag than to Env probably caused by the increased presence of Gag antigen compared to Env antigen in the lymph nodes of HIV infected individuals and also by the persistence of p24 antigen in lymph nodes after long-term ART. Thus, this lack of specificity of Tfh to the HIV envelope may be one of the factors contributing to the slow development (and failure) of bNAbs generated after several years of infection (reviewed in [237]).

Besides the unclear role of Tfh cells in bNAb development there are some evidences demonstrating that these cells may contribute to HIV neutralization as showed by the positive correlation between the frequency (and quality) of Tfh subsets and effective humoral responses against HIV, measured by the development of bNAbs [240, 254-256]. Recently, Tfh cells from elite controllers were found to have a stronger *ex vivo* capacity of inducing B cell maturation and antibody switch compared with normal HIV progressors suggesting that the immune responses in this small subset of individuals are mediated by better helper activity compared with progressors[257]. Thus, due to the crucial interactions between Tfh cells and B cells, Tfh should be considered a potential target for vaccine strategies aiming to induce neutralizing antibodies.

Within follicular cells, a subset of cells with regulatory functions- T follicular regulatory cells (Tfr) - seems to moderate the role of Tfh cells in driving antibody production by B cells (Figure 9) [258-260]. These cells are also a crucial component of the GC response as they are able to inhibit GC expansion and regulate Tfh and GC responses to limit autoantibody production [259-261]. Similar to Tfh, Tfr express high levels of CXCR5, ICOS and PD-1, but they also express FOXP3 which contributes for its regulatory functions [259-261]. Tfr develop independently of Tfh from natural Treg precursors but they share similar signals with Tfh, such as CD28 and ICOS, in order to differentiate [260]. The role of immune tolerance has also been implicated in the development of bNAbs, with evidences of a negative correlation between Tfr and the presence of bNAbs in HIV-1-infected individuals compared with HIV-1 infected individuals without bNAbs[262]. Several studies reported that higher quality Env-specific (gp120) antibodies in SIV-infected rhesus macaques were correlated with a lower frequency of Tfrs [263]. Also neutralizing antibodies to HIV were negatively correlated to $FOXP3^{+}$ Env-specific follicular T cells in $SHIV_{A28}$ (Simian-Human immunodeficiency virus) infected rhesus macaques [256]. Together these data suggest that Tfrs increase during chronic HIV infection and may contribute for Tfh dysfunction resulting in disruption of proper B cell differentiation and SHM. In mice, circulating Tfrs were shown to be expanded after viral infection with influenza or lymphocytic choriomeningitis virus (LCMV) and could potently suppress Tfh function without requiring specific antigens[264]. As Tfrs frequency has been found to negatively correlate with bNAb generation it is important to consider this subset when developing new vaccine strategies [256, 263]. Moreover, recent studies have shown that the function of Tfr and/or a skew in the balance of Tfh/Tfr frequency can lead to impaired humoral immunity [264, 265]). Increased understanding of the biology of Tfh and Tfr cells may lead to improved vaccination strategies against HIV.

3.2.2 Humoral responses

The humoral immune response is characterized by the production of antibodies by B lymphocytes and their progeny, plasma cells [266].

3.2.2.1 B cells

B cells development includes a first stage of antigen independency followed by a stage dependent of antigen presence. The first stage occurs in the bone marrow, where precursor B cells develop from hematopoietic cells into the immature B cell stage with the help of stromal cells. [267]

Maturation of the B cells starts when the stem cell differentiates into the progenitor B cell (pro-B cell) (Figure 10). These cells proliferate and differentiate into precursor B cells (pre-B cells). Interactions between pre-B cells and stromal cells, which provide growth factors that stimulate lymphocyte differentiation and proliferation, are necessary to the development of immature B cells[267, 268]. Immature B cells undergo selection for self-tolerance and ability to survive in the peripheral lymphoid tissues where they continuously circulate among all lymphoid tissues (including lymph and blood) becoming mature B cells (naïve B cells)[267].

Effector cells (mature and fully functional B cells) migrate from the bone marrow to the peripheral lymphoid organs where they can find an antigen (antigen-dependent process). When a B cell with a specific receptor encounters a specific antigen it activates and starts to proliferate and differentiate into plasma and memory B cells (Figure 10). Plasma cells are non-dividing, terminally differentiated and immobile antibody-secreting cells of the B cell lineage. Plasma cells localized in the spleen and lymph nodes are mainly short-lived plasma cells (SLPCs) and produce antibodies during a short period (*e.g.* few weeks). In contrast, long-lived plasma cells (LLPC) migrate to the bone marrow, where they produce antibodies for extended periods (*e.g.* years). Moreover, plasmablasts are plasma cells precursors that produce functional antibodies but, in contrast to plasma cells, are able to divide[177]. These cells can develop from any type of activated B cells (*e.g.* naïve, follicular, memory)[267]. Finally, memory B cells are generated in GC reactions in the course of T cell-dependent immune responses and are distinguished from naive B cells by an increased lifespan, faster and stronger response to stimulation and expression of somatically mutated and affinity matured immunoglobulin (Ig) genes [267, 269].

Activation of B cell can be achieved with or without T cell help. Thymus-independent (TI) antigens can activate B cells and induce antibody production in the absence of helper T cells. B cells activated by TI antigens proliferate outside the lymphoid follicles in the

secondary lymphoid organs (without GCs formation) [267, 270]. In contrast, Thymus-dependent antigens need T cell help to successfully activate B cells resulting in the production of both high-affinity memory B cells and LLPC [218, 267].

Differentiation of B cells into memory cells and their affinity maturation and differentiation into long-lived plasma cells both take place in germinal centers of secondary lymphoid follicles [267, 271].

During an acute viral infection, naïve B cells remain in the marginal zone and differentiate into SLPCs producing low-affinity antibodies which provide the first wave of defense against pathogens or, alternatively, with CD4 T cell help in B cell follicles, initiate germinal center reactions which produce both high-affinity memory B cells and LLPCs secreting high-affinity antibodies. These high-affinity antibodies are necessary to efficiently neutralize pathogens and toxins and for a persistent protection [271]. LLPCs migrate to the bone marrow where they can reside for extended periods maintaining these high affinity antibody levels in the serum and mucosa (reviewed in [272]).

CD4 T cells play an essential role in helping B cells mediated immune responses. Tfh has been shown to specialize in providing B cell help (Figure 9). Tfh, by expressing the CXCR5 chemokine receptor, relocate into B cell follicles and provide cognate help to B cells in GCs. These cells are initially activated by antigen presented on dendritic cells outside the follicle. Then, B cells migrate into the B cell follicles to form GCs, where they proliferate and undergo somatic hypermutation and affinity maturation [235, 240, 242, 273]. Of note, germinal centers develop after the activation of follicular B cells by TD antigens and consists of a specialized site within secondary lymphoid organs where antibody diversification and affinity maturation occur during an immune response to an infection[273].

In contrast with T cells, B cells can circulate in the blood but also can reside in tissues as specialized lymphoid tissues (*e.g.* spleen, lymph nodes, tonsils and bone marrow). Moreover, B cells are present in the mucosa-associated lymphoid tissue (MALT) in the genitourinary and gastrointestinal tracts where they may play a critical role in host defense from pathogens [274-276].

B cell dysfunctions have been associated with HIV-1 infection and they may occur at several different stages of B cell development resulting from HIV-induced immune

activation, HIV-induced lymphopenia (*i.e.* abnormal low level of lymphocytes in the blood) or other factors [277, 278]. As humoral responses become compromised by defects in B cell function, opportunistic infections may arise [278, 279].

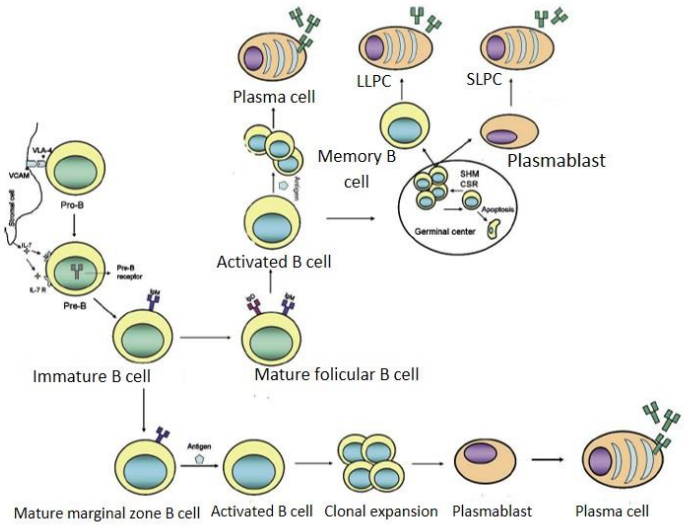

Figure10-Generation of plasma cells and memory B cells. Mature B cells become activated in secondary lymphoid tissue after interaction with antigen leading to the generation of plasma cells and long-lived memory cells. Antigen-specific naïve B cells can differentiate within secondary lymphoid tissue into short-lived low-affinity antibody secreting plasma cells or undergo GC reaction. B cells that survived the GC reaction proceeds to form either memory B cells or high-affinity antibody secreting plasma cells. Memory B cells are re-activated by specific antigen proliferating and differentiating into the long-lived high affinity antibody producing plasma cells. CSR, antibody class switch recombination; IL-7, interleukin 7 (hematopoietic growth factor); IL-7R, receptor pre IL-7; pre-B, precursor B cell; pro-B, progenitor B cell; SHM, somatic hypermutation; VCAM, vascular cell adhesion molecule; VLA-4, very late antigen 4

Features of B-cell dysregulation associated with HIV-1 infection include hypergammaglobulinemia, extensive expansion of B-cell areas in lymphoid tissues, increased expression of activation, proliferation and terminal differentiation markers on circulating B cell, decrease in memory B cell frequencies and expansion of aberrant B cell populations and B cell exhaustion [135, 136, 280-282]. Despite hypergammaglobulinemia (*i.e.* high levels of gamma globulin) described in HIV-1 infected patients, the frequency of HIV-specific B cells is quite low (<1.5%)[135, 136]. This is justified by the fact that plasmablasts, the terminally differentiating B cells responsible for the hypergammaglobulinemia condition, are maintained at abnormally high levels in HIV infection but the majority of these cells are not HIV specific[136]. Moreover, these unspecific plasmablasts are thought to arise early in the infection which may be associated with the inefficient antibody response in HIV-1 infection [136].Also, the delay in neutralizing antibody production following HIV-1 transmission may be

explained by HIV-1 associated damage in lymphoid tissues in gastrointestinal tract and the loss of $CD4^+$ T help in those sites. This is because the gastrointestinal tract $CD4^+$T cells are the main site for HIV-1 replication and the depletion of those cells occurs in the early stages of the disease [275, 276, 283].

3.2.2.2 Antibodies

Antibodies, also known as immunoglobulins, are large glycoproteins molecules found in the blood and tissue fluids[177]. They are presented on the surface of B cells acting like antigen receptors or can be secreted into the extracellular space where they can bind their antigen targets [266]. An antigen is the first element that drives all adaptive immune responses aiming to eliminate it. Antibodies bind to a restricted part of an antigen called epitope[177]. Antibody and cellular responses are generally directed against different determinants on the antigen.

An antibody consists of four protein chains, two heavy chains and two light chains that are bound by disulfide bounds (Figure 11). The antigen-binding fragment (Fab) is composed of one variable domain from each heavy and light chain and is involved in antigen binding. The constant domains of the heavy chains are less variable and are brought together and form the crystallizable fragment (Fc) which is responsible for the effector functions of the antibody through binding to Fc receptors (FcRs)[177, 266].The two heavy chains and the two light chains of an immunoglobulin molecule are identical and for that reason an antibody has two identical antigen binding sites being able to bind simultaneously to two identical structures[284]. Based in their Fc region, antibodies can be classified in five isotypes that are distinguished according to the C-terminus regions of heavy chain which do not participate in antigen binding: IgM, IgD, IgG, IgA, and IgE. IgG antibodies, the most abundant isotype in plasma and with longer lifetime, are subdivided in four isotypes: IgG1, IgG2, IgG3 and IgG4 [266]. Antigen specificity is determined by the antigen binding domain whereas effector functions are determined by antibody isotype and binding affinities of activating and inhibitory FcR on immune cells. Effector functions include: 1) neutralization of their targets (*e.g.* virus), 2) activation of macrophages and other immune cells by binding to Fc receptors that recognize the Fc

region of specific antibody isotypes (*e.g*. IgG1 and IgE) and 3) activation of the complement system (*e.g.* IgM and IgG3) [266, 285].

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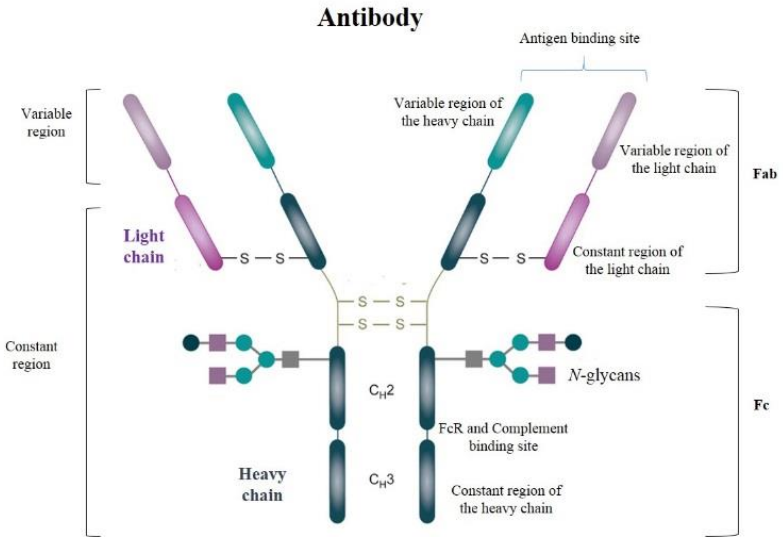

Figure 11- General structure of an immunoglobulin G1 (IgG1).The Fab portion comprise the antigen-binding region and include the light chain and part of the heavy chain. Fc consists in two identical CH2 and CH3 fragments derived from the constant domains of the heavy chains. Fc fragments do not recognize the corresponding antigen and instead bind to cell receptors and complement proteins. All antibodies have in the Fc region an N-glycosilation site N297 (adapted from [255].

Naïve B cells, *i.e.* before antigen exposure, express IgM and IgD on their surface. After antigen contact, $CD4^+$T cells induce B cells maturation causing isotype switch to IgG, IgA or IgE. Somatic hypermutation and subsequent clonal selection in the GCs under Tfh influence lead B cells to express high-affinity antibodies optimizing antibody-antigen affinity and selectivity. When isotypes switch random mutations are inserted in the antigen-binding coding sequences of the IgG and the higher affinity mutants to the antigen are selected [177, 218].

During acute and chronic stages of HIV-1 disease, antibodies against Env are mainly from type IgG1 with higher titers of these antibodies being associated with better control of viral replication [286, 287]. IgG3 is also detected in HIV-1 infected patients, namely in HIV-1 controllers, although to a lesser extent compared with IgG1 [286]. IgA is predominant in mucosal surfaces being associated with protection against HIV-1 infection in individuals who are highly sexually exposed to HIV-1 but remain uninfected [288, 289].

3.2.2.3 Neutralizing antibodies

The initial antibody response to HIV-1 can be detected as early as 1 week after infection and is directed to non-neutralizing epitopes in Env. The first antibodies are against gp41 region followed by the appearance of anti-gp120 antibodies a few weeks later and targeting the V3 loop [274, 290]. These binding antibodies mainly form immune complexes and don´t seem to have impact in viral replication suggesting that they are not responsible for the initial decline in plasma viral load [290]. However, although the first antibodies are not able to neutralize the virus, they may play a protective role in HIV-1 infection. For instance, a study in macaques has shown that Tat and Env binding antibodies confer some degree of protection against SHIV challenge despite the lack of neutralizing antibodies [291]. Moreover, the modest protection observed in RV144 (31.2%) trial may be in part due to binding antibodies that mediated antibody-dependent cell-mediated cytotoxicity (ADCC) and/or other non-neutralizing humoral effector functions [118, 131, 292]. Recently, a study performed in humanized mice using a replication-competent HIV-1 reporter virus expressing a heterologous HA-tag on the surface of infected cells and virions demonstrated that non-neutralizing antibodies can actually alter the course of HIV-1 infection, apply immune pressure on the infecting virus and also achieve modest levels of protection by a Fc receptor mediated mechanism. In this particular study, non-neutralizing antibodies were found to clear HIV-1 infected cells *in vivo* [293].

Neutralizing antibodies act by binding cell-free virus preventing the virion to infect the host cell [130]. However, it is still not clear (due to the variability in the experimental approaches) the relative activity of NAbs in cell-to-cell transmission, a major mechanism of HIV infection where HIV replicates more efficiently and rapidly through direct contact between cells[294, 295]. Overall, several studies have consistently demonstrated that NAbs are able to block HIV-1 infection at synapses, supporting the notion that cell-to-cell infection occurs through the direct transfer of virions accessible to the external environment. However, higher concentrations of specific NAbs (*e.g.* b12 and VRC01 against the CD4bs) are needed to inhibit cell-to-cell infection[296]. In addition, neutralizing polyclonal sera, which represent better the nature of the antibody response

during natural infection, is less effective in inhibiting cell-to-cell infection compared with cell-free infection, although it varies significantly according to the sera[294, 297].

The first NAbs, targeting the variable regions of the HIV-1 envelope, arise several months post-infection and, although often potent, are directed to the infecting strain (*i.e.* autologous virus) being unable to neutralize more divergent viruses (*i.e.* heterologous virus) [130, 132, 298-300]. Despite the high specificity of autologous NAbs, they exert a selective pressure on the virus that rapidly evolves to escape neutralization [130, 300]. Thus, it seems that NAbs appears sequentially and demonstrate temporal fluctuations consisting with the emerging of new escape variants [128, 300, 301]. An evidence that supports neutralization escape driven by autologous NAbs is the fact that contemporaneous viruses are less sensitive to autologous neutralization in comparison with earlier viruses[128]. Viral escape to avoid recognition is mediated by several features of Env that limit or block the access of antibodies to conserved neutralizing epitopes (Figure 12). Such features include carbohydrate shielding and shifting, conformation masking, steric occlusion, temporary epitope exposure (*e.g.* pre-fusion state form of gp41) and non-functional envelope spikes which are not expressed by mature functional spikes (*e.g.* gp120-gp41 monomers, gp41 stumps or uncleaved gp160 precursors) and may deviate the immune response from functional targets (reviewed in [298]). In fact, gp120 carries about 18 to 32 N-linked glycans which constitutes about 50% of the total molecular weight and function as a glycan armor avoiding antibody recognition[147, 302]. Also, the very limited number of gp160 glycoproteins per virion (between 21-42 SU molecules or 7-14 trimers per particle) likely reduce the ability of antibodies to bind simultaneously to two Env molecules (bivalent antibody binding) [303, 304]. Single amino acid substitutions, insertions and deletions in Env also contribute to viral escape [130, 300].

Despite of all these features some individuals are able to develop bNAbs targeting conserved functional sites on gp120 and gp41. In fact, during the course of infection, between 10-50% of HIV-1 infected individuals (depending on the definition of breadth and potency) develop broadly neutralizing antibody responses against diverse heterologous HIV-1 variants [305, 306]. Among them, few individuals (1%) called elite neutralizers develop highly potent and broad neutralizing responses.

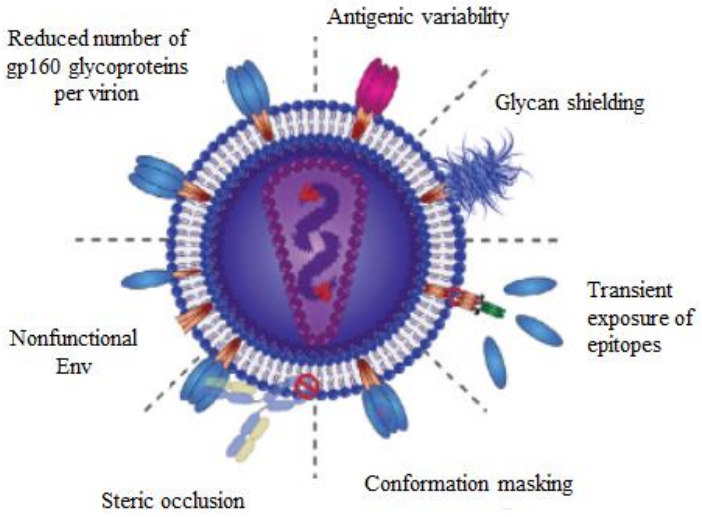

Figure 12- Envelope features defense mechanism against antibody recognition (adapted from [298]).

According to Simek *et al*, this elite activity is defined by the ability to neutralize, on average, more than one pseudovirus at an IC50 titer of 300 within a clade group and across at least four clade groups [307]. bNAbs generally arise only 2-4 years after seroconversion (in the chronic stage) and are absent in newly infected individuals suggesting that persistent infection and antigen exposure are required for their development [262, 308]. However, in rare cases bNAbs can arise 1 year following infection [309]. Development of bNAbs in HIV-1 infection is associated with the duration of infection, high viral load, low $CD4^+$ counts and a higher frequency of T cell help (Tfh) in the periphery [254, 305]. Also, the early preservation of both Tfh and B cells is associated with the later development of breadth of neutralizing antibody responses in chronic HIV-1 infection [310]. All of these factors are consistent with the requirement of high levels of antigenic stimulation of B cells and the T cell help in the maturation of breadth through the infection. Nonetheless, individuals with low viral loads can also develop bNAbs suggesting that high viral load alone is not a determinant of breadth [311, 312]. In addition, viral genetic subtype may also be a contributing factor as exemplified by the more potent and broad humoral responses observed in subtype C chronically infected individuals compared with subtype B infected individuals [313, 314]. Interestingly, infants who acquired HIV-1 *in utero*, during delivery, or via breastfeeding can also mount bNAb responses as early as 20 months after infection suggesting that the development of bNAbs is not an inherent trait of adults and that even in early life there is an adequate B cell-functionality able to develop bNAbs against HIV-1. However, in this

study, bNAbs epitopes were unclear with only a modest response observed in one infant against MPER in gp41[315].

Despite their protective role *in vitro*, bNAbs are unable to suppress viral replication in infected individuals due to viral escape [130, 300]. Demonstration that elicitation of bNAbs can be sufficient to prevent HIV-1 infection is now well documented based on passive immunization studies performed in animal models with human monoclonal antibodies (HuMAbs) with broadly neutralizing activity. In nonhuman primates protection was observed against viral challenge via intravenous, rectal and vaginal routes after passive immunization with broadly neutralizing monoclonal antibodies (*e.g.* F105, 2G12, 2F5, VRC01, PGT121, PGT126, b12, VRC07) [316-324]. Another study in macaques showed that the administration of PGT121 conferred sterilizing immunity against vaginal SHIV challenge at low concentrations [319]. Li and colleagues recently demonstrated that VRC01 was able to protect against HIV-1Ada vaginal and rectal challenge in hu-BLT mice (humanized mice) [318]. Also in humanized mice, passive administration of b12 and PGT126 provided sterilizing protection against repeated intravaginal HIV challenge [324].

3.2.2.4 Neutralizing epitopes in Env

Using novel antibody cloning techniques several potent and broadly neutralizing antibodies have been isolated from HIV-1 infected individuals in recent years. Mapping and structural definition of bNAb epitopes have allowed the identification of several sites of vulnerability on the HIV-1 envelope spike that are shared between subtypes and isolates. These conserved epitopes include: CD4 binding site (CD4bs), N-glycan-associated epitopes in the V1/V2 loops and V3 loop (N-glycans and tip of the loop), gp120/gp41 interface, the MPER regions and the fusion peptide in the N terminus of the Env-gp41 subunit [298, 325-337].

Recognition of neutralizing epitopes by multiple bNAbs is illustrated in figure 13.

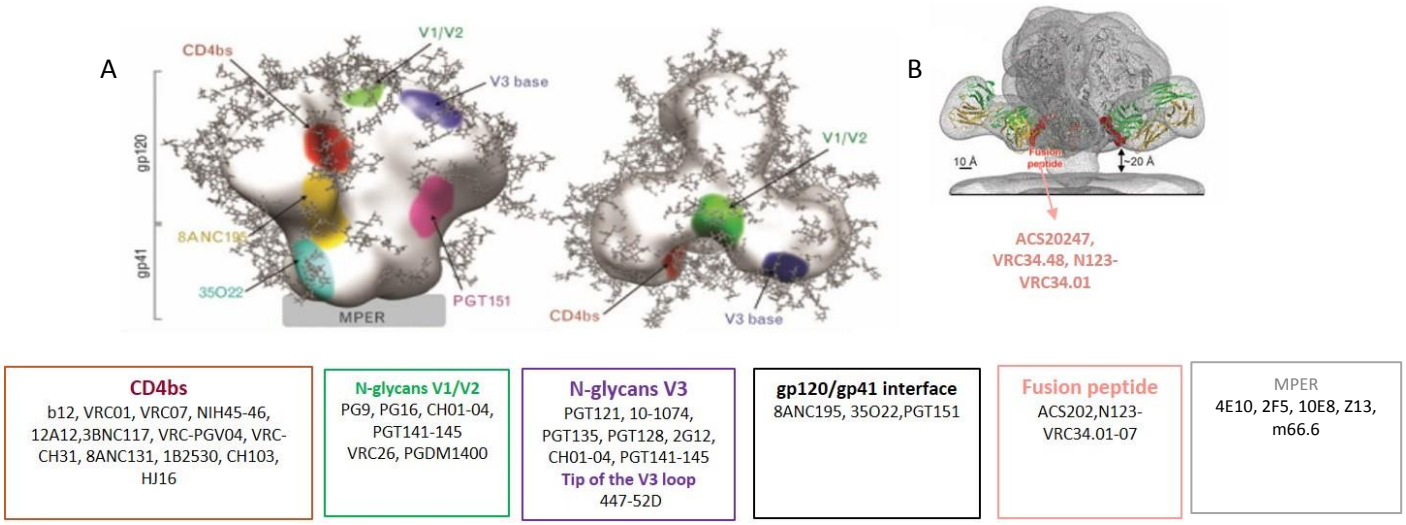

Figure 13- Representation of bNAbs epitopes on HIV-1 trimer. A-Env structure obtained by electron microscopy, structure EMD-5782; CD4 binding site epitope (red), N-glycans V1/V2 loops (green), N-glycans V3 loops (blue), gp120/gp41 interface (yellow, cyan and pink), MPER (grey); N-linked glycans regions are shown as grey sticks. For each epitope there is a set of representative bNAbs. B- Structure of the BG505 SOSIP.664-VRC34.01 complex representation the fusion peptide site (salmon); Heavy chains are colored in green and light chains in yellow. CD4bs-CD4 binding site; MPER- membrane proximal external region (adapted from [298, 337, 338].

V1/V2 region constitutes an important target for autologous NAbs and can serve both as a direct antibody target but also as a shield for other vulnerable sites due to its location and extensive glycosylation (Figure 14)[339-341]. Sequence conserved regions of the V1/V2 domain and V3 loop form the V2 site at the apex of HIV-1 trimer which is protected by densely packed glycans. Multiple bNAbs recognize epitopes that are only present in the trimeric envelope (located within the V2 domain at the trimer apex) meaning that these epitopes are conformation-dependent [342]. bNAbs recognizing these epitopes include PG9, PG16, PGT145, CAP256-VRC26 and PGDM1400. PG9 and PG16, which were isolated from a clade A chronically infected individual with exceptional plasma neutralization breadth, are quite frequent in sera from individuals who develop bNAbs[128, 153]. In fact, Burton and colleagues demonstrated that bNAbs present in the sera of 19 donors who exhibits a potent cross-clade neutralizing activity were directed against an epitope defined by PG9 and PG16 bNAbs[343]. With the exception of the CAP256-VRC26, all known bNAbs targeting the V1/V2 region interact with N156 and N160 glycans[150]. Moreover, these antibodies through its long CDR H3 (third heavy chain complementary determining regions) loop have the ability to penetrate the glycan shield and recognize a conserved β-strand in V2 [344, 345]. PGDM1400 is

one of the most potent bNAbs that has been isolated so far with cross-clade neutralization coverage of 83% [346].

V3 is a highly immunogenic region and anti-V3 antibodies have been found to neutralize up to 50% of the viruses in various multi-clade panels, including the broad and potent bNAb 447-52D [325, 326, 329, 330, 347, 348]. The cross-clade neutralizing activity of this particular bNAb is determined by the highly conserved motif GPGR (residues 312-315 in the HXB2) at the tip of the V3 loop. In fact, this antibody was able to neutralize 92% of 38 primary isolates carrying the motif GPGR from clades A, B, F or H. In contrast, none of the viruses with a GPGQ or other non-GPGR/Q motif was neutralized by this antibody[330]. More recently, maternal plasma antibodies targeting amino acid residues in the C-terminal region of the V3 loop crown were also associated with reduced peripartum HIV transmission risk in U.S. Woman and Infant Transmission Study (WITS) cohort suggesting the importance of this particular region in vaccine design [349, 350].

The target of the largest and most diverse group of bNAbs that recognize N-linked glycan containing epitopes is the region adjacent to the highly conserved N-linked glycosylation site at N332 which includes the V3 loop base and nearby glycans[351]. These bNAbs, in contrast with others, are able to approach the region centered on N332 from several different angles and include 2G12, PGT128, PGT121/10-1074 and PGT135[335, 352-354]. Whereas 2G12 recognize a cluster of high-mannose glycans attached to N332, N295 and N339, PGT128 recognize N-glycans attached to N332 and N301 in association with amino acid residues at the end of the V3 loop[351]. PGT121, PGT122 and 10-1074 are the most potent among the N332-dependent antibodies and target the glycan attached to N332 in combination with amino acid residues at the base of V3 loop and a glycan in V1/V2 regions[354]. Thus, despite the fact that the HIV-1 Env glycan shield blocks antibody access to potential epitopes it´s evident that some bNAbs overcame this obstacle by including the glycans as part of their target.

Because CD4bs is a highly conserved site present in all HIV-1 clades needed for the initial host-CD4 receptor interaction it represents one of the most attractive targets for vaccines that aim to induce neutralizing responses. NAbs against this conserved site act by blocking HIV-1 trimer binding to the CD4 receptor. In contrast with bNAbs described above, CD4bs antibodies don´t interact much with glycans. Although CD4bs is highly conserved it´s not an easy target due to the CD4-binding pocket size limitation[355].

Nevertheless, there are two distinct ways to produce antibodies that can reach this site. The first way is based on the mimetization of CD4 by positioning the antibody domain V_H to bind gp120 by using the CDRH2 loop and framework residues and engaging the CD4 binding loop on gp120 with backbone atoms in the C strand of V_H. Because the V_H domain occupies the same location as the terminal immunoglobulin-like domain of CD4 and both use the C strand these antibodies are CD4 mimetics.

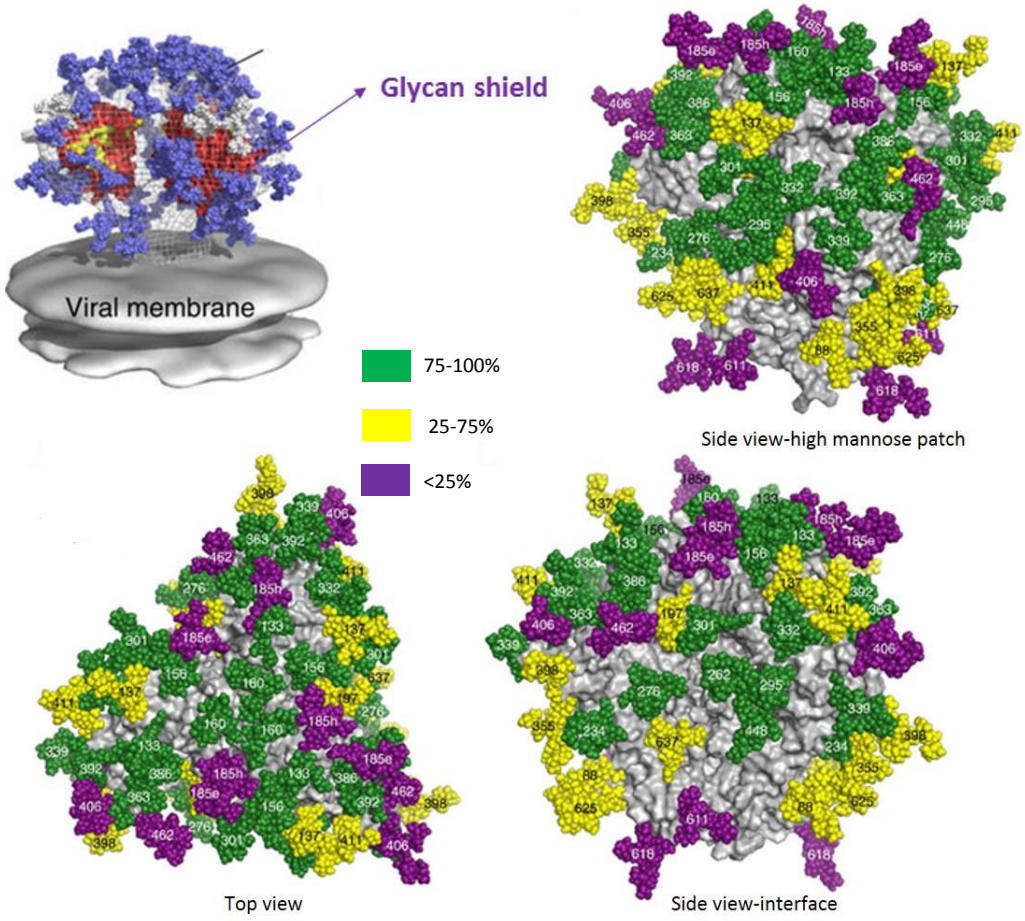

Figure 14- Site-specific glycosylation of the BG505 SOSIP.664 trimer. Trimer is shown as a grey surface and the glycans are shown as colored spheres according to the proportion (%) of oligomannose content (represented by green, yellow and blue colors) (adapted from [356]).

Within the groups that mimic CD4, bNAbs can derive from the IgH V_H1-2 germline gene segment (*e.g.* VRC01) or from V_H1-46 gene segment that have unusually short CDRL3 loops to avoid interactions with N276 glycan in the D loop of gp120 (*e.g.* 8ANC131) [357-360]. VRC01 is capable of neutralizing 91% of HIV-1 isolates and is also protective when used in a topical gel, protecting vaginally challenged humanized mice against HIV-1 infection [358, 361]. In the second way, the mode of recognition does not mimic CD4

but instead uses the CDR H3 loop region to reach CD4-binding loop (*e.g.* HJ16 and CH103) [325, 351, 362]. CH103 binds to the outer domain of gp120 by using a CDR H3 dominated mode of interaction and is able to bind its donor autologous transmitted-founder Env with high affinity (Figure 15) [362].

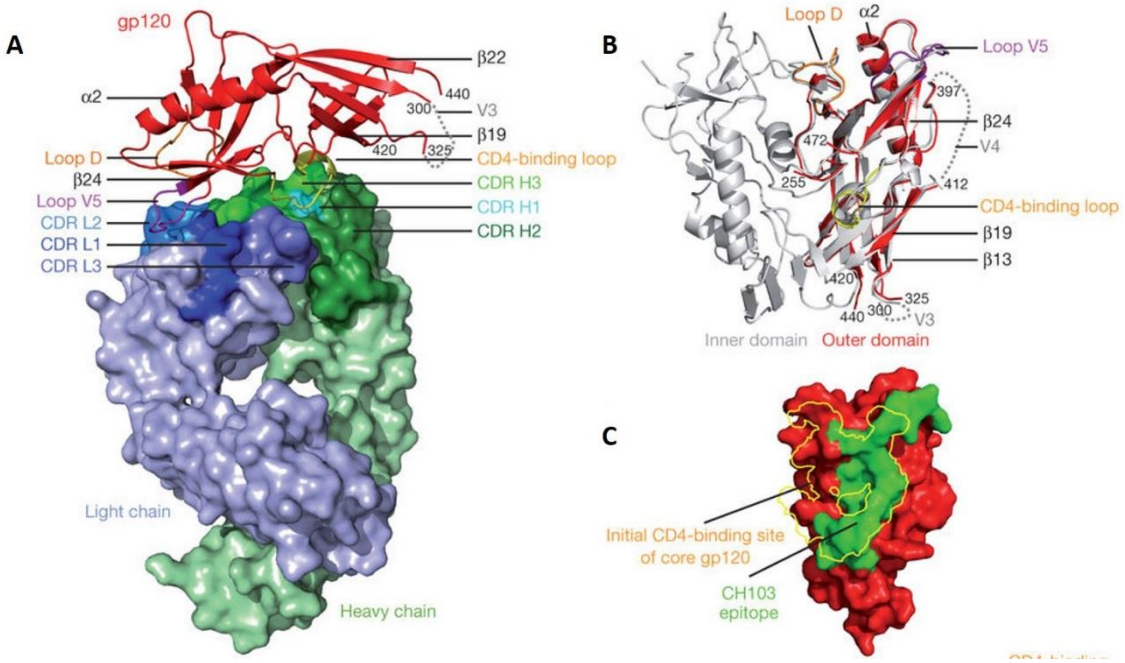

Figure 15- Structure of antibody CH103 in complex with the outer domain of gp120 demonstrating the non-mimetic mode of recognition of the CD4bs. A- overall structure of complex with gp120 polypeptide depicted in red ribbon and CH103 as a molecular surface (heavy chain in green and light chain in blue). CH103 bind to loop D (orange), CD4bs (yellow) and loop V5 (purple). B- Superposition of outer domain bound by CH103 (red) and core gp120 bound by VRC01 (gray). C- CH103 epitope in green on the outer domain (red) with the initial CD4bs superposed (yellow lines); (adapted from [362]).

gp120-gp41 interface was recently identified as a target for bNAbs that interact with both gp120 and gp41 subunits as well as glycans being for that reason trimer specific [363]. These antibodies probably neutralize the virus by stabilizing the pre-fusion state and/or by interfering with the conformational modifications necessary for fusion [364]. Examples of this class of bNAbs are PGT151-158, 35O22 and 8ANC195 [336, 364]. Of note, PGT151 and PGT152 don´t recognize uncleaved Env trimers being specific only for cleaved Envs which is in contrast with 8ANC195 that bind both to gp120 monomers and gp140 trimers [359, 364, 365]. PGT151 was able to neutralize 66% of 117 cross-clade isolates whereas 8ANC195, isolated from an HIV-1 elite controller, neutralized

57% of 118 isolates of different HIV-1 clades and 2/5 of VRC01-resistant isolates[359, 366].

Linear epitopes in the MPER of gp41, the region closer to the viral membrane, are also a target for several bNAbs like 4E10, 2F5, Z13, 10E8 and m66.6 [153, 325, 333, 334, 351].

This region contains conserved residues which have a critical role in the fusion process. Moreover, several factors like transient exposition, steric elements, hydrophobicity and host mimicry interfere negatively with the induction of bNAbs against this region [351]. Because 2F5 and 4E10 bNAbs recognize self-antigens, most autoreactive B cells are depleted which may justify the low frequency of this antibodies in infected patients and vaccines [367]. However, 4E10 possesses a potent and broad neutralizing activity with neutralization of more than 90% of different viral clades [153, 154, 325]. 2F5 also has neutralization activity although less potent and broad compared with 4E10 [153, 334].

Finally, the HIV-1 fusion peptide, comprising 15 to 20 hydrophobic residues at the N terminus of the Env-gp41 subunit, is a target for some bNAbs like N123-VRC34.01-07 and ACS202 [337, 368, 369]. VRC34.01 recognizes a unique trimer-specific cleavage-dependent epitope in the gp120-gp41 interface that consists of the gp41 fusion peptide and glycan N88. VRC34.01 was able to neutralize 16/25 pseudoviruses and 49% of 208 HIV-1 strains [337]. In addition, ACS202 was able to neutralize about 45% (34/75) of the viruses from all subtypes [368].

Because not all the bNAbs activities and specificities have been studied new important neutralizing epitopes on HIV-1 Env are yet to be discovered. Moreover, it's not completely understood if neutralization breadth is the result of a single or multiple specificities of bNAbs. Wu *et al.,* have demonstrated that individual antibodies targeting the CD4bs were sufficient to induce broad HIV-1 neutralization [358]. In contrast, another study has shown that B-cell memory response in a selected group of patients with broad serum neutralizing activity is comprised of multiple clonal responses against several epitopes on gp120 [370]. More recently, Moore *et al*. demonstrated that the potent and broad neutralizing response from sera of one participant in CAPRISA Acute infection cohort was due to the sequential and transient appearance of three distinct broadly neutralizing specificities in different Env epitopes during the first 4.5 years of infection[301]. Another interesting issue is whether escape from bNAbs occurs as fast as seen in autologous NAbs. Although this question remains undefined, it´s clear that the

generation of multiple bNAbs in response to a constantly evolving viral population that escape from earlier NAbs contribute to the exposition of new neutralizing targets[301].

bNAbs have acquired specific features through multiple rounds of affinity maturation that allowed them to overcome the Env defenses against antibody recognition: extensive hypermutation, long and hydrophobic CDR H3 loops, heavy chain domain-swapping, sulphated tyrosines and polireactivity (*i.e.* the ability to bind to a variety of structurally dissimilar antigens with moderate affinity) [128, 153, 371, 372]. Regarding hypermutation, HIV-1 bNAbs carry more than 40% amino acid substitutions in their heavy chains and nucleotide insertions and/or deletions in heavy and light chains[373]. In addition, the unusually long CDR H3 loops that characterize bNAbs are necessary to achieve a cryptic region of their epitope by penetrating the glycan shield or by extracting it from the membrane [351, 374]. All these features explain the difficulty in inducing bNAbs by immunization.

3.3 Search for an HIV-1 vaccine

3.3.1 Primer of vaccinology

Immunization is the process in which a person is made immune or resistant to an infectious disease, typically by the administration of a vaccine. Currently, vaccines are one of the most effective methods of controlling infectious disease. The benefits of successful vaccination strategies are clear, providing not only direct protective effects but also indirect effects among unvaccinated individuals who cannot be vaccinated[177, 375].

Immunization can be either passive or active. Passive immunity occurs with the transfer of antibodies to an unimmunized individual and can be classified as naturally or artificially acquired. Natural passive immunization is exemplified by the passage of maternal antibodies through the placenta to the fetus or through the colostrum or milk to the infant[376, 377]. Artificial passive immunization includes the administration of immunoglobulin preparations to susceptible individuals. Immunoglobulins currently approved for use in infectious disease prophylaxis and immunotherapy include Human Tetanus Immunoglobulin and Human Hepatitis B immunoglobulin [378-380]. Passive

immunization provides protection only for a short-period of time because the immune system is unable to develop memory [378].

Active immunization consists in the (natural or artificial) administration of an antigen in order to stimulate the host immune system to develop adaptive immunity against a certain pathogen and is based in two important features: specificity and memory[177]. Unlike passive immunity, active immunity usually lasts for many years or even for the whole life. Natural active immunization occurs with the exposure of an unimmunized individual to a pathogenic agent [377, 381]. In this case, the exposure to the pathogen induces clonal expansion of specific T and B cells and leaves a population of memory B cells that enable the induction of a rapid and effective secondary response upon further encounter with the same antigen[382]. Another way to produce active immunity is through antigen vaccination which produces an immune response similar to that produced by the natural infection but without the disease and related complications. Moreover, the majority of vaccines are able to produce immunological memory similar to that acquired from natural disease[377]. Immune response to vaccination may be influenced by several factors like maternal antibodies, nature and dose of antigen (more antigens lead to better response), route of administration and the presence of an adjuvant. Also, host factors as age, genetics and immunological status may affect the response to vaccination[383]. In addition, vaccine antigens must be safe, induce the right subset of immune responses and must be accessible by the target population. For many diseases this has been achieved with success but, for others like AIDS, there is still no effective vaccine available[384]. In this particular case, an ideal vaccine immunogen should be able to deal with the remarkably high diversity of the HIV-1 envelope and induce in the host immune system the capacity to cross-react with contemporaneous heterologous viruses.

Vaccines can be divided into two major types: live attenuated and killed/inactivated vaccines (Table I). Live attenuated vaccines contain usually attenuated viruses (whole virus) that are unable to cause disease when administered[385]. Attenuation may be achieved via the continuous passage of the virus through a series of cell cultures or animal embryos. The resulting attenuated virus loses its capacity to replicate in humans but is still recognized by the human immune system[386]. Vaccinia virus, the live replicative virus used for smallpox vaccination in humans, was naturally attenuated through its repeated passages in cows and other large animals (it causes Cowpox)[387]. Due to the high error rate of RNA viruses and selective pressures of the host environment, there is

some safety concerns regarding the fact that attenuated viruses can eventually revert back to wild-type virulence[388, 389]. However, this is very unlikely as the vaccine virus´s ability to replicate is limited. Killed or inactivated vaccines consist in the inactivation (destruction) of a pathogen (whole bacteria/virus or subunits) using heat or chemicals like formaldehyde or formalin[390]. Examples and the main characteristics of inactivated and live attenuated vaccines are described in table I.

Table I- Characteristics of inactivated and live attenuated vaccines

<table>
<tr><td rowspan="3">Vaccines</td><td colspan="2">Innactivated vacines</td><td colspan="2">Live Attenuated vacines</td></tr>
<tr><td>Type</td><td>Examples</td><td>Type</td><td>Examples</td></tr>
<tr><td>1.Whole organism</td><td>Polio and hepatitis A[377]</td><td>Whole organism[377]</td><td rowspan="2">measles, mumps, rubella, Vaccinia, varicella, zoster, yellow fever, rotavirus, intranasal influenza, oral Polio

BCG, oral typhoid</td></tr>
<tr><td>2.Fractional

-protein-based[391, 392]
-toxoid
- subunit

-polysaccharide based
[378, 391]
-pure
-conjugated</td><td><u>Toxoid</u>
diphteria and tetanus

<u>Subunit</u>
hepatitis B, influenza and human papilloma virus

<u>Pure polysaccharide</u>
pneumococcal disease, meningococcal disease and Salmonella Typhi

<u>Conjugated polysaccharide</u>[393, 394]
pneumococcal and meningococcal disease</td><td>1.Viral

2.Bacterial</td></tr>
<tr><td>Doses [378]</td><td colspan="2">Multiple doses</td><td colspan="2">One dose required</td></tr>
<tr><td>Stability</td><td colspan="2">More stable</td><td colspan="2">Less stable</td></tr>
<tr><td>Reversion to virulent form[391]</td><td colspan="2">No (safer)</td><td colspan="2">possible</td></tr>
<tr><td>Administered to immunodeficient individuals[395]</td><td colspan="2">yes</td><td colspan="2">no</td></tr>
<tr><td>Immune responses[378]</td><td colspan="2">Mostly humoral</td><td colspan="2">Humoral and cellular
(similar to natural infection)</td></tr>
</table>

BCG- Bacille Calmette-Guérin

Apart from the antigen, all vaccines may contain preservatives (*e.g.* thimerosal), stabilizers (*e.g.* gelatin, human serum albumin), residual substances derived from production processes (*e.g.* antibiotics, acidity regulators) and adjuvants[396]. Thimerosal

is a mercury-based preservative (ethylmercury) that has been used for decades in multi-dose vials of vaccines. Despite the good safety profile thimerosal has been removed from the majority of vaccines due to concerns related with the possible effects of pediatric exposure to mercury[397]. An adjuvant is a substance that is added to a vaccine in order to increase the adaptive immune response to a specific antigen[398]. Some vaccines containing attenuated or inactivated virus are able to stimulate an efficient immune response without the presence of the adjuvant. Examples of adjuvants used in licensed vaccines are described in Table II [399-403].

Table II- Characteristics of adjuvants used in licensed vaccines

Adjuvant	Composition	Immune effects	Vaccines
Aluminum	aluminium salts mixed with antigens	Increases local inflammation, antigen update by APCs and antibody production	Diphteria, tetanus, pertussis, inactivated poliomyelitis vaccine, hepatitis A and B, HPV, meningococcal and pneumococcal
Virosomes	Vesicles where antigens in aqueous volume are enclosed within a standard phospholipid cell membrane bilayer	Increases uptake by APCs and interaction with B cells leading to T-cell activation.	Hepatitis and influenza
AS04	3-deacyl-monophosphoryl lipid A) derived from lipopolysaccharide from *Salmonella Minnesota*, *Aluminum salts)*	Stimulates the TLR4 and increases Th1 responses	Hepatitis B, HPV
MF59	Squalene	Increases APC recruitment and activation. Promotes antigen uptake and migration of cells to lymph nodes.	Influenza-seasonal and pandemic
AS03	Vitamin E (α-Tocopherol); Surfactant polysorbate80; Squalene	Cytokines enhancement and recruitment of innate cells	Influenza-pandemic
ISA51	Mineral oil DRAKEOL 6VR Surfactant mannide-mono-oleate	Strongly immunogenic	therapeutic vaccine to non-small cell lung cancer (NSCLC)
GLA-AF	Glucopyranosyl Lipid Adjuvant (GLA) and synthetic surfactant DPPC (dipalmitoylphosphatidylcholine)	Stimulates TLR4	Pandemic influenza

APC- antigen presenting cells; TLRs-Toll-like receptors; HPV- Human Papilloma virus;

3.3.2 Recombinant vaccines

Genetic engineering technology has been used to produce several candidate antigens for vaccines [386]. These recombinant vaccines are based on the capacity of one or multiple

defined antigens to induce immunity against the pathogen when administered with adjuvants or when expressed by plasmids or bacterial and viral vectors. The development of efficient vaccines requires the combination of several strategies including different delivery systems and adjuvants in order to present the antigen in a way it can elicit an efficient immune response against these antigens [391].

There are several expression systems for antigenic protein components, such as bacteria, yeast, mammalian and insect cells where DNA encoding the antigen determinant can be inserted and further expressed [404-406]. Features as the level of expression obtained using each specific expression vector and promoter, the selection marker of choice and the presence or absence of post-translational modification by the recombinant vector must be taken into account in the choice of the system for antigen expression. Due to easy handling and high level of expression, bacterial expression systems are widely used. Mammalian or insect cells are also used but mainly for antigens in which post-translational modifications (*e.g.* glycosylation) are needed [391, 404].

Vaccine vectors can significantly influence the magnitude and breadth of vaccine-induced immune responses. In addition, the type of delivery vector in combination with the route of vaccine administration can determine the persistence of vaccine-specific immune responses in systemic and mucosal compartments [407]. Apart from the immune potential to induce specific, strong and persistent responses, other factors as pre-existing immunity must be taken into account. Pre-existing anti-vector immunity can prevent transduction and expression of vaccine genes leading to a reduced immunity. In addition, it can alter the natural course of infection leading to catastrophic consequences such as enhanced HIV acquisition and accelerated disease progression as was observed in an adenovirus-based HIV vaccine trial (Step study) [408]. Thus, the development of strategies that maximize vaccine immunogenicity and safety is an urgent priority especially for HIV/AIDS vaccine (reviewed in [407, 409]). Currently, recombinant vaccine strategies include recombinant protein vaccines (protein subunit), live recombinant bacterial or viral vector vaccines, DNA vaccines or the combination of all these in prime-boost strategies.

3.3.2.1 Recombinant protein vaccines

Recombinant protein vaccines, include highly purified recombinant proteins or subunits of pathogens [410, 411]. Currently, hepatitis B vaccine is an example of recombinant protein vaccine and is produced by expressing the hepatitis B surface antigen in yeast cells which assembles into virus-like particles (VLP) being extremely immunogenic[412]. Advantages of the yeast expression system includes the secretion of the antigen into the culture supernatant facilitating its purification. As eukaryotes, they are able to perform post-translational modification of proteins (e.g. glycosylation)[405]. Two human papilloma virus (HPV) vaccines (Gardasil® and Cervarix®) are also based on recombinant VLP technology expressing L1, the major protein capsid of the HPV virus, in yeast and insect cells, respectively[413]. Administration of L1-based VLPs allow the immune system to produce antibody titers 100 fold greater than those occurring in natural infection. Also, passive transfer studies using L1-VPL vaccinated animal serum into naïve animals confers protection against infection (reviewed in [414].

3.3.2.2 Live recombinant bacterial or viral vector vaccines

Live recombinant vectors are capable of delivering heterologous antigens by the introduction of antigen encoding genes. In addition, the capacity of infection and the immunological properties of the live vector can elicit an immune response against the heterologous proteins[407]. Live-attenuated bacterial vaccines are an attractive system for heterologous antigen presentation as they can elicit humoral and/or cellular responses, can be administered orally eliciting mucosal immunity, are antibiotic sensitive allowing antibiotic treatment if necessary and, finally, are of low cost. Examples of bacteria used as vectors include *Mycobacterium bovis* BCG , *Listeria monocytogenes* , *Salmonellae spp* and *Shigellae spp*[415]. Among these bacterial vectors, *M. bovis BCG* and *S. typhi* are the most common vectors used eliciting both humoral and cellular responses[391, 415-417]. Recombinant BCG expressing HIV antigens elicit specific antibodies, production of IFN-γ, T- helper cells and CTLs (reviewed in[407]).

Recombinant viral vectors are available for vaccine development and include Vaccinia (Poxvirus), modified Vaccinia virus Ankara (MVA), NYVAC (derived from the Copenhagen Vaccinia strain), adenovirus (Ad), adeno-associated virus (AAV), canarypox (ALVAC), fowlpox (TROVAC), Cytomegalovirus (CMV) and others[391]. Viral vectors can be either replicative (*e.g.* Vaccinia and CMV) or non-replicative/defective (*e.g*. Ad, MVA, NYVAC). Due to safety issues, replicative defective viruses are the most tested viral vectors in clinical trials and vaccine studies against HIV, TB and malaria [418, 419]. However, because replicative viruses are more effective in protection against infection as they elicit stronger humoral and cell-mediated immune responses, they have also been explored by some groups [387, 420-422].

Non-replicating recombinant viral vectors (adenoviruses, MVA, NYVAC and ALVAC)

- **Adenovirus vaccine vectors**

Adenoviruses are non-enveloped icosahedral viruses containing a linear double strand DNA being able to infect and replicate in different parts of the body, such as the respiratory tract and the bladder[391]. The stable non-replicative Ad serotype 5 (Ad5) is the most used as a delivery vector. Ad5 has the capacity to receive large segments of foreign DNA (8kb) in its genome and can be obtained easily and in high titers. Besides the induction of potent adaptive immune responses (e.g. effector memory $CD8^+$ T cell responses), adenoviruses also stimulate the innate immunity via inflammatory responses involving TLR2, TLR9, NOD-like receptors and the type 1 interferon pathways[423, 424]. On the other hand, Ad vectors can induce both systemic and mucosal immune responses[407]. Replicative competent Ad vectors are also being developed due to several features as its longer persistence in the host and the fact that lower doses are needed to induce immune responses. However, they have more limited clone capacity (3-4kb) compared with non-replicative Ad[421].

In the HIV field, Ad5 vectors expressing gag, pol, env and nef have been widely used showing promising results in diverse animal models. In NHPs, Ad5 expressing SIV Gag and Env proteins conferred protection against viral challenge with SHIV-89.6P [425]. In the STEP study, Ad5 was used as a booster immunization following DNA priming, inducing potent and strong $CD8^+$T cell responses in vaccinated individuals[408, 426]. However, pre-existing antibodies against Ad5 and cellular immunity may have

significantly compromised the clinical efficacy of this vaccine. In fact, an increase was observed in the rate of HIV infection in individuals with pre-existing immunity against Ad5 and this trial was interrupted. To overcome this problem, new immunization strategies with other adenovirus serotypes with lower pre-existing immunity (*e.g.* Ad26 and Ad35) and heterologous prime-boost regimens have been developed [427, 428].

- **Recombinant MVA and NYVAC vaccine vectors**

With the exception of Vaccinia virus, that has been used for almost two centuries providing cross-protection against smallpox, no other completely natural organism has ever been used in standard vaccination [387]. Besides their excellent safety profile, inherent adjuvant properties and easy manufacture, recombinant Vaccinia virus vectors have large genomes (~190 kb in length) making it possible to introduce large foreign genes and achieve their expression at high levels [429, 430]. In addition, poxvirus vectors can induce mucosal immune responses when administered by the mucosa route [431]. Unlike other DNA viruses, poxviruses have their own transcription machinery, viral DNA-dependent RNA polymerase and post-transcriptional modifying enzymes allowing a self-sufficient cytoplasmic replication in the host[432].

MVA is a highly attenuated strain of Vaccinia virus derived from Vaccinia strain Ankara. Attenuation of MVA was obtained through 500 passages in embryo fibroblast cells resulting in six major deletions corresponding to the loss of 10% of the whole genome. In addition, MVA does not replicate in humans [407, 433]. The potency of MVA to induce immune responses is largely due to the absence of genes that are involved in immune evasion (*i.e.* those who interfere with IFN-α, IFN-β, and TNF-α) which allows the generation of stronger innate immunity[434]. Recombinant MVA when used as a boost following DNA, fowlpox or Ad5 priming, has been shown to be highly immunogenic inducing strong and protective cellular and antibody responses against malaria antigens (reviewed in[407]). More recently, a heterologous prime-boost vaccination with DNA and MVA expressing HIV-1 mosaic Gag has shown to be highly immunogenic in mice inducing Gag $CD8^+$ and $CD4^+$ T cell responses[418].

Attenuated NYVAC vector is derived from a plaque-cloned isolate of the Copenhagen Vaccinia strain by the deletion of 18 genes involved in host range virulence. Like MVA, NYVAC cannot replicate in humans due to deletions in C7L and K1L genes[407]. In contrast to MVA, NYVAC induces mainly $CD4^+$T cell responses [435]. However, in a

recent study in chronically infected patients on ART, NYVAC-based vaccine expressing Gag/Pol/Nef/Env from an HIV-1 clade B isolate induced broad and polyfunctional $CD4^+$ and $CD8^+$ T cells[436]. When used in prime-boost combinations, NYVAC elicits better immune responses. Priming with DNA followed by NYVAC boost elicits broad and potent T cell responses in more than 90% of vaccinated individuals[437]. Thus, the potential of this vector to stimulate and boost potent immune responses should be taken into account when considering therapeutic HIV vaccines. In order to improve the immunogenicity of poxvirus vectors several strategies have been developed: heterologous prime-boost regimens, use of co-stimulatory molecules, deletion of viral immunomodulatory genes present in poxvirus genome, enhancement of virus promoter strength and vector replication capacity, optimization of the expression of foreign heterologous sequences, and the combined use of adjuvants(reviewed in[430]).

- **Canarypox (ALVAC) vaccine vectors**

ALVAC is a safe attenuated Canarypox virus obtained through the repeated passage in chick embryo fibroblasts replicating only in avian species[438]. In human cells, ALVAC replication is aborted early in the viral replication cycle prior to DNA synthesis[439]. Despite being less immunogenic compared with MVA and NYVAC, ALVAC has no potential pre-existing immunity in humans making it an attractive vaccine delivery vector. Currently, ALVAC is already licensed in veterinary use including feline leukemia virus (FeLV) and feline rabies vaccine (PUREVAX)[440]. Using ALVAC expressing SIV Gag/Pol/Env antigens protection against low-dose oral SIVmac251 challenge of neonate rhesus macaques was achieved [441]. The only HIV vaccine trial (RV144) that has demonstrated some degree of protection against HIV acquisition was based in a regimen including a priming with ALVAC-HIV(vCP1521) followed by a boost with (AIDSVAX B/E rgp120)[118]. Because the AIDSVAX vaccine (recombinant gp120 protein) alone showed no efficacy in earlier trials, the success of RV144 was in part associated with the immunostimulatory potential of canarypox virus vector and also to the prime-boost strategy. Thus, the combination of live viral vector-priming with protein-boost constitute a promising approach that should be optimized for increased efficacy. ALVAC is currently being used in the ongoing HIV vaccine trial HVTN 702 [119].

Live replicative viral vectors

Replicating vectors are able to induce persistent immune responses due to continuous antigen stimulation that may inhibit viral infection before the viral reservoir establishment and evolution of the initial infecting virus [426]. Although there are many advantages for using replicative-competent viral vectors in the vaccine field, there are several challenges concerning this issue. These includes the achievement of the adequate immunogen expression, engineering of genetically stable vectors, and more importantly, the achievement of the right balance between replicative capacity and attenuation ensuring both immunogenicity and safety[442]. Ad4 and Ad7, replicative competent adenoviruses replicating in mucosal tissues, have been successfully used for the prevention of respiratory and enteric illness[443]. In addition, Ad4 expressing HIV-1 clade C gp120, gp140 and gp120 elicited envelope-specific T cell in mice and antibody responses in rabbits. Moreover, serum from rabbits neutralized tier 1 clade C pseudovirus MW965.26 and the autologous and heterologous tier 2 pseudoviruses to a lesser extent[444]. In mice, replicative competent-vector NYVAC-C-KC induce better cellular and humoral immunity compared with non-replicative NYVAC [445]. Other vectors used in HIV vaccine development and that have shown strong immunogenicity include rhadinovirus, yellow fever virus, rabies virus and Semliki Forrest virus (reviewed in[407]). Also, Vaccinia virus has shown good results against HIV, hepatitis B virus and influenza virus in several animal models[446, 447]. A recent assay in rabbits using a prime with recombinant Vaccinia virus expressing full Env gp160 and subunit gp120 protein (gp120 monomer) from HIV-1 clade B was able to induce cross-reactive binding antibodies against V1/V2 fusion proteins, ADCC activity and neutralizing responses against heterologous tier 2 HIV-1 pseudoviruses (mostly from clade B) although with limited breadth and low titers (1:20-1:100) titers [422]. Also, oral immunization with recombinant Vaccinia virus (priming) expressing full-length HIV-1 SF162 Env or SIV Gag-Pol proteins followed by intramuscular protein boost with SF162 gp120 protein provided protection against intrarectal challenge with SHIV-SF162-P4 in macaques[448]. In addition, mice primed with Vaccinia virus expressing a truncated gp125 and boosted with recombinant polypeptides comprising the C2, V3, and C3 envelope regions developed a strong and broad neutralizing response against HIV-2 isolates[2]. Currently, several clinical trials using replicating viruses expressing HIV-1 proteins are underway. For instance, an oral replicating adenovirus (Ad26) vector expressing HIV-1 Env

(NCT02366013)[427] and a VSV vector (Vesicular stomatitis virus) expressing HIV-1 Gag (NCT01438606)[428] have recently completed phase 1 of clinical trials.

3.3.2.3 Recombinant DNA vaccine vectors

DNA vaccines consist in the direct injection of a naked DNA plasmid into the host with the capacity to induce an immune response and protection. Usually, the plasmid contains one origin of replication of *E.coli*, a strong promoter, multiple cloning sites and a selection marker (*e.g.* antibiotic) so that the antigen can be expressed directly by the host cells similar to a response to a natural infection. Advantages of DNA vaccines include stability, safety and easy production[407]. In addition, DNA plasmid vaccines can induce both T and B cell immune responses. Moreover, they can be used repetitively as a boost without immune interferences as is the case of pre-existing immunity[449]. DNA vaccines have been widely used to express antigens from influenza, HIV, malaria, TB and others [437, 450, 451]. However, they have been shown to be poorly immunogenic inducing sub-optimal immune responses. Improvements in DNA delivery have been performed in order to improve DNA vaccine immunogenicity. Such improvements include intramuscular, skin or intradermal electroporation and gene gun and biojector devices. In addition, the use of cytokines adjuvants (*e.g.* IL-2, IL-12, IL-15) have contributed to improve the immunogenicity of these vaccines[449]. Importantly, delivery of DNA vaccines by electroporation induced long-lived immune responses (cellular and humoral) without further heterologous boosting [452]. Despite the improvements in recent DNA vaccines, it is well established that they show much better immunogenicity when used as priming components in conjunction with viral vectors in heterologous prime boost regimens[419, 451-455].

3.3.2.4 Prime-boost strategies

Currently, vaccination with several doses (prime-boost regimen) is known to be the most effective way to achieve a potent and strong immune response. Prime-boost regimens can be autologous, when prime and boost vaccines consist of the same formulation or heterologous when immunization regime involves different formulations used

sequentially in more than one administration[391]. Several evidences demonstrate that heterologous prime-boost immunization can be more effective than autologous immunization, especially against intracellular pathogens[456]. In this case, the strategy involves the administration of the same antigens but formulated in different ways (*e.g.* purified proteins, live recombinant viral or bacterial vectors or DNA vaccines). The main objective of this approach is to combine both humoral and cellular immunity in an attempt to enhance the immune response against a specific antigen. Several studies have consistently demonstrated the potential of heterologous prime-boost strategies. For instance, prime-boost regimens comprising Ad and MVA have recently been shown to induce both cellular and humoral responses against SIV and malaria antigens [427, 457, 458]. Barouch *et al* have demonstrated protection from both acquisition and disease progression by the induction of systemic and mucosal immune responses when using Adenovirus/poxvirus and adenovirus/adenovirus-vector-based vaccines expressing SIV(SME543) Gag, Pol and Env antigens[457]. Moreover, another study including three doses of plasmid DNA followed by Ad5 delivery of immunogens comprising SIV-Gag, SIV-Env mosaic immunogens or SIVmac239 Env led to the induction of cellular and antibody responses (bNAbs and ADCC) that were associated with protection against intra-rectal challenge of SIV_{smE660}[459].

Distinct mechanisms can be attributed to the success of prime-boost regimens. First, the use of different immunization strategies reduces anti-vector immunity. Second, and most important, the induction of high-avidity T cell responses[460]. In fact, during priming immunization, T cells against the most immunodominant epitopes of the antigen are induced. Then, heterologous boosting, which shares the relevant antigen with the priming, will induce immune responses that focus on the expansion of the immunodominant T cells that were previously induced by the priming. This is particular true when live recombinant vectors such as MVA and Ad5 are used because they are especially efficient in boosting pre-existing memory immune responses [391].

3.3.3 HIV-1 completed efficacy trials

Almost four decades have passed since the discovery of HIV-1 and there is still no vaccine available. The development of an HIV-1 vaccine faces several obstacles: 1) the

extraordinary genetic diversity of the virus and the high mutational rate, 2) the early destruction of $CD4^+$ T cells, 3) the early establishment of latent viral reservoirs, 4) the short timeframe of opportunity for the host immune system to eradicate the initial viral infection, 5) the unclear immune correlates of protection, 6) safety concerns regarding the use of attenuated viruses in humans, and finally 7) the limited exposure of neutralizing epitopes in the outer surface of the virus and also the difficulty in exposing these epitopes in vaccine candidates [461-463]. On the other hand, the fact that there has been only one reported case of effective eradication of the virus (*i.e*. the Berlin patient) makes the development of an HIV-1 vaccine an even greater challenge[464]. Despite the urgent need for a vaccine, only six HIV-1 vaccine candidates have completed efficacy trials (Table III).

Table III- Completed HIV-1 vaccine efficacy trials in humans.

Trial	Vaccine immunogens	Immune responses observed	Main Results / observations
VAX003	AIDSVAX B/E gp120 in alum	Non-neutralizing responses No NAbs were induced	No efficacy
VAX004	AIDSVAX B/B gp120 in alum	Non-neutralizing antibody responses ADCVI No NAbs were induced	No efficacy
HVTN 502/ Step trial	Ad5 clade B expressing gag/pol/nef	HIV-1 specific $CD4^+$ and $CD8^+$ T cell responses	No efficacy Increased infection risk in Ad5 seropositive individuals
HVTN 503 (Phambili trial)	Ad5 clade B expressing gag/pol/nef	HIV-1 specific $CD4^+$ and $CD8^+$ T cell responses	HIV-1 specific $CD4^+$ and $CD8^+$ T cell responses
RV144	ALVAC-HIV (recombinant canarypox vector)/vCP1521 expressing env/gag/pro + AIDSVAX B/E rgp120 boost	non-neutralizing antibodies to variable loops (V1V2), high levels of ADCC and HIV-1 Env specific IgG3 responses;low titers of NAbs against tier 1 viruses	31.2% efficacy at 42 months 60% efficacy at 12 months
HVTN 505	DNA Gag, Pol, and Nef (clade B) and Env (clades A, B and C) + rAd5 subtype B gag/pol/env (clades A,, B and C)	Cellular and immune responses T- cell responses to HIV-1 potential T-cell epitopes;$CD4^+$ HIV gag responses	No efficacy
HVTN 702 (phase 2b/3)	ALVAC-HIV (vCP2438) expressing env /gag/pro + Bivalent Subtype C gp120	first results expected in 2020	Not applicable

ADCVI- antibody-dependent cell-mediated virus inhibition; ADCC-antibody-dependent cellular cytotoxicity

The first trials VAX003 and VAX004 used Env subunit gp120 proteins derived from subtypes B and E adjuvanted in alum that were administered intramuscularly at months 0,1, 6, 12, 18, 24 and 30 with a final follow-up at month 36. Despite the induction of binding antibodies, no NAbs were induced and no significant protection efficacy was achieved [465-468]. Taking into account the crucial role of $CD8^+$ T cells in controlling

HIV replication a new study was designed to promote cellular immune responses. The Step study (HVTN 502) used an Ad5 viral vector expressing HIV-1 gag, pol and nef from clade B administered at day 1, week 4 and week 26. However, despite the induction of specific cellular responses in the first stages of the trial (HIV-1 specific $CD4^+$ and $CD8^+$ T cell responses) no efficacy and/or virological control was observed. In addition, increased rates of infection were observed in individuals who were Ad5 seropositive as described above [408, 469]. A possible explanation for this may be the fact that the simultaneous presence of antibodies anti-Ad5 and the virus could have led to the activation of T cells which in turn favored HIV replication and decreased the response capacity of these cells. The Phambili trial (HVTN 503) used the same strategy as the Step trial and the same results were obtained. The HVTN 505 trial tested a multi-clade DNA prime (clades A, B and C) and a recombinant Ad5 boost in order to generate both cellular and humoral responses but with no success. Finally, RV144 trial conducted in Thailand from 2003 to 2006 was the only immunization strategy that demonstrated some degree of protection [118, 468]. This trial was based on clade B and CRF01_AE sequences that matched the prevailing HIV-1 strains in Thailand and consisted of four priming injections of an attenuated recombinant canarypox vector vaccine (ALVAC/vCP1521) expressing *env*, *gag* and *protease* genes with combination of two booster injections of a recombinant glycoprotein 120 subunit vaccine (AIDSVAX B/E)[118, 468]. Immune responses observed in RV144 associated with a reduced risk of HIV-1 infection included non-neutralizing antibodies to V1/V2, high levels of ADCC and HIV-1 Env specific IgG3 responses[118, 468, 470]. Although all RV144 recipients produced binding antibodies to gp120 they developed low titers of neutralizing antibodies that were mainly against tier 1 isolates which may explain the modest efficacy of this trial [131]. Based on the immune correlated of protection observed in RV144, there are several new HIV-1 vaccine trials ongoing. HVTN 702, the most recent phase 2b/3 trial, involves a new version of the immunogens and strategy used in RV144 but with some modifications [471]. Vaccine regimen consists of two immunizations of ALVAC-HIV (vCP2438) plus three immunizations with Bivalent Subtype C gp120. This trial started at November 2016 and will evaluate the preventive vaccine efficacy, safety and tolerability in 5400 HIV-seronegative South African adults with the first results expected in 2020 [472].

3.3.4 Vaccination strategies to induce bNAbs

Because broadly neutralizing antibodies are considered the best correlate of antibody protection against HIV infection, the development of immunogens that induce bNAbs is currently the main priority for the HIV-1 vaccine field [131, 319, 320, 422, 473-475]. So far, generation of immunogens that can elicit bNAbs is one of the unachieved goals in HIV-1 vaccine development and until now no vaccine candidate has been able to induce consistent bNAbs against heterologous tier 2 virus from different clades [422, 453, 476-479]. There are several strategies to elicit or deliver bNAbs by immunization which include immunogen design in order to mimic bNAb epitopes, immunogens that bind germline precursors of bNAbs (B-cell lineage vaccines), replicating viral vectors expressing Env antigens, DNA-based vaccinations, gene transfer of bNAbs (vectored immunoprophylaxis) and passive administration of bNAbs [375, 407, 480, 481].

Immunogen design to mimic Env epitopes include gp120 monomer-lineages, non-native gp140 proteins, gp120 core proteins, gp120 OD (outer domain) define proteins, epitope-specific scaffolds, epitope-based peptides and native-like Env trimers [482]. gp120 monomers are thought to induce limited bNAb responses against HIV-1compared with trimers due to specific features like the exposure of non-neutralizing epitopes and the lack of NAb epitopes that are only present in the quaternary structure or in the presence of gp41[483, 484].This hypothesis is based on the idea that although the presence of a bNAb epitope on an Env immunogen does not necessary means that bNAbs will be raised against it, the absence of an epitope or its presence in a conformational innapropriate form will probably reduce the chances of an efficient bNAb induction[484]. However, several evidences have demonstrated that HIV-1 envelope trimers and monomers induce comparable immune responses with oligomers showing only marginal improvements over monomers [342, 485, 486]. Env-based immunogens, including gp120 monomers and gp140 trimers or oligomers, have been widely used in animal models (and in RV144 human trial) inducing similar levels of neutralizing responses [152, 422, 487-490].

A successful HIV-1 vaccine should take into account the native trimer, the interaction of bNAbs with the trimer and the evolution of bNAbs and the Env trimer in natural infection[372]. For that propose, a new generation native-like Env trimeric proteins such as the soluble cleaved BG505 SOSIP.664 gp140 have been developed. This trimer is relatively stable, similar to the native virus spikes and expresses multiple epitopes for

bNAbs. However, despite the attempts to improve their stability, significant challenges remain. Although trimers can be relatively stable in solution they adopt different conformational states including those who are less relevant to induce bNAbs. Moreover, trimers expose irrelevant immunodominant regions that may distract the immune response from the conserved neutralization epitopes [491]. The majority of studies using HIV-1 trimers alone have failed in inducing consistent broad and potent neutralizing responses against heterologous tier 2 HIV-1 viruses. [478-480, 492]. An exception came very recently when four cows immunized with a single trimer immunogen (BG505 SOSIP) developed an extraordinary broad and potent serum antibody response against 117 cross-clade isolates [1]. However, with the exception of this study, Env monomers and trimers have been shown to be much more effective at inducing broad and potent neutralizing responses when administered in combination with other immunogens in prime-boost strategies. The majority of HIV vaccine regimens aiming to induce bNAbs consists in prime-boost regimens that use combinations of different immunogens, namely, recombinant virus expressing HIV envelope [2, 422, 493], and/or plasmid DNA constructs[454], and/or various HIV purified proteins (*e.g.* gp120 monomers and trimers) [342, 422, 453, 478, 479, 494]. As mentioned before, recent studies have shown that is possible to induce NAbs against tier 2 viruses although sporadically, with limited breadth and at low levels [422, 453, 476-479]. Table III includes a compilation of some of the prime-boost HIV vaccine-regimens performed in small animal models aiming to induce NAbs.

In conclusion, despite the role of antibodies in HIV protection is not completely clear it is widely accepted that anti-Env antibodies should be a critical element in a preventive vaccine for HIV-1. Therefore, it is expected that the achievements provided by all of the previous and ongoing vaccine regimens aimed to induce tier 2 neutralizing responses, either in humans or non-human animal models, will lead to an optimization and improvement of the ongoing strategies providing crucial insights for the future development of a neutralizing antibody vaccine.

Table III- Compilation of several prime-boost HIV vaccine-regimens performed in small animal models aiming to induce NAbs**.**

Authors	Immunogens	Immunization schedule	Main Results
Quinnan *et al.*, 2006[495]	**$gp140_{R2}$** (soluble, oligomeric form of HIV-1 *Env*) **$gp120_{R2}$** (surface component of HIV-1 *Env*)	4 immunizations of 30µg of each protein per group Days 1, 21, 42, 196	$gp140_{R2}$ immunogen induced antibodies that achieved 50% neutralization of 48/48 and 80% neutralization of 43/46 primary strains of diverse subtypes;$gp120_{R2}$ induced antibodies that neutralized 9/48 of the same strains
Klasse *et al.*, 2016[479]	SOSIP.664 trimers based (clade A,B, C and D)	Various trimers delivered simultaneous (clade A+B) at days 1, 28, 140, 168 or sequentially at days 252, 336, 420, 511 30 µg/dose for all groups except one that received 90 µg/dose	-Autologous, tier-2 NAbs -Heterologous tier-2 NAb responses albeit inconsistently and with limited overall breadth.
Moore *et al.*, 2015[478]	BG505 SOSIP.664 gp140 BG505 gp120 YU2gp140-Fd B41SOSIP.664gp140 B41SOSIP.664-D7324gp140	Days 1, 30, 140, 280; 30 µg/dose Days 1, 30, 168; 40 µg/dose Days 1, 30, 140; 30 µg/dose	-BG505SOSIP.664 induced NAbs potently against the sequence-matched tier-2 virus -Tier 1 cross-reactive NAbs -B41SOSIP.664 induced a strong autologous tier-2 NAb response
Sanders *et al.*, 2015[476]	AMC008 SOSIP.664 trimers	22 µg of trimers at days 1, 30 and 140; NAb response assessment at week 22.	-Autologous Tier-1B or Tier-2 NAbs Low levels of V3-directed Tier-1A NAbs.
Kong *et al.*, 2016[453]	Prime/boost immunization strategy gp120 DNA and various V1V2-scaffold proteins.	DNA prime at days 1, 15 (or 56), 30 and 2 protein boosts at days 70 and 98 (or 126)	-Cross clade anti-V1V2 antibodies remain detectable ≥1 year after the last immunization dose
Shiu-Lok Hu *et al.*, 2016[422]	Poxvirus prime-gp120 boost strategy recombinant Vaccinia virus expressing full Env gp160 and subunit gp120 protein	Priming at days 1 and 56 with 10^8 PFU of rVV and boosting at days 245 and 336 with the cognate gp120	-Induction of cross-reactive NAbs >50% of tier 2 global HIV-1 isolates V1/V2 directed antibodies -ADCC.
Whalen *et al.*, 2013[454]	DNA-prime protein-boost regimen with the gp120 subunit of HIV-1 JR-CSF envelope (Env)	Priming with 400 µg plasmid DNA construct JR-CSFgp120 DNA and boosting with JR-CSFgp120 purified protein;	Strong autologous neutralization of the primary isolate JR-CSF

ADCC- Antibody-dependent cell-mediated cytotoxicity; NAb- Neutralizing antibody; rVV- Recombinant Vaccinia virus

Aims and work plan

Preventing altogether new HIV-1 infections will require the development of an effective vaccine. To this end, new immunogens that elicit the production of broadly neutralizing antibodies are needed. To date, prime- boost approach used in RV144 trial is still the only immunization strategy that has demonstrated some level of protection against HIV-1 [118, 468]. However, with the exception of the recent results in cows, no vaccine candidate has been able to induce potent bNAbs against heterologous tier 2 virus from different clades [1, 478, 479].

Recently, work done in our lab has demonstrated that mice primed with Vaccinia virus expressing a truncated gp125 and boosted with recombinant polypeptides comprising the C2, V3, and C3 envelope regions developed a strong and broad neutralizing response against HIV-2 isolates[2]. The main aim of this thesis was to determine if a similar vaccine strategy would elicit the production of bNAbs against HIV-1 leading to a new type of HIV-1 vaccine.

Several approaches have been adopted to produce the best HIV-1 envelope vaccine candidate including the production of consensus envelopes and chimeric envelopes [118, 418, 422, 448, 496-501]. For our vaccine immunogens it was decided to use envelope glycoproteins from isolates of all clades representative of an "old" and stable epidemic such as the one ongoing in Angola. Our hypothesis was that ancestral R5 viruses would be better at eliciting bNAbs against highly heterologous viruses as they correspond to an ancestor of all circulating HIV strains thus encompassing key epitopes and conformational determinants that are conserved within the contemporaneous strains. In addition, the use of R5 isolates is adequate for vaccination because almost all transmitted isolates use CCR5 and a successful vaccine should elicit antibodies that neutralize the isolates that are most likely transmitted.

Specific objectives of this thesis were: 1) characterize HIV-1 samples derived from HIV-1 isolates from Angola (1993) as well as their *env* genes as a paradigm of the ancestral viruses we intended to use in our vaccine; 2) express envelope genes from Angolan and Portuguese isolates in Vaccinia virus 3) produce cognate gp120 and recombinant polypeptides encompassing the V3 region and flanking C2 and C3 sequences 4) immunize BALB/c mice and rabbits with these envelope constructs using different prime-

boost regimens; 5) characterize the binding and neutralizing antibody responses in these animals; 6) characterize the Tfh and Tfr cell responses in immunized mice.

Angola had a crucial role in the early dissemination of the HIV-1 epidemic; still, at the time there was no genomic data from HIV-1 infected patients from Angola. Currently, there are only three full-length genomes of subtype J and four of subtype H available in the Los Alamos sequence database. We therefore decided to sequence the full-length genomes from three Angolan patients (obtained in 1993) in order to better understand the origin of HIV-1 subtypes and recombinant forms (chapter 2). Maximum likelihood phylogenetic tree inference and analyses of potential recombination patterns were performed to evaluate the sequence classifications and origins.

In chapter 3, a total of seven HIV-1 strains obtained from HIV-1 infected individuals from Portugal and Angola were included in the study in order to account for the extensive genetic diversity of HIV-1. For all samples, the full-length envelope genes were amplified, sequenced and subtyped by phylogenetic analysis. Co-receptor usage of HIV-1 isolates was determined *in vitro* in TZM-*bl* cells ($CD4^+$, $CCR5^+$, and $CXCR4^+$) and with the geno-2-pheno software. To produce the truncated gp120 (gp120t), a stop codon was inserted at the end of the reverse primer resulting in the amplification of the gp120 region lacking 78 bases at the carboxyl terminus of the C5 region. The use of a truncated protein instead of a full protein has been previously associated with better immunogenicity and more broad and potent neutralizing responses against HIV-2 [2].

Recombinant Vaccinia viruses expressing the truncated surface gp120 glycoprotein from R5 isolates of HIV-1 subtypes B, C, CRF02_AG and J were successfully produced and selected to be used as vaccine immunogens. The antigenicity of truncated gp120 proteins was analyzed by Western Blot and Enzyme-Linked Immunosorbent Assay (ELISA) assays using sera from HIV-1 infected individuals and HuMAbs directed against gp120. We additionally produced, purified and characterized recombinant polypeptides comprising the C2, V3 and C3 envelope regions of all HIV-1 subtypes. Antigenic reactivity of the purified polypeptides was analyzed by Western blot with plasma from HIV-1 infected patients from Angola and quantified in an ELISA assay.

The combined immunogenicity of the new recombinant Vaccinia viruses and envelope proteins was determined in BALB/c mice (chapter 3) and New Zealand white rabbits (chapter 4).

First, a pilot study was performed in BALB/c mice using the immunization strategy that was proven effective in HIV-2 vaccination [2]. In this study, animals were immunized with immunogens derived from HIV-1 clades C, CRF02_AG and J. In order to check for potent cross-reactive binding antibodies against autologous and heterologous gp120, serum antibody responses from all timepoints were analyzed using an ELISA assay. Moreover, in order to detect neutralizing antibodies in mice, we performed neutralization assays against a panel of 16 HIV-1 viruses including 11 tier 2 HIV-1 [502], 3 HIV-1 primary isolates (2 subtypes J and 1 CRF02_AG in *Env*) and 2 tier 1 HIV-1. Neutralizing activity of mice serum was tested using a single-round viral infectivity assay using a luciferase reporter gene assay in TZM-*bl* cells.

In order to test new immunization strategies and immunogens, a second study was performed in BALB/c mice in a different facility. In this study we tested three different prime-boost strategies with immunogens derived from HIV-1 clades B, C and CRF02_AG. Binding and neutralizing responses were assessed as described previously.

Finally, a third vaccination assay (chapter 4) was performed in New Zealand White rabbits. The aim of this work was to investigate if the new prototype vaccine also induced heterologous neutralizing antibodies in a larger animal that is commonly used in HIV vaccine development studies [479, 494, 503]. Binding antibody responses against autologous and heterologous gp120 and gp140 proteins were determined in an ELISA assay using rabbit sera from different timepoints. Neutralization activity (Ab titer) was determined against a panel of 18 HIV-1 viruses, including tier 1 and tier 2 viruses.

Tfh cells, a recently characterized subset of helper T cells, have been described to be essential for antibody class switch and affinity maturation, an important process during the production of NAbs [218, 235-238]. Furthermore, Tfr cells, seem to moderate the role of Tfh cells in driving antibody production by B cells [258-260]. In order to investigate and better understand the role of Tfh and Tfr cells involved in NAbs induction we took advantage of spleens from a subset of immunized mice and analyzed the levels of Tfh ($CD4^+$ $CXCR5^+$ PD^-1^+) and Tfrs ($CD4^+$ $FOXP3^+$ $CXCR5^{hi}$ $PD\text{-}1^{hi}$ $CD25^{low}$ T-cell population) cells by flow cytometry. Moreover, correlations between Tfh and Tfr

numbers and binding and neutralizing response were studied in order to evaluate if this vaccine strategy was able to target the right cellular subsets behind the induction of an effective NAb response.

Chapter 2

Rare HIV-1 subtype J genomes and a new H/U/CRF02_AG recombinant genome suggests an ancient origin of HIV-1 in Angola

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Abstract

Angola has an extremely diverse HIV-1 epidemic fueled in part by the frequent interchange of people with the Democratic Republic of Congo (RDC) and Republic of Congo (RC). Characterization of HIV-1 strains circulating in Angola should help to better understand the origin of HIV-1 subtypes and recombinant forms and their transmission dynamics. In this study we characterize the first near full-length HIV-1 genomic sequences from HIV-1 infected individuals from Angola.

Samples were obtained in 1993 from three HIV-1 infected patients living in Cabinda, Angola. Near full-length genomic sequences were obtained from virus isolates. Maximum likelihood phylogenetic tree inference and analyses of potential recombination patterns were performed to evaluate the sequence classifications and origins. Phylogenetic and recombination analyses revealed that one virus was a pure subtype J, another mostly subtype J with a small uncertain region, and the final virus was classified as an H/U/CRF02_AG recombinant. Consistent with their epidemiological data, the subtype J sequences were more closely related to each other than to other J sequences previously published. Based on the *env* gene, taxa from Angola occur throughout the global subtype J phylogeny.

HIV-1 subtypes J and H are present in Angola at low levels since at least 1993. Low transmission efficiency and/or high recombination potential may explain their limited epidemic success in Angola and worldwide. The high diversity of rare subtypes in Angola suggests that Angola was part of the early establishment of the HIV-1 pandemic.

According to the World Health organization (WHO) there are currently about 35 million people living with HIV/AIDS worldwide and it is estimated that approximately 39 million people have died of AIDS since 1981 [504]. HIV can be divided into two types: HIV-1, which is responsible for the worldwide AIDS pandemic, and HIV-2, which is less prevalent and virulent [55]. Based on extreme genetic diversity, HIV-1 variants can be classified into four major groups: M (main), O (outlier), N (non-M, non-O), and more recently P [505]. The M group, which is responsible for the pandemic, includes nine different subtypes (A-D, F-H, J and K), more than 70 circulating recombinant forms (CRFs), and many unique recombinant forms (URFs) [506]. Groups O, N and P have infected a relatively small number of patients worldwide [35].

In sub-Saharan Africa almost all subtypes, CRFs, and URFs circulate; the highest genetic diversity has been observed in West Central Africa where the HIV-1 pandemic is believed to have originated [35, 49, 507]. On a global perspective, the most prevalent HIV-1 subtypes are C (50%), A (12%), B (11%), followed by CRF02_AG (8%), G (5%), CRF01_AE (5%) and D (2%). The remaining subtypes and recombinant strains represent less than 1% of HIV-1 infections [35, 507, 508]. Currently there are only three full-length genomes of subtype J and four of subtype H available in the Los Alamos HIV sequence database [506]. Full-length subtype J genomes are from Sweden and Cameroon (GenBank accession numbers AF082394, AF082395, GU237072) whereas subtype H genomes are from Belgium, United Kingdom and Central African Republic (AF190127, AF190128, FJ711703, AF005496). In addition, subtype J appears as fragments in several CRFs and shorter sequences [506].

Angola is a South-western African country surrounded by Namibia, Zambia, Democratic Republic of Congo (DRC), and Republic of Congo (RC). According to the UNAIDS report, HIV/AIDS prevalence among adults in Angola was 2.4% in 2014 [509]. Despite this low prevalence, all subtypes except B and many CRFs and URFs have been detected in Angola, in particular in the Provinces of Luanda and Cabinda where most studies have been done. This high diversity is a direct consequence of the long-standing presence of HIV-1 in the country and the frequent interchange of people with the DRC and RC [67, 68, 510-512]. In this study we describe the first near full-length HIV-1 genomic sequences from HIV-1 infected individuals from Cabinda, a province of Angola which is an exclave surrounded by DRC in the south and east, and by the RC in the north.

Blood samples from HIV-1 infected individuals were collected in 1993 from Hospital Distrital de Cabinda, Cabinda, Angola. Samples were collected anonymously. The study was approved by the ethics committees of the participating institutions. Virus isolates were obtained using the co-cultivation method as described previously [513]. All three isolates used the CCR5 co-receptor [514]. Viral genomic RNA was extracted from cell culture supernatant and RT-PCR was performed using Titan One Tube RT-PCR System (Roche Diagnostic Systems). All amplifications were performed using the Expand Long Template PCR system (Roche Diagnostic Systems) according to the manufacturer's instructions. New primers were designed to amplify the full-length genomes (Supplementary material, Table 1). Amplified DNA fragments were purified using JETQUICK Gel Extraction Spin Kit (Genomed) and sequenced. The nucleotide sequence data was deposited in GenBank with accession numbers KU310618-20. Genomic sequences were aligned with a set of reference sequences representative of all HIV-1 group M subtypes obtained from the Los Alamos HIV Sequence Database[515]using Clustal X 2.1 (http://www.clustal.org/clustal2/) and the alignment was manually edited with GeneDoc (http://iubio.bio.indiana.edu/soft/molbio/ ibmpc/genedoc-readme.html).

Maximum likelihood (ML) trees were inferred with program PhyML using the Seaview software (http://pbil.univ-lyon1.fr/software/seaview.html) using the best-fit substitution model identified by Modeltest v3.7 using the Akaike information criterion. To find the ML tree an iterative heuristic method combining nearest neighbor interchange and subtree pruning and regrafting tree rearrangement methods was used. The reliability of the obtained tree was estimated with the approximate likelihood-ratio test (aLRT). Potential recombination patterns of our new near full-length sequences were analyzed by bootscanning using SimPlot with a sliding window of 500 bp advanced in 100 bp increments (http://sray.med.som.jhmi.edu/SCRoftware/simplot/). For each window 100 bootstrap replicates were generated. Potential recombination breakpoints between subtypes were considered when the percentage of permuted trees for a given subtype was above 70%. We also used RIP, jpHMM and the branching index (BI) to confirm the bootscanning results [516-519].

An overall genome-wide tree analysis of 93AOHDC250 and 93AOHDC253 showed that they clustered with subtype J reference sequences and were more closely related to each other than to the other J isolates, which is consistent with their epidemiological data (Figure 1). Note that this tree is not a true phylogeny as it cannot depict the evolutionary

history of all recombinant sequences; we used it here merely to investigate the overall sequence-based similarity for our classification purpose.

Bootscanning analysis showed no evidence of recombination in isolate 93AOHDC253 (Figure 2A). Isolate 93AOHDC250 displayed an untypable region between positions 1450 and 2050 corresponding to the end of *gag* gene, the protease (PR) region, and the first 192 nucleotides of reverse transcriptase (RT), which was further confirmed by phylogenetic analysis (Figure 2B). BI results confirmed that 93AOHDC253 was a pure subtype J and that 93AOHDC250 had a genomic region with no known subtype in this *gag/pol* region (Figure 5).

Comparing our new J sequences to existing J sequences in the Los Alamos HIV database revealed that subtype J is quite diverse (Figure 3). In the region where most J sequence fragments exist (HXB2 positions 7041-7358), 93AOHDC250 and 93AOHDC253 cluster together with a previous sequence from Angola, 93AOHDC247 [67]. Interestingly, Angolan J sequences occur throughout the phylogeny of this *env* region, suggesting that the J epidemic in Angola is either the origin of subtype J or that there has been a lot of influx of subtype J from other geographic regions. The great diversity of subtype J has been previously noted in analyses of J-containing CRF11 and CRF13 genomes [520, 521].

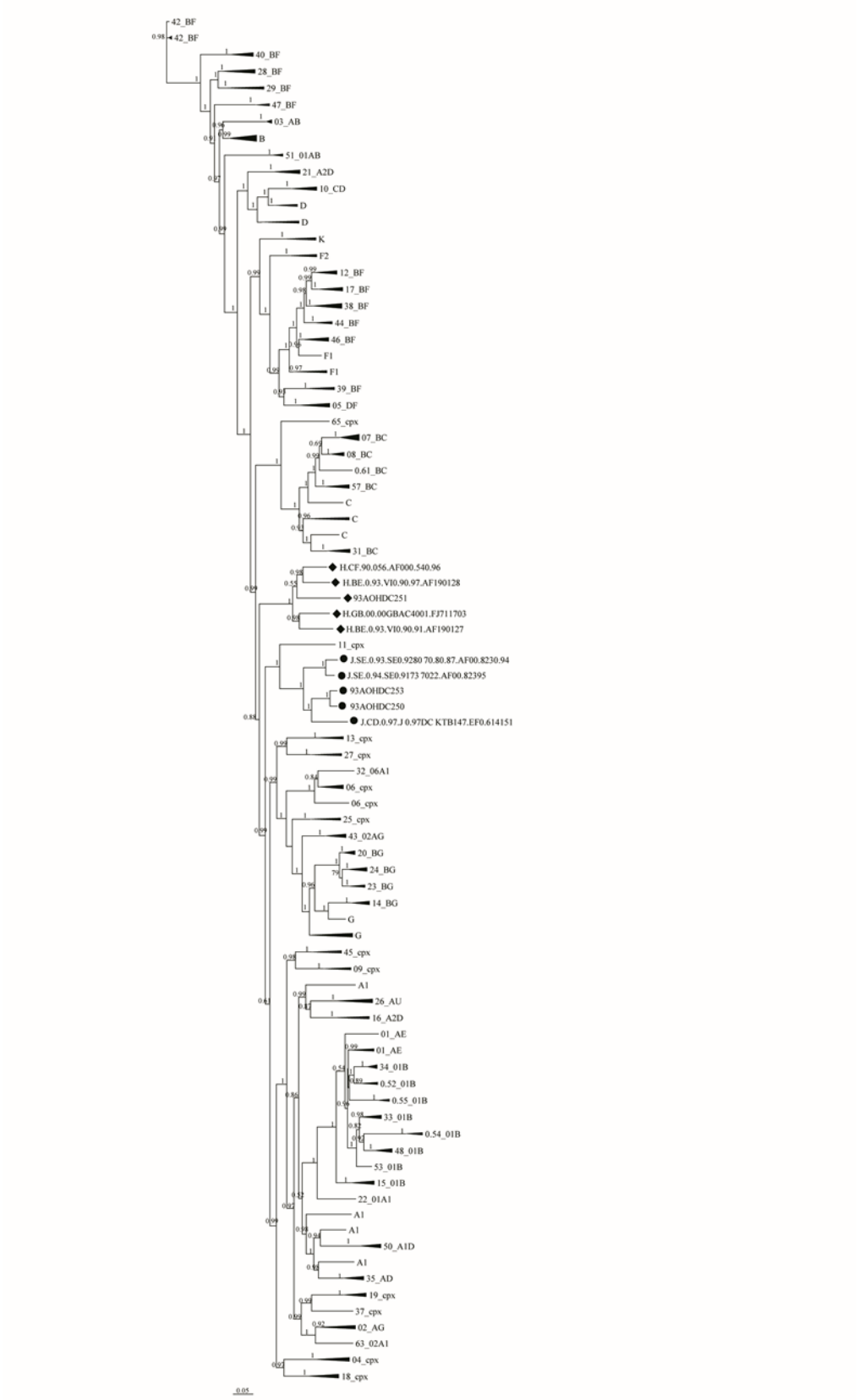

Figure 1 – Overall tree classification of HIV-1 near full-length genomes of isolates 93AOHDC250, 93AOHDC251 and 93AOHDC253. ML trees were constructed with our sequences (highlighted by colored dots) and 136 reference sequences representative of all HIV-1 group M subtypes. Cladistics support is indicated by aLRT values. The scale is in units of substitutions per site.

The overall tree analysis showed that 93AOHDC251 clustered with subtype H reference sequences (Figure 1). Bootscanning analysis indicated an unclassifiable (U) region between positions 4936 and 5167 (corresponding to *vpr* gene), and a region between positions 8151 and 8888 (corresponding to the *nef* gene and 3´LTR) appeared to cluster between subtypes A1 and G (Figure 2C). BI analyses again confirmed these results, and suggested that the *nef*/LTR region was below the subtype-defining threshold for subtype G (Figure 5). Upon closer inspection using RIP, comparing this region to subtypes A1, G and CRF02, it became clear that this region was in fact derived from CRF02; 93AOHDC251 was closer to CRF02 than either A1 or G in all parts of this region, which included a CRF02 A/G breakpoint (Figure 4). Together, these analyses led to a classification of this genome sequence as the first H/U/CRF02_AG recombinant.

In this report we describe the first three HIV-1 genomic sequences from Angola, a country that with the DRC and RC had a crucial role in the early dissemination of the HIV-1 epidemic [49, 67]. Sequences were derived from isolates obtained in 1993 from patients living in Cabinda, a province in the North that borders both DRC and RC.

One isolate was a pure subtype J, another a J with an at this point unclear segment, and one was an H-based recombinant. The unclear region in 93AOHDC250 may be a divergent J segment or a segment of an as yet undiscovered subtype. As mentioned above, subtype J has previously been described as very diverse and may hide further sub-subtypes or recombinants of sub-subtype nature [520].

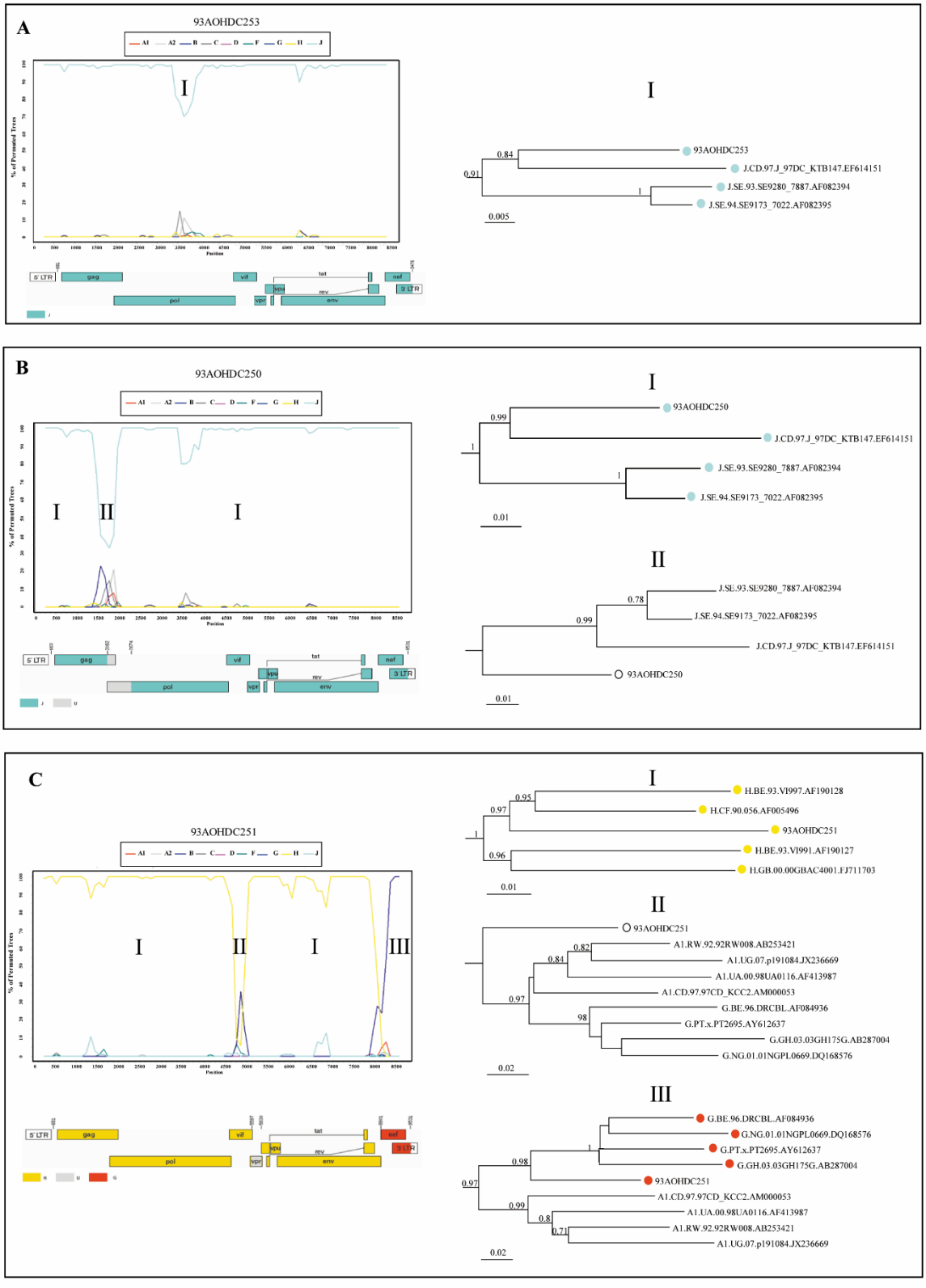

Figure 2 – Genomic segment analysis of near full-length genomes. Panels show bootscanning and tree analyses of isolates 93AOHDC253 (A), 93AOHDC250 (B) and 93AOHDC251 (C). Tree analysis was done for regions suggested by bootscanning to belong to different subtypes. A schematic genome map of the subtype composition of the isolates was produced based on the results obtained by bootscanning.

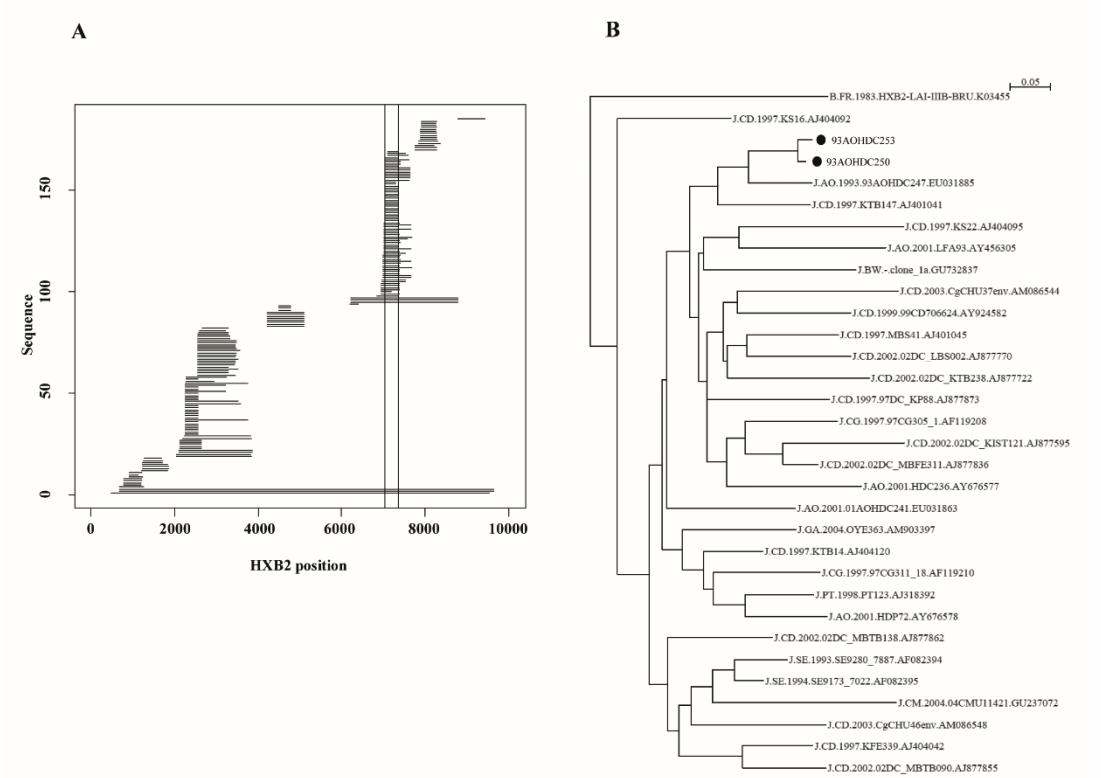

Figure 3 – Subtype J *env* fragment comparison. At the time this report was written 185 subtype J sequence fragments were available in the LANL HIV database. Their HXB2 coordinates covered various parts of the HIV-1 genome (A). Previous to our new sequences, only 3 full subtype J genomes have been described (long horizontal lines at bottom of graph). The most sequenced region was a part of *env* (HXB2 coordinates 7041 to 7358), indicated by the vertical lines in the graph. A ML phylogeny of this region revealed that subtype J is very diverse (B). Our new sequences are highlighted by black dots.

Despite being in circulation in Cabinda since 1993, as of February 2015 only 27 J and 73 H sequences from Angola had been deposited in the Los Alamos HIV Sequence Database (3.1 and 8.3% of respective subtype sequences from Angola). Globally, pure subtypes J and H are also very rare which suggests low biological fitness, low transmissibility, high recombination potential, and unsuccessful introductions into high-risk populations. However, the isolates described herein replicate to normal levels in different cell types suggesting that their biological fitness is no different from other subtypes [514]. Interestingly, we have found that our J and H isolates are much more sensitive (about nine times) to the CCR5-antagonist TAK-779 when compared with isolates from other subtypes that are more prevalent in Angola and Portugal [514]. This suggests poor binding to the main HIV co-receptor (CCR5) which may indicate transmissibility problems of these subtypes. On the other hand, J and H subtypes have been found in many recombinants such as CRF04_cpx, CRF06_cpx, CRF11_cpx, CRF13_cpx, CRF18_cpx, CRF27_cpx, and CRF49_cpx[515].

Thus, the rarity of pure subtype J and H, together with the large HIV-1 diversity in Angola and neighboring countries, which suggests they have been around for long times, suggests that these subtypes are less fit to transmit or establish infection by themselves. Similar to how latent virus may survive within a host through recombination with non-latent immune escaping plasma virus, subtype J and H virus may do better if they recombine with more fit subtypes [522].

HIV-1 subtypes J and H are present in Angola at low levels since at least 1993. The high diversity among Angolan subtype J *env* sequences and the fact that the rare subtype H has recombined in Angola together suggest that Angola is either the origin of subtype J or, more complicated, that there has been a lot of influx of subtype J from other geographic regions. Low transmission efficiency and/or high recombination potential may explain their limited epidemic success in Angola and worldwide.

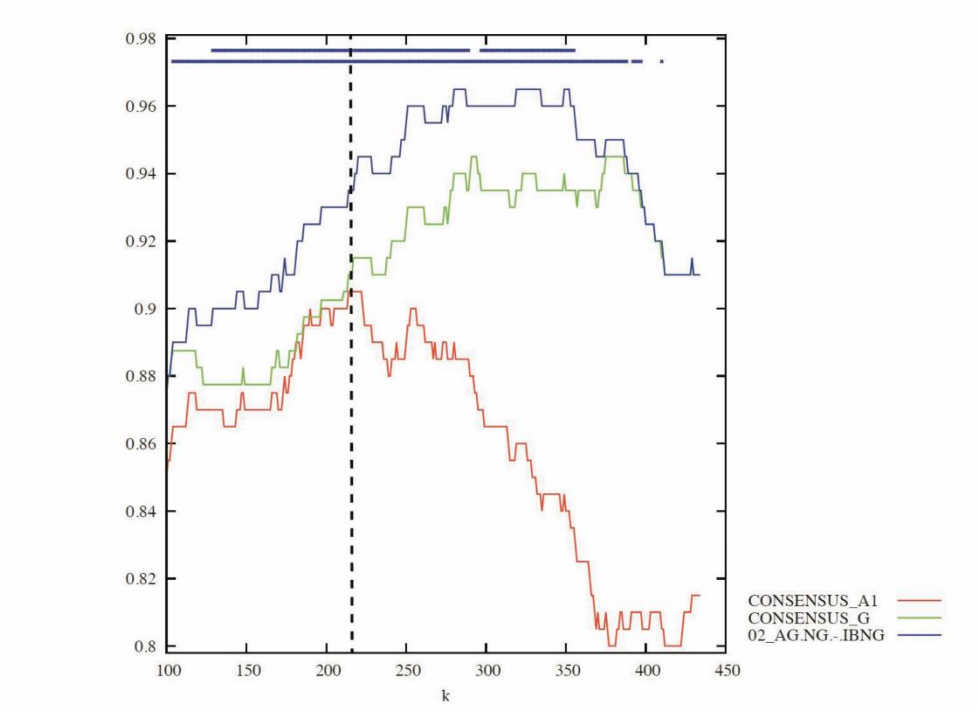

Figure 4 – RIP analysis of segment III of 93AOHDC251. A sliding window analysis (window size 200 nt, step 1 nt; significance threshold 0.90) comparing 93AOHDC251 to consensus subtype sequences A1 and G, and CRF02 prototype sequence IBNG showed that this region was closer to CRF02 in both the A1 and G parts. The CRF02 A1/G breakpoint is indicated by a bashed vertical line. The top bar indicates statistical significance, and the lower bar best match.

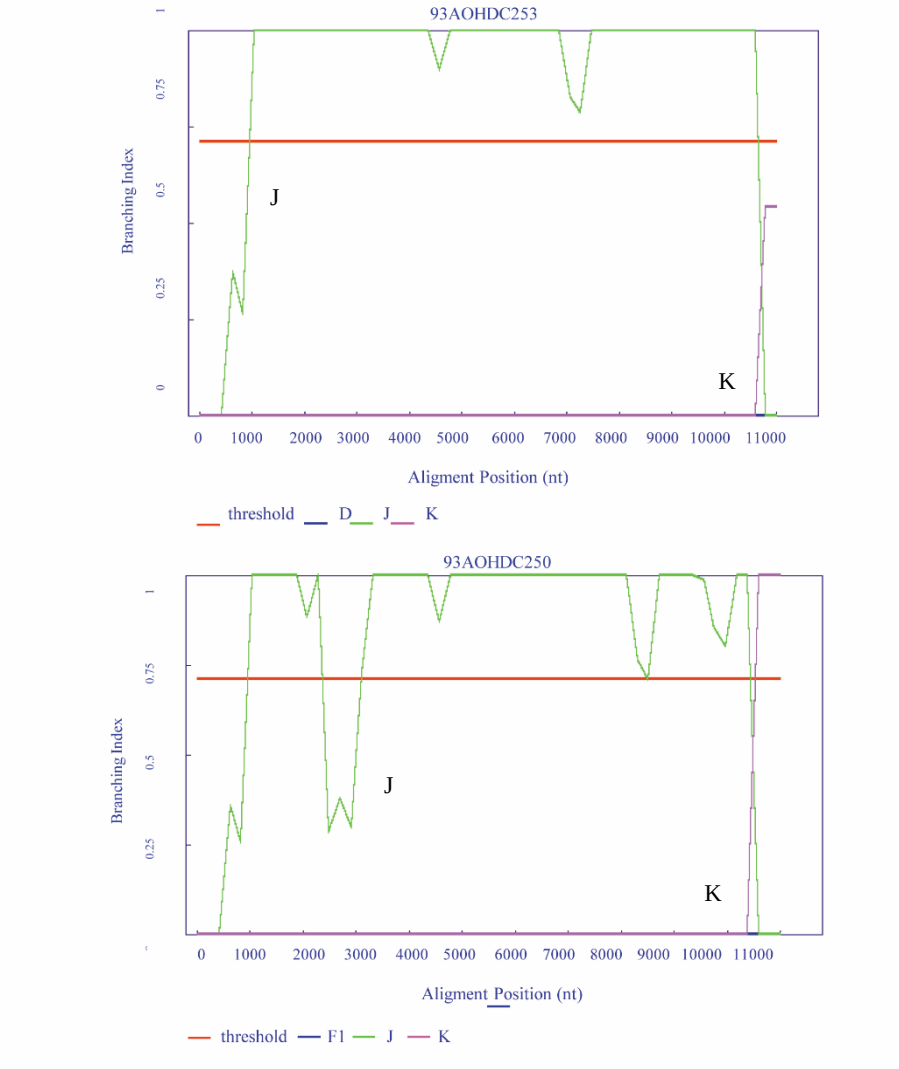

Figure 5 - Branching index (BI) analysis of 93AOHDC253 and 93AOHDC250.

Competing interests

The authors have no commercial or other type of association that might pose a conflict of interest.

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Sequence data

Sequences have been assigned with GenBank accession numbers KU310618, KU310619 and KU310620

Authors' contributions

Conceived and designed the experiments: IB, TL and NT. Performed the experiments: IB, PB, TL and RC. Analyzed the data: IB, TL, RC and NT. Wrote the paper: IB, RC, TL and NT. The final text was read and approved for submission by all authors.

Supplementary Material

Table S1- Primers used for polymerase chain reaction amplification of HIV-1 near full-length genomes

Name	***HXB2 Position***	***Region***	***Sequence (5´-3´)***
JA153	683-707	p17	CTCTCGACGCAGGACTCGGCTTGCT
P17-894F	894-913	p17	ATGGGCAAGCAGGGAGCTGG
P17-913R	913-894	p17	CCAGCTCCCTGCTTGCCCAT
P24-1300F	1300-1324	p24	ATACCCATGTTT(A/T)CAGCATTATCAG
JA155 (R)	1324-1300	p24	CTGATAATGCTGAAAACATGGGTAT
P24-1818F	1818-1838	p24	AGAAGAAATGATGACAGCATG
P24-1838R	1838-1818	p24	CATGCTGTCATCATTTCTTCT
IBPR1.1	2008-2030	gag NC	AAAAGGGCTGTTGGAAATGTGG
IBPR2.2	2733-2712	RT	GCAAATACTGGAGT(A/G)TT(G/A)TATG
IBPR3.1	2119-2140	gag P1-P6	AGGCCAGGGAATTT(T/C)C(T/C)TCAGA
IB2621PR4	2591-2621	PR	AATGCTTTTATTTT(C/T)TCTTCTGTCAATGGC
IBRT1	2487-2505	RT	CCTACACCTGTCAACATAA
IBRT2	3649-3630	RT	TGTTTTACATCATTAGTGTG
IBRT3	2542-2560	RT	TAAATTTTCCAATTAGTCC
IBRT4	3579-3560	RT	TAAATTTGATATGTCCATTG
RT3482FI	3482-3509	RT	AGAACCAGTACATGGRGTATATTATGA
IB3626RT2	3626-3593	RT	TCCGTTAA(C/T)TGT(C/T)TTACATCATTAGTGTG(A/G)GCA
RT3864F	3864-3881	RT	CAACAAATCA(A/G)AAGACTG
P15-3881R	3881-3864	p15	CAGTCTT(T/C)TGATTTGTTG
P15-4176F	4176-4194	p15	GGAGGAAATGAACAAGTAG
P15-4194R	4194-4176	p15	CTACTTGTTCATTTCCTCC
P31-4658F	4658-4675	p31	CAATCCCCAAAGTCAAGG
P31-4675R	4675-4658	p31	CCTTGACTTTGGGGATTG
Vif5041F	5041-5059	vif	ATGGAAAACAGATGGCAGG
Vif5059R	5059-5041	vif	CCTGCCATCTGTTTTCCAT
Vif5461F	5461-5476	vif	AAGGTAGGATC(T/C)(T/C)TAC
Vif5476R	5476-5461	vif	GTA(A/G)(A/G)GATCCTACCTT
PBENV1	5968-5986	rev	CTATGGCAGGAAGAAGCGG
Rev5986R	5986-5968	rev	CCGCTTCTTCCTGCCATAG
Env6223RI	6223-6203	rev	CCACTGTCTTCTGCTCTTTC
PBENV2	6203-6223	rev	GAAAGAGCAGAAGAYAGTGGC
Env8637FI	8637-8657	gp41	CAGGAACTAAAGAATAGTGC
PBENV4	8797a8817	nef	TTTTGACCACTTGCCHCCCAT
PBENV3	9036-9016	nef	AGTCATTGGTCTTARAGGTAC
Nef9532RI	9532-9511	3´LTR	GCGAAAAGCRGCTGCTTATAT

Chapter 3

A prime-boost immunization strategy with Vaccinia virus expressing novel envelope gp120 glycoproteins from non-B subtypes induces cross-clade tier 2 HIV-1 neutralizing antibodies in mice

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Abstract

Development of immunogens inducing broadly neutralizing antibodies is currently the main priority for the HIV-1 vaccine field. Recently, we have demonstrated that bNAbs against HIV-2 can be elicited in mice using a Vaccinia vector-prime C2V3C3-boost vaccination strategy. Here we tested if a similar vaccination strategy could be effective for HIV-1. We produced a set of immunogens consisting of Vaccinia virus vectors expressing a truncated form of gp120 derived from four primary isolates belonging to subtypes B, C, CRF02_AG and J from Portugal and Angola, and the cognate truncated monomeric gp120 glycoproteins and C2V3C3 recombinant polypeptides. BALB/c mice were immunized with different combinations of these immunogens. Mice developed potent cross-reactive binding antibodies against autologous and heterologous gp120 antigens. Unlike previous vaccination studies in HIV-2, the C2V3C3 polypeptides were found to be poor vaccine antigens. Remarkably, a cross-reactive neutralizing response (>50%) was observed against three primary isolates and six heterologous tier 2 HIV-1 pseudoviruses from different clades in mice immunized with CRF02_AG based immunogens. In addition, gp120AG immunogen bound strongly to mAb 447-42D (anti-GPGR) both in ELISA and Western Blot suggesting that V3 could be an important target of bNAbs induced by our best vaccine regimen. Follicular helper T (Tfh) cells provide crucial help to germinal center B cells for proper antibody production, and a specialized subset of regulatory T cells, follicular regulatory T (Tfr) cells ($CD4^+PD\text{-}1^+CXCR5^+$ $CD25^+$ $Foxp3^+$) modulate this process. There was a positive correlation between Tfh cell number and binding antibody activity against all gp120 immunogens [(r(gp120C)=0.764; r(gp120AG)=0.770; r(gp120J)=0.768, p=0.0001)] and a negative correlation (r=-0.6606, p= 0.0438) between the frequency of Tfr cells and neutralization potency. Hence our vaccination strategy is able to drive follicular T cells to provide adequate help for germinal center B cells to produce broadly neutralizing antibodies. Ongoing studies will determine whether this strategy leads to similar results in rabbits.

Keywords: HIV-1 infection, neutralizing antibodies, BALB/c mice, HIV vaccines, B cells

Introduction

Since the discovery of HIV-1, more than 70 million people have been infected and about 35 million have died with HIV-1 related disease. At the end of 2015, there were approximately 36.7 million people living with HIV[523]. Effective antiretroviral drugs can control the virus, prevent disease progression and prevent HIV-1 transmission [524]. However, in 2015 <50% of HIV infected adults and children were accessing treatment and 2.1 million people became newly infected with HIV[117]. Developing a safe, effective and affordable vaccine to prevent HIV infection is the best hope for controlling or ending the HIV epidemic.

The search for a preventive vaccine faces enormous challenges, namely i) the absence of well-defined immune correlates of protection against HIV in humans, ii) the lack of adequate animal models that can be used to predict human responses to vaccines , iii) the failure in the induction of bNAbs by different antigens, and most importantly, iv) the extraordinary sequence diversity of HIV-1 and its capacity to constantly mutate, evolve and escape from the host immune response[52, 373, 471, 473, 478, 525, 526]. There are at least nine HIV-1 genetic subtypes as well as multiple recombinant forms worldwide. On a global perspective five HIV-1 strains dominate the global epidemic: C (50%), A (12%), B (11%) followed by CRF02_AG (8%), G (5%) and CRF01_AE (5%). Other subtypes, like J and H, represent less than 1% of infections [35, 66, 507, 508, 527]. Most vaccine research is based on subtype B. However, there is no guarantee that a subtype B vaccine could protect against other HIV-1 subtypes that are much more prevalent, as subtype C which is dominant in India and Southern African countries [35, 528]. An ideal vaccine immunogen should be able to contend with the remarkably high diversity of HIV-1 and induce an immune response able to cross-react with contemporaneous heterologous viruses. Although correlates of protection from HIV-1 infection are not completely defined, there are several studies that support the crucial role of neutralizing antibodies in preventing HIV-1 infection [320, 324, 373, 473, 475, 529-532]. In some individuals, broad neutralizing antibodies emerge after few years of infection and these antibodies are able to block a diverse range of viruses including tier 2 viruses, that dominate human transmissions, or even tier 3 viruses with a higher resistance profile [473, 474]. Thus, a major goal of HIV-1 vaccine development is to identify immunogens that are capable to induce these broadly neutralizing antibodies against primary isolates of the virus [478].

Proof-of-concept passive immunization studies in nonhuman primates have demonstrated that administration of bNAbs can protect from infection [319, 320, 324, 532-535]. These antibodies are directed to several epitopes located in HIV-1 envelope glycoprotein (Env) and are capable of binding to the virus and prevent infection [536]. Neutralizing epitopes on HIV-1 includes the CD4 binding site, V1/V2 loops, V3 loop, gp120/gp41 interface region and the fusion peptide and MPER (Membrane-proximal external region) in gp41 [298, 325-332, 337]. In contrast with the CD4 binding site which is highly conserved, V1, V2 and V3 are variable regions [347]. V3 is the most conserved region of the three variable regions and it harbors a highly conserved motif, GPGR/Q (residues 312-315 in the HXB2). V3 is a highly immunogenic region and anti-V3 antibodies such as the broad and potent bNAb 447-52D have been found to neutralize up to 50% of the viruses in various multiclade panels [325, 326, 329, 330, 347, 348].

Despite the urgent need for a vaccine, only six HIV-1 vaccine candidates have completed efficacy trials. To date, the prime-boost regimen used in RV144 trial is still the only immunization strategy that has demonstrated some level of protection against HIV-1 infection [118, 468]. This trial was based on clade B and CRF01_AE sequences and consisted of four priming injections of a recombinant canarypox vector vaccine (ALVAC/vCP1521) expressing *env*, *gag* and *protease* genes in combination with two booster injections of a recombinant glycoprotein 120 subunit vaccine (AIDSVAX B/E). Immune responses observed in RV144 associated with a reduced risk of HIV-1 infection included non-neutralizing antibodies to V1/V2, high levels of antibody-dependent cellular cytotoxicity (ADCC) and HIV-1 specific IgG3 responses[118, 468, 470]. Importantly, all RV144 recipients developed low titers of neutralizing antibodies that were mainly against tier 1 isolates which may justify the modest efficacy of this trial [131]. Despite these limitations, RV144 trial was very helpful to inform new vaccination strategies. HVTN 702 [119, 471], the only phase 2b/3 HIV vaccine trial ongoing involves a new version of the immunogens and strategy used in RV144 but with some modifications: HIV-1 subtype, number of immunizations and type of adjuvant. Vaccine regimen consists of two immunizations of ALVAC-HIV (vCP2438) plus three immunizations with Bivalent Subtype C gp120[472]. Because bNAbs have been considered the best correlate of antibody protection against HIV infection, the development of immunogens that induce broadly neutralizing antibodies is currently the main priority for the HIV-1 vaccine field [131, 319, 320, 422, 473-475]. So far, no

vaccine candidate in humans has been able to induce bNAbs against heterologous tier 2 viruses from different clades [478, 479]. The majority of HIV vaccine regimens consists of prime-boost regimens that use different combinations of immunogens, namely, recombinant virus expressing HIV envelope[2, 422, 493], or/and plasmid DNA constructs[454], or/and various HIV purified proteins (*e.g*. native-like trimers) [342, 422, 453, 478, 479, 494]. Recent studies have shown that it is possible to induce NAbs against tier 2 viruses although sporadically, with limited breadth and at low levels [478, 479]. A recent study using a poxvirus prime-gp120 boost strategy based on clade B Envs demonstrated the induction of cross-reactive binding antibodies against V1/V2 fusion proteins and neutralizing responses against heterologous tier 2 isolates, mostly from clade B [422]. In line with this, we previously demonstrated that bNAbs against HIV-2 can be elicited in mice using a Vaccinia vector-prime C2-V3-C3-boost vaccination strategy [2].

The effective production of NAbs is highly dependent on an effective T cell response. Broadly neutralizing antibodies exhibit high somatic mutation frequencies suggesting that bNAbs generation is likely to be highly dependent on the activity of $CD4^+$ follicular helper T cells (Tfh), and may be constrained by host tolerance controls such as follicular regulatory T cells (Tfr). Tfh have been described to be essential for antibody class switch and affinity maturation, an important process during the production of NAbs [235, 240]. These cells are defined by the expression of high levels of surface markers program death-1 (PD-1) and chemokine CXCR5, especially within germinal centers (GCs)[244]. The interplay between HIV infection and Tfh is complex and still remains to be fully defined. However, studies report a positive correlation between the frequency of Tfh subsets and effective humoral responses against HIV, as measured by the development of bNAbs[240], suggesting Tfh as a potential target for vaccine strategies aiming to induce neutralizing antibodies. On the other hand, some studies have shown a negative correlation between regulatory $CD4^+$ T cells ($CD25^+$ $Foxp3^+$ $CD4^+$) and bNAbs in HIV-1 infected individuals[537]. Recently, Tfr cells were identified as a unique subset of CD4 T cells that controls and regulates GC responses. Similar to Tfh, Tfrs express high levels of CXCR5 and PD-1, expressing also Foxp3 which contributes for its regulatory functions [259-261]. These cells are important modulators of the GC response as they inhibit GC expansion and regulate Tfh and B cell numbers. Studies reported that the avidity of Env-specific (gp120) antibodies in SIV-infected rhesus macaques correlated with a lower frequency of Tfrs[263]. Also neutralizing antibodies to HIV were negatively correlated

to Foxp3^{+} Env-specific follicular T cells in SHIV-infected rhesus macaques [256] . As Tfrs frequency has been found to negatively correlate with bNAb generation [256, 263] it is important to consider this subset when developing new vaccine strategies against HIV.

In the present study, we aimed to investigate if the vaccine concept used previously against HIV-2[2] could be effective in the induction of bNAbs against HIV-1 in mice. For this purpose, we produced for priming immunization a new set of recombinant Vaccinia virus vectors expressing a truncated form of gp120 from several non-B clades of HIV-1 primary isolates from Angola and Portugal and for boosting the cognate C2V3C3 polypeptides and truncated gp120 protein. We have also analyze Tfh and Tfr cell subsets in immunized animals. We were able to induce cross-reactive binding antibodies in mice against all gp120 proteins tested and neutralizing responses against HIV-1 heterologous tier 2 isolates from different clades. Tfh cells were positively correlated with binding antibodies levels and Tfr cells were negatively correlated with neutralizing antibody responses.

Material and Methods

Ethics statement

BALB/cByJ mice were maintained under specific pathogen free (SPF) conditions at the Instituto de Higiene e Medicina Tropical and Instituto de Medicina Molecular, where all animal work was performed in accordance with Directive 2010/63/EU. All experimental procedures were approved by the institutional Animal Welfare Body. Experimental animals were females between 8–10 weeks.

Cells, plasmids, viruses, and antibodies

Rat2 (TK^{-}) cells were purchased from American Type Culture Collection (Rockville, MD). HeLa cells (ATCC® CCL-2™) were obtained from American type Culture Collection. TZM-*bl* cells were provided by the AIDS Research and Reference Reagent Program, National Institutes of Health. HeLa, Rat2 and TZM-*bl* cells were cultured in complete growth medium that consists of Dulbecco´s minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin-

streptomycin (Gibco/Invitrogen, USA), 1mM of sodium pyruvate (Gibco/Invitrogen, USA), 1mM of L-glutamine (Gibco/Invitrogen, USA) and 1mM of non-essential amino acids (Gibco/Invitrogen, USA). All cell cultures were maintained at 37ºC in 5% CO_2. The following items were obtained from the NIH AIDS Reagent Program: Western Reserve Strain of Vaccinia virus (VV_{WR}), a Panel of Global tier 2 HIV-1 Env Clones (cat#12670) designed to assess neutralization responses and HuMAbs 447-52D (anti-V3), VRC01 (anti-CD4bs), 3BNC117 (anti-CD4bs), HJ16 (anti-CD4bs), PG16 (anti-V2), 2G12 (anti-N-linked glycans in C2, C3, V4, C4), b12 (anti-CD4bs), and 2F5 (anti-MPER).

Cloning of envelope genes from primary isolates

Envelope genes expressed in this study (Table S1) were obtained by PCR amplification from five primary isolates derived from HIV-1 infected individuals from Portugal (n=2) and Angola (n=3) and from plasma samples from HIV-1 infected patients (n=2) from Portugal and Angola. Viral genomic RNA was extracted from plasma and/or cell culture supernatant and reverse transcription (RT) was performed using Titan One Tube RT-PCR System (Roche Diagnostic Systems). Full-length *env* genes were amplified by nested PCR as described previously [538], purified using JETQUICK Gel Extraction Spin Kit (Genomed) and sequenced (Table S2 and S3). For subtyping, the nucleotide sequences were aligned with reference sequences using ClustalX4 ((http://www.clustal.org/clustal2/) and manually edited with GeneDoc (http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc-readme.html). Find model tool (https://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html) was used to estimate the best nucleotide substitution model and PhyML v3.1 program in SeaView version 4.6.2. was used to compute maximum likelihood phylogenetic trees[539].
CCR5 and /or CXCR4 usage of primary isolates was determined in TZM-*bl* cells ($CD4^+$, $CCR5^+$, $CXCR4^+$) in the presence of excessive amounts of the CCR5 antagonist TAK-779 (10µM) and/or of the CXCR4 antagonist AMD3100 (1.2µM) as previously described[514]. For two HIV-1 samples coreceptor usage was determined based on the sequence of V3 using the geno2pheno algorithm[540].

For each subtype, a group of newly selected primers based on *env* sequences were designed in order to amplify the gp120 region lacking 78 bases at the carboxyl terminus of the C5 region. Forward and reverse primers included a restriction site for *SalI* (5´-

GTCGAC-3´) and Reverse primers included a stop codon (CTA) before the restriction site. PCR primer numbers and positions are described in Table S2 (Supplemental material). All amplifications were performed using the Expand Long Template PCR system (Roche Diagnostic Systems) according to the manufacturer's instructions. gp120 truncated fragments (named gp120t) were amplified and cloned into the *Sal*I site of the Vaccinia virus insertion vector pMJ601. In this vector, protein expression is driven by the strong late Vaccinia virus promoter present in pMJ601[541].

Production of recombinant Vaccinia viruses and expression of Env glycoproteins

Recombinant Vaccinia viruses expressing the glycoprotein gp120t were obtained as described[2].Briefly, HeLa cells were transfected with recombinant pMJ601 plasmids and simultaneously infected with Vaccinia virus strain VV_{WR} and recombinant Vaccinia viruses resistant to 5-bromodeoxyuridine and expressing β-galactosidase were selected in Rat2 cells and then propagated in HeLa cells. The recombinant viruses were titrated in Rat2 cells using the method of Reed and Muench[542].

The method of Rose *et al*[543] was used to produce gp120t . Briefly, HeLa cells were infected with 5 PFU of recombinant Vaccinia virus per cell and incubated for 3 hours. Medium containing the infecting virus was replaced with DMEM supplemented with 2.5% FBS at 3h post infection. After 24 hours of infection medium containing gp120t was collected, clarified by centrifugation at 3000xg for 10 minutes and filtered with a 0.2µM pore size filter to remove Vaccinia virus. Cells were washed with cold PBS (Phosphate buffered saline) and lysed using RIPA+DOC buffer (0.15M NaCl, 0.05M Tris-HCl, 1% Triton X-100, 1% DOC, 0.1% SDS). Lysates were centrifuged at 35.000 rpm for 60 min at 4°C and the supernatant containing the proteins was collected. Antigenic reactivity of gp120 obtained from cell lysates and cell-free supernatants was quantified by an ELISA assay and by Western-Blot as described below.

Analysis of HIV-1gp120t proteins

Proteins obtained from the cell lysate and from the cell-free supernatant were analyzed on 7.5% SDS-PAGE followed by Coomassie Brilliant Blue staining. For Western blot analysis, proteins were prepared in Laemmli sample buffer (125mM Tris, pH 6.8, 2%

SDS and 20% glycerol) with β- mercaptoethanol and denaturated by heating for 5 min at 96ºC, loaded within the acrylamide gel and then transferred from the gel to nitrocellulose membranes 0.45µM (BioRad). After protein transfer, the membranes were treated with blocking buffer (1X TBSt with 4% w/v nonfat dry milk) and incubated with serum from HIV-1 and HIV-2 infected individuals diluted (1:200) in primary antibody buffer (1X TBSt with 4% w/v nonfat dry milk and 5% goat serum) and/or HuMAbs 447-52D (anti-V3), VRC01 (anti-CD4bs), 3BNC117 (anti-CD4bs), HJ16 (anti-CD4bs), PG16 (anti-V2), 2G12 (anti-N-linked glycans in C2, C3, V4, C4), b12 (anti-CD4bs), and 2F5 (anti-MPER) at 1µg/ml. Next, the membrane was washed with TBSt 0.25% and incubated with Anti-human IgG- alkaline phosphatase antibody produced in goat (Sigma) and/or Anti-Human IgG-Peroxidase antibody produced in goat (Sigma). Colorimetric detection of proteins was performed by AP conjugate Substrate Kit (BioRad) and chemiluminescent detection was performed with Pierce™ ECL Western Blotting Substrate (ThermoFisher Scientific). Quantification of glycoproteins was done using Kodak image analysis software.

Binding specificity of the immunogens (gp120) was analyzed by an ELISA assay against the eight HuMAbs described above at a final concentration of 10µg/ml. For this purpose, Immuno MaxiSorp 96-well microplates (Nunc) were coated with the gp120t supernatant (~2.3 to 4µg per well) in 0.05M bicarbonate buffer. Envelope gp120 glycoprotein expressed by vPE16, SF162 gp140 (2µg/ml) and M.CON.S D11 gp120 (2µg/ml) were used as protein positive controls. Of note, vPE16 is a recombinant Vaccinia virus obtained from the plasmid pPE16 (isolate HTLV-IIIB, clone BH8) that expresses gp120 and gp41 glycoproteins. HIV-1 plasma samples at a final dilution of 1:200 were used as positive antibody control and 2F5 antibody (anti-gp41) was used as a negative control. The clinical cut-off value of the assay was calculated as the mean OD value of 2F5 reactivity plus 2 times the SD. Cut-off value of sera from HIV-1 plasma samples and SF162gp140 (which reacts with 2F5) was calculated as the mean OD value of HIV-seronegative samples plus 2 times the SD.

Production, expression and analysis of C2V3C3 polypeptides

A DNA fragment of 534 nucleotides comprising the C2, V3 and C3 coding regions from all isolates (6858-7392 in HXB2) was amplified using primers described in Table S2 and cloned into the bacterial expression vector pTrcHis (Invitrogen). Expression of C2V3C3 polypeptides in *Escherichia coli* strain TOP10 were induced with isopropyl-β-D-

thiogalactopyranoside (IPTG) according to the manufacturer´s instructions and protein purification was performed using Dynabeads® His-tag Isolation & Pulldown (Life Technologies). Bradford assay (Bio-Rad) was performed to determine protein concentration. Purified recombinant polypeptides were analyzed by SDS-12% PAGE and the antigenic structure of the purified polypeptides was analyzed by an ELISA assay with plasma samples from HIV-1 infected patients from Angola (n=28). Briefly, Immuno MaxiSorp 96-well microplates (Nunc) were coated with recombinant polypeptides from HIV-1 subtypes C, CRF02_AG, J, G and H (2.5µg/ml) diluted in 0.05M bicarbonate buffer (pH 9.4) by overnight incubation at 4°C and blocked with 2% gelatin (Bio-Rad). Plasma from HIV-1 infected patients (starting at 1:100) was added to the microplates and, after 2h of incubation at room temperature, anti-Human IgG (Fc specific)−Alkaline Phosphatase antibody produced in goat (Sigma) was added as a secondary antibody. Colorimetric reaction was developed with SIGMAFAST™ p-Nitrophenyl phosphate (pNPP) Tablets and read at 405 and 492 nm against a reference wavelength of 620 nm on a microplater reader. The clinical cut-off value of the assay, calculated as the mean OD value of HIV-seronegative samples plus 2 times the SD, was determined using samples from healthy HIV-seronegative subjects. Binding antibody titer was calculated as the highest plasma dilution giving a positive reaction (OD / cut-off> 1).

BALB/c mice immunizations

Recombinant Vaccinia viruses expressing gp120t (VVgp120), soluble gp120t (Sgp120) and recombinant polypeptides C2V3C3 described above were used as vaccine immunogens in two separate immunization experiments. In the first experiment, herein considered a pilot study, we used the immunization strategy that was proven efficient in HIV-2 vaccination but with some minor modifications [2]. A total of 20 six week BALB/mice (BALB/c ByJ (H-2d, BALB/c)) divided in six groups (Groups 1-6, mice 1-20) were first immunized intraperitoneally (IP) with $2x10^7$ PFU of recombinant Vaccinia viruses from clades C, CRF02_AG and J in 100 µl of PBS (Table 1). Mice were boosted three times (IP route) at days 15 and 30 with 10µg of C2V3C3 from the cognate subtype and at day 45 with gp120 supernatant also from the cognate subtype. Groups 1 and 2 were control groups. Group 1 didn´t received any immunogen and group 2 was immunized with VV_{WR}. Three to four mice were used in each group with the exception of group 1

that included only 2 mice. Of note, although groups 3 and 4 received exactly the same immunogens, C2V3C3 administered to group 4 was purified with urea instead of imidazole.

Table 1. BALB/c mice immunization scheme in the pilot study

Groups	ID	HIV-1 env clade	Priming	Boost I (IFA)	Boost II (IFA)	Boost III (CFA)
			Day 1	Day 15	Day 30	Day 45
1	M1-M2	X	X	X	X	X
2	M3-M5	VWR (control)	VV_{WR}	X	X	X
3	M6-M8	C	VVgp120C	C2C3C[a]	C2C3C[a]	Sgp120C
4	M9-M11	C	VVgp120C	C2C3C[b]	C2C3C[b]	Sgp120C
5	M12-M16	CRF02_AG	VVgp120AG	C2C3AG	C2C3AG	Sgp120AG
6	M17-M20	J	VVgp120J	C2C3J	C2C3J	Sgp120J

A total of 20 female BALB/mice (BALB/c ByJ (H-2d, BALB/c)) divided in six groups were first immunized intraperitonealy (IP) with $2x10^7$ PFU of recombinant Vaccinia viruses expressing gp120t from clades C, CRF02_AG and J and boosted with 10µg of C2V3C3 and 7-12.9 µg of gp120 supernatant from the cognate subtype. Each animal was immunized at days 1, 15, 30 and 45. Mice sera was collected fifteen days after immunization. All mice were sacrificed at day 60. Groups 1 and 2 were control groups; ID- Animal Identification; M- Mice; CFA- Complete Freunds Adjuvant; IFA- Incomplete Freund´s Adjuvant; [a] C2C3 eluted in imidazole; [b] C2C3 eluted in urea

In the second experiment, called the main study, 30 female BALB/c ByJ (H-2d, BALB/c) between 8-10 week old were included. Animals were divided in eleven groups (1-5B) according to the immunogen clade and immunization strategy (Table 2).

Animals from groups 3, 4 and 5 were primed (IP route) with $2x10^7$ PFU of recombinant Vaccinia virus expressing gp120t in 100µl of PBS and boosted two times with 10µg of recombinant polypeptides C2V3C3 and one time with 100 µl of soluble gp120t. Animals from groups 3A, 4A and 5A were primed with $2x10^7$ PFU of recombinant Vaccinia virus expressing gp120t in 100µl of PBS and boosted three times with 100 µl of soluble gp120t. Finally animals from groups 3B, 4B and 5B were primed with 100 µl of soluble gp120t and boosted two times with 10µg of recombinant polypeptides C2V3C3 and one time with 100 µl of soluble gp120t. The amount of protein included in the 100 µl was 9.1µg of gp120B, 12.9 µg of gp120C and 7 µg of gp120AG.

Groups 3, 3A and 3B were immunized with immunogens derived from HIV-1 clade C, groups 4, 4A and 4B with immunogens derived from clade CRF02_AG and groups 5, 5A and 5B with immunogens derived from clade B (Table 2). Groups 1 and 2 were control groups. Group 1 didn´t received any immunogen and group 2 was immunized with VV_{WR}. Complete Freund's adjuvant (CFA) (Sigma) was used for priming in groups 3B, 4B and 5B whereas Incomplete Freund's adjuvant (IFA) (Sigma) was used for all the boosts. In

both studies, the schedule of immunization consisted of one priming and three boosts at days 15, 30 and 45. Mice sera were collected immediately before each immunization to access for binding antibodies and 15 days after the last immunization. Mice were sacrificed on day 60.

Table 2. BALB/c mice immunization scheme in the main study

					Boost I (IFA)	Boost II (IFA)	Boost III (IFA)
Groups	**ID**	**HIV-1 env clade**	**Priming**	**Priming adjuvant**	**Day 15**	**Day 30**	**Day 45**
1	M1-M2	X	X	X	X	X	X
2	M3-M4	VWR (control)	VVvwr	X	S_{VVWR}	S_{VVWR}	S_{VVWR}
3	M5-M8	C	VVgp120C	X	C2C3C	C2C3C	Sgp120C
3A	M9-M11	C	VVgp120C	X	Sgp120C	Sgp120C	Sgp120C
3B	M12-M13	C	Sgp120C	CFA	C2C3C	C2C3C	Sgp120C
4	M14-M17	CRF02_AG	VVgp120AG	X	C2C3AG	C2C3AG	Sgp120AG
4A	M18-M20	CRF02_AG	VVgp120AG	X	Sgp120AG	Sgp120AG	Sgp120AG
4B	M21-M22	CRF02_AG	Sgp120AG	CFA	C2C3AG	C2C3AG	Sgp120AG
5	M23M26	B	VVgp120B	X	C2C3C	C2C3C	Sgp120B
5A	M27-M28	B	VVgp120B	X	Sgp120B	Sgp120B	Sgp120B
5B	M29-M30	B	Sgp120B	CFA	C2C3C	C2C3C	Sgp120B

A total of 30 female BALB/cByJ divided in 11 groups were immunized intraperitonealy with different combinations of immunogens:$2x10^7$ PFU of recombinant Vaccinia virus expressing gp120t from HIV-1 subtypes B, C and CRF02_AG in 100µl of PBS; 10µg of recombinant polypeptides C2V3C3 C and C2C3AG and 7-12.9 µg of gp120t. Each animal was immunized at days 1, 15, 30 and 45. Mice sera was collected fifteen days after immunization. All mice were sacrificed at day 60. Groups 1 and 2 were control groups; ID- Animal Identification; M- Mice; CFA- Complete Freunds Adjuvant; IFA- Incomplete Freunds Adjuvant

Envelope-specific antibody binding in mice sera

In order to analyze serum antibody responses, Immuno MaxiSorp 96-well microplates (Nunc) were coated with gp120 supernatant (clades B, C, AG and J) and recombinant polypeptides C2V3C3 (clades C, AG and J) as described above. After overnight incubation at 4°C microplates were blocked with 2% gelatin (Bio-Rad). Mouse anti-serum from days 0, 15, 30, 45 and 60 was heat inactivated for 1 hour at 56ºC added to the microplates (1:200 final dilution in a total volume of 100µl) and incubated for 2 hours at room temperature. Anti-Mouse IgG-Alkaline Phosphatase antibody produced in goat (Sigma) at 1:2000 dilution was added to the microplates as a secondary antibody. Colorimetric reaction was developed as previously described. Negative controls were preimmune serum and serum from mice immunized with VV_{WR}. Positive control was serum from HIV-1 infected individuals. In this case, the secondary antibody was Anti-human IgG- Alkaline Phosphatase antibody produced in goat (Sigma). Sera with an optical density (OD) above the preimmune controls were considered positive.

Neutralization assays

A panel of sixteen HIV-1 viruses including eleven tier 2 HIV-1 from a reference panel [502], three HIV-1 primary isolates (two subtypes J and one CRF02_AG in *Env*) and two tier 1 HIV-1 viruses (NL4.3 and SG3.1) were used in neutralizing assays in order to evaluate the neutralizing antibody response in mice (Table S4). Primary isolates were obtained from HIV-1 infected patients by co-cultivation with peripheral blood mononuclear cells (PBMCs) from seronegative subjects. Env-pseudotyped viruses were produced by transfection of Env-expressing plasmids in 293T cells using pSG3.1Δenv as backbone and titrated in TZM-bl cells as described [502, 544]. Neutralizing activity of mice serum was tested using a single-round viral infectivity assay using a luciferase reporter gene assay in TZM-*bl* cells, as described previously [2, 300]. Briefly, the cells (10.000 cells in 100µl of DMEM supplemented with 10% heat-inactivated fetal bovine serum) were added to each well of 96-well flat-bottom culture plates (Nunc) and allowed to adhere overnight. Next, 100 µl of each virus (200 TCID50/well) were incubated for 1h at 37ºC with heat-inactivated mice sera (56ºC, 1 hour) in a total volume of 200 µl of growth medium (DMEM supplemented with 10% heat-inactivated fetal bovine serum) containing DEAE-Dextran (19.7 µg/ml) and added to the cells. Final serum dilution used in the assays was 1:40. After 48h, culture medium was removed from each well and plates were analyzed for luciferase activity on a luminometer (TECAN) using Pierce™ Firefly Luciferase Glow Assay Kit (ThermoFisher scientific). Wells with medium were used as background control and virus-only control were included as infection control. The effect of pre-immunized serum on infection was used as baseline neutralization. To monitor the amount of neutralization activity that was not HIV specific, each serum sample was tested against a pseudovirus carrying the vesicular stomatitis virus (VSV).

Sera from HIV-1 infected individuals and HuMAbs PG9 and VRC-CH31 in a final concentration ranging between 0.05-0.5µg/ml were used as positive controls of the assay [502]. Percent neutralization was determined by calculating the difference in average RLU (Relative light units) between test wells containing post-immune sera and test wells containing pre-immune sera after the normalization of the results using the average RLU of cell controls[545]. Results were considered valid if the average RLU of virus control wells was ≥10 times the average RLU of cell control wells. Neutralization assays were performed with sera obtained fifteen days after the last immunization (day 60).

Characterization of Tfh and Tfr cells from mice spleen

To better understand the correlation between Tfh/Tfr subsets and NAb production spleen cells were harvested from the animals and red blood cells were lysed. Cells were washed in PBS with 0.01% NaN_3, 2% FBS. Cells were stained for flow cytometry analysis with fluorochrome-labeled monoclonal antibodies anti-CD4-APC, anti-CD8-APC-Cy7, anti-anti-CD25-FITC, anti-Foxp3-Pacific Blue, anti-PD-1-PE-Cy7 and anti-CXCR5-PercP (BioLegend®, San Diego) following the standard procedures for surface and intracellular stainings. Tfh cells were identified as $CD4^+$ $PD\text{-}1^+$ $CXCR5^+$ cells and Tfrs as $CD4^+$ $PD\text{-}1^+$ $CXCR5^+$ $CD25^+$ $Foxp3^+$ cells[262, 263].

Statistical analysis

Statistical analysis was performed with GraphPad Prism 5.0. Medians of C2V3C3 binding responses were estimated with interquartile range (IQR). Kruskal-Wallis test and Dunn´s multiple comparison test was used to compare median binding antibody responses against recombinant polypeptides C2V3C3 from different subtypes. Non parametric Mann Whitney test was used to compare antibody binding titers. Spearman's rank correlation coefficient (r) was used to analyze the association between IgG binding responses of mice sera and neutralization percentage and the association between Tfh and Tfr counts and binding antibodies levels and neutralization activity. Statistical significance between two groups was determined using the two tailed non-parametric Mann-Whitney *U* test. Linear regression was calculated to determine the correlations between parameters. *P* values <0.05 were considered significant.

Results

Generation of recombinant Vaccinia Viruses expressing HIV-1 gp120t from different subtypes

Phylogenetic analysis showed that the envelope gene sequences belonged to subtypes B, C, CRF02_AG, G, H and J thereby representing the most frequent subtypes worldwide (Figure 1). In addition, all HIV-1 isolates used the CCR5 co-receptor.

Recombinant Vaccinia viruses expressing the truncated surface glycoprotein gp120 from subtypes B, C, CRF02_AG, H and J were obtained with infectious titers in the range of

10^7-10^9 PFU/ml. To investigate the expression of truncated gp120 proteins, HeLa-CCL2 cells were infected with recombinant Vaccinia (5 PFU/cell) and cell lysates and cell-free supernatants were obtained and analyzed. Western-blot analysis with plasma from HIV-1-infected patients demonstrated the presence of gp120t both in cell lysate and in cell supernatant (~120kDa) albeit at different levels (Figure 2A-B). The amount of gp120t recovered from cell supernatant ranged from 63 to 129 µg/ml. No gp160 or gp41 was observed as expected. The presence of a highly reactive second band above the 120kDa band in Western Blot analysis is consistent with different patterns of glycosylation.

gp120t binds to V3 and CD4bs-specific neutralizing monoclonal antibodies

To gain further insights into their antigenic structure, the binding reactivity of gp120 immunogens used in the main immunization assay (Sgp120B, Sgp120C and Sgp120AG) was tested in an ELISA assay against sera from HIV-1 infected patients and Human bNAbs targeting the CD4bs, V3 and N-linked glycans in C2, C3, V4 and C4 regions. C2V3C3 immunogens were not included in this analysis because we assumed that they were not responsible for the neutralization observed. Antibodies from HIV-1 infected patients and HuMAb 447-52D bound to all glycoproteins (Table 3).

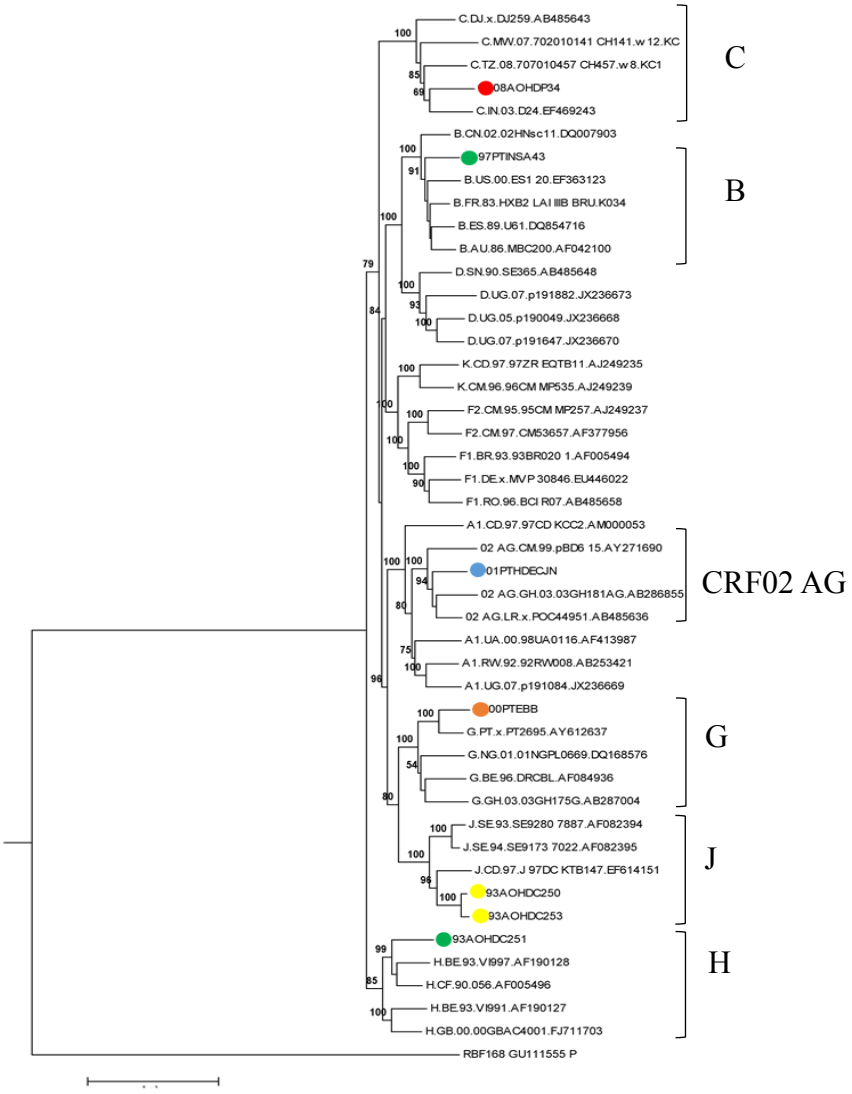
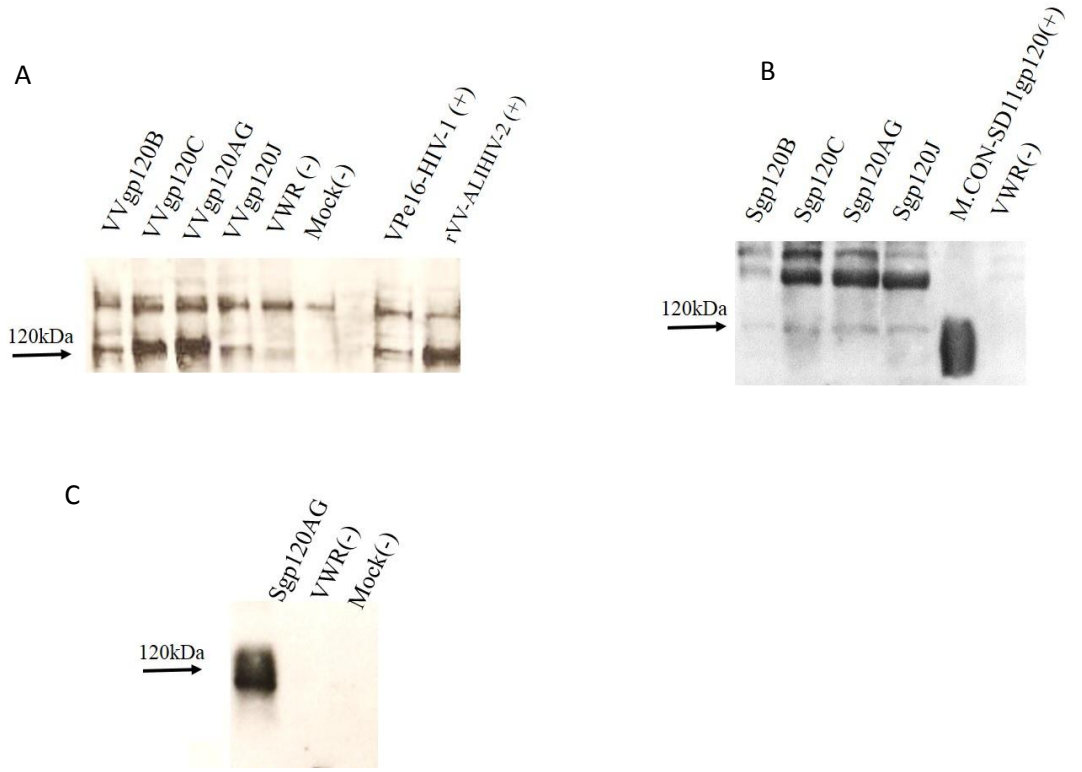


Figure 1- Phylogenetic analysis of HIV-1 *env* genes used as vaccine immunogens. The maximum likelihood phylogenetic trees were constructed with alignments of reference sequences representative of all HIV-1 group M subtypes and the *env* gene sequences from Angolan and Portuguese patients. Env sequences used to produce vaccine immunogens are represented with colored dots. Sequence RBF168 GU111555 belonging to HIV-1 group P was used as outgroup. Only bootstrap values above 70% are shown.

For gp120AG, which was the protein expressed to higher levels, this result was further confirmed by Western-blot analysis (Figure 2C).The epitope of 447-52D is the crown sequence GPGR of the V3 loop [546]. HJ16 (CD4bs-specific) bound to all glycoproteins in the ELISA assay, but not in WB analysis (Table 3).

These results demonstrate that the novel glycoproteins present adequately the continuous V3 loop neutralizing epitope and suggest that it fails to form the neutralizing epitopes of a conformational nature.



F**igure 2- Western blot analysis of gp120t expressed by recombinant Vaccinia virus VVgp120B, VVgp120C, VVgp120AG and VVgp120J.** Cell lysate (A) and cell supernatant (B) were incubated with HIV positive sera. Cell supernatant from clade AG (Sgp120AG) was incubated with V3 HuMAb 447-52D (C). vPE16, a recombinant Vaccinia virus expressing the HIV-1 envelope, rVV/ALI, a recombinant Vaccinia virus expressing the HIV-2ALI envelope and M.CON.S-D11 a gp120 subunit Env from the group M consensus sequence, were used as HIV positive controls. Mock-uninfected cells; VWR- cells infected with the WR strain of Vaccinia virus used (negative controls).

gp120AG has a GPGR motif in V3 crown region and a high probability of glycan occupancy on position N332

Analysis of the amino acid sequence of all our immunogens revealed that all isolates (immunogens) differ at sequence level and number of amino acids (Figure S2). For instance, V2 (126C-196C, HXB2), V3 loop (296C-331C, HXB2), V4 hypervariable region (385C-418C, HXB2) and C4Dbs beta sheet 23/24 (451G-471G, HXB2) were the most divergent regions regarding sequence and number of amino acids, whereas CD4 binding loop (345S-374H, HXB2), loop D (275V-283T) and gp120/gp41 interface (56S-82Q) were the most conserved regions. Interestingly, we found that gp120AG had a GPGR motif in a conserved epitope in V3 crown region (GPGQ/R) in contrast with GPGQ motif presented in the majority of the tested viruses, including tier 1 and 2 viruses from clades A, C, CRFO7, G and J. The data also revealed that gp120AG had a high

probability of glycan occupancy (Pg=0.79) in position 332 (HXB2) as demonstrated by the presence of the motif NVS in this position (Figure S2)[547].

Table 3. Binding activity of selected human monoclonal bNAbs against the new monomeric gp120 glycoproteins and reference gp120 and gp140 glycoproteins

HuMAbs	Epitope	SF162gp140	M.CON.S D11gp120	Sgp120B	Sgp120C	Sgp120AG
3BNC117	CD4bs	3.82	11.47	0.81	1.05	0.86
HJ16	CD4bs	0.31	11.47	1.08	1.71	1.10
PG16	V2	0.20	2.37	0.91	1.24	0.86
2G12	N-linked glycans	3.82	11.47	0.68	0.97	0.91
b12	CD4bs	3.82	11.47	0.76	0.97	0.63
VRC01	CD4bs	2.96	31.62	0.94	0.91	0.87
447-52D	V3	2.96	31.62	2.32	2.62	2.81
HIV+ sera	unknown	3.82	7.53	3.18	1.67	2.59

Green values correspond to positive results as defined by OD/cut-off ≥ 1; The clinical cut-off value of the assay was calculated as the mean OD value of 2F5 reactivity plus 2 times the SD. For the positive control of the assay (HIV+) as well as SF162gp140 (which reacts with 2F5) the cut-off value was calculated as the mean OD value of HIV-seronegative samples plus 2 times the SD. HuMAbs were used at a final concentration of 10µg/ml;

Immunogenicity of the C2V3C3 polypeptides

Recombinant polypeptides comprising the C2V3C3 envelope region were obtained for subtypes C, CRF02_AG, G, H and J with concentrations ranging between 80-120 µg/ml. Antigenic reactivity of the purified polypeptides was analyzed by Western blot (Figure 3) with plasma from HIV-1 infected patients from Angola and quantified in an ELISA assay.

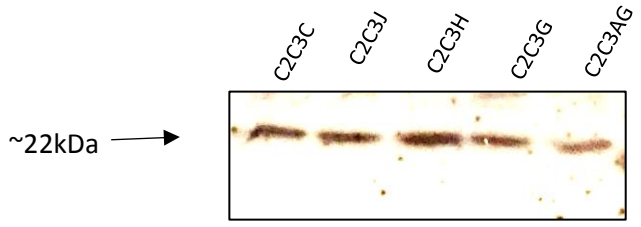


Figure 3- Western-Blot analysis (12%) of HIV-1 C2V3C3 recombinant polypeptides from clades C, J, H, G and CRF02_AG expressed in *Escherichia coli* strain TOP10. All recombinant polypeptides were incubated with serum from HIV-1 infected individuals.

The majority of plasma samples (n=28) reacted with the recombinant polypeptides indicating that the polypeptides present a conformation that was recognized by anti-HIV-1 antibodies. Median binding antibody titers (log_{10}) ranged from 2.151 to 2.903 (Figure 4B). Higher titer of binding antibodies was observed for subtype C (C2V3C3C) and the lower for subtype G (C2V3C3G) ($p<0.05$) which can be related with the predominance of subtype C in Angola [527, 528].

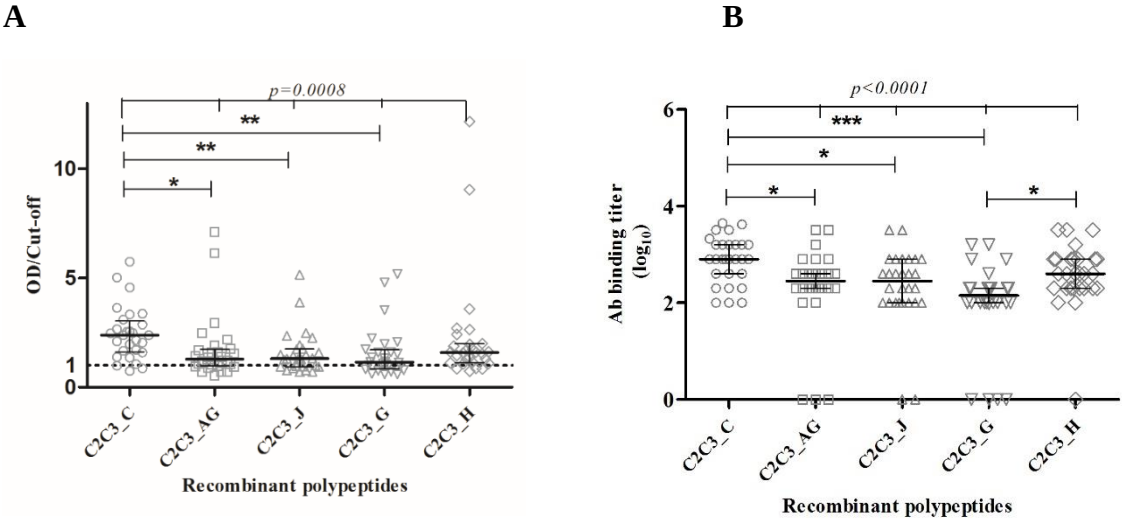


Figure 4- Antibody reactivity (A) and antibody binding titer (B) of 28 plasma samples against recombinant polypeptides C2V3C3 from different subtypes. A- Dot-plot graphic showing the median binding antibody reactivity (OD/Cutoff) and interquartile range of 28 plasma samples against five different recombinant polypeptides from clades C, CRF02_AG, J, G and H. B- Dot-plot graphic showing the median binding antibody titers and interquartile range of 28 plasma samples against the different recombinant polypeptides. Kruskal-Wallis test and Dunn´s multiple comparison test was used to compare the median values. *p* values < 0.05 were considered statistically significant. The black line in each group represents the median value. *$p=\leq 0.05$, **$p\leq 0.01$, ***$p\leq 0.001$.

Envelope glycoproteins elicit cross-reactive gp120 binding antibodies and neutralizing antibodies in mice

To investigate the combined immunogenicity of our HIV-1 immunogens, BALB/c mice were inoculated with several combinations of recombinant VV expressing gp120t (VVgp120), soluble gp120t (Sgp120t) and recombinant polypeptides C2V3C3 from different clades (Table 1 and Table 2).

Binding antibody responses were analyzed in mice sera drawn at five different timepoints (T0-T4) using an ELISA assay with Sgp120t and recombinant polypeptides C2V3C3 as capture antigens. For all immunized groups, binding antibody responses were higher than

those from control groups. In the pilot study, the majority of immunized mice developed binding antibodies against C2V3C3 only after the third immunization and at low levels (Figure S1). Moreover, strong antibody responses against this polypeptide were only observed in mice immunized with clade C immunogens (Figure S1). In contrast, all immunized mice developed antibodies that reacted with autologous and heterologous Sgp120t soon after the priming with no significant differences between subtypes. Neutralizing antibodies against tier 2 viruses from different clades were produced in at least one animal per group (Table 4, Figure 5A). At 1:40 dilution, animals immunized with clade C, were able to neutralize >50% two heterologous tier 2 pseudoviruses from clades A and C and one primary isolate from clade J. In addition, one animal immunized with clade J neutralized at more than 50% only one heterologous tier 2 pseudovirus from clade C. Remarkably, one animal immunized with AG immunogens (M13, Group 5) elicited neutralizing antibodies against four tier 2 pseudoviruses and 2 primary isolates from different clades (Table 4). Overall, the percentage of neutralized tier 2 viruses per group was 23% (3/13) for group 3 (immunized with clade C), 46% (6/13) for group 5 (immunized with clade AG) and 8% (1/13) for group 6 (immunized with clade J).

To confirm and extend these results we performed a second immunization experiment in a different facility and with some modifications. For this study, 11 groups of 30 female BALB/cByJ 8-10 weeks old were included. All groups (except control groups) were immunized with different combinations of our immunogens, namely recombinant Vaccinia virus expressing gp120t from clades B, C and CRF02_AG, C2V3C3 polypeptides and soluble gp120t (Table 2). All immunized mice generated IgG antibodies against autologous and heterologous Sgp120t but only some of them produced antibodies against C2V3C3 (Figure 6). In all groups, mice produced cross-reactive antibodies against autologous and heterologous Sgp120t soon after the priming. Like in the pilot study priming followed by three boosts was strictly necessary to achieve a potent binding antibody response against gp120t (subtypes B, C and AG). Overall, mice primed with gp120t produced the strongest IgG response against gp120t from different clades comparing with groups primed solely with Recombinant Vaccinia virus (groups 3B, 4B and 5B- Figure 6).

Similar to the pilot study we observed that mice immunized with clade C immunogens developed the strongest response against C2V3C3 polypeptides although only after the third immunization indicating that C2V3C3 antibodies arise slowly.

A- Pilot study

B- Main study

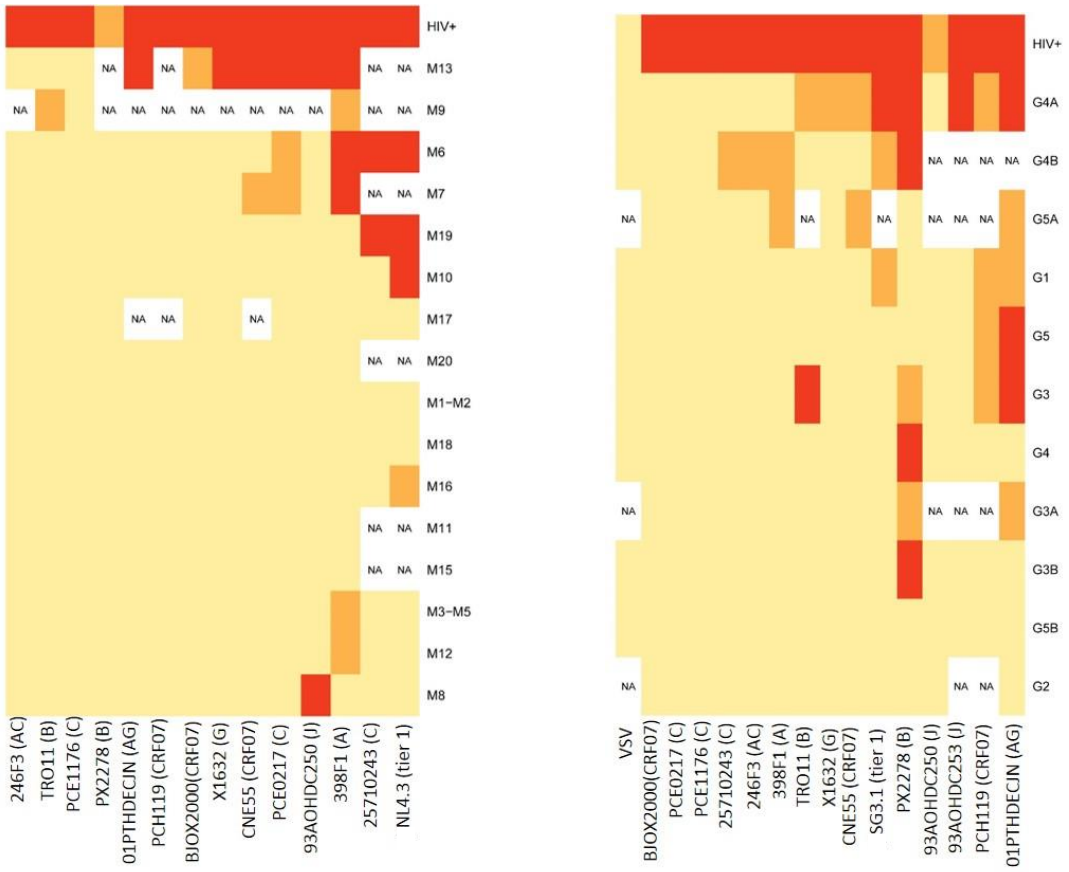
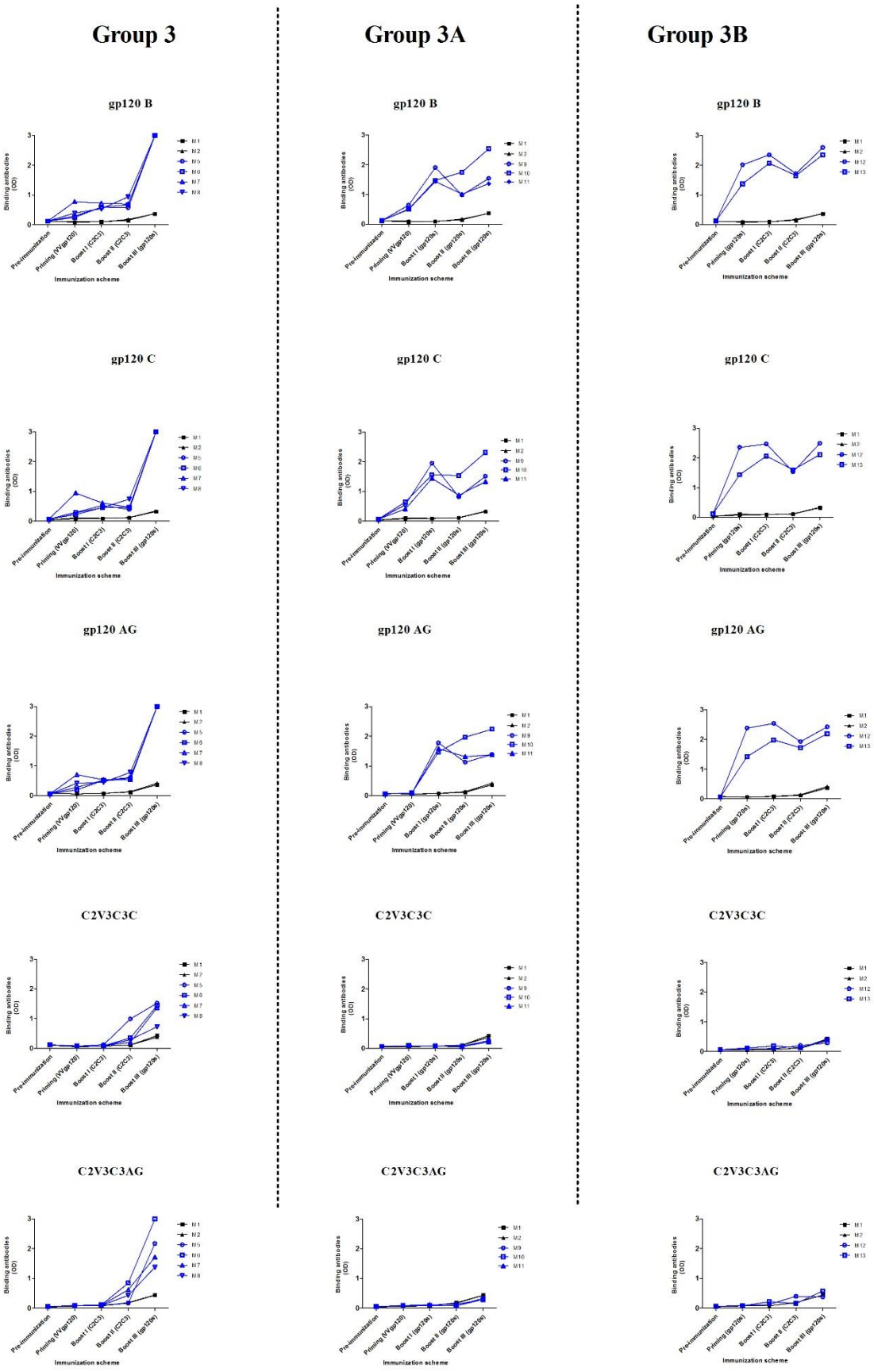


Figure 5- Heatmap graphic representation of sera neutralization potency and virus sensitivity from immunized mice from the pilot study (A) and main study (B) against 12 tier 2 pseudoviruses, 3 primary isolates (93AOHDC250, 93AOHDC253, 01PTHDECJN) and two tier 1 viruses (SG3.1 and NL4.3). The color key histogram summarizes the percentage of neutralization. More potent sera is represented by red. To monitor the amount of neutralization activity that is not HIV-1 specific each serum (final dilution 1:40) was tested against a pseudovirus carrying the VSV (only for main study); light yellow indicates <30% neutralization; orange indicates neutralization values between 30-48%; red indicates >49% of neutralization. Missing neutralization data are indicated by white cells (NA) in the heatmap

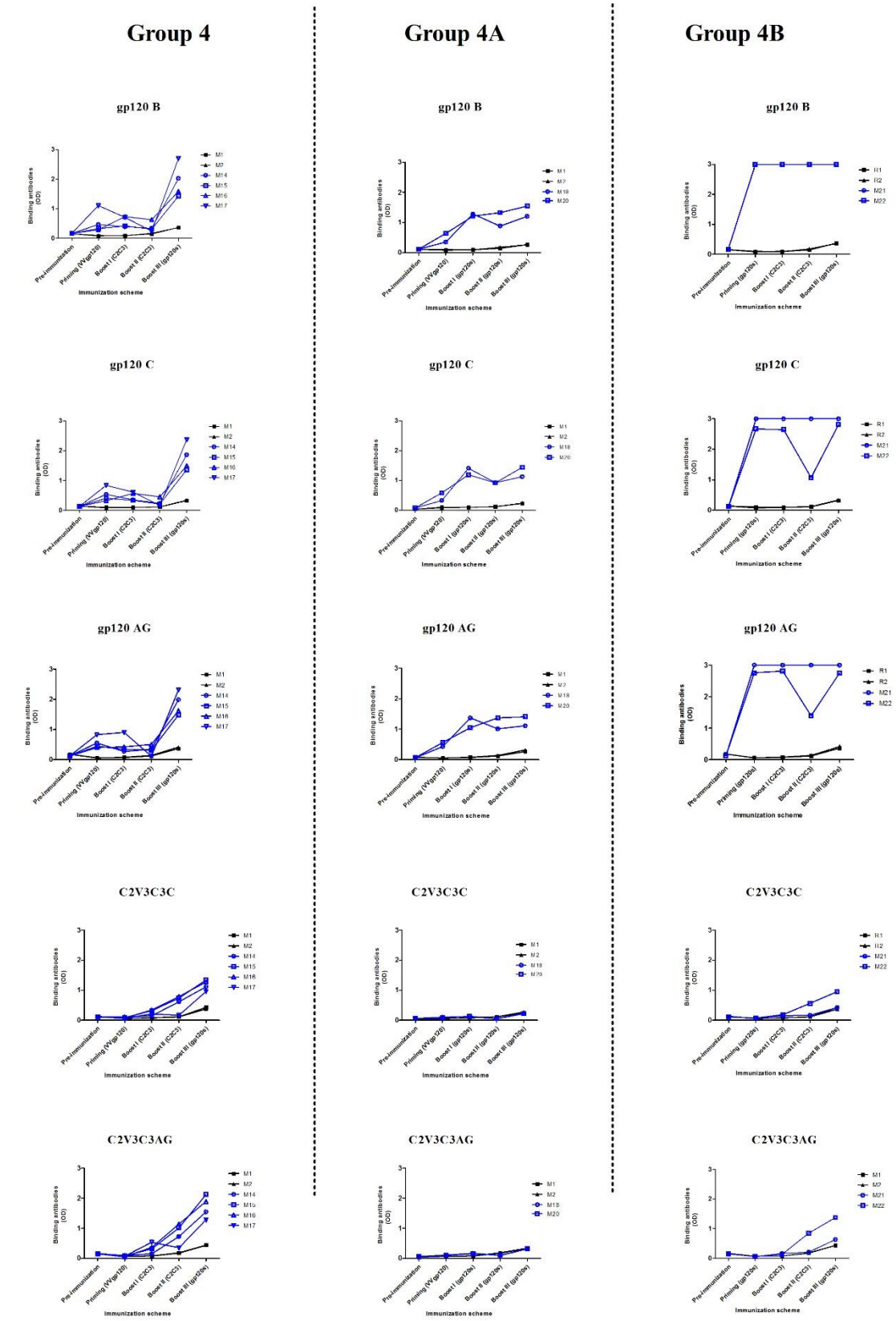
Moreover, in all groups, mice immunized with C2V3C3 polypeptides developed a moderate binding IgG response against C2V3C3 polypeptides from different clades. Interestingly, mice immunized with VV expressing gp120 and boosted three times with cognate gp120t did not produced antibodies against C2V3C3 indicating that epitopes present in the C2V3C3 polypeptides were not presented in gp120 immunogens (Groups 3A, 4A and 5A-Figure 6). Overall, there were no significant differences between immunogen responses, *i.e.*, groups that were immunized with an immunogen from a specific subtype didn´t produce more binding antibodies to the autologous immunogen compared with the heterologous one. Regarding antibody neutralization, at 1:40 dilution

at least one animal per main group (immunized with subtypes C, AG, and B) neutralized at least one tier 2 virus at more than 50%, including viruses from different clades (Table 5; Figure 5B). However, there were significant differences between subtype immunogen concerning neutralization efficacy. Mice primed with VV expressing gp120t AG and boosted with the cognate gp120t (Group 4A) produced a cross-reactive neutralizing response (>49%) against two HIV-1 primary isolates, one heterologous (clade J) and one autologous (clade AG), and two heterologous tier 2 pseudoviruses (clades B and CRF07_BC). As expected, mice sera from this group potently neutralized the autologous virus (01PTCJN) (70% neutralization) (Table 5). Mice immunized with gp120 from clade B plus C2V3C3 from clade C (Groups 5, 5A and 5B) had the weakest neutralizing response with only one group (M23-M26, Group 5) neutralizing one virus at more than 50% (Table 5). Sera from Group 3 (immunized with clade C immunogen), neutralized one tier 2 virus (Clade B) and one primary isolate from clade AG while sera from group 4 (clade AG) neutralized only one tier 2 virus from clade B (>53%) (Table 5; Figure 5B). Both groups 3 and 4 were primed with VV expressing gp120t and boosted with C2V3C3 polypeptides and gp120t. The weakest neutralizing responses were observed in animals primed solely with gp120t (groups 3B, 4B and 5B) instead of recombinant VV. Curiously, these groups achieved the higher IgG binding response against all gp120t indicating that the majority of antibodies generated were of non- neutralizing type (Figure 6). Tier 2 clade B virus PX2278 was the most neutralized virus followed by primary isolate 01PTHDECJN (Table 5, Figure 5B). In fact, PX2278 was neutralized at more than 50% by all mice from groups immunized with clade AG immunogens (groups 4, 4A and 4B) which suggests that this immunogen may be useful for the development of a subtype B vaccine. Overall, the total percentage of neutralized tier 2 viruses per group were 21% (3/14) for mice immunized with clade C (groups 3, 3A, 3B), 43% (6/14) for groups immunized with clade AG (groups 4, 4A and 4B) and 7% for groups immunized with clade B+C (5, 5A, 5B). Tier 2 viruses BJOX2000 (clade CRF07), PCE1176 (clade C) and 246F3 (clade AC) were not neutralized by any sera in both studies. Both experiments (pilot and main study) generated coherent and reproducible results demonstrating that groups immunized with clade AG immunogens produced the best neutralizing response.

A


Group 3
Group 3A
Group 3B
gp120 B
gp120 C
gp120 AG
C2V3C3C
C2V3C3AG
Binding antibodies (OD)
Immunization scheme
Pre-immunization
Priming (VVgp120)
Boost I (C2C3)
Boost II (C2C3)
Boost III (gp120e)
Priming (gp120e)
Boost I (gp120e)
Boost II (gp120e)
Boost III (gp120e)
M1
M2
M5
M6
M7
M8
M9
M10
M11
M12
M13

B


Group 4
Group 4A
Group 4B
gp120 B
gp120 C
gp120 AG
C2V3C3C
C2V3C3AG
Binding antibodies (OD)
Immunization scheme
Pre-immunization
Priming (VVgp120)
Priming (gp120e)
Boost I (C2C3)
Boost II (C2C3)
Boost III (gp120e)
M1
M2
M14
M15
M16
M17
M18
M20
R1
R2
M21
M22

C

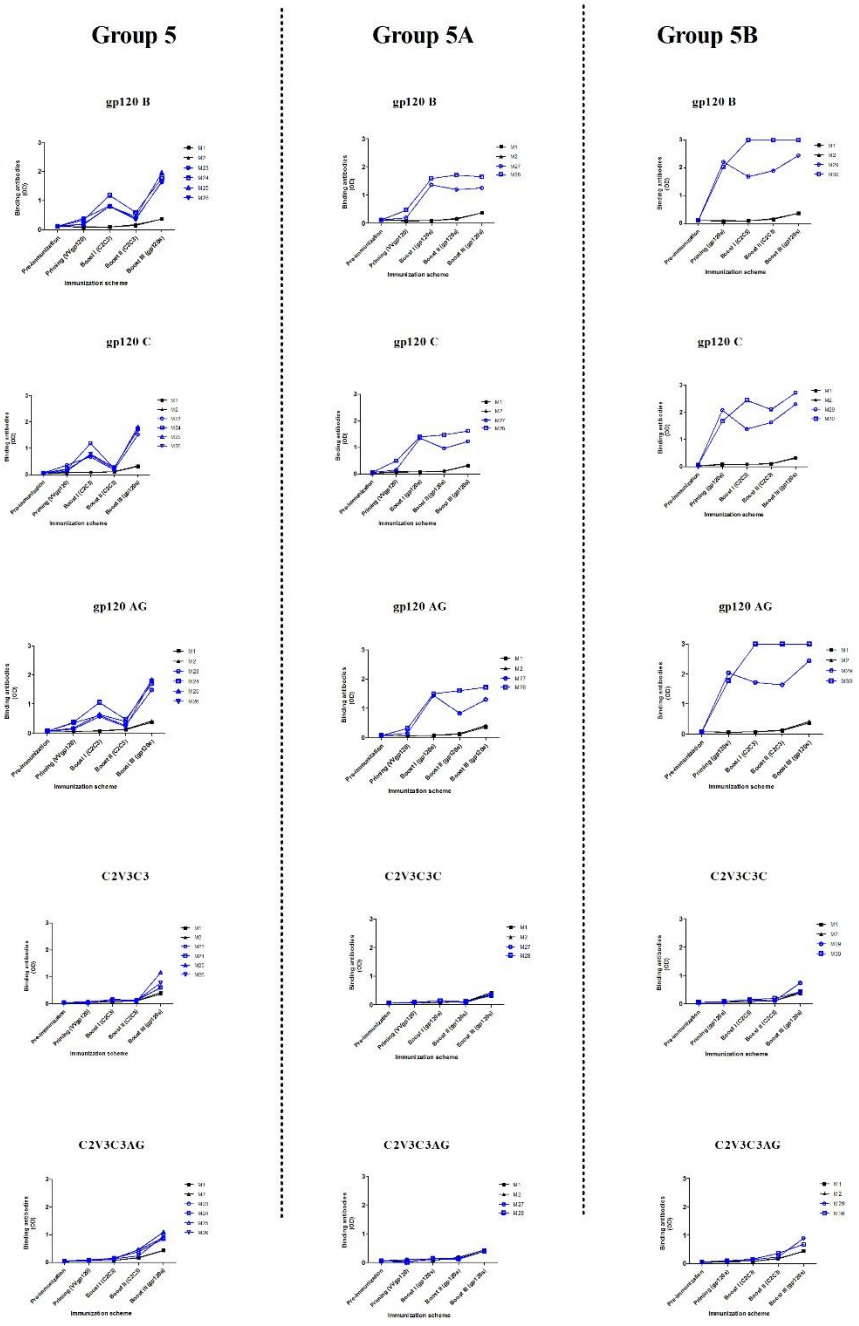


Figure 6- Binding IgG response (OD values) over time against gp120t supernatant (B, C, AG) and C2V3C3 polypeptides (C,AG) in BALB/c mice immunized with different combinations of HIV-1 immunogens (main study): Groups 3, 4 and 5- mice primed with VV expressing gp120t and boosted with C2V3C3 polypeptides and gp120; Groups 3A, 4A and 5A- mice primed with VV expressing gp120t and boosted with gp120; Groups 3B, 4B and 5B- mice primed with gp120t and boosted with C2V3C3 polypeptides and gp120 (A)- Mice immunized with clade C immunogens; (B)- Mice immunized with clade AG immunogens; (C)- Mice immunized with clades B (gp120) and C (C2V3C3) immunogens. For all mice, the schedule of immunization included one priming and three boosts at days 15, 30 and 45. Fifteen days after each immunization, sera were collected and assayed for the presence of binding antibodies against HIV-1 immunogens.Blue lines represent immunized mice from the respective group; black lines represent mice from control group (G1-M1-M2).

Table 4- Neutralizing activity in sera of mice immunized in the pilot study.

Immunization schedule	Mice ID	Group	Immunogen clade	tier1	tier 2													
				B	A	J	C	C	CRF07	G	CRF02_AG	CRF07	B	B	C	AC	CRF07
				pNL4.3	398F1	93AO250	25710243	PCE0217	CNE55	X1632	01PTCJN	BJOX2000	PX2278	TRO11	PCE1176	246F3	PCH119
Uninfected control	M1-M2	1	_	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
$VV_{VWR}+S_{VWR}$	M3-M5	2	_	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
V VVgp120+C2C3+C2C3+Sgp120	M6	3	C	58	69	<30	59	35	<30	<30	<30	<30	<30	<30	<30	<30	<30
	M7			ND	61	<30	ND	47	41	<30	<30	<30	<30	<30	<30	<30	<30
	M8			<30	<30	50	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
	M9	4	C	ND	47	ND	ND	ND	ND	ND	ND	ND	ND	31	<30	ND	ND
	M10			49	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
	M11			ND	<30	<30	ND	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
	M12	5	CRF02_AG	<30	37	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
	M13			ND	76	55	ND	56	57	50	49	47	ND	<30	<30	<30	ND
	M15			ND	<30	<30	ND	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
	M16			<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
	M17	6	J	<30	<30	<30	<30	<30	ND	<30	ND	<30	<30	<30	<30	<30	ND
	M18			<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
	M19			73	<30	<30	54	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
	M20			ND	<30	<30	ND	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
Positive control (HIV-1 neutralizing sera)				95	78	87	92	63	67	90	51	78	48	69	91	75	75

Columns subheading indicate virus designation, clade and tier; Percent neutralization was determined in TZM-bl cells measuring the reduction in number of RLU relative to wells with the corresponding preimmune sera. Beige highlighting indicate less than 30% of neutralization; salmon highlighting indicate ≥ 31-49%; red highlighting indicate equal or more than 50% of neutralization; ND indicate the groups where neutralization activity was not measured. HIV-1 positive control was used at 1:20 final dilution; mice sera was used at 1:40 final dilution.

Table 5- Neutralizing activity in sera of mice immunized in the main study.

Immunization schedule		Groups	tier1	tier 2													VSV
			B	B	B	CRF02_AG	CRF07	J	CRF07	A	G	C	C	C	CRF07	AC	J	
			pSG3.1	PX2278	TRO11	01PTCJN	PCH119	93HDC253	CNE55	398F1	X1632	PCE1176	25710243	PCE0217	BJOX2000	246F3	93HDC250	
Uninfected control		G1	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
$VV_{VWR}+S_{VWR}$		G2	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	ND
	VVgp120+C2C3+C2C3+gp120	G3	<30	40	52	54	34	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
	VVgp120+gp120+gp120+gp120	G3A	<30	42	<30	46	ND	ND	<30	<30	<30	<30	<30	<30	<30	<30	ND	ND
C	gp120+C2C3+C2C3+gp120	G3B	<30	55	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
	VVgp120+C2C3+gp120	G4	<30	53	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
	VVgp120+gp120+gp120+gp120	G4A	56	52	44	70	49	50	36	<30	<30	<30	<30	<30	<30	<30	<30	<30
CRF02_AG	gp120+C2C3+C2C3+gp120	G4B	44	51	<30	ND	ND	ND	<30	38	<30	<30	<30	<30	<30	<30	ND	ND
	VVgp120+C2C3+gp120	G5	<30	<30	<30	58	48	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
	VVgp120+gp120+gp120+gp120	G5A	<30	<30	ND	44	ND	ND	35	43	<30	<30	<30	<30	<30	<30	ND	ND
B+C	gp120+C2C3+C2C3+gp120	G5B	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
Positive control (HIV-1 neutralizing sera)			99	93	69	51	57	75	95	67	80	91	92	94	78	95	38	<30

Columns subheading indicate virus designation, clade and tier; Percent neutralization was determined in TZM-bl cells measuring the reduction in number of RLU relative to wells with the corresponding preimmune sera. Beige highlighting indicate less than 30% of neutralization; salmon highlighting indicate ≥ 31-49%; red highlighting indicate equal or more than 50% of neutralization; ND indicate the groups where neutralization activity was not measured. HIV-1 positive control was used at 1:20 final dilution; mice sera was used at 1:40 final dilution; VSV was used as the negative control.

Vaccine strategy affects Tfh and Tfr balance

In order to investigate which $CD4^+$ T cell subsets were involved in NAbs induction we determined the number and proportion of Tfh and Tfr cells using flow cytometry. There was a positive correlation between Tfh cell number and binding antibody reactivity against all gp120 immunogens [(r(gp120C)=0.764; r(gp120AG)=0.770; r(gp120J)=0.768, P=0.0001)] (Figure 7A). However, no correlation was found between Tfh levels and neutralization activity. Tfr proportion (%) inversely correlated with neutralization potency (r=-0.6606, P=0.0438) and breadth (r=-0.9710, P= 0.028) (Figure 7B).

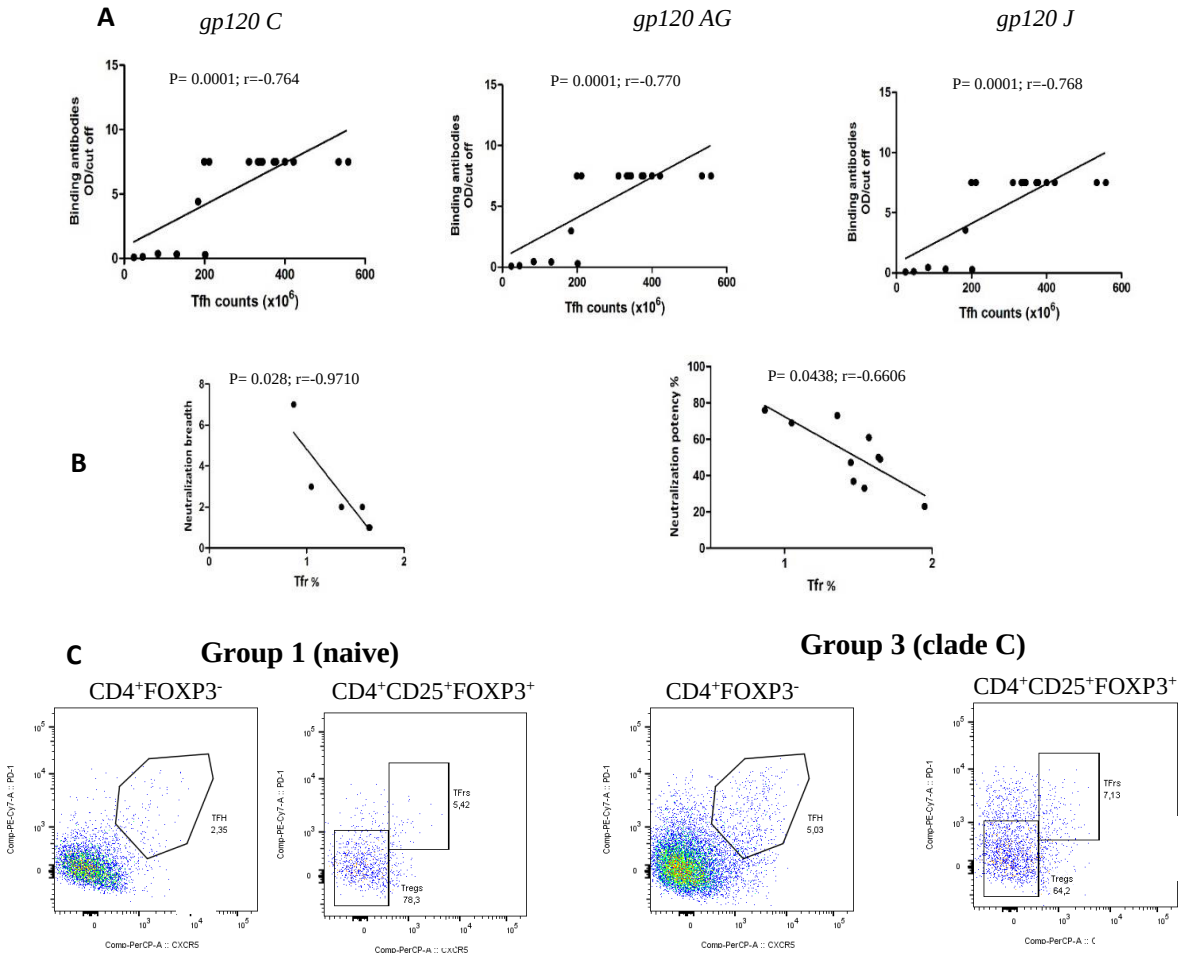


Figure 7 – Tfh and Tfr cell counts significantly correlate with levels of binding antibodies in mice primed with Vaccinia viruses expressing gp120t and boosted with C2V3C3 polypeptides and gp120t from clades C, AG and J (pilot study). A- The figure represents the linear regression between serum antibody levels (OD/cut off) of immunized mice (day 60) detected by ELISA assays against the different HIV-1 immunogens (C, AG and J) and total Tfh counts ($x10^6$) from mice spleens. **B.1**-Tfr cells percentage measured within CD4 T cell subset inversely correlate to neutralization potency (% of neutralization) induced by the same mouse serum (p=0.0438). **B.2**- Tfr cells percentage measured within CD4 T cell subset inversely correlate to the number of viruses neutralized (neutralization breadth by the same mouse serum (p=0.028). **C**- Flow cytometry plots of Tfh (within $CD4^+FOXP3^-$) and Tfr (within $CD4^+CD25^+FOXP3^+$) cell percentage representative of control group (G1, M1) and immunized animal with neutralization activity (G3, M6).

Discussion

We show here that mice primed with VV expressing gp120AG and boosted with the cognate gp120AG developed autologous and heterologous neutralizing antibodies against different HIV-1 tier 2 clades which is the main goal of a global HIV vaccine.

Immunogens used in this study were derived from the envelope genes of primary isolates originated from Portugal and Angola belonging to clades C (Angola, 2008), J (Angola, 1993), B (Portugal, 1997) and CRF02_AG (Portugal, 2001). Of note, the "Portuguese" CRF02_AG viral isolate was obtained from a child of Angolan origin that was in Portugal at the time of the sample collection.

We hypothesized that envelope glycoproteins from viruses from an old epidemic such as the HIV epidemic in Angola would be better at eliciting bNAbs against heterologous viruses as they comprise key epitopes and conformational determinants that should be conserved within the contemporaneous strains due to functional constraints [46, 66, 67]. All envelope glycoproteins were derived from R5 isolates because R5 isolates dominate the early stages of HIV disease being preferentially transmitted over X4 isolates. To our knowledge, this is the first vaccine study using envelope immunogens from viruses coming from an old epidemic and from HIV-1 clades CRF02_AG and J where neutralizing antibodies against tier 2 HIV-1 isolates were produced [471].

Our results demonstrate clear differences in neutralization efficacy related with the immunogen clade. Immunogens derived from clade CRF02_AG were able to elicit bNAbs against six heterologous HIV-1 tier 2 pseudoviruses and three primary isolates. The ability to raise neutralizing antibodies against primary isolates is noted because primary isolates are produced in PBMCs and unlike envelope-pseudotyped viruses their glycosylation profile is similar to clinically relevant isolates[548, 549].

Interestingly, mice immunized with gp120 from subtype B, the most common subtype used in vaccine trials, raised the weakest neutralizing responses with only one group neutralizing one tier 2 virus at more than 50% [408, 466-468]. The fact that an AG-based immunogen could induce broadly neutralizing responses against HIV-1 tier 2 viruses from different clades (including clade B) supports the urgent need of new vaccine immunogen design studies that have non-B subtypes in consideration.

Unlike previous vaccination studies in HIV-2[2], the C2V3C3 polypeptides did not contribute for the neutralizing responses in vaccinated animals indicating that they are

unable to effectively present the V3 neutralizing epitopes and confirming that there are major differences in the antigenic structure of this variable region between HIV-1 and HIV-2[550]. Another interesting finding was the higher IgG binding response against the different gp120 (B, C and AG) in groups that were primed with gp120t solely instead of recombinant VV (Figure 6- Groups 3B, 4B and 5B). However, this high IgG levels didn´t correlate positively with increased neutralization activity. In fact, these three groups achieved the weaker neutralizing response. These results are consistent with several studies and clinical trials demonstrating that the administration of monomeric gp120 or other Env proteins alone is not enough to induce broad and potent neutralizing antibodies against HIV infection [2, 408, 467, 468, 478, 487-489, 551]. However, elicitation of bNAbs by the same monomeric gp120 protein was achieved in animals primed with recombinant VV expressing truncated gp120.

The observation that only groups primed with Vaccinia virus developed bNAbs emphasize the importance in the choice of the prime-boost regimen suggesting a possible role for the use of replication-competent virus in HIV-1 vaccine design [2, 422, 448]. Poxvirus vectors are among the most used in vaccine development due, in part, to the success of the Vaccinia virus vaccine in eradicating smallpox [387, 420]. However, due to safety issues they have been replaced by safer non-replicating human poxvirus vectors such as MVA, NYVAC and Canarypox (ALVAC) [118, 420, 421]. Although these vectors are able to induce humoral and cellular immune responses in the host they fail in inducing consistent bNAb responses against tier 2 viruses when used as vaccine candidates[118, 419, 455, 552-554].

Replicative-competent viral vectors, have been described as being more effective in conferring protection against infection as they elicit stronger humoral and cell-mediated immune responses when compared with highly attenuated non-replicative viral vectors [387, 420, 421, 555]. For instance, Shiu-Lok Hu *et al,* [422] using a prime-boost approach based on a recombinant VV expressing full Env gp160 and subunit gp120 protein, was able to induce in rabbits cross-reactive neutralizing activities against >50% of tier 2 global HIV-1 Env-pseudotyped viruses. The use of a live recombinant Vaccinia virus vector in our immunization strategy may have been determinant for the generation of a strong Tfh mediated cellular response which in turn enabled an effective NAb production. The positive association between Tfh cells response and gp120-specific binding antibody response provide additional support for Tfh cells as important players in the induction of

an HIV-specific antibody response, regardless of the lack of correlation with neutralization[240, 254-256]. Furthermore, Tfrs which are in fact involved not only in the inhibition of Tfh function, but also in B cell maturation and antibody production processes, were inversely correlated to our neutralization parameters, including potency and breadth (percentage of neutralization and number of viruses neutralized), when analyzed individually for each mice serum [259, 260, 262]. Therefore, the efficiency of this new vaccine strategy including the induction of neutralizing antibodies may rely on the triggering of these specific subsets of the T cell response. Overall, our results provide additional support for the use of replicating competent recombinant Vaccinia viruses as a component of an HIV vaccine as they can induce the cell-mediated immune responses that are important for antibody production and HIV-specific neutralizing antibodies.

In order to better understand why CRF02_AG immunogens induce more potent and broad neutralizing responses compared with the immunogens from other clades we analyzed the amino acid sequence of all our immunogens (Figure S2). The observation that gp120AG had a GPGR motif in a conserved epitope in V3 crown region (GPGQ/R) and the majority of the tested viruses had the GPGQ motif was interesting since GPGR is uncommon outside subtype B. For subtype B the consensus sequence for the V3 crown motif is GPGR, while for non-B subtypes is often GPGQ [330, 556-559]. Of note, 447-42D was the only monoclonal antibody that recognized our immunogen both in ELISA and Western Blot analysis. 447-52D binds to and neutralizes preferentially virus with GPGR motif in the V3 crown with limited capacity against those with the GPGQ motif [329, 330, 556]. Another interesting observation related with this was that the PX2278 tier 2 HIV-1 from clade B, which has a GPGR motif, was neutralized by all AG immunized groups (4, 4A and 4B). Overall, these results suggest that unlike the other envelope constructs gp120AG induces the production of potent neutralizing antibodies directed against the V3 crown. Importantly, the g120AG immunogens were able to induce bNAbs against B and non-B Env-pseudoviruses and HIV-1 primary isolates that did not present the GPGR motif indicating that NAbs targeting other epitopes were also elicited. Broadly neutralizing antibody HJ16 [325] (obtained from a clade C infected patient), bound to all gp120 immunogens in ELISA assay (albeit at lower levels) suggesting some exposition of CD4 binding site epitopes. On the other hand, the lack of binding to other NAbs targeting conformational epitopes is consistent with the monomeric nature of our immunogens. In sum, the demonstration that antibodies induced by the novel vaccine

immunogens neutralized tier 2 HIV-1 isolates from different clades despite targeting essentially the V3 crown provides further support for the V3 crown as a crucial vaccine target [325, 326, 330].

Although our results support the possibility that the main target of the NAbs produced by our immunogens is the V3 crown, other epitopes, may also have been involved in neutralization. Position N332 in gp120 is known to be a vulnerable site for neutralization by some bNAbs [547, 560, 561]. Several studies have demonstrated that the second position (X) in NXS/NXT motifs can indicate whether or not a glycan will be attached [547, 562, 563]. The observation that gp120AG had a high probability of glycan occupancy (*Pg*= 0.79, motif NVS) together with the fact that PX228, the tier 2 HIV-1 pseudovirus neutralized by all AG immunized mice, also have a high probability of glycan occupancy (Pg= 0.86, motif NIS) suggests that AG immunogens could also have induced glycan-dependent bNAbs directed to this region in immunized mice. However, further studies will be necessary to clarify the epitope specificities of NAbs induced by the new immunogens.

In conclusion, our results confirm that a prime-boost immunization strategy with recombinant Vaccinia virus expressing the monomeric envelope gp120t (priming) and the cognate gp120t (boost) can induce in mice a heterologous HIV-1 tier 2 neutralizing response, albeit with limited breadth and potency. Remarkably, however, CRF02_AG-based immunogens were able to induce a potent envelope binding antibody response and a broad neutralizing response against B and non-B subtypes. Antibody responses were associated with adequate Tfh and Tfr responses indicating that our vaccine strategy is targeting the cellular subsets required for the induction of an effective NAb response. Thus, our findings provide support for testing these new HIV-1 immunogens and vaccine strategy in other animal models. Finally, our study provides proof-of-concept for a new type of HIV-1 vaccine based in non-B subtypes that uses a replication-competent virus for priming. Ongoing studies will determine whether this strategy leads to similar results in rabbits.

Supplementary Material

Table S1. Characterization of HIV-1 primary isolates included in the study

Sample	Year	Origin	Co-receptor usage	*Env* subtype
93AOHDC250	1993	Angola	CCR5	J
93AOHDC251	1993	Angola	CCR5	H
93AOHDC253	1993	Angola	CCR5	J
01PTHDECJN	2001	Angola (obtained in Portugal)	CCR5	CRF02_AG
08AO34HDP	2008	Angola	CCR5	C
97PTINSA43	1997	Portugal	CCR5	B
00PTEBB	2000	Portugal	CCR5	G

Table S2- Primers used for polymerase chain reaction amplification of HIV-1 *Env*, C2V3C3 and truncated gp120

Amplified fragment	Name	Orientation	Sequence (5´- 3´)[1]	Clade	HXB2 position
Full-length Env	PBENV1	Forward	CTATGGCAGGAAGAAGCGG	All	5968-5986
	PBENV2	Forward	CCACTGTCTTCTGCTCTTTC	All	6203-6223
	PBENV3	Reverse	AGTCATTGGTCTTARAGGTAC	All	9036-9016
	PBENV4	Reverse	TTTTGACCACTTGCCHCCCAT	All	8797A8817
Truncated gp120	RC1gp120*SalI*	Forward	TTGTGTGTCGACGAAAGAGC AGAAGAYAGTGGC	All	6203-6223
	RC2gp120BC*SalI*	Reverse	TTCTGTGTCGAC**CTA**ATATTT ATATAATTCACTTCTC	B,C	7661-7682
	RC2gp120GAG*SalI*	Reverse	TTCTGTGTCGAC**CTA**ATACT TATATAATTCACTTCTC	G,CRF02_AG	7661-7682
	RC2gp120J*SalI*	Reverse	TTCTGTGTCGAC**CTA**ATATTT ATATAACTCACTCCTC	J	7661-7682
	RC2gp120H*SalI*	Reverse	TTCTGTGTCGAC**CTA**ATATTT ATATAGCTCACTTCTC	H	7661-7682
C2-V3-C3	HIV1EPIT11	Forward	TGTGGATCCCCAATTCCYAT ACATTATTG	All	6858-6878
	HIV1EPIT12	Reverse	TGAAAGCTTTCATCAGAAAA ATTCYCCTCYAC	All	7374-7392

[1] Underlined letters indicate the restriction site for SalI; the stop codon **CTA is** indicated in bold letters

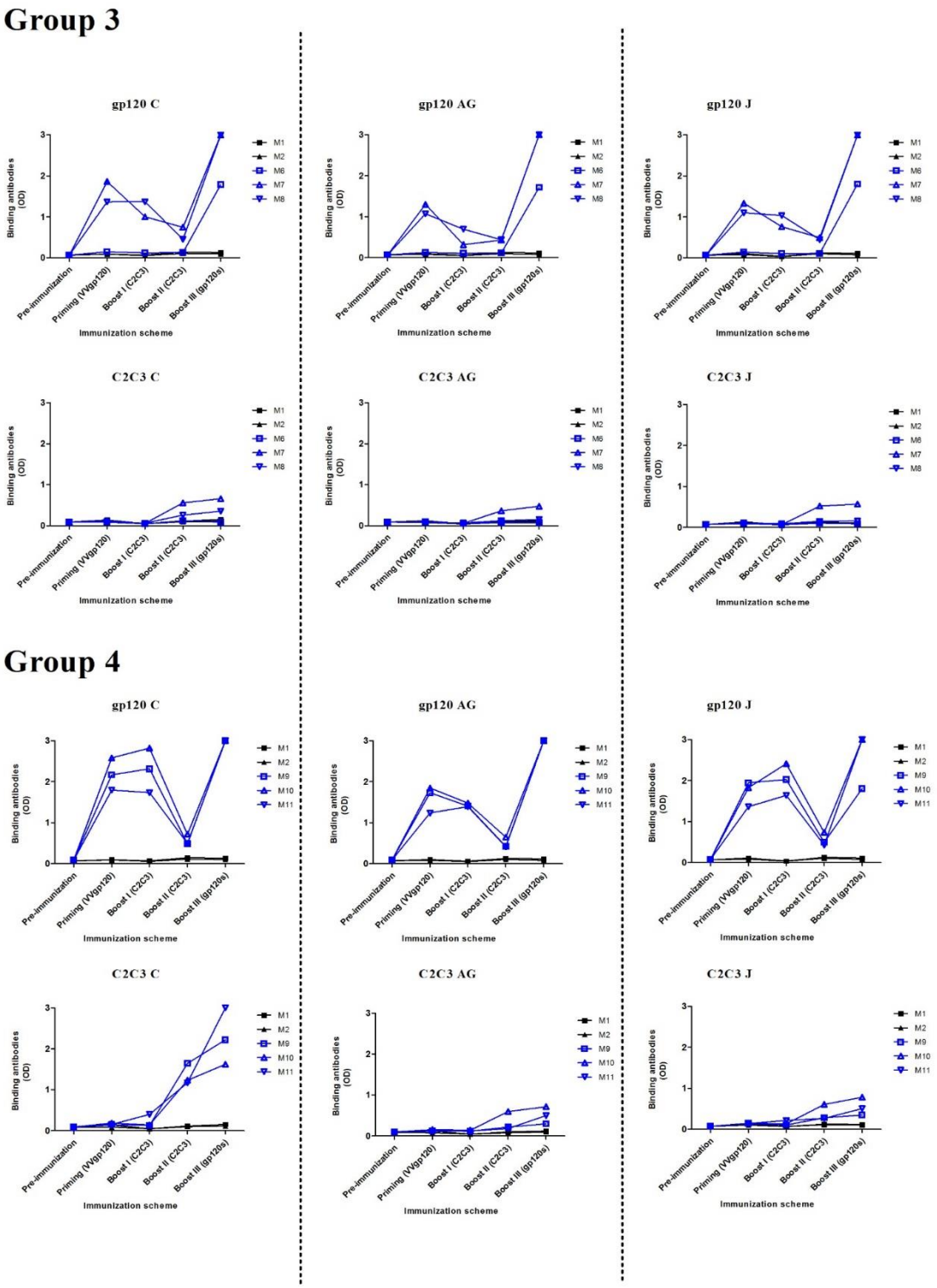
Table S3. Primers used for sequencing of HIV-1 *env* gene segments

Primer	Orientation	Sequence (5´- 3´)	HXB2 position
PBSEQ1	Forward	AGCYTAAAGCCATGTGT	6567 – 6583
PBSEQ2	Reverse	ACACATGGCTTTARGCT	6567 – 6583
PBSEQ3	Forward	CAGTACAATGTACACA	6955 – 6970
PBSEQ4	Reverse	TGTGTACATTGTACTG	6955 – 6970
PBSEQ5	Forward	CATAGTTTTAATTGTRGAGG	7344 – 7363
PBSEQ6	Reverse	CCTCYACAATTAAAACTATG	7344 – 7363
PBSEQ13	Forward	GGACAATTGGAGAAGTGAA	7652 – 7670
PBSEQ7	Forward	GAGAGAAAAAAGAGCAGT	7745 – 7762
PBSEQ8	Reverse	ACTGCTCTTTTTTCTCTC	7745 – 7762
PBSEQ9	Forward	ATCTGCACCACTAATGT	8031 – 8047
PBSEQ10	Reverse	ACATTAGTGGTGCAGAT	8031 – 8047
PBSEQ11	Forward	CCTGTGCCTCTTCAGCTACC	8510 – 8529
PBSEQ12	Reverse	GGTAGCTGAAGAGGCACAGG	8510 – 8529

Table S4. Global panel of tier 2 HIV-1 Env-pseudoviruses and HIV-1 primary isolates used in neutralization assays.

Env- pseudoviruses (n=11)	**HIV-1 Clade**	**Origin**	**Year**
pTRO11 env	B	Italy	1995
p25710 env	C	India	1999
p398F1 env	A	Tanzania	2001
pX2278 env	B	Spain	2007
pBJOX2000 env	CRF07_BC	China	2007
pX1632 env	G	Spain	2004
pCE1176 env	C	Malawi	2004
p246F3 env	AC recomb	Tanzania	2001
pCH119 env	CRF07_BC	China	2004
pCE0217 env	C	Malawi	2007
pCNE55 env	CRF01_AE	China	2007
Primary isolates (n=3)	**Subtype**	**Origin**	**Year**
01PTCJN	CRF02_AG	Angola (obtained in Portugal)	2001
93AOHDC250	J	Angola	1993
93AOHDC253	J	Angola	1993

Figure S1


Group 3
gp120 C
gp120 AG
gp120 J
C2C3 C
C2C3 AG
C2C3 J
Group 4
gp120 C
gp120 AG
gp120 J
C2C3 C
C2C3 AG
C2C3 J
Binding antibodies (OD)
Immunization scheme
Pre-immunization
Priming (VVgp120)
Boost I (C2C3)
Boost II (C2C3)
Boost III (gp120s)
M1
M2
M6
M7
M8
M9
M10
M11

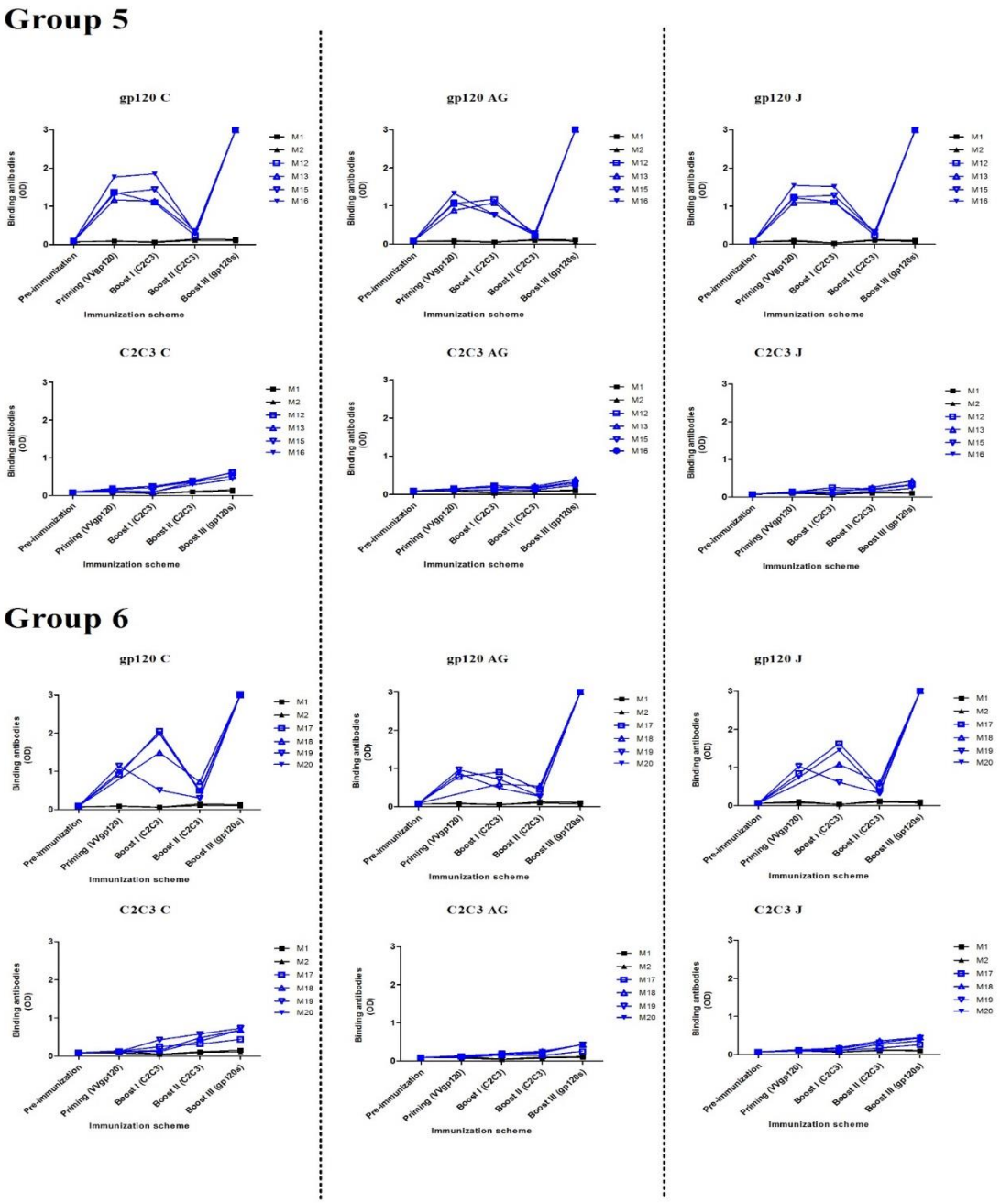
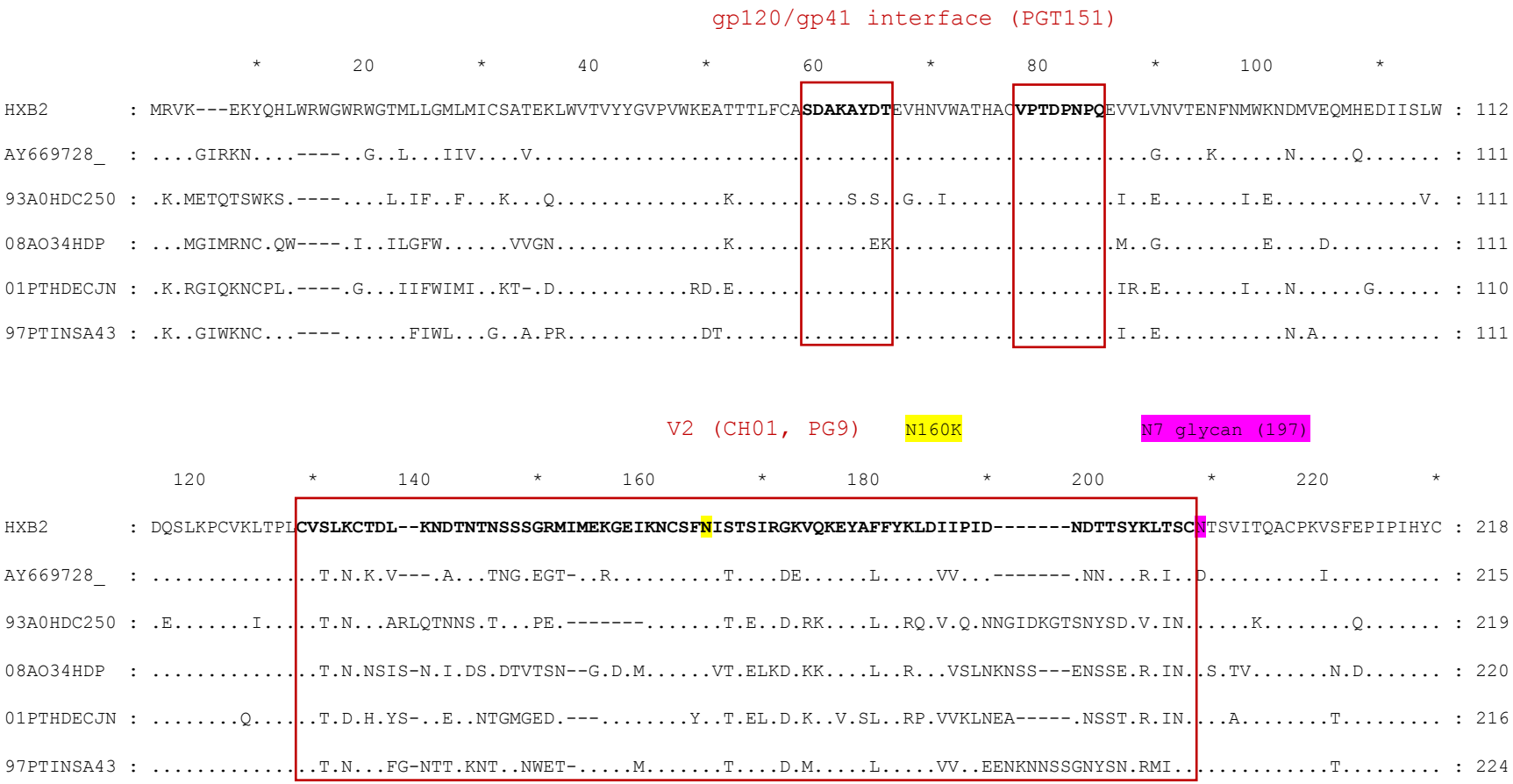
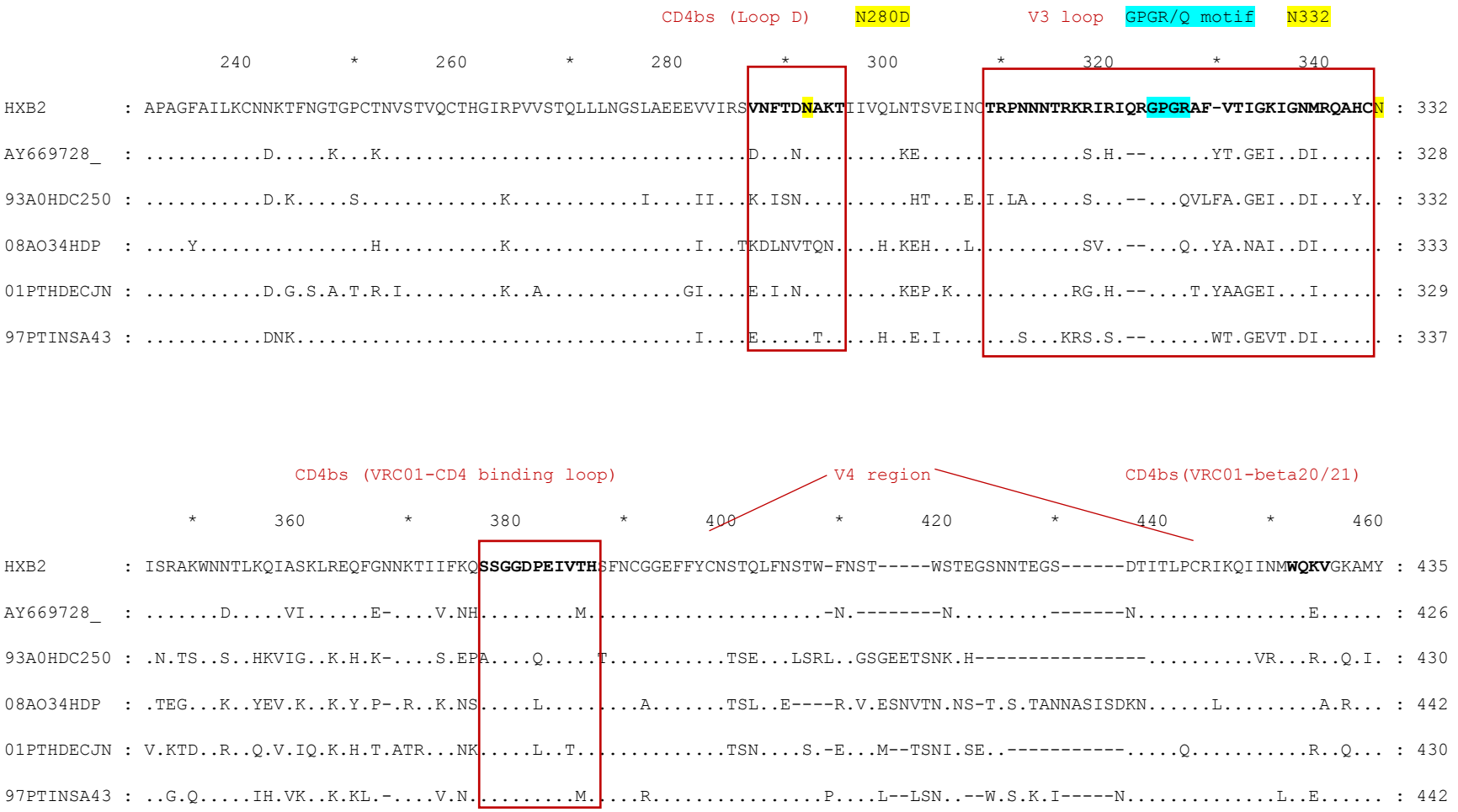


Figure S1 - Binding IgG response (OD values) overtime against gp120t supernatant (C, AG, J) and C2V3C3 polypeptides (C,AG, J) in BALB/c mice in the pilot study: Groups 3 and 4-mice primed with VV expressing gp120t and boosted with C2V3C3 polypeptides and gp120 from clade C; Group 5- mice primed VV expressing gp120t and boosted with C2V3C3 polypeptides and gp120 from clade AG; Group 6- mice primed with VV expressing gp120t and boosted with C2V3C3 polypeptides and gp120 from clade J. For all mice, the schedule of immunization included one priming and three boosts at days 15, 30 and 45. Fifteen days after each immunization, sera were collected and assayed for the presence of binding antibodies against HIV-1 immunogens.Blue lines represent immunized mice from the respective group; black lines represent mice from control group (G1-M1-M2).

Figure S2


gp120/gp41 interface (PGT151)
V2 (CH01, PG9)
N160K
N7 glycan (197)
HXB2 : MRVK---EKYQHLWRWGWRWGTMLLGMLMICSATEKLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNVWATHACVPTDPNPQEVVLVNVTENFNMWKNDMVEQMHEDIISLW : 112
AY669728_ : ....GIRKN...----..G..L...IIV....V.....G....K......N.....Q..... : 111
93A0HDC250 : .K.METQTSWKS.----....L.IF..F...K...Q.....K.....S.S..G..I.....I..E.....I.E.....V. : 111
08AO34HDP : ...MGIMRNC.QW----.I..ILGFW......VVGN.....K.....EK.....M..G.....E....D..... : 111
01PTHDECJN : .K.RGIQKNCPL.----.G...IIFWIMI..KT-.D.....RD.E.....IR.E.....I...N......G..... : 110
97PTINSA43 : .K..GIWKNC...----......FIWL...G..A.PR.....DT.....I..E.....N.A..... : 111
HXB2 : DQSLKPCVKLTPLCVSLKCTDL--KNDTNTNSSSGRMIMEKGEIKNCSFNISTSIRGKVQKEYAFFYKLDIIPID-------NDTTSYKLTSCNTSVITQACPKVSFEPIPIHYC : 218
AY669728_ : .....T.N.K.V---.A...TNG.EGT-..R.....T....DE.....L.....VV...-------.NN...R.I..D.....I..... : 215
93A0HDC250 : .E.....I.....T.N...ARLQTNNS.T...PE.-------.....T.E..D.RK....L..RQ.V.Q.NNGIDKGTSNYSD.V.IN.....K.....Q..... : 219
08AO34HDP : .....T.N.NSIS-N.I.DS.DTVTSN--G.D.M.....VT.ELKD.KK....L..R...VSLNKNSS---ENSSE.R.IN..S.TV.....N.D..... : 220
01PTHDECJN : .....Q.....T.D.H.YS-..E..NTGMGED.---.....Y..T.EL.D.K..V.SL..RP.VVKLNEA-----.NSST.R.IN...A.....T..... : 216
97PTINSA43 : .....T.N...FG-NTT.KNT..NWET-.....M.....T....D.M.....L.....VV..EENKNNSSGNYSN.RMI.....T..... : 224


CD4bs (Loop D)
N280D
V3 loop
GPGR/Q motif
N332
240 * 260 * 280 * 300 * 320 * 340
HXB2 : APAGFAILKCNNKTFNGTGPCTNVSTVQCTHGIRPVVSTQLLLNGSLAEEEVVIRSVNFTDNAKTIIVQLNTSVEINCTRPNNNTRKRIRIQRGPGRAF-VTIGKIGNMRQAHCN : 332
AY669728_ : ..........D.....K...K.....D...N.....KE.....S.H.--.....YT.GEI..DI..... : 328
93A0HDC250 : ..........D.K.....S.....K.....I....II...K.ISN.....HT...E.I.LA.....S...--...QVLFA.GEI..DI...Y.. : 332
08AO34HDP : ....Y.....H.....K.....I...TKDLNVTQN....H.KEH...L.....SV..--...Q..YA.NAI..DI..... : 333
01PTHDECJN : ..........D.G.S.A.T.R.I.....K..A.....GI....E.I.N.....KEP.K.....RG.H.--....T.YAAGEI...I..... : 329
97PTINSA43 : ..........DNK.....I....E.....T....H..E.I.....S...KRS.S.--.....WT.GEVT.DI..... : 337
CD4bs (VRC01-CD4 binding loop)
V4 region
CD4bs(VRC01-beta20/21)
* 360 * 380 * 400 * 420 * 440 * 460
HXB2 : ISRAKWNNTLKQIASKLREQFGNNKTIIFKQSSGGDPEIVTHSFNCGGEFFYCNSTQLFNSTW-FNST-----WSTEGSNNTEGS------DTITLPCRIKQIINMWQKVGKAMY : 435
AY669728_ : .....D.....VI.....E-....V.NH.....M.....-N.--------N.....-------N.....E..... : 426
93A0HDC250 : .N.TS..S..HKVIG..K.H.K-....S.EPA....Q.....T.....TSE...LSRL..GSGEETSNK.H----------.....VR...R..Q.I. : 430
08AO34HDP : .TEG...K..YEV.K..K.Y.P-.R..K.NS.....L.....A.....TSL..E----R.V.ESNVTN.NS-T.S.TANNASISDKN.....L.....A.R... : 442
01PTHDECJN : V.KTD..R..Q.V.IQ.K.H.T.ATR...NK.....L..T.....TSN....S.-E...M--TSNI.SE..----------.....Q.....R..Q... : 430
97PTINSA43 : ..G.Q.....IH.VK..K.KL.-....V.N.....M.....R.....P....L--LSN..--W.S.K.I-----N.....L..E..... : 442

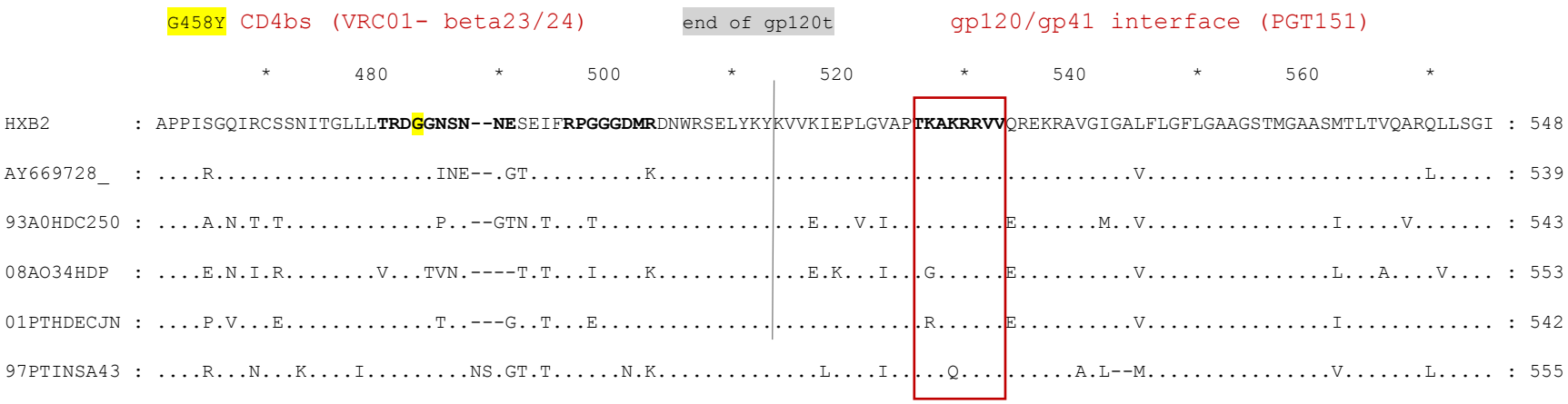

G458Y CD4bs (VRC01- beta23/24)
end of gp120t
gp120/gp41 interface (PGT151)
480
500
520
540
560
HXB2 : APPISGQIRCSSNITGLLLTRDGGNSN--NESEIFRPGGGDMRDNWRSELYKYKVVKIEPLGVAPTKAKRRVVQREKRAVGIGALFLGFLGAAGSTMGAASMTLTVQARQLLSGI : 548
AY669728_ : ....R.....INE--.GT.....K.....V.....L..... : 539
93A0HDC250 : ....A.N.T.T.....P..--GTN.T...T.....E...V.I.....E.....M..V.....I.....V..... : 543
08AO34HDP : ....E.N.I.R.....V...TVN.----T.T...I....K.....E.K...I...G.....E.....V.....L...A....V.... : 553
01PTHDECJN : ....P.V...E.....T..---G..T...E.....R.....E.....V.....I..... : 542
97PTINSA43 : ....R...N...K....I.....NS.GT.T.....N.K.....L....I.....Q.....A.L--M.....V.....L..... : 555
N611A gp120/gp41 interface (PGT151)
580
600
620
640
660
680
HXB2 : VQQQNNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQQLLGIWGCSGKLICTTAVPWNASWSNKSLEQIWNHTTWMEWDREINNYTSLIHSLIEESQNQQEKNEQELLEL : 663
AY669728_ : .....RM.....V.....G.....DR...NM.....E...D....E.YT..... : 654
93A0HDC250 : ....S...K.....K.....V.....T....S.....Y.E..DNM..L..EK.....GI.YN....A..T.....D..A. : 658
08AO34HDP : ....S.....T.VV.I.....S.....Q.D...NM...Q.....S...YT.YQ.L...HI.....KD..A. : 668
01PTHDECJN : ....S.....K.....V..L.A.....T....S.....TYDS..GNM..LQ..K..S...YT.YD...K.....D..A. : 657
97PTINSA43 : ....K...N.....V.....N....D.....DT..HNM.....E...D...D..YT.L.K..... : 670

Figure S2- Amino acid sequence alignment of four envelope sequences included in the present study and location of neutralizing epitopes.
Amino acid sequences of the envelope glycoproteins of isolates 93AOHDC250 (clade J), 08AO34HDP (clade C), 01PTHDECJN (clade CRF02_AG) and 97PTINSA43 (clade B) used to produce the immunogens of the study. Epitopes of representative broadly neutralizing human monoclonal antibodies were extracted from several publications [153, 154, 365, 564-569].The location of the neutralizing epitopes is indicated in relation to the reference HIV-1 strains HXB2 (KO3455) and JR-FL (AY669728) also included in the alignment. Location and name of the neutralizing epitopes and corresponding NAbs are shown in red. Aminoacids corresponding to an epitope are shown in boldface letters. Specific mutations and/or motifs and respective sequence are shown in yellow. N7 glycan (position 197 in HXB2) is shown in purple.

Chapter 4

Induction of tier 2 cross-clade neutralizing antibodies in rabbits by a prime-boost immunization strategy with Vaccinia virus expressing novel HIV-1 gp120 glycoproteins from clade CRF02_AG

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Manuscript in preparation

Abstract

A vaccine to prevent HIV infection is the best hope for controlling or ending the HIV-1 epidemic. Despite years of research there is still no vaccine for HIV-1. Broadly neutralizing antibodies are considered the best correlate of protection against HIV infection but their induction by vaccination remains a difficult task. Poxvirus-based heterologous prime-boost immunization regimens have been widely used in HIV vaccine trials as they provide a strong T and B cell immune response, occasionally inducing the production of neutralizing antibodies. We previously demonstrated that bNAbs against HIV-2 could be elicited in mice using a Vaccinia vector-prime C2-V3-C3-polypeptide boost vaccination strategy [2]. More recently, using novel clade CRF02_AG gp120 glycoproteins and a recombinant Vaccinia virus prime-gp120 boost immunization regimen we were able to induce heterologous tier 2 HIV-1 cross-clade neutralizing antibodies in mice. Here, New Zealand White Rabbits were immunized with the same CRF02_AG derived immunogens in order to examine if NAb responses against tier 2 viruses of all clades could also be elicited. All immunized rabbits generated antibodies binding to autologous and heterologous gp120 and gp140 proteins and one rabbit generated antibodies that neutralized at more than 50% 13/16 tier 2 global HIV-1 pseudoviruses tested. These results confirm our previous results in mice and support the use of CRF02_AG based immunogens as a component of a new preventive HIV-1 vaccine.

Introduction

HIV infection is still a major global public health issue. Since the beginning of HIV epidemic about 70 million people have become infected and about 35 million people have died of AIDS. At the end of 2015 about 2.1 million people became newly infected resulting in a total of 36.7 million cases of HIV infected individuals until that year [570]. Therefore, prevention of new HIV-1 infections will depend on the development of an effective and safe vaccine. However, despite years of research in this field, there is still no vaccine available for HIV. This incapacity in producing an effective vaccine is related with the lack of adequate animal models that can be used to reliably predict human responses to vaccines, the extraordinary genetic diversity and high mutational rate of HIV and the limited exposure of neutralizing epitopes in the outer surface of the virus as well as the incomplete knowledge of all the vulnerable sites of HIV [35, 52-54, 461, 462]. On the other hand, no vaccine antigen produced until now has been able to induce a protective effect against HIV in vaccinated animals and/or humans.

The most promising vaccine trial that was able to induce some degree of protection (31%) was the RV144 conducted in Thailand from 2003 to 2006 [118, 468]. This trial was based on clade B and CRF01_AE sequences and consisted of four priming injections of a attenuated recombinant canarypox vector vaccine (ALVAC/vCP1521) expressing env, gag and protease genes plus two booster injections of a recombinant glycoprotein 120 subunit vaccine (AIDSVAX B/E)[118, 468]. Interestingly, as shown in previous trials none of the immunogens alone induced protective responses [465-468]. Immune responses observed in this trial correlated with protection from HIV-1 infection included antibodies to variable loops (V1 and V2), high levels of antibody-dependent cellular cytotoxicity (ADCC) [118, 468, 470]. Low titers of neutralizing antibodies were also observed but only against tier 1 isolates which may justify the modest degree of the protection observed in this trial [131].

Broadly neutralizing antibodies are considered the best correlate of antibody protection against HIV infection. Passive administration of bNAbs has been shown to protect humanized mice and macaques against high-dose challenge with HIV or SHIVs viruses [316-324]. In addition, bNAbs have been shown to have an active role in therapy contributing to the decline of plasma viremia to undetectable levels in HIV-1 infected humanized mice and SHIV-infected macaques, especially when combined with other

bNAbs and/or ART [571-574]. Recently, Nussenzweig and colleagues have demonstrated that the passive administration of HuMAb 3BNC117 was safe and effective in reducing viral load in HIV-1 infected individuals[575]. Thus, bNAbs can be used to prevent HIV-1 infection but also disease progression.

bNAbs are directed to several epitopes located in HIV-1 envelope glycoprotein and are capable of binding to the virus preventing infection [536]. Therefore, bNAbs should be a crucial element in a preventive or therapeutic HIV vaccine as they are able to neutralize primary isolates from different clades [131, 316, 318-320, 322, 324, 422, 473-475]. Recent studies in rabbits have shown that it is possible to induce bNAbs against heterologous tier 2 viruses although sporadically, with limited breadth and at low levels [478, 479]. However, the induction of efficient and consistent bNAb responses against heterologous tier 2 virus from different clades has been a difficult task for the majority of vaccine candidates. Remarkably, the recent immunization of four cows with the trimer BG505 SOSIP resulted in the rapid elicitation of broad and potent serum antibody responses in all immunized animals [1]. In addition, one monoclonal antibody with an ultra-long CDR H3 of 60 amino acids isolated from one cow neutralized 72% (n=117) of HIV-1 cross-clade isolates. The induction of these potent and broad NAbs to HIV may provide important clues regarding the quality and nature of the new generation of immunogens currently used in vaccine trials.

Due to its surface exposure and immunogenicity, gp120 has been a major target for HIV vaccine development. However, with the exception of the recent experiments in cows [1], monomeric and trimeric preparations of gp120 have repeatedly failed to induce broad and potent neutralizing responses against tier 2 viruses when administered alone [342, 478, 487-490, 492, 576]. The recent success of the trimer BG505 SOSIP immunization in cows is related with unique features of their immune system which allowed the rapid elicitation of potent and broad neutralizing responses. In fact, cows produce antibodies with exceptionally long CDR H3 at a much higher frequency and faster (months *versus* several years in humans) than HIV-1 infected individuals who produce bNAbs [1, 351, 374, 577, 578].

Heterologous prime-boost regimens based in a combination of different immunogens have been widely used in the majority of HIV vaccine regimens aiming to induce bNAbs as they provide a stronger overall immune response [422, 453, 454, 479, 494, 579]. One

of the most common approaches is the use of attenuated live viral vector vaccines (replicating and non-replicating) in combination with various HIV envelope proteins as exemplified by the RV144 trial described above [118]. Viral vector vaccines are able to elicit a wide range of immune responses including antibodies, $CD4^+$ T cells and $CD8^+$ T cells [207, 418, 421, 553, 579-581]. Moreover, replicative-competent viral vectors (*e.g.* Vaccinia virus strain Western Reserve and Cytomegalovirus) can act like adjuvants activating the immune system as they are able to actively invade and replicate in host cells leading to an increase and continuous exposure of the antigen [207, 387, 420, 421, 582]. In addition, replicating vaccines can elicit life-long protective immunity[421]. However, due to safety issues, non-replicative attenuated vaccine vectors as ALVAC, NYVAC and MVA are preferentially used in pre-clinical HIV/SIV vaccine studies being also able to induce cellular and humoral immune responses although in a less extent compared with replicating vectors[391, 418, 553, 581, 583]. For instance, Williamson and colleagues using a heterologous prime-boost vaccination with DNA and MVA expressing HIV-1 mosaic Gag were able to elicit Gag $CD8^+$ and $CD4^+$ T cell responses in mice. However, no antibodies were induced [418].

Within prime-boost immunization regimens with live viral vaccines, Vaccinia viruses are among the most exploited for vaccine development. This is because they have large genomes allowing the insertion of more than 10kb of foreign DNA and consequently high levels of gene expression [387, 420]. Other advantages of Vaccinia viruses include its easy manufacturing, the capacity to infect efficiently a wide range of different cell types both *in vivo* and *in vitro* and the possibility to be handled in BSL-2 facilities [584, 585].

We previously demonstrated that bNAbs against HIV-2 could be elicited in mice using a Vaccinia vector-prime C2-V3-C3-polypeptide boost vaccination strategy [2]. More recently, using novel clade CRF02_AG gp120 glycoproteins and a recombinant Vaccinia virus prime-gp120 boost immunization regimen we were able to induce heterologous tier 2 HIV-1 cross-clade neutralizing antibodies in mice (chapter 3) [586]. Specifically, mice primed with Vaccinia virus expressing gp120 from clade CRF02_AG and boosted three times with the cognate gp120 protein developed autologous and heterologous neutralizing antibodies (>50%) against four tier 2 HIV-1 isolates from different clades.

Rabbits are an animal model frequently used in HIV vaccine development assays constituting a preferential host for testing immunogens aimed at inducing broadly

neutralizing responses [479, 494, 503]. Rabbit antibodies have high affinity, specificity and can recognize unique epitopes[587]. In fact, monoclonal antibodies derived from rabbits have the potential to cross-react with human, non-human primate and mouse antigens [588]. More importantly, the CDR H3 length distribution in rabbit antibodies is more similar to humans than to mouse antibodies which is a very important feature since human bNAbs have unusual long CDR H3 loops necessary to penetrate the Env glycan shield [351, 374, 589]. Also, like human antibodies, rabbit antibodies are able to recognize a large variety of epitopes that may not be immunogenic in mice [589]. Finally, the possibility of obtaining large amounts of sera compared with mice make rabbits an attractive animal model for vaccination studies.

The aims of the present study were to confirm and extend our previous results in mice in order to examine if NAb responses against tier 2 viruses representative of all HIV-1 clades could also be elicited in rabbits.

New Zealand White rabbits were primed with a live recombinant VV expressing a truncated form of gp120 from clade CRF02_AG and boosted with the cognate truncated gp120 protein. Using this immunization strategy we were able to induce the production of antibodies binding potently to the autologous and heterologous gp120 glycoproteins and antibodies that potently neutralized tier 2 HIV-1 envelope-pseudoviruses from multiple clades. As far as we know, no other vaccine study has been able to induce this type of response using clade AG based immunogens and so the present study represents a major contribution for HIV vaccine immunization field aiming to induce bNAbs.

Material and methods

Cells, viruses and antibodies

Rat-2 (TK^-) cells were purchased from American Type Culture Collection (Rockville, MD). HeLa cells (ATCC® CCL-2™) were obtained from American type Culture Collection. TZM-*bl* cells were provided by the AIDS Research and Reference Reagent Program, National Institutes of Health. HeLa and TZM-*bl* cells were cultured in complete growth medium that consists of Dulbecco´s minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin-streptomycin (Gibco/Invitrogen, USA), 1mM of sodium pyruvate (Gibco/Invitrogen, USA), 1mM of

L-glutamine (Gibco/Invitrogen, USA) and 1mM of non-essential amino acids (Gibco/Invitrogen, USA). All cell cultures were maintained at 37ºC in 5%CO_2. Plasmid pMJ601was a gift from Bernard Moss[541]. The following items were obtained from the NIH AIDS Reagent Program: a Panel of Global tier 2 HIV-1 Env Clones (cat#12670) designed to assess neutralization responses, the Western Reserve Strain of Vaccinia virus (VWR), recombinant proteins M.CON.SD11gp120 and SF162 gp140 Trimer and HuMAbs PG6, HJ16 and VRC01.

Production of recombinant Vaccinia viruses and expression of env glycoproteins

Recombinant Vaccinia viruses expressing the glycoprotein gp120t from clade CRF02_AG were obtained by transfection of recombinant plasmids as described previously (chapter 3). The method of Rose *et al*[543] was used to produce gp120t . Briefly, HeLa cells were infected with 5 PFU of recombinant Vaccinia virus per cell and incubated for 3 hours. Medium containing the infecting virus was replaced with serum-free DMEM at 3h post infection. After 24 hours of infection medium containing gp120t was collected, clarified by centrifugation at 3000xg for 10 minutes and filtered with a 0.2µM pore size filter to remove Vaccinia virus. In order to collect cell lysates we performed the same assay in parallel and after 48h, cells were washed with cold PBS and lysed using RIPA+DOC buffer (0.15M NaCl, 0.05M Tris-HCl, 1% Triton X-100, 1% DOC, 0.1% SDS). Lysates were centrifuged at 35.000 rpm for 60 min at 4°C and the supernatant containing the proteins was collected. Antigenic reactivity of gp120 obtained from cell lysates and cell- free supernatants was analyzed by Western-Blot using sera from HIV-1 infected individuals. For Western blot analysis, proteins were prepared in Laemmli sample buffer (125mM Tris, pH 6.8, 2% SDS and 20% glycerol) with β-mercaptoethanol and denaturated by heating for 5 min at 96ºC, loaded within the acrylamide gel and then transferred from the gel to nitrocellulose membranes 0.45µM (BioRad). After protein transfer, the membranes were treated with blocking buffer (1X TBSt with 4% w/v nonfat dry milk) and incubated with serum from HIV-1 and HIV-2 infected individuals diluted (1:200) in primary antibody buffer (1X TBSt with 4% w/v nonfat dry milk and 5% goat serum). Next, the membrane was washed with TBSt 0.25% and incubated with Anti-Human IgG Peroxidase antibody produced in goat (Sigma).

Chemiluminescent detection was performed with Pierce™ ECL Western Blotting Substrate (ThermoFisher Scientific).

Immunization

Eight female New Zealand White rabbits were purchased and maintained at the Faculdade de Medicina Veterinária, Lisboa (FMV), from October 2016 to March 2017. All animal care and handling were approved by Ethics and Animal Welfare Committee (CEBEA-Comissão de Ética e Bem-Estar Animal da FMV). Rabbits were divided in three groups: Group 1 (placebo) was immunized with 500 µl of PBS, Group 2 (control) was primed with 500 µl of $2x10^7$ PFU of Vaccinia virus strain WR (VV_{WR}) and boosted three times with 500 µl of VV_{WR} supernatant (S_{VVWR}) and group 3 was primed with 500 µl of $2x10^7$ PFU of recombinant VV expressing gp120t from HIV-1 CRF02_AG clade (VVgp120AG) and boosted three or four times with 500 µl of VVgp120AG supernatant ($S_{gp120AG}$) corresponding to 35 µg of the cognate gp120 protein (Table 1). For all groups boosts were performed at days 35, 63 and 121 (boost I, II and III). Two rabbits of the main group (R6 and R8) received an extra boost at day 150 (boost IV). S_{VVWR} and $S_{gp120AG}$ boosts were emulsified in IFA in a 1:1 ratio. All immunizations were administered subcutaneously and blood samples were collected from the marginal ear vein. Rabbit sera was collected immediately before each immunization plus two times between boost II and III to access for binding antibodies. All rabbits were sacrificed on day 154.

Envelope-specific antibody binding reactivity in sera of immunized rabbits

HIV-1 gp120 specific binding antibodies were analyzed during the experiment by ELISA using rabbit sera from six time points (T0-T5). Briefly, Immuno MaxiSorp 96-well microplates (Nunc) were coated with 2.3 µg of autologous $S_{gp120AG}$ and heterologous recombinant gp120 derived from a consensus HIV-1 group M envelope gene sequence (M.CON.SD11) at 1 µg/ml. Rabbit sera from the last timepoint (day 154) was also tested against the heterologous gp140 trimer (SF162) at 1 µg/ml. All proteins were diluted in 0.05M bicarbonate buffer. After overnight incubation at 4°C microplates were blocked with 2% gelatin (Bio-Rad). Rabbit anti-serum (1:300 final dilution) from days 1, 35, 63, 99, 121, and 154 was heat inactivated for 1 hour at 56ºC [590], added to the microplates

and incubated for 2 hours at room temperature. Anti-rabbit IgG-Alkaline Phosphatase antibody produced in goat (Sigma) was added at 1:2000 dilution to the microplates as a secondary antibody. Colorimetric reaction was developed with SIGMAFAST™ p-Nitrophenyl phosphate (pNPP) tablets and read at 405 and 492 nm against a reference wavelength of 620 nm on a microplate reader. In order to confirm the reactivity of autologous and heterologous proteins we use positive (serum from HIV-1 infected individuals) and negative (serum from healthy donors) controls at 1:200 final dilution. Sera from pre-immunized rabbits and from rabbits immunized with VV_{WR} were used as negative controls of the assay. Sera with an optical density (OD) above the cutoff (mean OD of rabbits immunized with VWR immunogens plus four times the standard deviation) were considered positive. Binding antibody titer of rabbit sera from the last timepoint against autologous and heterologous gp120 proteins was calculated using the function $\log_{10}$ of the highest serum dilution at which a positive reaction (OD / cut-off> 1) was obtained.

Reactivity of gp120 truncated proteins against rabbit sera from the last time point was also analyzed by western blot analysis as described above. For this propose rabbit sera was used at 1:200 final dilution in primary antibody buffer and anti-rabbit IgG-Alkaline Phosphatase antibody produced in goat was used as a secondary antibody at 1:2000. Colorimetric detection of proteins was performed by AP conjugate Substrate Kit (BioRad).

Neutralization assays

A panel of 17 HIV-1 viruses including 12 tier 2 HIV-1 from different clades [502], four HIV-1 primary isolates (clades CRF02_AG, J and Untypable) and one tier 1 pseudovirus were used in the neutralization assays in order to detect neutralizing antibodies in rabbits. Primary isolates were obtained from HIV-1 infected patients by co-cultivation method with PBMCs from seronegative subjects. Env-pseudotyped viruses were produced by transfection of Env-expressing plasmids in 293T cells using PSG3.1Δenv as backbone and titrated in TZM-bl cells as described [502, 544].

Neutralizing activity of rabbit serum was tested using a single-round viral infectivity assay using a luciferase reporter gene assay in TZM-bl cells, as described previously [2,

300]. Briefly, cells (10 000 cells in 100µl of DMEM supplemented with 10% heat-inactivated fetal bovine serum) were added to each well of 96-well flat-bottom culture plates (Nunc) and allowed to adhere overnight. Next, 100 µl of each virus (200 TCID50/well) were incubated for 1h at 37ºC with heat-inactivated rabbit sera (56ºC, 1 hour) in a total volume of 200 µl of growth medium (DMEM supplemented with 10% heat-inactivated fetal bovine serum) containing DEAE-Dextran (19.7 µg/ml) and added to the cells. Final serum dilution used in the assays was 1:20. Neutralizing antibody titers were also obtained for rabbit serum with positive neutralization responses (>50%). In that case, 100 µl portions of 2-fold serial dilutions beginning at 1:20 were mixed with 100 µl of each virus and incubated for 1 hour before added to the cells. After 48h, culture medium was removed from each well and plates were analyzed for luciferase activity on a luminometer (TECAN) using Pierce™ Firefly Luciferase Glow Assay Kit (ThermoFisher scientific). Wells with medium were used as background control and virus-cells wells were included as infection control. The effect of pre-immunized serum on infection was used as baseline neutralization. To monitor the amount of neutralization activity that was not HIV specific, each serum sample was tested against VSV. Sera from HIV-1 infected individuals (1:40) and HuMAbs PG16, HJ16, VRC01 in a final concentration between 0.001-2µg/ml were used as positive controls of the assay [591]. PG16 was used against 246F3 (0.08 µg/ml), BJOX2000 (0.04 µg/ml), X1632 (0.04 µg/ml), SG3.1 (2 µg/ml), PCNE8 (2 µg/ml), PCE1176 (0.01 µg/ml), PX2278 (0.004µg/ml), 25.710.243 (0.02 µg/ml) and HIV-1 primary isolates 01PTCJN, 93HDC252, 93HDC253 and 93HDS249 (1 µg/ml); HJ16 against PCE0217 (0.2 µg/ml), Tro11 (0.16 µg/ml), PCH119 (0.28 µg/ml); VRC01 against PCNE55 (1.2 µg/ml), 398F1 (0.4 µg/ml). Percent neutralization of rabbit sera was determined by calculating the difference in average RLU between test wells containing post-immune sera and test wells containing pre-immune sera after the normalization of the results using the average RLU of cell controls[545]. Results were considered valid if the average RLU of virus control wells was ≥10 times the average RLU of cell control wells. Neutralization titer (ID50) was defined as the highest dilution for which 50% of neutralization was achieved. Neutralization percentage of the positive controls was determined by calculating the difference in average RLU between test wells (cells + sample + virus) and cell control wells (cells only), dividing this result by the difference in average RLU between virus control (cell + virus) and cell control wells, subtracting from 1 and multiplying by 100[592]. Neutralization assays were performed with sera from the last timepoint (day 154).

Purification of HIV gp120 envelope

Purification of gp120 AG from cell culture supernatant was performed based on Dr. Jim Arthos's protocol (Laboratory of Immunoregulation, NIAID, NIH) [593]. This technique allows the concentration of recombinant gp120 from cell culture supernatant using lectin affinity chromatography. In this case, agarose bound Galanthus nivalis lectin (GNL) (Vector Laboratories) was used which binds to the terminal D-mannose groups of HIV envelope glycoprotein gp120. For purification 1000 ml of HeLa cell culture supernatant containing gp120 AG in complete DMEM medium without FBS was used. Briefly, a Sigma 1.0x10 cm chromatography column was used with 4 ml of GNL slurry, settle and washed with 40 ml of PBS at 0.2ml/min. Then, cell culture supernatant was pumped through the lectin column at 1 ml/min and the flow-through was collected to determine the efficiency of the binding. After washed with ten volumes of cold PBS 5 ml of a mannose solution (0.5 M methyl alpha-D-manno-pyranoside, Sigma) was added at 0.25 ml/min. Eluted proteins were collected with a Frac-100 collector (Pharmacia) in 2 ml fractions and absorption at 280nm was measured (Nanodrop UV spectrophotometer). Finally contaminating proteins from the eluted fractions were removed through metal-affinity chromatography using TALON Superflow (GE) charged previously with 200mM cobalt chloride (2 ml on a Sigma 1.0x10 cm chromatography column). Flow-through fractions containing gp120 were collected and concentrated using Vivaspin 6® >100.000 MWCO PES according with the manufacturer´s instructions. Proteins obtained were analyzed on SDS- 7.5% PAGE followed by BlueSafe protein stain (Nzytech) and quantified using Nanodrop.

IgGs purification

Rabbit's sera were purified by affinity chromatography using a 5-ml Protein A HiTrap column (GE Healthcare) attached to an ÄKTA start protein purification system (GE Healthcare). PBS was used as equilibration and washing buffer and 0.5 M acetic acid for elution. Eluted fractions were neutralized immediately using 0.5 volumes of 1M Tris-HCl (pH 9.0), pooled, and dialyzed against PBS using PD-10 Desalting Columns (GE Healthcare). Protein purity was analyzed by SDS-PAGE followed by Coomassie Blue

staining. Protein quantification was determined using the classic Bradford reagent (Bio-Rad).

Data analysis

Data analysis and graphs were performed with GraphPad Prism 5.

Table 1- Rabbit immunization schedule

		T0		T1		T2		T3	T4			T5
		Blood Colection	**Priming**	**Blood Collection**	**Boost I**	**Blood Collection**	**Boost II**	**Blood Collection**	**Blood Collection**	**Boost III**	**Boost IV**	**Blood Collection**
Groups	ID	Day 1	Day 1	Day 35	Day 35	Day 63	Day 63	Day 99	Day 121	Day 121	Day 150	Day 154
1	R1	●	-	●	-	●	-	●	●	-	-	●
	R2	●	PBS	●	PBS	●	PBS	●	●	PBS	-	●
2	R3	●	VV_{WR}	●	S_{VVWR}	●	S_{VVWR}	●	●	S_{VVWR}	-	●
	R4	●	VV_{WR}	●	S_{VVWR}	●	S_{VVWR}	●	●	S_{VVWR}	-	●
3	R5	●	$VV_{gp120AG}$	●	$S_{gp120AG}$	●	$S_{gp120AG}$	●	●	$S_{gp120AG}$	-	●
	R6	●	$VV_{gp120AG}$	●	$S_{gp120AG}$	●	$S_{gp120AG}$	●	●	$S_{gp120AG}$	$S_{gp120AG}$	●
	R7	●	$VV_{gp120AG}$	●	$S_{gp120AG}$	●	$S_{gp120AG}$	●	●	$S_{gp120AG}$	-	●
	R8	●	$VV_{gp120AG}$	●	$S_{gp120AG}$	●	$S_{gp120AG}$	●	●	$S_{gp120AG}$	$S_{gp120AG}$	●

VV_{WR}- wild-type Vaccinia virus strain Western Reserve; S_{VVWR}- supernatant of cells infected with VV_{WR}; VVgp120AG- Vaccinia virus expressing gp120 from HIV-1 clade CRF02_AG; $S_{gp120AG}$- gp120 supernatant of cells infected with VVgp120AG ; PBS- phosphate-buffered saline; ●- done; ID- animal identification; R- rabbit; T0-T5 refer to time of blood collectio

Results

Immunization

Throughout the procedure all animals were maintained in a good health status in ventilated cages in a temperature controlled room. Changes in behavior and physical status were monitored regularly. The majority of animals demonstrated some degree of stress/anxiety at the time of immunogen administration. With the exception of one animal (R7), all infected and immunized rabbits developed a small lesion in the site of injection after priming which constitutes a typical side effect of Vaccinia virus vaccination. Blood collection was performed at six time points during the assay (table 1) in order to monitor antibody responses against the heterologous and autologous gp120.

All immunized rabbits developed cross-reactive gp120 binding antibodies

In order to monitor the kinetics of antibody production in vaccinated animals binding antibody activity to the autologous and heterologous gp120 glycoproteins was analyzed with an ELISA assay using rabbit sera collected at the different time points (T0-T5). As expected, binding antibody responses of the vaccinated animals (G3) were much higher than those from animals in the control groups (G1 and G2) (Figure 1). All immunized rabbits from group 3 developed specific antibodies against the autologous $gp120_{AG}$ and heterologous gp120 M.CON.SD11 soon after the priming (OD/cutoff >1) with the exception of two animals (R7 and R8) that only developed binding antibodies against the heterologous protein after the first boost (Figure 1). For group 3, binding antibody responses against the autologous $gp120_{AG}$ were always very potent (overflow levels or OD>3) even at high sera dilutions.

In general, vaccinated animals showed an increase in IgG binding response against M.CON.SD11 up to boost II (day 99) followed by a slight decline of antibody response at day 121 which matches the period of time where animals didn´t receive any boost immunization. In fact, after the last immunizations (boost III for R5-8 and boost IV for R6 and R8) all the animals presented a considerable IgG binding response against the autologous (Ab titer between 4.408 and 5.311, mean 4.784) and heterologous gp120 immunogens (Ab titer between 3.204 and 4.408, mean 3.881) suggesting that the last

immunization strongly contributed to the establishment of a sustained antibody response (Figure 1, table 2).

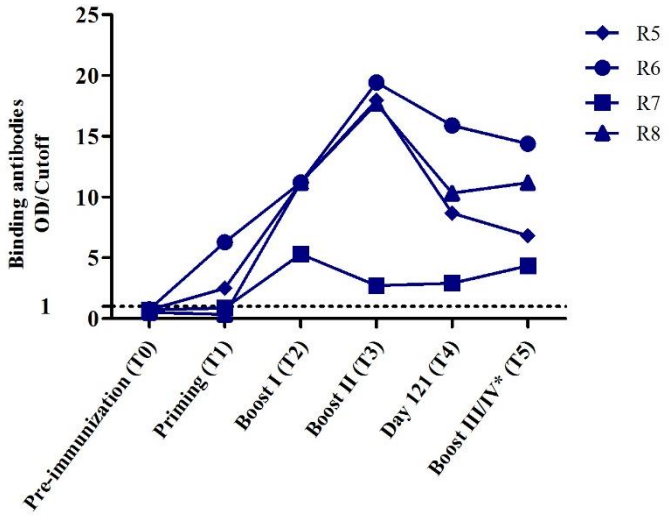

Figure 1- Total binding IgG response of immunized rabbit sera against heterologous protein HIV-1 group M consensus gp120 envelope M. CON-S D11. Animals R5-58 (group 3) represent animals primed with VVgp120AG and boosted with $S_{gp120AG.}$ Priming was administered at day 1 and boosts I, II and III at days 35, 63 and 121, respectively; *Animals R6 and R8 received an extra boost at day 150 (boost IV); All animals were sacrificed at day 154 (T5). Cut-off value was calculated as the mean OD of rabbits immunized with VWR immunogens (control group 2) plus four times the standard deviation. OD/cut-off≥1 was considered positive and is defined by the dashed line.

Rabbit R7 developed a positive binding response through all the experiment albeit at a lowest level compared with the animals from the same group. In addition, at the last time point (day154) R6 and R8 who received an extra boost at day 150 (boost IV) developed the highest IgG binding response which can be due to the extra antigen stimulation provided by the fourth boost. Moreover, rabbit sera from the last time point also reacted with HIV-1 SF162 gp140 trimer (OD/cut-off= 4.284, 4.284, 1.010 and 3.992 for R5, R6, R7 and R8, respectively).

Rabbit antibodies also reacted strongly with the autologous gp120AG and multiple heterologous gp120s in Western blot (Figure 2). Similar to our previous results (Chapter 3), we observed a characteristic pattern of two major bands with approximate molecular weight marker of ~120 kDa and ~150kDa. The extra band with 150kDA may reflect the presence of protein aggregates or alternative glycosylation profiles relative to gp120 [2]. This two band pattern was also observed in SDS-PAGE and Western blot analysis with rabbit antisera after lectin purification of gp120 (Figure 2). Overall, these results demonstrate that the novel CRF02_AG immunogens elicit the production of HIV specific

antibodies in rabbits that are able to recognize and bind strongly to different HIV-1 Env trimers and monomers.

Table 2- Binding antibody titer of sera from vaccinated rabbits against autologous and heterologous gp120 glycoproteins at day 154

Ab titer (log10)		
Rabbit serum	M.CON-SD11	Sgp120AG
R5	3.806	4.408
R6	4.408	5.311
R7	3.204	4.408
R8	4.107	5.010

Rabbit anti-serum from day 154 was used at a 1:300 final dilution; heterologous gp120 (M.CON.SD11) was used at 1µg/ml and the autologous gp120 glycoprotein (Sgp120AG) was used at 2.3 µg/ml;

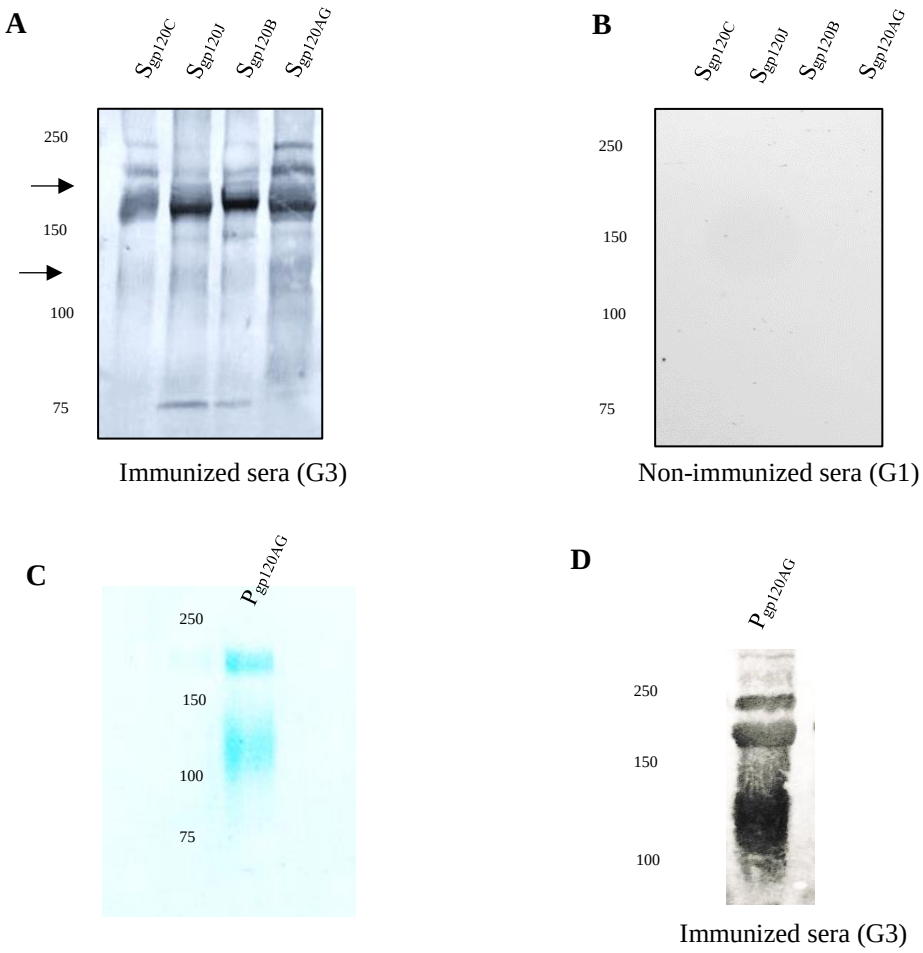

Figure 2 Western blot analysis of gp120t expressed by recombinant Vaccinia virus VVgp120B, VVgp120C, VVgp120AG and VVgp120J. A-gp120 supernatant ($S_{gp120AG}$) incubated with immunized rabbit sera and B- non-immunized rabbit sera; C- SDS-PAGE of gp120AG after lectin affinity chromatography purification ($P_{gp120AG}$); D-Western-blot analysis of $P_{gp120AG}$ against immunized rabbit sera; Precision Plus Protein All Blue Standards (10-250 kDa) was used as a molecular weight marker.

Induction of heterologous tier 2 HIV-1 neutralizing antibodies

In order to determine the neutralizing capacities of the antibodies produced in immunized rabbits, sera from day 154 was tested against a panel of 17 HIV-1 viruses including 12 tier 2 env-pseudotyped viruses from different clades [502], four HIV-1 primary isolates (clades AG, J and untypable) and one tier 1 lab-adapted env-pseudotyped virus (SG3.1). At a 1:20 dilution, none of the sera from control animals showed neutralizing activity (Table 3). Sera from 2 of 4 animals of group 3 neutralized one or more tier 2 viruses whereas the remaining two failed to do so. This neutralization was HIV-specific as determined by the lack of neutralization of VSV-pseudotyped viruses. Notably sera from one vaccinated animal (R8) was able to neutralize at more than 50% almost the entire virus panel (13/16) at 1:20-1:80 titers. In addition, four of the viruses from the panel were neutralized at more than 80% by this particular sera (table 3). These results demonstrate that the new CRF02_AG based immunogens induce in rabbits a potent and broad neutralizing response against HIV-1 primary isolates and tier 2 HIV-1 Env-pseudotyped viruses from all clades.

Table 3- Neutralization of tier 1 and 2 HIV-1 viruses by rabbit sera

Immunization schedule	Animal ID	tier/clade	tier 1	tier 2															
			B	C	CRF01_AE	CRF07	B	CRF07	C	AC	CRF07	G	B	A	C	AG	U	J	U	
		virus	pSG3.1	PCE0217	PCNE8	PCH119	TRO11	PCNE55	PCE1176	246F3	BJOX2000	X1632	PX2278	398F1	25 710 243	01PTCJN	93HDC252	93HDC253	93HDC249	VSV
Non-immunized animals	R1-R2	Group 1	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
$VV_{WR}+S_{VVWR}$	R3-R4	Group2	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
Priming (VVgp120) Boosts (Sgp120)	R5-R6		<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30	<30
	R7		36	<30	<30	<30	<30	<30	<30	39	<30	45	<30	<30	<30	33	33	<30	<30	<30
	R8	Group3	63	30	90	36	32	58	54	80	61	77	79	66	86	71	91	65	75	<30
Controls	huMAb		95	92	70	85	82	62	<30	66	<30	<30	93	79	<30	90	85	84	<30	<30
	HIV+		82	96	100	98	97	95	95	86	79	81	92	99	99	97	91	92	96	<30

Columns subheading indicate virus designation, clade and tier; Group1-non-immunized rabbits; Group2- rabbits primed with VV_{WR} and boosted with SVWR; Group 3- rabbits primed with VVgp120Ag and boosted with Sgp120AG. Percent neutralization was determined in TZM-bl cells measuring the reduction in number of RLU relative to wells with the corresponding preimmune sera. Beige highlighting indicate less than 30% of neutralization; salmon highlighting indicate ≥ 31-49%; red highlighting indicate equal or more than 50% of neutralization; HuAb s refer to PG16, HJ16, VRC01 in a final concentration between 0.02-1µg/ml; HIV^+ refers to HIV-1 positive sera at 1:20 final dilution; rabbit sera was used at 1:20 final dilution. VSV was used as a negative control.

Discussion

Here it was demonstrated that one out of four rabbits primed with a live recombinant Vaccinia virus expressing gp120 from a clade CRF02_AG isolate and boosted with the cognate gp120 developed potent neutralizing responses against nearly the entire panel of tier 2 HIV-1 viruses tested (13/16), although at low titers (1:20-1:80). A similar strategy using a replication-competent Vaccinia virus expressing the full Env (gp160) plus two gp120 subunit boosts was also able to induce cross-reactive neutralizing activities against >50% of a panel of tier 2 HIV-1 isolates in rabbits [422]. Like in our study, the neutralizing response was observed only in some animals and at low titers (1:20-1:100).
The choice of these immunogens and immunization strategy was based in a previous study where mice developed neutralizing responses against a number of heterologous tier 2 isolates albeit with more limited breadth and potency (chapter 3) [586]. Whereas mice developed heterologous neutralizing responses of more than 49% against 4/14 (29%) tier 2 HIV-1 isolates of clades B, CRF07_BC, J and CRF02_AG (autologous virus), rabbit developed bNAbs against 13/16 (81%) tier 2 isolates of clades CRF01_AE, CRF07, CRF02_AG, C, AC, G, B, A, J and U (untypable).
In addition, antibody binding response was seven fold higher in rabbits, with all immunized animals maintaining strong responses against the autologous gp120 through the whole experiment. In the previous study, mice immunized with the same immunogen combination developed a positive antibody response against $S_{gp120AG}$ but at significant lower levels and with a decline between boost I and II [586].
Despite differences in binding antibody titers between the two assays, both studies demonstrated that gp120 immunizations strongly boosted antibody reactivity which in turn may have contributed to the neutralization observed. Interestingly, Shiu-Lok Hu and colleagues [422] have recently demonstrated that repeated immunization with gp120 have little effect in neutralization against heterologous tier 2 isolates.

SF162, a gp140 trimer, was recognized in an ELISA assay by antibodies present in sera of all immunized rabbits (Group 3) from the last time point but not by mice sera from the corresponding time point in the previous study (chapter 3). This may explain in part the more effective neutralizing responses in rabbits as trimers closely resembles the native viral spikes.

Differences concerning the antibody binding response against autologous gp120 immunogen and the potency and breadth of neutralizing responses in mice and rabbits may be assigned to several issues that includes the immunization schedule, immunogen administration and, most important, natural features inherent to the animal model. In fact, it is well known that rabbit antibodies have a higher specificity and affinity being able to recognize epitopes that are not immunogenic in mice thus justifying the significant difference between neutralization breadth observed in both studies [587-589].

In conclusion, in this study we have shown that in rabbits a prime-boost vaccination strategy based on recombinant Vaccinia viruses expressing a novel CRF02_AG gp120 and the cognate gp120 is able to induce the production of antibodies potently binding to monomeric and trimeric autologous and heterologous envelope glycoproteins as well as potent and broadly neutralizing antibodies. These results are consistent with previous findings in mice and provide strong support for additional vaccination studies in NHPs. The results obtained in both studies suggest that the novel CRF02_AG-based immunogens and prime-boost immunization strategy may be able to induce the type of response intended in a preventive HIV-1 vaccine.

Chapter 5

General Discussion and Conclusions

About 30 years ago, when HIV-1 was discovered, people strongly believed that a vaccine would be developed in a short period of time. Today, after years of intense work in the vaccine field there is still no safe and efficient vaccine for HIV. Although the introduction of ART has contributed to turn HIV infection into a manageable disease, a vaccine for HIV is currently the best hope for the control and eventual cure of HIV infection.

Genetic diversity of HIV-1 is huge with multiple subtypes and recombinants unevenly distributed all over the world and an effective vaccine will need to contend with this diversity. Several strategies such as the use of mosaic and consensus envelope glycoproteins have been pursued in the past but with no success [118, 418, 422, 448, 496-501]. Most current vaccine candidates are based on subtypes B and C because of their higher global prevalence relative to the other subtypes and recombinants [118, 119, 465-468, 507, 594-597]. However, it is highly unlikely that a subtype B-or C-based vaccine could provide significant protection against other HIV-1 genetic forms. Thus, candidate vaccines based on other non-B HIV-1 strains that are prevalent in developing countries with high HIV-1 burden need to be explored.

The work described in this thesis is a contribution for the development of a new generation of HIV vaccines based on live Vaccinia virus vectors and ancestral envelope immunogens obtained from primary R5 isolates from the Angolan and Portuguese HIV-1 epidemics. We based our work on these isolates to test the hypothesis that envelope glycoproteins from primary isolates present in highly complex and long-term epidemics could contend better with the genetic and antigenic diversity of HIV-1 compared with envelope glycoproteins from isolates from more recent and monovalent epidemics. In support of this, Hahn BH and colleagues have shown that immunization with ancestral envelope genes recognized a greater number of contemporary clade C antigens compared to immunization with a contemporary subtype C env control[598].

To better characterize H and J subtypes which were intended to be used in the new vaccine, the full-length genomes of three isolates from 1993 from Angola, previously identified as clustering with subtypes H and J based on small gene fragments [514, 538], were sequenced and analyzed phylogenetically (Chapter 2). We had previously shown that these isolates replicated well in culture and were highly sensitive to entry inhibitors [514, 538]. The evidence that HIV-1 pandemic first emerged in West Central Africa and that Angola had a crucial role in the early dissemination of the HIV-1

determined the genomic characterization and inclusion of these subtypes in the new HIV-1 vaccine [67, 68, 510-512].

Near-full-length envelope gp120 fragments from HIV-1 isolates from Angola and Portugal, obtained from 1993 to 2008 and belonging to subtype B and non-B clades (C, CRF02_AG and J) were expressed successfully in Vaccinia virus (chapter 3). Selected isolates to include in the vaccine used the CCR5 coreceptor because most transmitted isolates use this receptor [104, 166, 599].

Monomeric gp120 was used for our vaccine because 1) it encompasses most of the neutralizing domains in the HIV envelope [461, 600, 601] and 2) it was used with some success in HIV-2 and HIV-1 vaccines [2, 422, 453, 454, 479, 494, 579, 602]. In addition, C2V3C3 boosts were used in order to expand and direct the antibody response generated after the priming towards the central C2, V3, and C3 envelope regions. Moreover, C2V3C3 boosts were found to be crucial for the induction of high levels of bNAbs against HIV-2[2].

Antibodies neutralizing at least one heterologous tier 2 isolate were elicited in mice immunized with clades B, C, CRF02_AG, and J but there were significant differences between immunogens. Overall, mice immunized with Vaccinia virus expressing the envelope gp120t derived from clade CRF02_AG followed by a boost with the cognate truncated gp120 developed more potent and broad Env-binding and neutralizing antibodies relative to mice receiving immunogens from other clades. Subsequently, rabbits vaccinated with the CRF02_AG-based immunogens developed neutralizing antibodies against several heterologous tier 2 HIV-1 pseudoviruses and primary isolates from different clades. To the best of our knowledge, this was the first vaccine trial using an immunogen derived from clade CRF02_AG that was able to induce bNAbs against heterologous tier 2 HIV-1 pseudoviruses of all clades and against primary virus isolates. Neutralization of primary isolates is very important because they are produced in PBMCs and, unlike envelope-pseudotyped viruses, their glycosylation profile is similar to clinically relevant isolates [548, 549]. These results suggest that the new CRF02_AG derived immunogens may be useful as a new HIV-1 vaccine and should be further tested in NHPs. It should be noted however that even with the best immunogens the neutralization results were inconsistent as they were only observed in few vaccinated animals. Similar inconsistency has been described in recent studies using other vaccination approaches [422, 453, 476-479, 492]. Some explanations have been offered

to account for this problem such as behavioral features (*i.e.* stress) inherent to the animal that affect vaccine intake and subsequent immune response [422, 598, 603]. In our case, we believe that the main problem is likely related with the initial infection by the recombinant Vaccinia viruses which we did not control for. In the future we plan to closely monitor Vaccinia virus infection before boosting immunizations using a simple immunoassay to detect Vaccinia virus-specific antibodies.

The use of immunogens derived from ancestral primary isolates, *i.e.* isolates showing lower evolutionary distances to the most recent common ancestor (MRCA) defining each clade, may have been important for the induction of broadly binding and neutralizing antibody responses. In fact, envelope proteins derived from contemporaneous isolates have failed to elicit such broad antibody responses in rabbits; usually the antibodies produced only bind to and neutralize tier 1 isolates, the autologous isolate and some heterologous tier 2 isolates matched by subtype [422, 444, 448]. The use of a truncated gp120 protein instead of a full protein may also have contributed for the success of the immunization as it was previously associated with more broad and potent neutralizing responses against HIV-2 than the full-length cognate gp120 [2]. Compared to the full-length envelope glycoproteins, the truncated version of gp120 lacking the C5 region allows for higher levels of expression and shedding while exposing adequately the main neutralizing domains identified in our study, the V3 region and CD4-binding site. These two features are important for the antigenic stimulation of B cells towards the development of high affinity broadly neutralizing antibodies [604-609].

The use of a live Vaccinia vector in the priming immunizations has contributed crucially for the induction of bNAbs as demonstrated by the observation that animals primed with gp120 solely developed the weakest neutralizing response. Many other researchers have shown previously, in different animal models and in humans that priming with monomeric gp120 or vaccination with monomeric gp120 only does not lead to the production of bNAbs [2, 408, 467, 468, 478, 487-489, 551]. With the exception of a recent study in cows immunized with the SOSIP-BG505 trimer where broad and potent serum antibody responses were rapidly elicited [1], much better antibody responses have been obtained when monomeric or trimeric proteins are used in combination with other immunogens [2, 422, 453, 454, 479, 494, 579].

Advantages of using Vaccinia virus as vaccine vectors include their easy production, the capacity to infect efficiently a wide range of different cell types both *in vivo* and *in vitro*,

their large genomes allowing the insertion of large segments of foreign DNA and high levels of expression of the transgene [387, 420, 584, 585]. In addition, live Vaccinia virus vectors elicit stronger humoral and cell-mediated immune responses when compared with highly attenuated non-replicative viral vectors [387, 420, 421, 555]. This is because live replicative viral vectors act like adjuvants activating the immune system and continuously replicate in host cells leading to high level and continuous exposure of B and T cells to the vaccine antigen [207, 387, 420, 421, 582].

Despite these advantages, killed or highly attenuated non-replicative viral vectors are often preferred as vaccine vectors due to safety issues concerning possible adverse reactions with live viral vectors [391, 418, 553, 581, 583]. Most adverse events caused by live vectors are minor and occur primarily in immunocompromised patients [610, 611]. In this context it is perhaps important to note that adverse reactions to Vaccinia virus could potentially be interrupted and reversed with the administration of a CCR5 antagonist as Vaccinia uses the CCR5 molecule to enter into the cells [610, 612-614]. Recently we and others have shown that the CCR5-antagonist TAK-779 inhibits the replication of Vaccinia virus strain WR *in vitro* and *in vivo* (in mice) suggesting that this drug could be a useful safety net against adverse effects eventually caused by vaccination with live Vaccinia vectors (Diniz & Taveira, unpublished)[615].

The use of live recombinant Vaccinia virus vector in this immunization strategy may have been also determinant for the generation of a strong Tfh mediated cellular response which in turn enabled an effective Nab production. Tfh cells are a potential target for HIV vaccines aiming to induce bNAbs as they have been positively correlated in humans and NHPs with an effective humoral response against HIV [240, 254-256, 616-619]. In our mice studies, Tfh cell number correlated positively with binding antibodies against gp120 immunogens but no correlation was observed between Tfh levels and neutralization activity. Similarly, Crotty and colleagues have demonstrated that mice immunized with soluble BG505 SOSIP.664 trimers developed high-quality Tfh responses that positively correlated with Env-binding IgG antibodies but not with NAbs[620]. Also in our study, Tfr cell frequency were found to inversely correlate with neutralization potency and breadth in immunized mice. These cells are important modulators of the germinal center responses and their frequency have been inversely associated in NHPs with higher quality Env-specific antibodies and NAbs [256, 263]. Thus, the efficiency of the new vaccine strategy may, at least partially, be based on the triggering of the specific subsets of T

follicular cells that drive B cell responses to the right path. Overall, our results provide additional support for the use of replicating competent Vaccinia viruses as a component of an HIV vaccine.

Using the same prime-boost strategy and the same CRF02_AG based immunogens, one rabbit (chapter 4) was found to develop more potent and broad antibody responses comparing with mice (chapter 3). Whereas mice developed antibodies neutralizing 29% of tier 2 HIV-1 isolates (clades B, CRF07_BC, J and CRF02_AG), the rabbit developed antibodies neutralizing 81% of the tier 2 isolates including clades CRF01_AE, CRF07, CRF02_AG, C, AC, G, B, A, J and U (untypable). In addition, binding antibody response was 7-fold higher in rabbits with all immunized animals maintaining strong responses against the autologous gp120 throughout the whole experiment. Finally, antibodies present in sera of all immunized rabbits from the last time point but not in mice sera bound to SF162, a gp140 trimer, in an ELISA assay. This may explain in part the more effective neutralizing responses in rabbit as trimers closely resemble the native viral spikes.

Unlike previous vaccination studies in HIV-2 [2], boosting the mice with the C2V3C3-polypeptide did not contribute for the development of neutralizing responses implying that the most potent conformation-dependent neutralizing domains in the V3 region of HIV-1 are not adequately presented by this polypeptide. It is also possible that antibodies against this region are slow to develop as suggested by the increased levels of anti-C2V3C3 antibodies in the last timepoint. Overall, these results confirm that there are major differences in the antigenic structure of V3 between HIV-1 and HIV-2 [2, 550].

Interestingly, whereas the majority of the viruses tested in the neutralization assays had the GPGQ motif in V3 crown region, the gp120 from CRF02_AG clade used in vaccination had a GPGR motif. For subtype B the consensus sequence for the V3 crown motif is GPGR, while for non-B subtypes it is often GPGQ [330, 556-559]. Tier 2 clade B Env-pseudovirus PX2278, which has the canonical GPGR motif, was neutralized by all animals immunized with the CRF02_AG derived glycoproteins. Together with the strong binding of the anti-GPGR human monoclonal antibody 447-52D to gp120 AG this indicates that, unlike the C2V3C3-polypeptides, our CRF02_AG monomeric gp120 glycoprotein presents adequately this dominant neutralizing epitope in V3 and leads to the induction of potent neutralizing antibodies directed against the V3 crown. 447-52D is known to bind and neutralize preferentially viruses from clade B but it also neutralizes viruses from non-B clades although in a lesser extent [330, 348, 546, 621, 622]. NAbs

against heterologous HIV-1 viruses targeting the V3 loop of gp120 have been widely described by several authors highlighting the importance of this region [325, 326, 330, 347, 623]. In a recent study where 66 HuMabs specific to V3, CD4bs and V2 were tested against 41 tier 1, 2 and 3 Env-pseudoviruses neutralization and breadth of anti-V3 HuMabs were found to be significantly higher than those directed to other regions [624]. Importantly, only anti-V3 antibodies were able to neutralize tier 2 and 3 viruses. Overall, our results showing that antibodies induced by the novel CRF02_AG gp120-based vaccine neutralized tier 2 HIV-1 isolates from different clades despite targeting essentially the V3 crown, provide further support for the V3 crown as a crucial vaccine target.

Because g120AG immunogens were also able to induce NAbs against non-B Env-pseudoviruses and primary isolates that did not present the GPGR motif, NAbs targeting other epitopes must have also been elicited. In fact, HuMAb HJ16 (a CD4 binding site bNAb obtained from a clade C infected patient) bound in ELISA to all gp120 immunogens, although at low levels, suggesting some exposition of CD4 binding site epitopes which may have contributed to the neutralization observed [325]. CD4bs is a highly conserved site present in the gp120 of all HIV-1 clades needed for the initial virus-host interaction and therefore it represents one of the most attractive targets for vaccines that aim to induce neutralizing responses [422, 625-628].

The target of the largest and most diverse group of bNAbs that recognize N-linked glycan containing epitopes is the region adjacent to the highly conserved N-linked glycosylation site at N332 which includes the V3 loop base and nearby glycans [547, 560, 561]. Antibodies directed to V3 glycans are major contributors to neutralization breadth and potency [154, 335, 352, 354, 629]. The observation that the novel CRF02_AG based immunogen had a high probability of glycan occupancy in position N332 together with the fact that PX228, the tier 2 HIV-1 pseudovirus neutralized by all gp120 AG immunized mice, also had a high probability of glycan occupancy (Pg= 0.86, motif NIS) in this position suggests that these immunogens could also have induced glycan-dependent bNAbs directed to this region in immunized mice and rabbits. However, further studies will be necessary to clarify the epitope specificities of NAbs induced by the novel immunogens produced in this study.

Conclusions

The high diversity among Angolan subtype J sequences suggest that Angola is either the origin of subtype J or that there has been a lot of influx of subtype J from other geographic regions. In addition, the high diversity of rare subtypes in Angola is consistent with the important role of this region in the early establishment of the HIV-1 pandemic.

It was demonstrated that it is possible to induce neutralizing antibodies against heterologous tier 2 HIV-1 pseudoviruses and primary isolates in different animal models using a prime-boost immunization strategy based in a live replicative viral vector expressing the envelope gp120t derived from an ancestral CRF02_AG clade subtype. These results provide support for the use of Vaccinia virus as a vaccine vector for HIV-1 and for the a priori hypothesis that envelope immunogens from ancestral non-B strains could induce potent and broad antibody responses against contemporaneous isolates of all clades. Finally, the V3 crown was found to be one of the main targets of neutralizing antibodies induced by the novel immunogens supporting the V3 region as one of the constituents of a successful HIV-1 vaccine. Further studies will be necessary to formally identify the other neutralizing targets of our vaccine and to understand why only a fraction of the animals produced broadly neutralizing antibodies.

Future perspectives

Optimization of this particular vaccine, namely the nature of the immunogens and immunization regimen, may lead to a considerable improvement in antibody responses, namely in breadth and potency. In order to expand and elicit neutralizing responses against more conserved epitopes, such as CD4bs and glycan regions, new env constructs with deletions in some variable loops may be an important approach as it may ultimately turn the conserved regions of gp120 more accessible to neutralizing antibodies. In addition, shielding of irrelevant domains could also lead to the exposition of more important domains and consequently target the right set of NAbs to the neutralizing epitopes. Co-immunization of this new Env constructs in a prime-boost regimen with the same recombinant Vaccinia virus vector used in this study could induce higher antibody responses. Although this new vaccine concept was able to induce bNAbs against heterologous HIV-1 tier 2 pseudoviruses and primary isolates belonging to different

clades, the inclusion of immunogens from different non-B clades in the same prime-boost regimen (*i.e*. vaccine cocktail) could contribute to increase further the breadth of Nab responses. Our medium-term perspective if all goes well is to test the new vaccine (or its modifications as described above) in nonhuman primates.

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