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Broadly neutralizing antibodies against HIV : design of isolation strategies and application to two elite neutralizers

Romy Rouzeau

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THÈSE

Pour obtenir le grade de

DOCTEUR DE L'UNIVERSITE GRENOBLE ALPES

Spécialité : **Virologie, Microbiologie, Immunologie**

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Présentée par

Romy ROUZEAU

Thèse dirigée par **Pr Pascal POIGNARD, PuPh, CHUGA**
préparée au sein de l'**Institut de Biologie Structurale**
dans l'**École Doctorale Chimie Science du Vivant**

Anticorps Neutralisants à Large Spectre contre le VIH : mise au point de stratégies d'isolement et application à deux neutraliseurs d'élite

Thèse soutenue publiquement le **25 juin 2021**,
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Abstract

A fraction of HIV-1-infected individuals develop Broadly Neutralizing Antibodies (BNAbs). i.e. Abs that target the viral envelope glycoprotein (Env) and can block the replication of the majority of the highly genetically divergent global HIV-1 strains. A vaccine that would elicit such BNAbs would likely protect against HIV infection. However, to date, all vaccine approaches have failed in this regard. Our team focuses on understanding the mechanisms of development of BNAbs responses, notably in a large African longitudinal cohort of primary infection. The objectives of my PhD have been: i) to isolate and characterize the BNAbs of donor PC02, the 4th best neutralizer of the cohort; and ii) to complete the ontogeny of PC94 BNAbs, the 2nd best neutralizer of the cohort. Our work first aimed to map the Env epitopes targeted by the BNAbs in the PC02 serum, in order to use this information to isolate BNAbs through a differential B cell sorting approach. Despite the use of multiple mapping strategies, the PC02 BNAbs specificities could not be identified, suggesting either that Abs targeting diverse epitopes may be responsible for the serum breadth, or the presence of unknown specificity(ies). The results led us to reassess the cell sorting strategies commonly employed for BNAbs isolation in the laboratory, particularly when the broad specificity is known. We designed a new isolation strategy, based on the sorting of B cells able to bind multiple recombinant trimeric Env, followed by a cell activation step and a functional neutralization screening. The strategy was then applied to donor PC94. Indeed, previous work in the laboratory led to the isolation and characterization of two BNAbs lineages from this donor, both directed against the HM Patch at the base of the Env V3 loop (unpublished), but the corresponding Abs could not recapitulate the broad neutralizing activity of the donor serum. It was thus hypothesized that other Abs, from the same or different lineages, potentially targeting other Env epitopes, may explain the broad neutralization. To answer this question, the newly designed isolation strategy was applied to PC94 PBMC samples harvested at different time post-infection, yielding fifty-one pairs of heavy and light chains that were selected for cloning. Thirty-three Abs could then be produced and tested for neutralization. Thirteen Abs showed neutralizing activity with notable breadth. Ten Abs newly isolated were shown to belong to one of the previously identified lineages with some being slightly broader than their somatic variants. One Ab was part of the second lineage previously identified, although showing a weaker neutralizing activity. Two isolated Abs were part of BNAbs lineages different from those already characterized for this donor. The neutralization spectrum of these Abs was complementary, with one Ab neutralizing preferentially clade B viruses while the other neutralized in particular clade C viruses. The dynamic of the emergence of those

BNAbs lineages and the nature of their epitope on Env remain to be determined. In conclusion, the newly elaborated strategy enabled the successful isolation of novel PC94 BNABs, with notably Abs from a lineage already identified and the identification of 2 new lineages. However, the serum breadth still remained not entirely recapitulated by the activity of the Abs isolated. Our results illustrate the difficulty of explaining the broad neutralizing activity of the serum of some patients, despite new technical advances in BNABs isolation. They also suggest that the presence of several lineages of NABs might possibly explain the broad serum activity of some elite neutralizer.

Résumé

Une fraction des individus infectés par le VIH-1 développe des anticorps neutralisants à large spectre (AcNLS), dirigés contre la glycoprotéine d'enveloppe (Env) et capables de bloquer l'infection des cellules cibles malgré la grande diversité génétique du virus. Un vaccin qui induirait une telle réponse Ac pourrait probablement protéger contre l'infection par le VIH. Notre équipe étudie les mécanismes de développement des réponses AcNLS, notamment dans une large cohorte africaine de primo infection. Les objectifs de ma thèse ont été i) d'isoler et caractériser les AcNLS du donneur PC02, le 4^e meilleur neutraliseur de la cohorte et ii) de compléter l'ontogénie des lignées d'AcNLS de PC94, 2^e meilleur neutraliseur. Nos travaux ont d'abord visé à cartographier l'épitope de Env majoritairement ciblé par les AcNLS du sérum de PC02, afin d'utiliser cette information pour la réalisation d'un tri différentiel des lymphocytes B du donneur et l'isolement d'AcNLS. Malgré l'emploi de différentes méthodes, un épitope des AcNLS de PC02 n'a pu être identifié, suggérant l'existence d'une diversité d'AcN chez ce donneur ou de spécificités AcN nouvelles. Ces résultats nous ont amenés au remaniement des stratégies d'isolement d'AcNLS utilisées habituellement dans le laboratoire. Les nouvelles stratégies de tri, basées sur une sélection par de multiples Env trimériques recombinantes, couplée à de l'activation cellulaire B et à un criblage fonctionnel ont été appliquées à PC94. Pour ce donneur, des travaux du laboratoire avaient auparavant permis l'isolement et la caractérisation de deux lignées d'AcNL ciblant la région riche en mannose à la base de la boucle V3 de Env. Ces Ac ne récapitulant pas l'extraordinaire capacité de neutralisation du sérum de PC94, l'hypothèse a été faite que d'autres Ac des mêmes lignées, ou de lignées différentes, potentiellement dirigés contre d'autres épitopes de Env, devaient expliquer la neutralisation à large spectre. De nouveaux tris de cellules B mémoire spécifiques ont été effectués, basés sur la stratégie nouvellement établie. Ces tris ont permis l'isolement de cellules B porteuses d'Ac à potentiel de neutralisation LS. Cinquante-et-une paires de chaînes lourdes et légères ont été sélectionnées pour être clonées. Trente-six Ac ont été produits et testés pour leur capacité à neutraliser. Treize Ac ont montré une activité neutralisante avec une largeur de spectre notable. Dix Ac appartenaient à l'une des deux familles d'Ac identifiées antérieurement, présentant parfois des spectres légèrement plus larges. Un Ac appartenait à l'autre famille, en moins neutralisant. Enfin, 2 AcN isolés étaient de familles nouvelles. Les spectres de ces 2 AcNs, dont les épitopes restent à déterminer, semblent se compléter, avec l'un plutôt actif contre les virus de clade B et l'autre contre ceux de clade C. En conclusion, les nouvelles stratégies de tri mises en place ont été efficaces à isoler des AcN chez PC94, de lignées déjà identifiées et également de deux nouvelles lignées.

Leurs relations de coopération et dynamique d'apparition restent à mieux caractériser. Toutefois, le spectre de neutralisation du sérum de PC94 n'est toujours pas entièrement expliqué par les AC isolés. Ceci pourrait s'expliquer par la rareté des cellules B correspondant aux AcNLS ou l'utilisation pour le tri d'appâts dont la conformation serait trop éloignée des Env virales natives. Par ailleurs des cellules B d'intérêt pourraient avoir été sélectionnées mais les AcNLS correspondant non isolés dû au rendement imparfait des étapes d'activation, d'amplification génique, de clonage ou de production qui ont suivi. Nos résultats illustrent la difficulté à expliquer pour certains patients la neutralisation à large spectre de leur sérum, malgré les avancées techniques d'isolement des AcNLS. Ils montrent aussi comment l'évolution de plusieurs lignées d'AcN pourrait potentiellement expliquer le spectre d'activité du sérum d'un neutraliseur d'élite.

List of abbreviations

Ab : Antibody

ADCC: Ab-dependent cellular cytotoxicity

AID: Activation-Induced Cytidine Deaminase

AIDS : Acquired ImmunoDeficiency Syndrome

AZT : Azydothymine

BCR: B cell Receptor

BNAb : Broadly Neutralizing Antibodies

cART : combined Antiretroviral Therapy

CC: I201C-A433C

CD4-bs : CD4 binding site

CDC : Center for Disease Control

CDR: Complementary Determining Region

CMV : Cytomegalo Virus

CRF : Clade Recombinant Form

CTL: Cytotoxic T Lymphocytes

DF: Depleted Fraction

DMEMc: Complete DMEM

DNA : DeoxyriboNucleic Acid

EF: Elution Fraction

ELISA: Enzyme-Linked ImmunoSorbent Assay

EM: Electron Microscopy

Env : Envelop Glycoprotein

eOD-GT: engineered Outer Domain Germline Targeting

Fab: Antibody-Fraction

FACS: Fluorescence-Activated Cell Sorting

Fc: Crystallizable Fraction

FDC: Follicular Dendritic Cell

FP: Fusion Peptide

FR: Framework

GC: Germinal Center

GlcNac: N-acetylglucosamine

GnTI: N-acetylglucosaminyltransferase

Gp : glycoprotein

HAART : Highly Active Anti-Retroviral Therapy

HC : Heavy Chain

HEK: Human Embryonic Kidney

HIV : Human ImmunoDeficiency Virus

HLA: Human Leukocyte Antigen

HM Patch: High Mannose Patch at the base of Env V3 loop

HTLV : Human T-Lymphotropic Virus

HVTN: HIV Vaccine Trial Network

IC50: Index Concentration for 50%

ID50: Index Dilution for 50%

IgG: Immunoglobulin G

IMGT : ImmunoGeneTics database

InSTI : integrase strand transfer inhibitor

KC : Kappa Light Chain

KO: Knocked Out

LAV : Lymphadenopathy Associated Virus

LC : Lambda Light Chain

IC : Light Chain

LTR : Long Terminal Repeats

mAb : monoclonal Antibody

Mgp120: monomeric gp120

MHC: Major Histocompatibility Complex

MMWR : Morbidity and Mortality Weekly Report of the CDC

MPER: Membrane Proximal External Region

MPI: Month Post Infection

Mrgp120: monomeric recombinant gp120

NAb : Neutralizing Antibody

NFL: Native Flexible Linker

NGS: Next Generation Sequencing

NHP: Non-Human Primate

NK: Natural Killer

NN : Non Neutralizing

NNAb : Non Neutralizing Antibodies

NNRTI: non-nucleoside reverse transcriptase inhibitors

NRTI: nucleoside-analog reverse transcriptase inhibitors

PBMC : Peripheral Blood Mononuclear Cells

PCR: Polymerase Chain Reaction

PI: protease inhibitors

PIC : Pre Integration Complex

PrEP: Pre Exposure Prophylaxis

PSG: Penicilline-Streptomycin-Glutamine

RNA : RiboNucleic Acid

RSS: Recombination Segment Sequence

RT-PCR: Reverse Transcription PCR

SIV : Simian ImmunoDeficiency Virus

SMH: Somatic HyperMutations

SOSIP: structure platform to stabilize Env trimer with a disulfide bond between gp41 and gp120 and I559P mutation

TD: Trimer Derived

Tfh : T follicular helper

Th : T helper

UCA: Unmutated Common Ancestor

UFO: Uncleaved Fusion Optimized

V1, V2 and V3: Env Variable Loops 1, 2 and 3

VLP: Virus-Like Particle

WHO: World Health Organization

WT: Wild Type



Introduction

I. HIV, at the origin of the AIDS pandemic

1. AIDS: from the first cases to the global health threat

In June 1981, the *Center for Disease Control's* weekly report described 5 cases of a very rare form of *Pneumocystis carinii* pneumonia affecting homosexual men in Los Angeles (CA, USA) (Gottlieb et al, MMWR Morb Mortal Wkly Rep. June 1981). The following month, the CDC announced 26 cases over 30 months of Kaposi sarcoma, an epithelial cell cancer thought to also be extremely rare, in both California and New York (MMWR Morb Mortal Wkly Rep. July 1981). All together, these reports fitted the description of an emerging syndrome, with an unusual failure of the immune system, associated with various outcomes notably linked to opportunistic infections and cancers... (Brennan & Durack, 1981, Masur et al., 1981, M. S. Gottlieb et al., 1981...). Spreading like a wildfire within the American male homosexual community, the new disease was initially called “the gay cancer” or “the pink plague”. In 1982, following the description of several heterosexual cases (Masur et al., 1982)(Pitchenik et al., 1983), the pathology was officially named “Acquired Immune Deficiency Syndrome” (AIDS).

From an emerging unknown syndrome, AIDS became a worldwide pandemic in less than 5 years. In 2019, the number of deaths due to the syndrome since the beginning of the pandemic was estimated at 32,7 million, and the number of new cases at 1,7 million this same year (UNAIDS) (Fig. I-1).

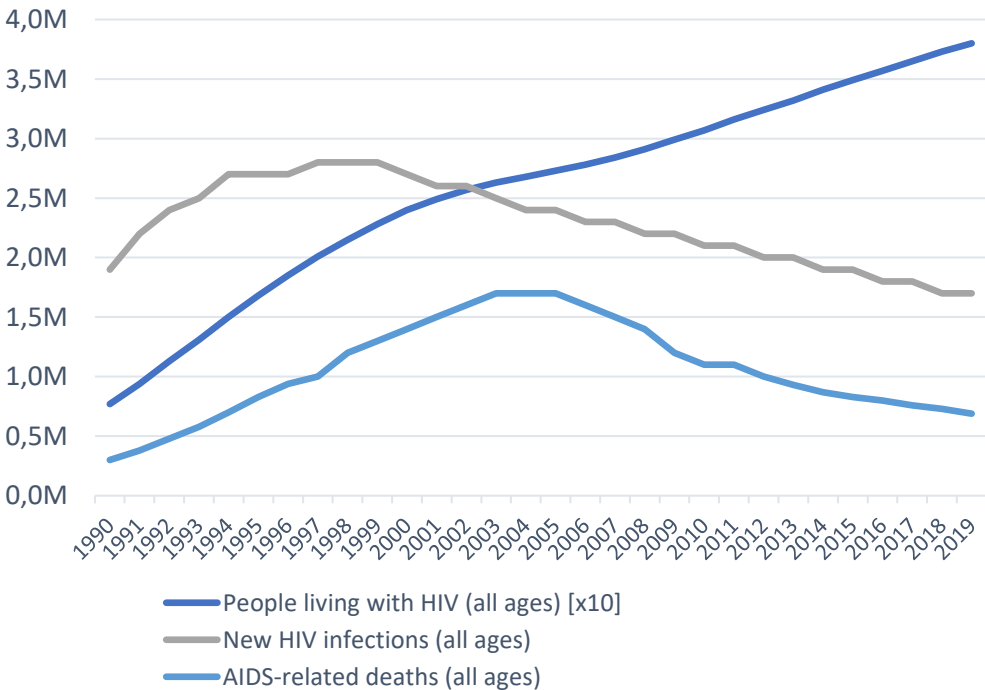


Figure I-1: Prevalence, new cases and deaths from HIV/AIDS worldwide. Data from UNAIDS.

AIDS was eventually found to be caused by infection by the Human Immunodeficiency Virus (HIV).

2. The discovery of HIV

HIV was originally isolated and characterized under the name of LAV (Lymphadenopathy Associated Virus) in 1983 by the French group of L. Montagnier (Barré-Sinoussi et al., 1983). Within the same year, the American team of R. Gallo published several papers about the virus HTLV-III, also claimed to be responsible for AIDS (Gallo et al., 1983)(Gelmann et al., 1983) (Popovic et al., 1984). In 1985, both LAV and HTLV-III genomes were sequenced and proven to be identical (reviewed in Norman, 1985). The virus was renamed HIV. The paternity of the discovery was ultimately attributed to the French group in 1987 and lately rewarded by a Nobel Prize in 2008.

3. Origin and diversity of HIV

There are two HIV types: HIV-1 (Barré-Sinoussi et al., 1983) and HIV-2 (Barin et al., 1985) (Clavel et al., 1986), presenting 40% nucleotide sequences homology for the more conserved *gag* and *pol* genes and 30 to 40% for the other viral genes and LTRs (Guyader et al., 1987).

HIV-1 is more commonly encountered than HIV-2 and is responsible for the AIDS pandemic with more than 37,2 million worldwide infections in 2019 (UNAIDS).

The simian origin of HIV-1 (Huet et al., 1990) (Keele et al., 2006) is supported by the phylogenetical analyses of both the HIV-1 and the Simian Immunodeficiency Virus (SIV)(Gao et al., 1999). These data indicate that the latter have been transmitted to humans on at least seven occasions, supposedly through blood exposures associated with monkey hunting (Hahn et al., 2000). The event that started the AIDS pandemics is roughly dated around the beginning of the XXe century (B. Korber et al., 2000). The oldest HIV sample identified to date is a 1959 blood sample from a male individual of the Democratic Republic of Congo (T. Zhu et al., 1998). It has been suggested that the popularization of transportations (planes, more roads...) and the return of the Haitians to Haiti from Congo when it became independant largely contributed to the spreading abroad, likely bringing HIV to Haiti and then to the USA (Gilbert et al., 2007).

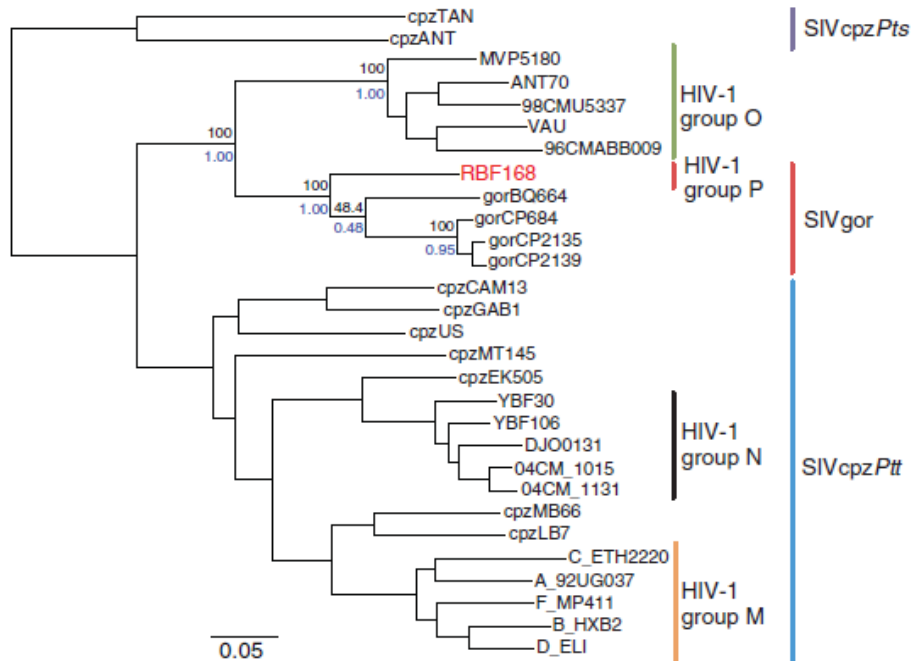


Figure I-2: Evolutionary relationship HIV-1, SIVcpz and SIVgor. Maximum likelihood phylogeny inferred from concatenated amino acid alignments corresponding to the partial sequences available for SIVgorBQ664; 1,052 amino acid positions remained after stripping gap-containing sites. The support values (indicated for key nodes only) in black above the branches are from 1,000 maximum likelihood bootstraps (shown as percentages), whereas posterior probabilities from amino acid Bayesian analysis are shown in blue below the branches (shown as proportions). From Plantier et al., 2009.

HIV-1 presents a tremendous diversity and can be classified into four groups (Fig. I-2):

- M (Major): This group is the principal cause of the current pandemic, as it is responsible of 98% of worldwide infections. Group M derived from a chimpanzee SIV strain (SIVcpz)(Gao et al., 1999). Within this group, there are nine pure subtypes, or clades (A, B, C, D, F, G, H, J and K) and more than 90 recombinant forms (CRF01_AE, CRF02_AG...). Subtype C is the most represented at the global scale (Thomson et al., 2002) while subtype B is the most geographically dispersed (Magiorkinis et al., 2016)(Fig. I-3).
- N (Non-M Non-O): Like the M-group, N-group viruses are likely derived from SIVcpz (François Simon et al., 1998). There are less than 20 N-group infections reported to date, all localized in Cameroon (DeLaugerre et al., 2011).
- O (Outlier): SIVgor (gorilla) was identified as the immediate source of O-group viruses (D’Arc et al., 2015). The O-group epidemic concern about 100,000 infections, localized in West-Central Africa (De Leys et al., 1990) (Bush & Tebit, 2015).
- P: This small group has likely derived from SIVgor. To date, only 2 individuals were identified with such an infection (Plantier et al., 2009) (Vallari et al., 2011)(Fig. I-2).

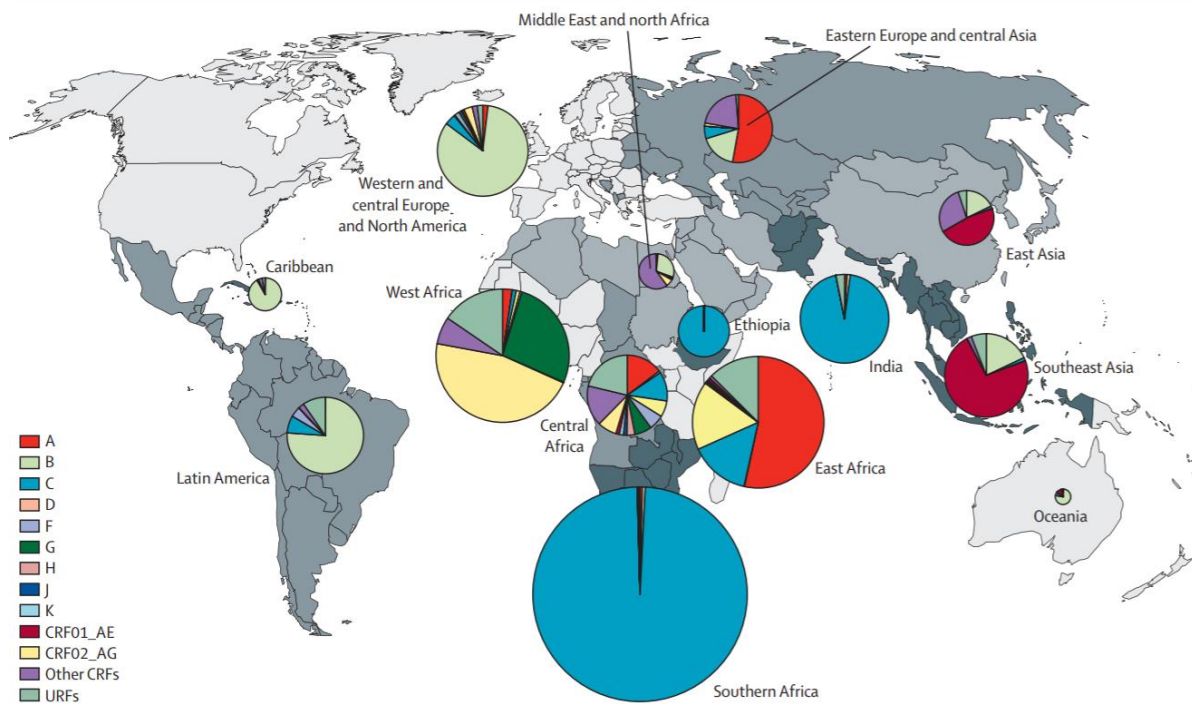


Figure I-3: Regional distributions of HIV-1 subtypes, CRFs, and URFs, 2010–15. From Hemelaar et al., 2019.

Unlike HIV-1, HIV-2 type was shown to be derived from a Sooty Mangabey SIV strain (SIVsmg) (Hirsch et al., 1989). HIV-2 infection only concerns 1 to 2 million individuals (G. S. Gottlieb et al., 2018), is mostly endemic to West Africa (Guinea-Bissau, Gambia, Senegal, Mali, Sierra Leone, Nigeria...), but can also be found in historically associated countries in Europe and India (Campbell-Yesufu & Gandhi, 2011). HIV-2 is divided in nine sub-groups (A to I) with only A and B being epidemic (Visseaux et al., 2016).

HIV variability is a major issue for the design of a preventive vaccine (the immune system having to potentially recognize a tremendous diversity of targets) and for the treatment of the infection (the extreme variability potentially leading to the emergence and selection of resistant variants).

4. HIV transmission

HIV can be transmitted upon exposure with infected body fluids (blood, semen, rectal fluid, vaginal fluid, and breast milk) of another individual's bloodstream or mucous tissues.

Transmission routes are divided in:

- Horizontal transmission, mainly by sexual routes (96% of infections worldwide), the remainder being transmitted through the parenteral route (See additional details in Table I-1).
- Vertical transmission, i.e. from an infected mother to her child: during the pregnancy, delivery or breast-feeding.

Exposure route	Risk per 10 000 exposures to an infected source	95% Confidence interval
Parenteral exposure		
Blood transfusion	9250	(8900–9610)
Needle-sharing injection drug use	63	(41–92)
Percutaneous needle stick	23	(0–46)
Sexual exposure		
Receptive anal intercourse	138	(102–186)
Insertive anal intercourse	11	(4–28)
Receptive penile–vaginal intercourse	8	(6–11)
Insertive penile–vaginal intercourse	4	(1–14)
Receptive oral sex	Low	(0–4)
Insertive oral sex	Low	(0–4)
Vertical transmission		
Mother-to-child transmission	2260	(1700–2900)

Table I-1: Estimated per-act probability of acquiring HIV from an infected source, by exposure route (Patel et al., 2014). Sexual exposure is estimated i) to an HIV-infected partner and ii) assumed no condom use.

5. Overall structure of the HIV-1 virion

HIV belongs to the genus *Lentivirus*, of the subfamily *Orthoretrovirinae*, of the family *Retroviridae*.

Its genome is composed of two identical single-stranded positive-sense RNA (+ssRNA) of approximately 9kb each (Fig. I-4). Both RNA strands are closely bound to nucleocapsid proteins (p7, *not represented*) and kept together with three viral enzymes – a protease (p10), a reverse transcriptase (p64) and an integrase (p32) – in a protein capsid (p24).

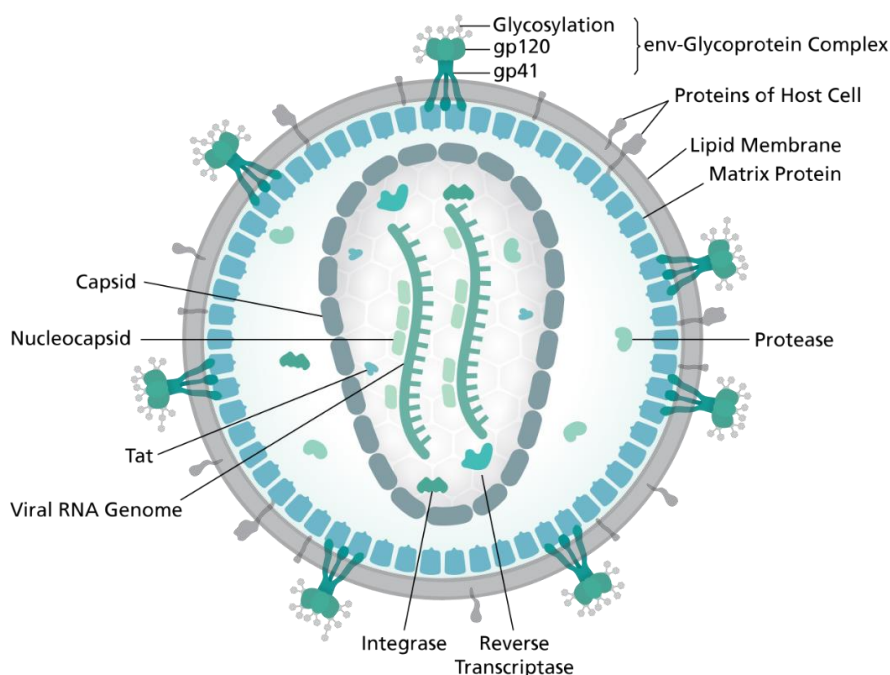


Figure I-4: Schematic HIV structure, adapted from an illustration of Thomas Spletstößer (scistyle.com)

The capsid is itself contained in a matrix (p17) which is enveloped by a lipid membrane derived from the cell in which the virus replicated.

The only HIV protein displayed on the surface of the virus is the envelope glycoprotein (Env).

Six to 20 Env maximum are estimated to be included on the surface of HIV, depending on the viral strain (P. Zhu et al., 2003)(P. Zhu et al., 2006), and only a few (1 to 3) are required for viral entry according to current estimates (Brandenberg et al., 2015). Env is a trimer of heterodimers composed of gp41-transmembrane and gp120-surface subunits. Its structure is more extensively detailed further, in part II-C-1.

6. HIV-1 genomic organization

Each HIV-1 RNA strand is flanked by two Long-Terminal Repeat (LTR). One strand includes nine Open Reading Frame (ORF), enabling the production of 15 different proteins (Frankel & Young, 1998) (Fig. I-5).

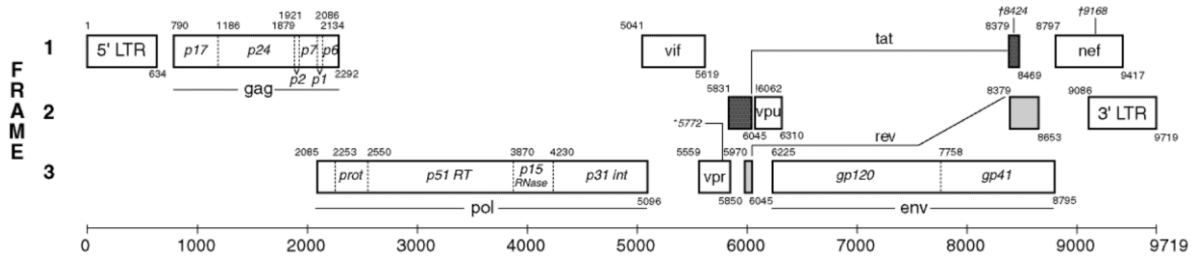


Figure I-5: HIV-1 gene map. From the HIV sequence database of Los Alamos National Laboratory.

HIV-1 genes are classified in 3 different categories (HIV InSite Knowledge Base Chapter, Hope and Trono, The Salk Institute, 2000):

Structural	<ul style="list-style-type: none"> - Gag (Group specific-antigen) encodes proteins of the viral matrix (p17), capsid (p24) and nucleocapsid (p7-p9) - Pol (DNA polymerase) segment encodes three viral enzymes: a protease (p10), a reverse transcriptase (p64) and an integrase (p32) - Env encodes the envelope glycoprotein (gp160 cleaved into gp41 and gp120 subunits)
Regulatory	<ul style="list-style-type: none"> - Tat (Trans-Activator of Transcription) encodes the Tat protein (p14), - Rev encodes the crucial transactivating protein Rev (p19)
Accessory	<ul style="list-style-type: none"> - Vif (viral infectivity) for the identically named protein (also named p23) - Vpr (viral protein R) for the identically named protein - Vpu (viral protein U) for the identically named protein (also named p16) - Nef (negative factor) for the identically named protein (also named p27)

HIV-1 transcription is mediated by a single promoter in the 5' Long-Terminal Repeat (LTR).

7. Viral cycle

The viral cycle refers to the succession of steps which enable the virus to replicate in its host cell. The HIV cycle contains 12 main steps very briefly described below (reviewed by Engelman & Cherepanov, 2012)(Fig. I-6):

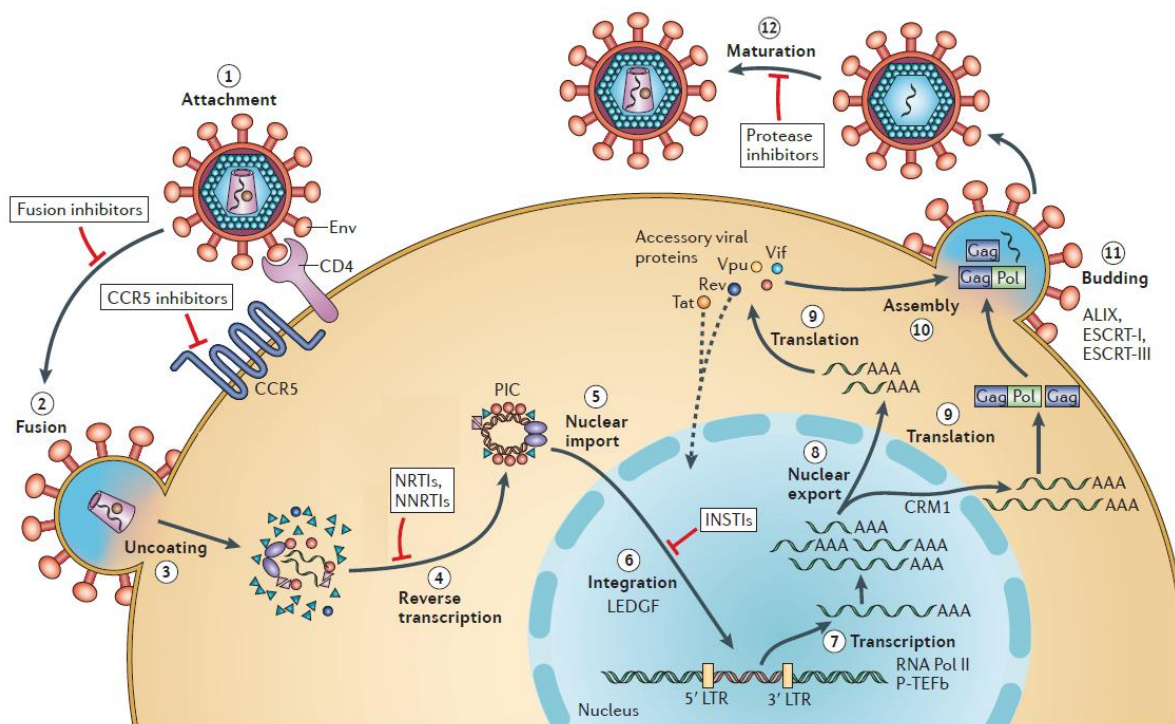


Figure I-6: Schematic overview of the HIV-1 replication cycle. Derived from Engelman & Cherepanov, 2012.

- 1. Attachment:** The CD4-binding site (CD4-bs) on gp120 subunits interacts with the CD4 receptor of the target cell which triggers a conformational change of gp120, allowing an interaction with a co-receptor, CXCR4 or CCR5, leading to further unfolding of the Env trimer and exposure of the fusion peptide.
- 2. Fusion:** The fusion peptide of the gp41 subunit then anchors itself in the host cell membrane, while gp41 folds into a six-helical bundle, further forcing the viral and cell membranes to eventually merge, forming a pore into the host membrane.
- 3. Entry:** The capsid containing the HIV genome and proteins is released in the host cell cytoplasm.
- 4. Reverse Transcription:** The viral reverse transcriptase, activated by the binding of cellular tRNA to the viral RNAs and an increase in dNTP concentration, retrotranscribes the viral RNA into DNA inside the capsid which, despite being already partially degraded by cellular proteases, migrates toward the host nucleus using microtubules.

5. **Nuclear import:** The newly formed viral DNA is imported inside the host nucleus thanks to the pre-integration complex – (the PIC: an association of cellular and viral proteins, among which the integrase) - through the nuclear pore complex.
6. **Integration:** The viral DNA is integrated into the host genome by the viral integrase and is either immediately active or remains latent for periods varying from days to years, depending on the cell activation state.
7. **Transcription:** The integrated viral DNA is transcribed by the host polymerase, as for host genes.
8. **Nuclear export:** The viral protein Rev guides the new viral RNA out of the nucleus.
9. **Translation:** The viral RNA is translated into viral proteins (precursor structural polyproteins, regulatory, accessory...). The new nucleocapsid proteins capture two RNA strands which will form the genome of a new viral particle.
10. **Assembly:** The new genome and viral proteins are aggregated at the membrane to assemble in a new viral particle. The cellular endosomal sorting complex required for transport (ESCRT) machinery is also recruited to allow the pinching of the membranes in the next step.
11. **Budding:** The new virion buds and separates from the host cell, taking a piece of the cell membrane.
12. **Maturation:** Separated from the host cell, the virion goes through a maturation step when the protease cleaves the capsid protein precursors allowing an internal structural rearrangement.

The total duration of the cycle is estimated at 1.2 days (Perelson et al., 1996).

8. Physiopathology of the HIV infection

As previously mentioned, HIV targets the cells carrying the CD4 molecule, i.e. CD4+ T lymphocytes, macrophages and dendritic cells. HIV infection is associated to the depletion of the activated CD4+ T lymphocytes, not only due to the direct attack by the virus but also the mechanisms of chronic immune activation and inflammation, pyroptosis and apoptosis (detailed in Vijayan et al., 2017). In resting CD4+ T lymphocytes or macrophages, the virus can remain in a latent state, building viral reservoirs in the circulation or in tissues (Finzi et al., 1997).

Notably, in targeting CD4+ T helper cells (Th), HIV infection has dramatic consequences, as Th cells are the main leader-managers of adaptive immunity. Indeed, Th cells help B cells to activate and secrete antibodies, macrophages to degrade microbes and cytotoxic T cells to destroy infected cells. HIV infection thus leads to the gradual depletion of major actors of the immune defenses, rendering the infected organism harmless against typically non-life threatening opportunistic infections (Gougeon, 2005).

Left untreated, HIV infection is typically characterized by three different clinical stages (Fig. I-7).

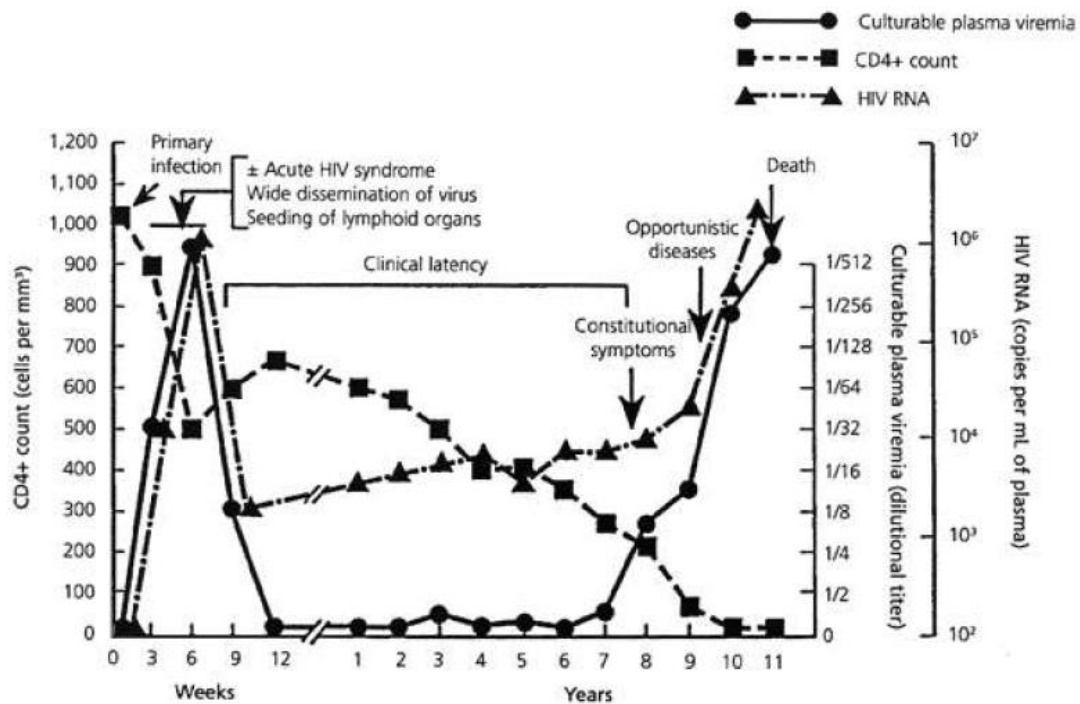


Figure 1-7: Natural history of human immunodeficiency virus (HIV) infection in the average patient without antiretroviral therapy, from the time of HIV transmission to death at 10 to 11 years. The initial burst of viremia with very high HIV-1 RNA levels (●) is followed by a prolonged period during which viral replication continues with lower but measurable HIV-1 RNA levels (*). With the decline of the CD4 lymphocyte count (■) and the collapse of the immune system, high-level viremia, symptoms and opportunistic infections develop, and death ultimately occurs. Adapted with permission from (Fauci et al., 1996).

During the initial acute infection, the recently transmitted virus replicates at a very high rate, with viral load reaching up to 10 million of RNA copies per mL of plasma. As a consequence, the number of CD4+ cells decreases drastically. Newly infected individuals eventually display flu-like symptoms, e.g. fever, fatigue, rash, headache, lymphadenopathy, pharyngitis, and weight loss. This primary infection stage typically lasts for 2 to 4 weeks (Guttman et al., 2012)(Kahn & Walker, 1998).

Following the acute stage, the host elicit immune responses against the infection: CD4+ T cell levels rebound while HIV replication decreases leading to the asymptomatic infection phase, also called clinical latency or chronic infection. This asymptomatic stage lasts 10 years in average, with variabilities from a couple of months to decades. Over this period, HIV continues to replicate until the viral load slowly starts increasing while CD4+ cells count gradually diminishes (Lyles et al., 2000).

The last stage arises when CD4+ cells drop to low levels making the organism fully vulnerable to opportunistic infections that eventually leads to death. Patients infected with HIV are defined as having AIDS if they have either:

- a CD4+ T-cell count below 200 cells/ μ l,
- a CD4+ T-cell percentage of total lymphocytes of less than 14%
- or one of AIDS-defining illness, opportunistic infections and cancers such as mycobacterium tuberculosis, invasive cervical cancer, lymphoma, CMV retinitis....

Without any treatment, the AIDS stage usually lasts 3 years, leading to the death of the patient.

To be noted, the two HIV types have different natural history and pathogenesis: HIV-2 infection has been shown to be less transmissible (Kanki et al., 1994) (G. S. Gottlieb et al., 2006), associated with a longer asymptomatic period, lower viral load (F. Simon et al., 1993) and lower mortality rate (Marlink et al., 1994) than HIV-1 infection. HIV-2 is also less responsive to the drugs used to treat HIV-1 (Tuailon et al., 2004).

Co-infections with HIV-1 and HIV-2 have been observed (Evans et al., 1988) and represent only 0.08% of the infections (Ingole et al., 2013).

9. HIV-infection treatment

The first molecule used to treat HIV infection was the azidothymine (AZT), a nucleoside-analog inhibitor of the reverse transcriptase, which appeared in 1985 (Yarchoan et al., 1986). However, AZT treatment was associated with a number of adverse side-effects (nausea, vomiting, diarrhea, headache, weight loss, loss of appetite...) and the emergence of resistant viruses (De Clercq, 1994) leading to reevaluation of its use in monotherapy.

In 1993, new drugs appeared, which could be used in combinations. These new treatments were first called Highly Active Anti-Retroviral Therapy (HAART) but were lately renamed Combined Anti-Retroviral Therapy (cART, in 2009).

The drugs approved by the Food and Drug Administration or the equivalent European Medicines Agency for the treatment of HIV infection (<https://hivinfo.nih.gov/understanding-hiv/fact-sheets/fda-approved-hiv-medicines>) are categorized in different classes, depending on their molecular mechanism: i) nucleoside-analog reverse transcriptase inhibitors (NRTIs), ii) non-nucleoside reverse transcriptase inhibitors (NNRTIs), iii) integrase strand transfer inhibitor (INSTI), iv) protease inhibitors (PIs), v) fusion inhibitors and vi) co-receptor antagonists.

The current recommendations from the World Health Organization (WHO) state that the optimal regimen is the use of three drugs with at least two different mechanisms of action. For the majority of patients, the tri-therapy is composed of: two NRTIs + one INSTI (updated recommendations reviewed in Günthard et al., 2016). However, several combinations of dual therapies have been tested in the recent years, in order to reduce the drug burden and toxicities. In this regard, a meta-analysis of fourteen studies evaluating the relative risk of failure of dual therapies compared to triple therapies in HIV-positive naïve patients revealed that dual therapy were as effective as those with three drugs, however, they were associated with a higher selection of resistance-associated mutations at 96 weeks of therapy (Pisaturo et al., 2021).

Combined ART allows the control of viral replication, decreasing the viral load down to undetectable levels (Attia et al., 2009) as well as a restoration of CD4+ T cells. The availability of treatment is estimated to provide a person infected with HIV a life-expectancy similar to the one of a non-infected individual (May et al., 2014). However, cART is still sometimes associated with side-effects and resistance emergence. Furthermore, a state of chronic inflammation remains in treated individuals, leading to a higher risk of various health problems typically associated with aging. Finally, cART cannot cure HIV, as the virus establishes a latent reservoir at the very early stages of infection (Chun et al., 2010) and the treatment has to be taken for life. Nevertheless, early treatment of the infection was shown to reduce both the overall size of the HIV reservoirs (Vanhamel et al., 2019) and drug resistance (Dorward et al., 2020) in most cases.

The antiretroviral drugs presented above are also now used in preventive strategies, known as Pre-Exposure Prophylaxis (or PrEP) to prevent transmission in HIV-negative at risk individuals, showing great efficacy in controlled trials (Grant et al., 2010)(Choopanya et al., 2013). PrEP adherence though somehow constraints this efficacy in real-life settings (Van Damme et al., 2012) (Minnis et al., 2013). Big hopes were placed in cabotegravir, a single injectable molecule thought to prevent HIV-1 infection with high efficacy. However, recent data showed that in rare cases, cabotegravir could hide infections and consequently fuel resistances (Maxmen, 2021).

10. HIV vaccine: a crucial necessity

In 1984, M. Heckler, the US secretary of Health and Human Services expressed in a now infamous press conference her “hope to have a [HIV] vaccine ready for testing in about two years [...]”. But 37 years and 32 million of AIDS-related deaths later, there is still no HIV vaccine available on the market, making this research one of the biggest challenges of our generation.

The drastic improvement and availability of treatment options (cART), as well as the substantial efforts in the prevention of transmission through various interventions (availability of condoms and clean needles, male circumcision, PrEP...) led to a notable decrease of the number of new infection and AIDS-related deaths within the past 20 years (Fig. I-1). The question about the necessity of an HIV vaccine was thus raised (Fauci & Marston, 2014). Certain factors will notwithstanding continue to limit the reach of non-vaccine prevention options such as social (male circumcision acceptance), legal (illegal homosexuality), logistical and financial (treatment accessibility), or functional factors (antiretroviral resistances). Moreover, even if the UNAIDS goal of “90-90-90” is reached by 2030 – i.e. 90% of the infected individuals aware about their status, 90% of infected individuals on cART, and 90% of those on treatment maintaining an undetectable viral load – there would still be around 200 000 new HIV infections annually (UNAIDS).

The crucial necessity of an HIV vaccine is also supported by modeling studies, in which predictions based over 1,000 model simulations project an important enhancement of the disease control, thanks

to an hypothetical vaccine. It is evaluated than an HIV vaccine, even with a moderate efficacy, could prevent 17 million new infections by 2035 (Medlock et al., 2017).

Hence the eradication of HIV will very likely not be feasible without a preventive vaccine, still missing among the panel of preventive and/or curative tools, despite the considerable efforts brought to its development (Fauci, 2017).

II. The humoral response to HIV infection

A. Clues for HIV vaccination

1. Basis of adaptative immunity

“Immunity” refers to the set of actors and mechanisms that confer an organism the ability to fight against infections by foreign bodies (fungi, bacteria, viruses...). The human immunity is divided into two different and complementary systems: the innate and the adaptive immunity.

The innate immunity is the first line of defense. It provides a fairly non-specific inflammatory and rapid response to the infection, within hours, that lasts for several days. As illustrated in Fig II-1, the cells of the innate immunity are the Natural Killer cells and $\gamma\delta$ T cells (lymphoid lineage), and the dendritic cells, macrophages, mast cells, neutrophils, basophils and eosinophils (myeloid lineage). The myeloid cells produce cytokines or interact with other cells in order to trigger the adaptive immunity.

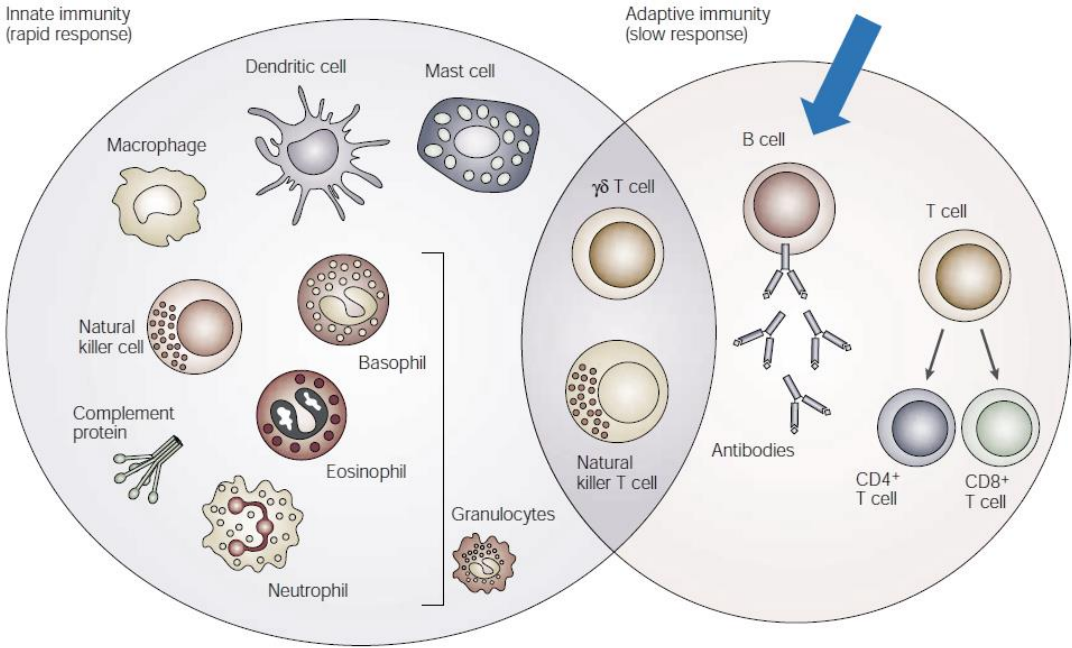


Figure II-1: Cellular actors of the innate and adaptive immune responses. From Dranoff, 2004.

The adaptive immune response is the second line of defense, slightly delayed (days) but lasting for weeks or longer. The cells of the adaptive system all belong to the lymphoid lineage and have at their surface specific antigen-receptors.

Those cells are:

- the T lymphocytes, among them the helper/auxiliary CD4+ (targets of HIV) and the cytotoxic CD8+, both responsible for the cell-mediated immunity.
- the B lymphocytes, which function is to produce immunoglobulins (Ig), responsible for the humoral immunity.

Lymphocytes that have not seen the antigen are in a naïve state, with a limited functional activity. When they meet the antigen that specifically bind to their receptor, some turn into effector lymphocytes (secretion of antibodies by B cells and killing of infected cells by T cells), while some will differentiate into memory cells, not actively engaged to respond but easily and specifically activable in case of a new encounter with the same antigen ([Molecular Biology of the Cell. 4th edition. Alberts, Johnson, Lewis, Raff, Roberts, and Walter. Garland Science, 2002](#)). This faculty of the adaptative response to generate a memory against pathogens is exploited by vaccination.

2. What is vaccination?

Vaccination is the introduction of antigens from a pathogen into an organism in order to elicit immune responses aimed at being protective against the disease that could arise from future exposure to the pathogen. It uses the memory capacity of the adaptative immune system to give rapid and strong responses against second encounters with specific intruders.

Even though the principle of immunization has been empirically used among early Asian and African populations for centuries, vaccinology foundations are commonly attributed to E. Jenner, who inoculated the first version of a smallpox vaccine to his gardener's son in 1796. The following massive implementation of smallpox immunizations eventually led to its complete eradication in 1979. In parallel, as knowledge about diseases causative agents and immunization principles rapidly developed, numerous vaccines were developed over the 19th and 20th centuries (reviewed in [Plotkin & Plotkin, 2011](#)).

3. HIV vaccination: B and T paradigms

Two main paradigms have been guiding the HIV vaccine research field since the beginning of the epidemic:

- The induction of neutralizing antibodies (NAbs), also called the "B cell strategy" started the HIV vaccine efforts. NAbs are immunoglobulins able to bind a virus and consequently prevent viral replication, mostly through inhibition of viral entry ([Plotkin, 2010](#)). NAbs are usually more effective at preventing infections by free viral particles than by infected cells (Fig. II-2). The interest in NAb started in the HIV field with the discovery of HIV NAbs in 1985 ([Robert-Guroff](#)

et al., 1985). Optimism was reinforced by the proof of the ability of NAbs to protect against viral challenge in diverse animal models when passively administered (Baba et al., 2000)(Mascola et al., 2000)(F. Klein et al., 2012). However, the paradigm notably faced a backlash in 2003, due to the negative results of the VaxGen gp120 vaccine candidate trial (detailed below). Nevertheless, the more recent characterization since 2009 of a growing number of highly potent and broad NAbs (BNAbs) steered the B cell ship back on course.

- The induction of cell-mediated immunity, or the “T cell strategy”, in which cytotoxic T lymphocytes (CTL) are activated to eradicate infected cells followed the early enthusiasm for B cell immunity in the HIV vaccine field (McMichael & Hanke, 2002). CTL can kill infected cells but however have no impact on free viral particles in contrast to Abs (Fig. II-2). The T cell paradigm began in the early 2000s and faded around 2007 with the failure of a T cell-based vaccine trial: the STEP trial (Buchbinder, S.P., 2009).

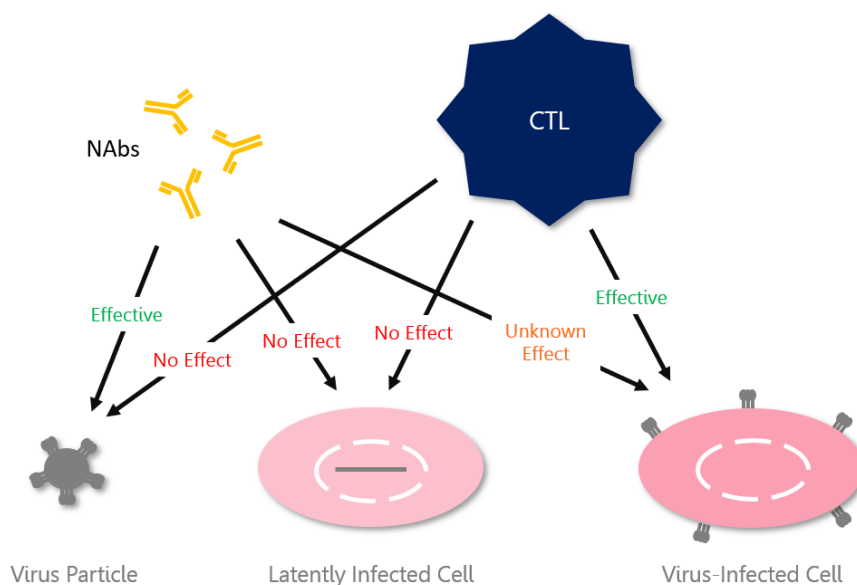


Figure II-2: Potential effects of the induction of Nabs or CTL on free virus, latently or active infected cells.

Those two trends in vaccine strategies eventually overlapped to ultimately combine, as it is now commonly thought that an effective HIV vaccine may need to elicit both NAbs and CTL responses.

To be noted, besides the potential neutralizing activity, Abs can exert other important immune functions, mediated by their Fc. They form bridges between the adaptive and the innate immunities thanks to Ab-dependent cellular cytotoxicity (ADCC), Ab-dependent complement-mediated lysis, and Ab-dependent phagocytosis (reviewed in Anand & Finzi, 2019 and better detailed below). These properties have recently attracted more interest in the vaccine fields following the potentially interesting results of the “Thai trial” (NCT00223080) (Montefiori et al., 2012) (Wren & Kent, 2011).

As this PhD subject focuses on the B cell strategy, here follows an overview of the human humoral response mechanisms as comprehensive basis for our project.

B. Immunological basis of the humoral response

1. Structure of immunoglobulins

Immunoglobulins (Ig) – also called Abs – are Y-shaped proteins composed of two identical heavy chains (HC; around 53kDa each) and two identical light chains (LC; 22 kDa) held together by disulfide bonds (Fig. II-3). One HC is made by one variable region and three constant regions whereas one LC is made by one variable and one constant region (Chiu et al., 2019).

Hence, the N-terminal part of the HC associated with the LC constitute the variable region of the antibody, also called the Fab fragment, responsible of the binding to the foreign organism, called *antigen* (for *antibody-generation*). The antigenic surface recognized by the Ab is called the epitope and the surface of the Fab that recognize the antigen is the paratope. Various non-covalent forces can hold the antigen-antibody complex, e.g. electrostatic forces, hydrogen bonds, Van Der Waals forces, hydrophobic forces. Having two Fabs provides bivalence to the protein, that is to say one Ab molecule is able to bind two target molecules.

The structure of the variable region of each chain is made by the alternate succession of relatively constant sequences (FR for FRamework, that ensure the global folding of the variable region) and variable sequences (CDR for Complementary Determining Region, responsible for the complementarity with the antigen). The order of the sequences is the following, starting from the N-term end: FRH1 – CDRH1 – FRH2 – CDRH2 – FRH3 – CDRH3 – FRH4. The letter “H” refers to the heavy chain; the equivalent sequences in the light chain are indicated with a letter “L”.

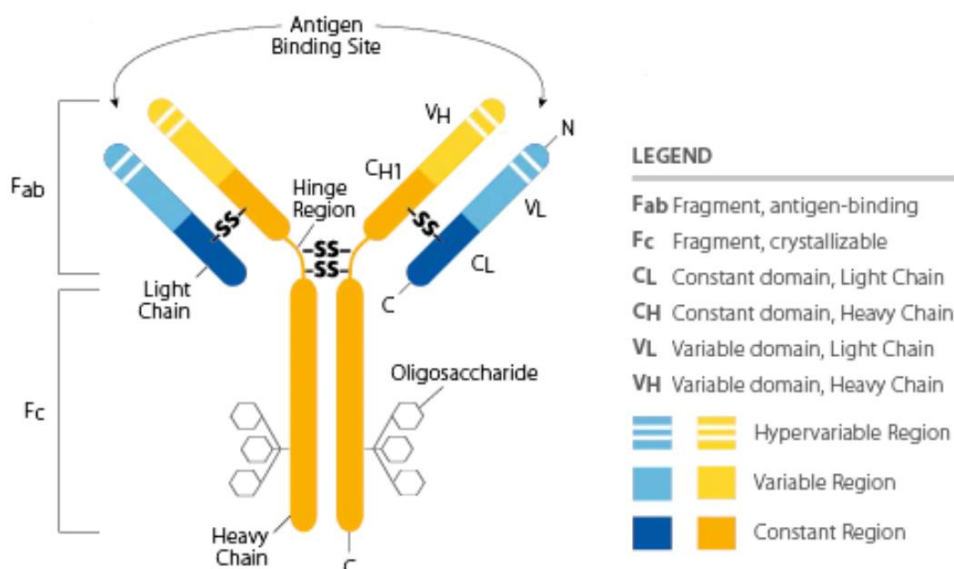


Figure II-3: Schematic antibody structure. Illustration from ThermoFisher Scientific website.

The C-terminal part of both chains constitute the constant region of the Ig, called Fc (for crystallizable fraction). The HC C-term parts determine the isotype/class of the antibody, of which there are five, better detailed in Table II-1.

Immunoglobulins can be expressed at the cell membrane as a receptor, the B cell receptor (BCR), notably in naïve and memory B cells. The binding of an antigen to the BCR activates the B cell which as a naïve cell will then differentiate into a plasma or memory B cell, as explained more in detail below. Immunoglobulins secreted by plasma cells have the same specificity than the BCR of the corresponding naïve cell.

2. Immunoglobulins: classification and functions

	<i>IgA</i>	<i>IgD</i>	<i>IgE</i>	<i>IgG</i>	<i>IgM</i>
HC	α	δ	ε	γ	μ
IC	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ
Molecular Weigh	165 kDa	180 kDa	200 kDa	150 kDa	900 kDa
Form	Monomer or dimer	Monomer	Monomer	Monomer	Pentamer
% of total Ig in serum	13%	1%	0,002%	80%	6%
Half-life	6 days	3 days	2 days	23 days	5 days
Subclasses	IgA1 and A2			IgG1, 2, 3 and 4 (different C regions)	
Characteristics	Main Ig in the mucous	-Membrane-bound Ig -low serum concentration -Co-expressed on B-cells with IgM		-Main Ig in the blood, lymph, cerebro-spinal fluid, perineal fluid -15% of total serum protein	-First Ig produced and expressed following exposure to an infectious agent
Fc binds to			Mast cells Basophils	Macrophages	
Role	- Agglutination - Neutralization of viruses	- proposed role in regulation of autoreactivity - role in B-cell activation	-Hyper-sensitivity reaction	- Opsonization - Agglutination - Activate complement - Neutralization of toxins and viruses - ADCC - Placental transfer	

Table II-1: Ig isotypes and associated characteristics.

The Ig isotypes have different functions because of their different constant Fc part. Those functions - detailed in [Lu et al., 2018](#) – can be classified in:

1) Fc-dependent functions (related to their binding to various Fc-receptors) such as:

- The ADCC - Antibody-Dependent Cell-mediated Cytotoxicity - is the process by which cells of the innate immune system (typically NK cells, but also neutrophils, eosinophils...) recognize the Ig Fc thanks to their Fc-receptors, get activated, and release cytotoxic granule to kill the unwanted microorganism.
- The complement is a cluster of innate immunity circulating precursor proteins which can be activated by binding of IgG (Fc), resulting in a cascade of cleavages, that will activate the cell-killing membrane attack complex and attract and stimulate phagocytes to fight the infection.
- The opsonisation is the process by which the binding of the antibody Fab to the infectious agent facilitates its recognition and consequently its phagocytosis by macrophages, which bind to the Fc part through specific receptors.
- The placental transfer of the IgG (except IgG2) is made possible by the presence of IgG receptors on the placenta after 3 to 4 months of pregnancy. Thereby, any mother gives her baby a primary protection at the very early stage of life, before the infant builds his own immune defenses.
- The agglutination refers to the ability of the Igs to clump particles together, which facilitates antigen elimination through macrophages phagocytosis.

2) Fc-independent functions :

- In particular, neutralization corresponds to the loss of infectivity of a virus due to the binding of the Ab (Fab), usually without involvement of any other Ab activity ([Burton & Mascola, 2015](#)). The neutralization mechanism will be better detailed part II.C.2. In our project we will focus on HIV neutralizing Abs of the IgG isotype.

3. Ig genetic organization and VDJ recombinations

HC, IC κ and IC λ genes are located at different loci on the chromosomes 14, 2 and 22 respectively. HC chain is encoded by several types of genes segments: V (Variable), D (Diversity), J (Joining) and C (Constant). IC is encoded by V, J and C only (Fig. II-4). Each segment contains several duplicate genes that are genetically unique. Moreover, the segments are separated by non-coding regions (introns) that are later removed during the splicing of the messenger RNA. Series of gene rearrangements take place in the bone marrow - where B lymphocytes are produced - and drive the evolution from a progenitor lymphoid cell to an immature B cell.

The HC recombination happens first (Fig. II-4, right panel). One J segment and one D segment recombine, then a V segment recombines with the DJ assembled segment, bringing the C region close to the newly formed VDJ segment. At this stage, this DNA assembly is transcribed into RNA, which is further spliced, removing the introns. The translation of this spliced RNA produces a transmembrane HC which is paired with a surrogate light chain, itself formed by the non-covalent association of $\lambda 5$ and VpreB proteins structurally mimicking the light chain constant and variable domain respectively. The progenitor cell is now called a precursor B-cell, carrying pre-BCRs.

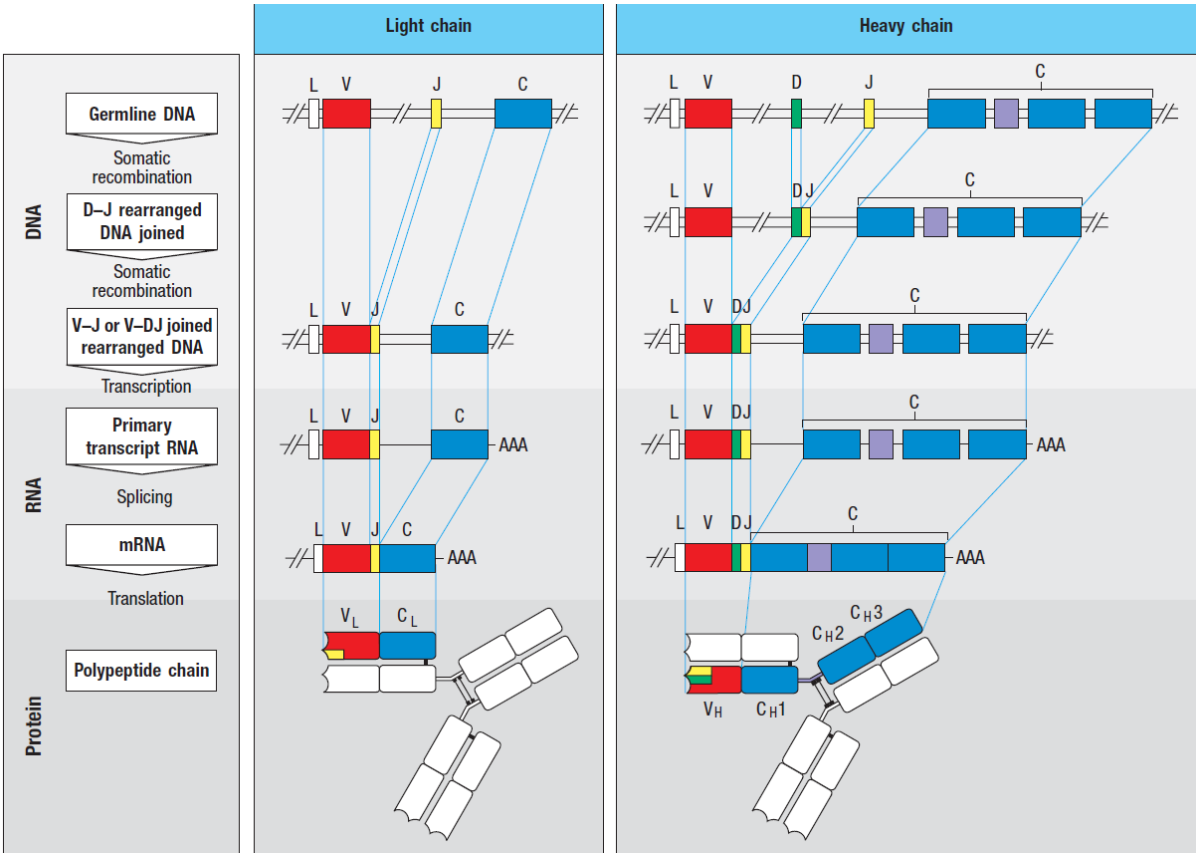


Figure II-4: Somatic recombinations of separate gene segments generates the sequences that encodes for the V-region. From the Janeway's Immunobiology – 9th edition.

Then occur the IC genes rearrangements (Fig. II-4, left panel). As mentioned earlier, IC genes do not contain any D segment such that there is only one recombination event between a V and a J segment, before transcription into RNA which, similar to the HC, is then spliced to remove introns, and translated into a full-length IC protein. The precursor cell is now an immature B-cell.

Here follows a brief description of the molecular mechanisms of recombination. Each V gene segment includes a specific motif called RSS for Recombination Segment Sequence at its 3' extremity. J gene segments bear it at their 5' end, and D gene segments on both extremity (Fig. II-5). Those RSS are composed by an heptamer and a nonamer of nucleotides, highly conserved among individuals, separated by a 12 or a 23-base-pair spacer. This leads to the 12/23 rule: only an RSS with a 12-base-pair spacer can recombine with another with a 23-base-pair spacer, and vice-versa.

V(D)J recombination starts with the binding of one of the RSS to a protein complex composed of RAG-1, RAG-2 and High-Mobility-Group proteins. The other RSS is then recruited by the complex. RAG proteins have an endonuclease activity, which allows the single-strand cleavage of the DNA, precisely between the coding region of the segment and the RSS. Numerous different protein complexes are then implicated in the re-ligation of both coding and signal joints.

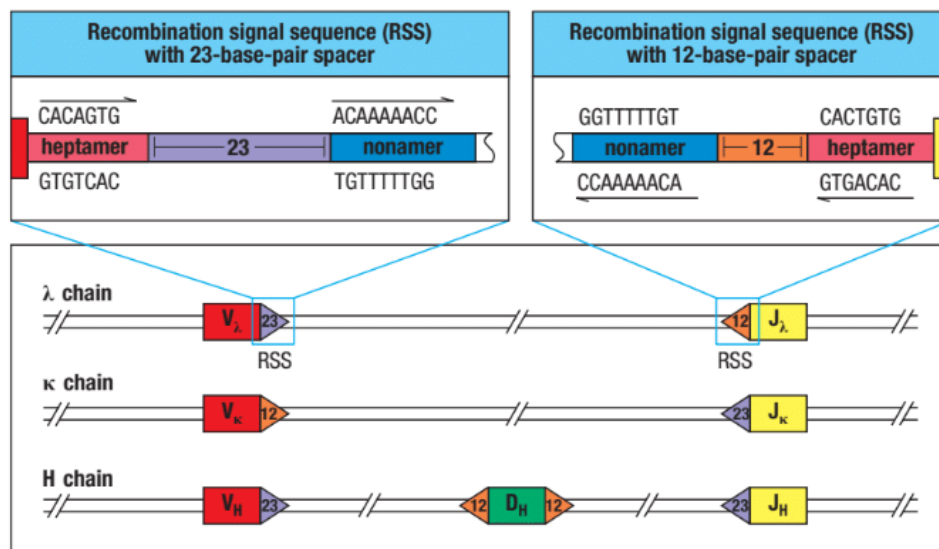


Figure II-5: Recombination signal sequences are conserved heptamer and nonamer sequences that flank the gene segments encoding the V, D and J regions of immunoglobulins. From the Janeway's Immunobiology – 9th edition.

Finally, the V segment encodes for the majority of the variability, from FR1 region to the beginning of CDR3. The CDR3 straddles all gene segments V, (D) and J. The rest of the J segment encodes for the FR4 (Fig. II-6).

4. Variable region diversity

Since each gene segment represents one of many variable duplicates (numbers in black, Fig. II-6), the random recombination between each V, D and J regions create a tremendous number of different unique Abs. This represents the combinatorial diversity.

In addition, the VDJ recombination involves the gain or the loss of nucleotides at the segments junctions generating non-templated regions during the rearrangement process thereby increasing the sequence diversity. This junctional diversity can lead to shifts in the Open Reading Frames (ORF) or even introductions of translational stop codons.

Moreover, the random pairing of HC with IC, that can be either κ or λ , brings another level of combinatorial diversity.

At this stage, the number of antibody specificities is estimated at 10^{12} (Fig. II-6).

Before exiting the bone marrow, cells carrying self-reactive immunoglobulins are eliminated, slightly reducing the diversity. However, the further differentiation of antigen-activated B cells in secondary lymphoid organs includes somatic hypermutations, generating even more sequence diversity. This latter process is detailed in a further paragraph.

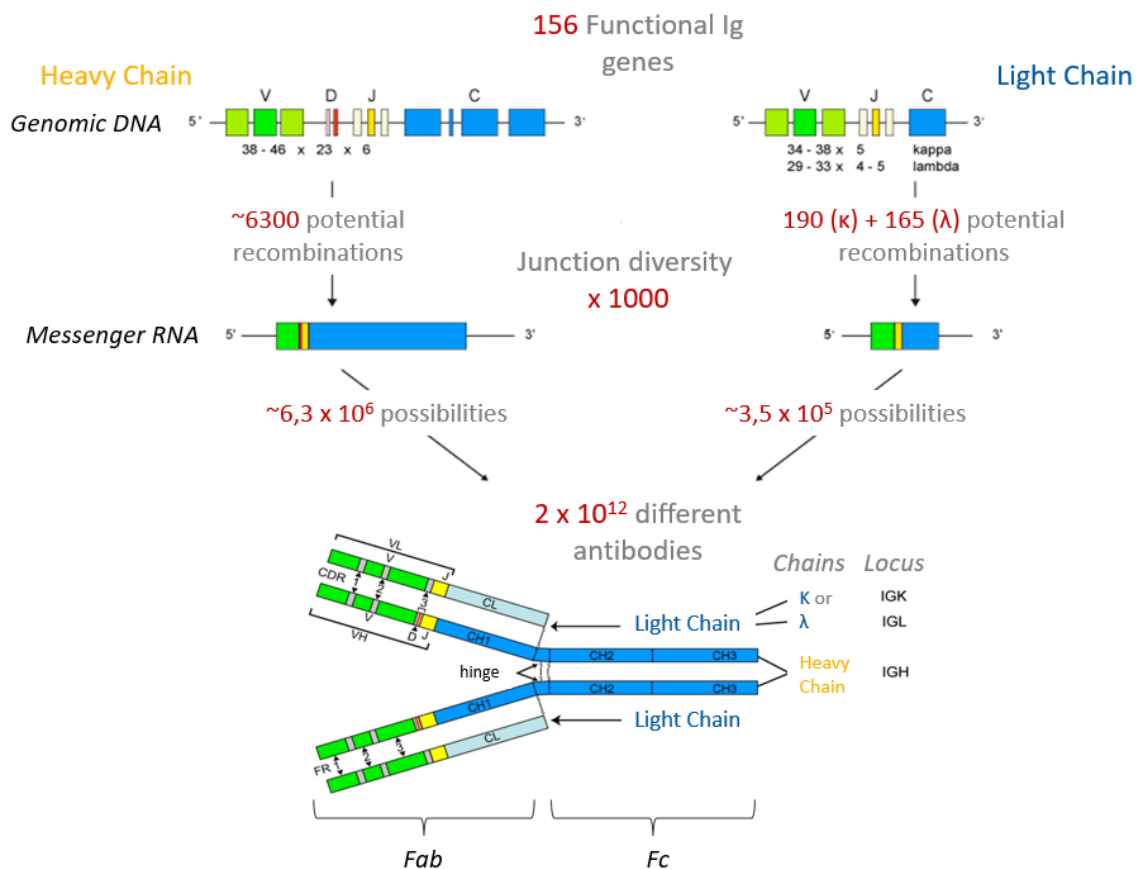


Figure II-6: Organization of the human Ig genes and the diversity it implies. Adapted from IMGT® website, the international ImMunoGeneTics information system®.

5. Immunoglobulins nomenclature

The Ig genes nomenclature includes:

- The chain: H for heavy, L for ICL and K for ICk
- The segment: V, D or J for HC, V or J for IC
- The family of the duplicate: indeed each V, D or J gene is affiliated to a family, depending to its genetic proximity with other members. Families were defined by genetic clusterization; each member of a family have more than 80% similarities in their nucleotide sequence. For instance, the V segments of the HC and the ICk are both distributed in 7 families and those of the ICL in 11.
- The polymorphism, classified by the concept of "allele": indeed, as there is a tremendous inter-individual polymorphism, the mutations of the nucleotide sequences are classified by reference to allele as in *01.

As an example, the J-gene of the heavy chain of an antibody belonging to the family #4 and allele #02 is indicated: IGHJ4*02 (Lefranc, 2011).

The international database ImMunoGeneTics (IMGT) catalogues many reported variants and known polymorphism but might be incomplete.

6. B lymphocytes activation and differentiation

The previous paragraphs described how a progenitor B-cell goes through the VDJ recombination inside the bone marrow and turns into an immature B-cell with a unique antibody – usually an immunoglobulin M. As mentioned previously, the immature B cell is tested in regard to its potential autoreactivity before leaving the bone marrow: autoreactive B cells are regulated during development through mechanisms, including editing of the BCR, clonal deletion, and anergy (Y. H. Wang & Diamond, 2008). Only the negatively selected migrate to the periphery, notably to secondary lymphoid organs – spleen or lymph nodes – via the afferent lymphatics (Fig. II-7). Once in the secondary lymphoid organ, the B-cell, now mature and naive expresses IgM and IgD and can be activated to become either an antibody-secreting plasma cell or a memory B-cell.

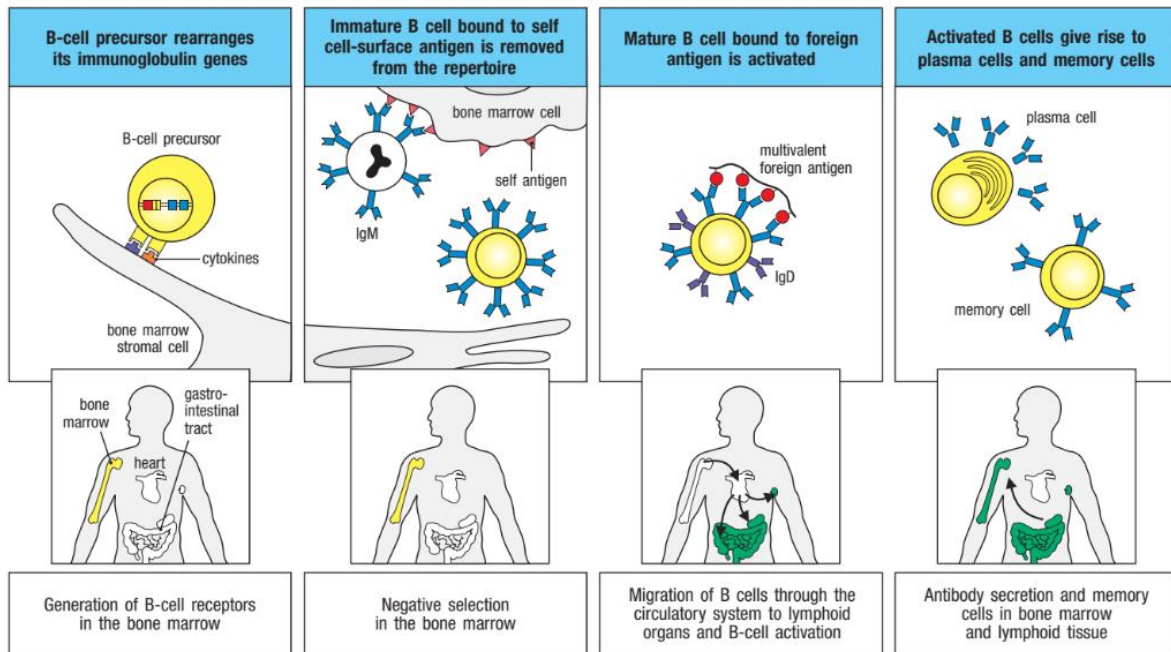


Figure II-7: B-cells develop in the bone marrow and migrate to peripheral lymphoid organs where they can be activated by antigens. From the Janeway's Immunobiology – 9th edition.

A cross-section of a lymph node shows different areas: the cortex (usually abundant in B cells), containing the Germinal Centers (GC), and the paracortex (usually abundant in T cells). GC are anatomical structures formed by the proliferation of B cells upon activation by their cognate antigen. A GC is typically composed of a dark zone and a light zone (Fig. II-8).

Naïve B-cells can be activated by free antigens or by antigens presented on the surface of follicular dendritic cells (FDC). The antigen is internalized through binding to the BCR, processed inside the lymphocyte, and resulting peptides are exposed at the surface on MHC-II molecules. Newly activated B-cells will migrate from the cortex to the paracortex where they must receive additional activation signals from a CD4 T-helper cells (Th) to start differentiating into effector cells (Fig. II-8, a). Both CD4-Tfh and B lymphocytes meet at the interface: the T cell recognize the antigen presented by the B cell and gives additional activation signals. The B lymphocyte starts proliferating and secreting IgM.

Some B cells will exit the follicle and become short-lived plasma-cell (Fig. II-8, b), others will become memory B cell, participating to the GC-independent humoral immunity (Fig. II-8, c). A third pool of B cell will form the GC dark zone where maturation occurs. An affinity selection is done in the light zone, with the help of FDCs and CD4⁺Tfh cells again. During the GC reaction, the intracellular enzyme Activation-Induced Cytidine Deaminase (AID) introduces point mutations, as well as deletions and insertions, on the variable region of the Ig DNA. The mutation rate is estimated at one base pair change per 10^3 base pairs per cell division, i.e. strongly higher than the mutation rate found in any other cell (estimated at one base pair change per 10^{10} base pairs). This process, called the Somatic HyperMutations (SHM), creates many new variants of the original B cell with different affinities for the cognate antigen (Fig. II-8, d). Those mutations are mostly detrimental, either by introducing stop mutations or introducing frameshifts consequently disrupting proper expression of folding. However, some SMH are advantageous and lead to an improved affinity of the Ig variable region to the cognate antigen. Affinity selection is done in the GC light zone, where the highly diverse centroblasts migrate to become centrocytes (with reduced proliferation rate) and compete for specific interactions with antigen-presenting FDC and CD4⁺T follicular helper cells (Tfh). The centrocytes for which the SMH confer a disadvantageous mutation (diminished recognition of the antigen) die by apoptosis. Those for which the SHM provide a better affinity for the antigen eventually proceed in the differentiation process. The B cells with the higher affinities will be recruited and turn either in memory B cells, either in long-lived plasma cells.

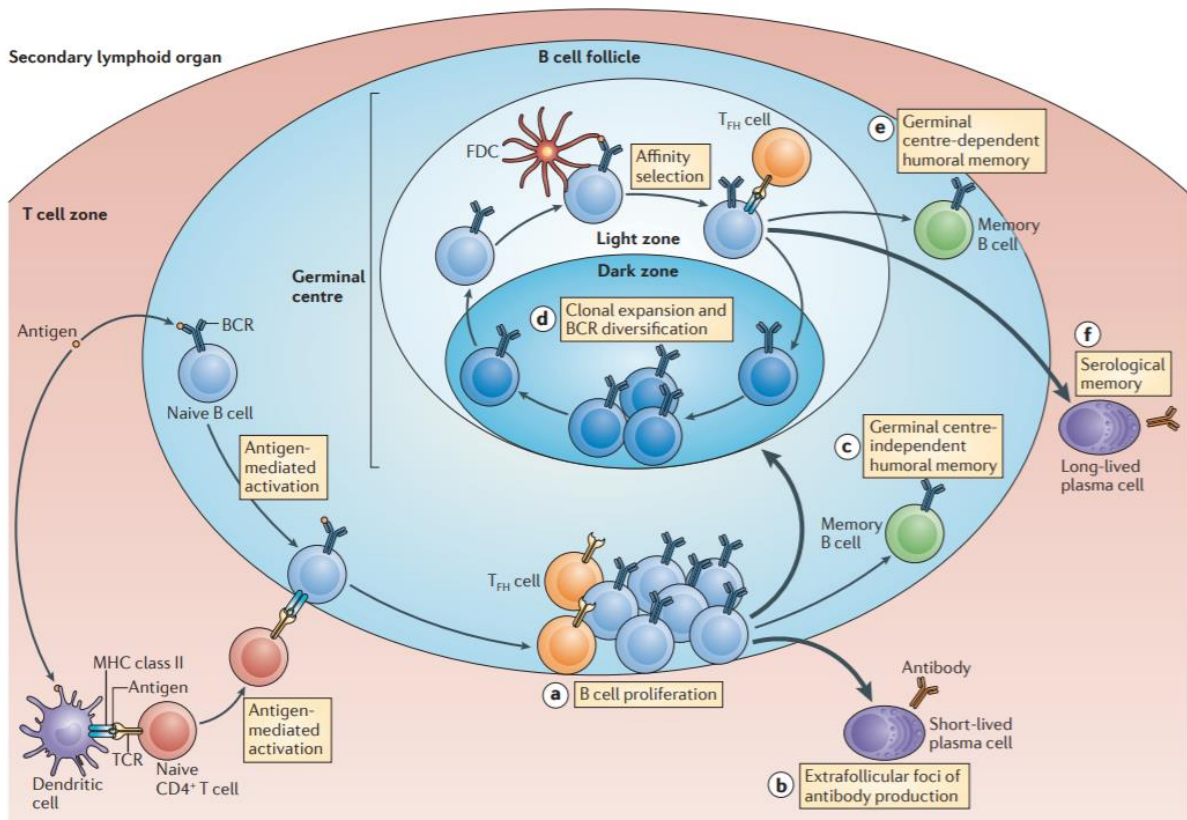


Figure II-8: T-cell dependent memory B-cell generation. From Kurosaki et al., 2015.

Class-switching is the process through which the constant region of the antibody is changed from isotype M or D to isotype G, E or A (see Table II-1), with no change of the variable part. This process is essentially regulated by the cytokine produced by CD4 Tfh.

Following class-switching, the centrocytes may have three different fates:

- differentiation into long-lived plasma cell, able to secrete soluble Abs which can exert their different functions, as explained above (Fig. II-8, f). These cells traffic to and reside in the bone marrow and maintain Ab production potentially for as long as the lifetime of an individual.
- re-entry in the GC to go through another mutation/replication cycle and accordingly the acquisition of a potentially better affinity for the antigen. Indeed B cells can enter in the maturation process several times.
- differentiation into memory B-cell with a quiescent metabolism and a long-lasting survival capacity, until reactivation by their cognate memory T-cell, providing a strong and fast response to the known re-infecting antigen (Fig. II-8, e).

C. Ab responses to HIV

An Ab response is eventually elicited by most HIV proteins upon infection. Notably, the presence of such Abs has been used from early on after the discovery of HIV as a marker of infection in serodiagnosis assays (ELISA, Western Blot).

We are specifically interested in Abs with neutralizing activity, i.e. able of blocking viral infection, as detailed below. Neutralization is of particular interest in the HIV vaccine field as there is a strong correlation between the ability of an Ab to neutralize *in vitro* and its capacity at preventing infection in animal models of HIV infection (detailed below). Therefore it is believed that a vaccine that would elicit Nabs could protect against HIV infection in humans. The only target for Abs at the surface of HIV are the Env glycoproteins (Arrildt et al., 2012), thus only Abs targeting Env can have a neutralizing activity.

1. Focus on HIV Env

- **Env structure**

The HIV-1 *env* gene codes for a precursor protein of 160kD, gp160, which undergoes folding, trimerization and glycosylation in the endoplasmic reticulum. The Env precursor is further cleaved in the Golgi by furin cellular proteases into gp41 (C-term, with a transmembrane and a cytoplasmic domain) and gp120 (N-term, completely extracellular) (Fig II-9, A). Both subunits remain non-covalently linked after cleavage. The association of three heterodimers of gp120 and gp41 thus constitutes the Env trimer (Fig II-9, C).

Gp120 is made of five hypervariable and five constant regions, alternating: C1 – V1 – V2 – C2 – V3 – C3 – V4 – C4 – V5 – C5. Variable regions are mostly exposed as loops at the surface of Env, with their basis maintained towards the core of the protein by disulfide bridges (Fig II-9, B). They form thereby the external domain of gp120, more variable and glycosylated than the internal domain (Starcich et al., 1986), which interacts with gp41. The V1 and V2 loops constitute the apex of Env while V3 is partially held under them.

To be noted, obtaining high-resolution structures of native full-length trimers Env has been particularly struggling and was achieved no sooner than 2013, allowing a better understanding of the trimer folding, the variable regions features, the glycosylations and the global plasticity of Env (Ward & Wilson, 2017). Structures of the currently used HIV Env recombinant soluble trimers are better detailed part IV-A-4.

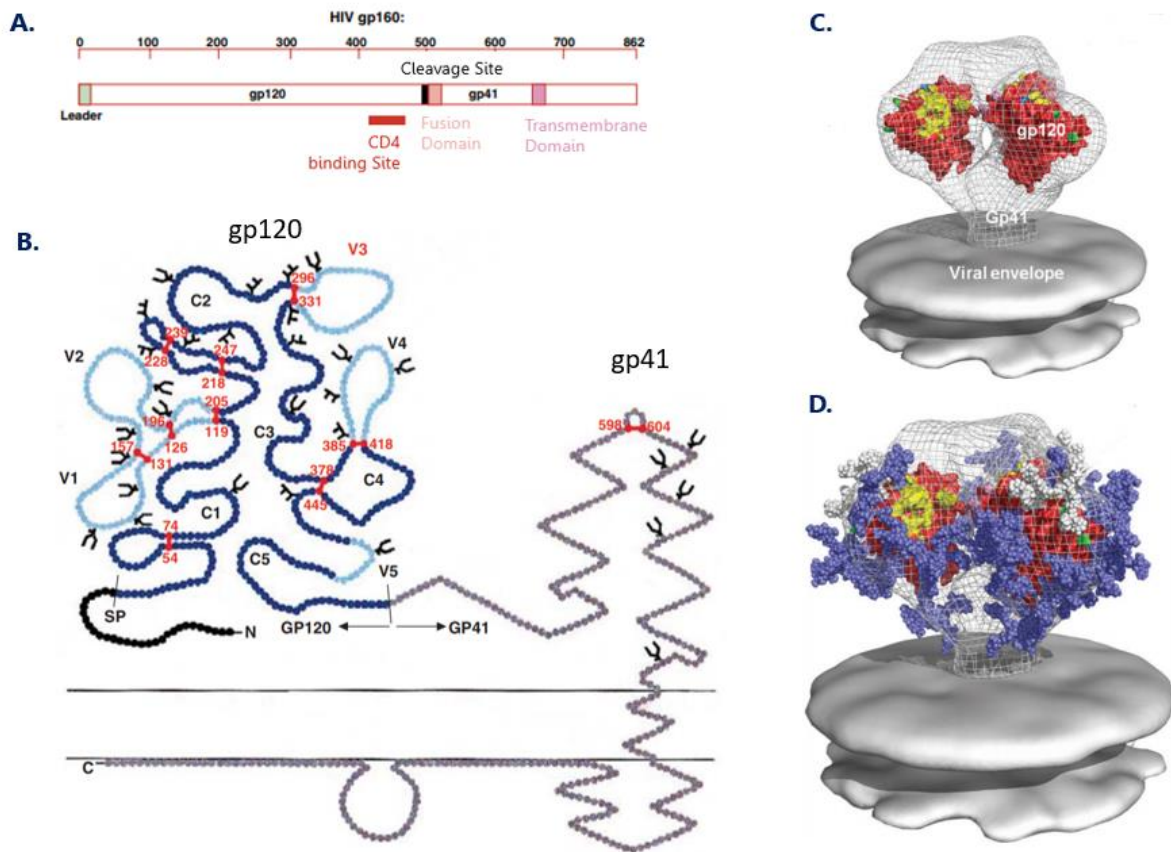


Fig. II-9: The HIV-1 Env, only protein at the surface of the virus. A. Structural organization of Env protein. B. Schematic representation of the precursor gp160. Gp120 subunit is in the left (blue). Signal Peptide is indicated in black; conserved regions in dark blue (C1-C5) and variable loops in light blue (V1-V5). Gp41 is in the left (grey) and contains one loop formed by a disulfide bridge (red). All cysteines and disulfide bridges are in red. Glycosylation sites are also indicated, F referring to high-mannose chains and Y to complex chains. From Pancera, 2005. C. Model of the HIV-1 Env, inspired from the 20 Å cryo-electron tomography resolution of Liu et al, Nature, 2008, shown as a transparent mesh, made of three gp120 molecules (red) non-covalently linked to three gp41 molecules on the surface of an HIV-1 virion (grey). D) Same model with the gp120 glycans (lavender blue), revealing the extensive glycosylation on Env. From Sattentau & McMichael, 2010.

- **Env glycosylation**

As already mentioned, Env is extremely glycosylated (Fig. II-10, D), as glycans participate to 50% of its mass (Leonard et al., 1990). Glycosylation is crucial to Env as it impacts folding (Shen et al., 2014). In addition glycosylation forms a protective shield against the immune system to the Env spike, impacting antigenicity and immunogenicity (Fig. II-10, D). They are 2 types glycosylations: O-linked and N-linked. Briefly, O-glycosylations consist in the addition of glycans on the oxygen atom of serine (Ser) or threonine (Thr) residues. For many years, O-glycosylations were thought to be lacking in HIV-1. Their presence has however recently been demonstrated both on virions and gp120, preferentially in the strains having long gp120 V1 domain (Silver et al., 2020). In parallel, N-glycosylations were more extensively studied. They correspond to the addition of sugars on asparagins (Asn) belonging to Potential-N-glycosylation Sites (PNGS), formed by the sequence: Asn-X-Ser/Thr-X (X being any amino

acid except for Proline). N-glycosylation forms overall more furnished glycans than O-glycosylation. Around 90 PNGS can be found on the Env trimer, largely on gp120 (Klasse et al., 2020). This number varies from one strain to another, as well as the glycosylation pattern, which is also dependent on the expression system used to produce recombinant Env (Pritchard, Harvey, et al., 2015). The post-translational process of N-glycosylation of Env is the same than any other cellular protein, as HIV uses the host cellular machinery to replicate its components. It begins by the transfer in the endoplasmic reticulum (ER) of a glycan precursor $\text{GlcNAc}_2\text{Man}_9\text{Glc}_3$ on Asn of the PNGS. Glucosidases I and II then trim the three last glucoses of the longest limb, while ER mannosidases remove some mannoses, leaving $\text{Man}_9\text{GlcNAc}_2$ and $\text{Man}_8\text{GlcNAc}_2$ trees, called oligomannoses (Fig. I-9). The protein is conveyed to the Golgi apparatus after proper folding, where another mannosidase, Man I, removes mannoses again until five remain: $\text{Man}_5\text{GlcNAc}_2$. N-acetylglucosaminyltransferase I (GnT-I) can consequently add N-acetylglucosamine (GlcNAc) residues, making hybrid glycans. Then, depending on the accessibility of the site to the enzymes, other residues can be added, like additional GlcNAc, fucoses or galactoses, to constitute more or less complex glycans (reviewed in Nagae et al., 2020).

Most of N-glycans on the Env surface are oligomannoses. This is particularly true for gp120, because of the higher glycan-density and compact folding of this subunit that reduce enzyme access to build hybrid or complex trees. Those glycans are particularly concentrated in the region at the base of the V3 loop, called the high-mannose patch (Doores et al., 2010). In parallel, most of gp41 glycans are complex glycans ending by a galactose (Pritchard, Vasiljevic, et al., 2015).

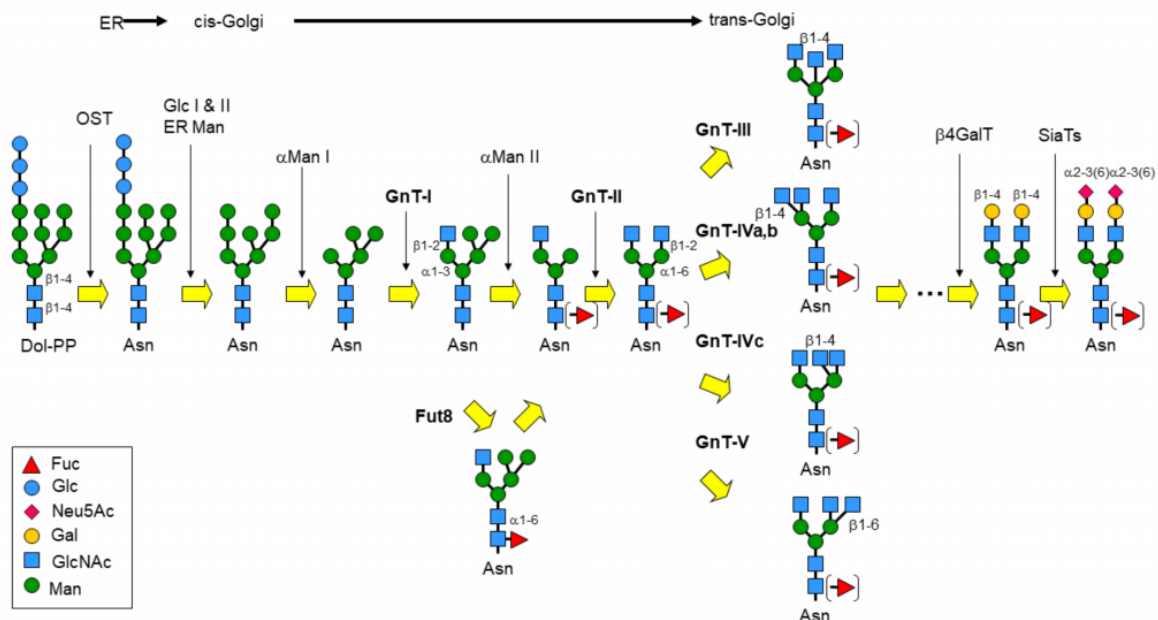


Fig. II-10: Schematic drawing of N-glycan processing. The oligosaccharides comprising 14 sugars were first transferred to an Asn residue, and the N-glycans were subsequently processed and matured by the ordered actions of various glycosyltransferases and glycosidases. From Nagae et al., 2020.

- **Env genetic diversity**

HIV-1 has a very high mutation rate, notably because of frequent errors of its reverse transcriptase, high efficiency replication and frequent genomic recombinations (Roberts et al., 1988) (Robertson & Sharp, 1995) (Perelson et al., 1996)(Robertson & Sharp, 1995). This leads to the tremendous diversity of HIV-1, that forms quasispecies, i.e. clouds of diverse variants that are genetically linked through mutation, interact cooperatively on a functional level, and collectively contribute to the characteristics of the population, as explained in Lauring & Andino, 2010.

The genetic diversity is particularly true for Env, the most variable part of HIV-1 (Geller et al., 2015). As HIV replicates, each new virus copy is slightly different from the original virus, making an individual not infected by one but millions of versions of the virus. As an example, the genetic diversity of HIV-1 Env in one single infected individual at one time point has been shown to be comparable to that of influenza virus hemagglutinin during one entire year worldwide (Bette Korber et al., 2001).

This tremendous HIV diversity is consequently true on a population level too, as shown by the diverse groups and clades spread worldwide (Fig I-3).

2. Neutralization : definition, mechanisms, stoichiometry

As explained earlier, we are interested in the Ab neutralizing responses against HIV-1 Env.

‘Neutralization’ is defined as the loss of infectivity that occurs when an Ab binds a virion (Burton & Mascola, 2015). The neutralizing activity is assessed *in vitro* by neutralization assays : briefly the virus is incubated with serial dilutions of test sample (polyclonal serum or mAbs) before the addition of target cells, on which infection can be recorded at each dilution of test sample (Montefiori, 2004). The potency of a NAb is usually expressed as the IC₅₀, i.e. the concentration required to inhibit 50% of cell infection by a given HIV strain. Herein, the Abs defined as neutralizing (NAbs) have mean IC₅₀ often well below 1 µg per milliliter.

Several neutralizing mechanisms have been described (Klasse, 2014). For HIV-1, neutralization can be due to the steric hindrance created by the binding of the Ab on Env. This is easily conceptualizable with Abs directed against the CD4-bands and preventing the primary interaction between Env and the CD4 receptor. The mechanism is also valid for Abs directed against the opposite site of gp120 – the V3 loop – by preventing the secondary binding of Env with the co-receptor CCR5 (ex Ab 2G12 (Platt et al., 2012)). In parallel, some NAbs allosterically modulate CD4 binding (ex Ab PGT121 (J. P. Julien et al., 2013)). Other NAbs neutralize HIV-1 by triggering gp120 shedding (ex Abs 2F5 and 4E10 directed toward the Membrane-Proximal External Region (MPER) of gp41 (Ruprecht et al., 2011)).

The stoichiometry of neutralization has not entirely been elucidated yet. Early studies on stoichiometry suggested that one NAb bound onto a spike can completely neutralize the trimer and that NAb recognition of one Env does not affect the surrounding spikes (Yang et al., 2005). Because of non-

functional forms of Env on the surface of HIV-1 virions (see below), total Ab occupancy per virion may not strictly correlate to neutralization, but those non-functional forms of Env do not appear to play a role in neutralization (Zwick & Burton, 2007). In addition, the epitope, angle of approach toward the epitope, the kinetics and dynamics of binding may make one NAb more potent than another, despite a similar affinity (Zwick & Burton, 2007).

In any case, studies suggest that the binding of an Ab to the functional native Env is the necessary and sufficient condition for neutralization.

3. Various types of Ab responses to HIV Env

Ab responses against HIV-1 Env can be classified into three groups (Burton & Mascola, 2015).

The first group includes Abs directed against Env but unable to neutralize the virus, because they recognize epitopes that are never exposed at the surface of the native Env trimer, the functional spike. The binding of such non-neutralizing Ab (NNAb) to virions is made possible by the presence of nonfunctional forms of Env on HIV-1 particles (Moore et al., 2006) (Fig II-11). Those NNABs can eventually provide an antiviral activity, via binding to nonfunctional forms of Env expressed on infected cells, through Fc-mediated functions, such as phagocytosis or sequestration of virions on Fc receptor-bearing cells. The NN responses are highly immunodominant, meaning that the humoral system tends to favor their elicitation.

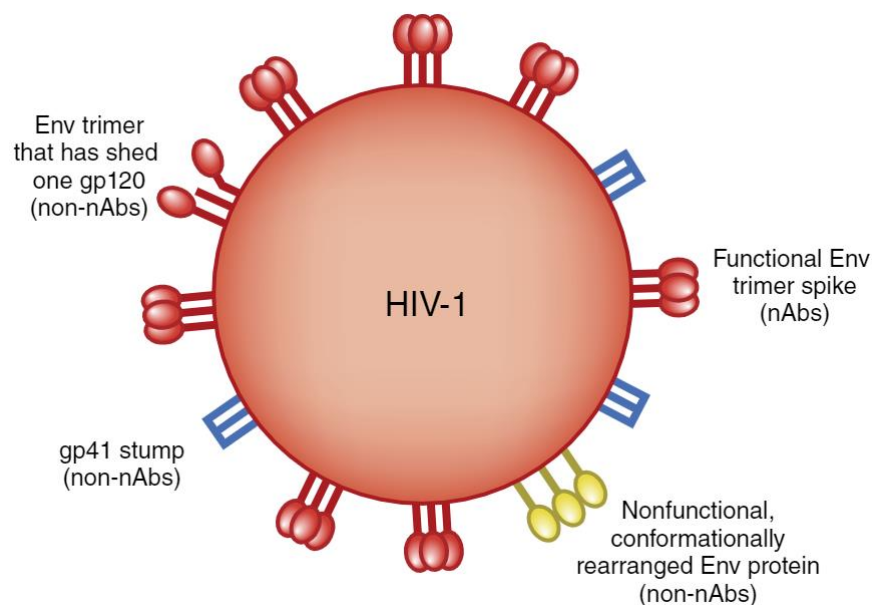


Figure II-11: Schematic of some of the forms of Env protein that may be present on infectious HIV-1 and available to elicit Ab responses. Only neutralizing Abs (NAbs) will bind to functional Env trimer spikes, although neutralizing Abs could, in principle, be elicited by other forms of Env protein. A range of non-neutralizing Abs (NNABs) and some NAbs will bind to nonfunctional Env proteins. NNABs could be elicited by the types of nonfunctional Env protein shown, but also by, for example, monomeric gp120 or Env debris from infected cells. The molecules shown on virions could also be expressed on infected cells. Other forms of Env protein may be expressed on HIV-1 (Burton & Mascola, 2015).

The second group contains Abs that recognize epitopes exposed at the surface of the conformationally proper native Env trimer and that have a strain-specific neutralizing activity. This narrow spectrum of activity comes from the fact that epitopes of such Abs are only exposed on a subset of viral strains or on viral strains with open Env conformation.

Indeed trimers on the virus surface are structurally dynamic and have the propensity to negotiate transitions to lower-energy states upon stimulation (Haim et al., 2011), exposing, depending on the viral strain, various proportions of open Env conformations, eventually targeted by Abs from this second group. The proportion of open Env conformation can help explain the notion of strain « tiers » which was defined to classify viral strains according to their sensitivity to Ab neutralization. Tier 1A viruses are the most sensitive and Tier 3 the least sensitive to neutralization by panels of sera from HIV-infected individuals. The sensitivity to neutralization of HIV strains can thus be seen as a consequence of the proportion of open Env at the surface of virions. Tiers 1A strains expose open Env conformations at a high frequency, making them easily neutralized. Tiers 1B have Env that are more frequently exposed in intermediate openness, making them moderately sensitive to neutralization. The most hardly neutralized viruses are tiers 2 and 3 strains, for which Env mainly exist in closed conformation (Montefiori et al., 2018) (Fig II-12).

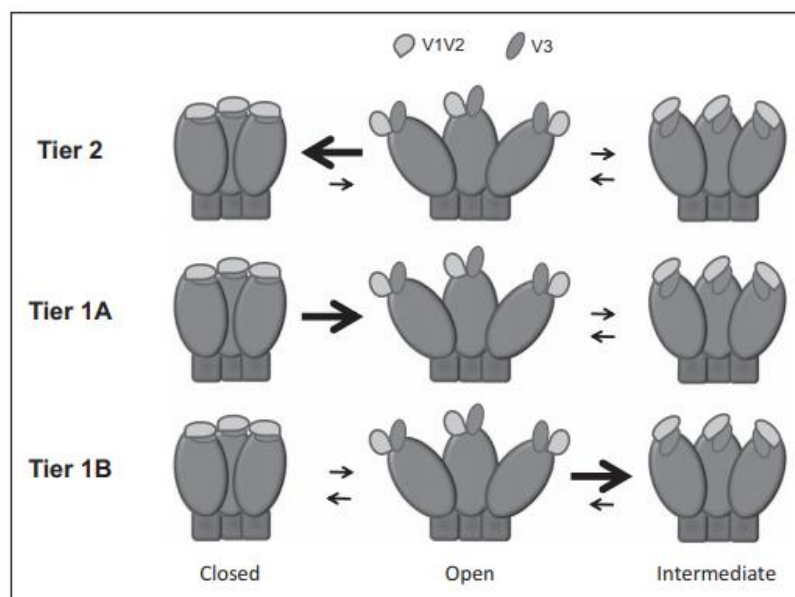


Figure II-12: Balance of conformational states in the HIV-1 Env trimer. From Montefiori et al., 2018.

Abs from the second group tend to be directed against the gp120 variable loops or other epitopes highly variable in sequence. These responses are also often immunodominant but during infection the virus manages to continuously escape such Abs by changes in their variable epitopes.

The third group of Abs directed against Env encompasses broadly neutralizing (BN) Abs, which are capable of neutralizing a large fraction of global circulating strains (breadth of coverage) (Burton & Hangartner, 2016). The Env targeted epitopes of such BNAbs are highly conserved in sequence and/or amino acid character. As explained above, a few BNAbs were first isolated in the early 1990s, which had limited breadth and potency. A second wave of BNAbs isolation started in 2009, yielding a large number of highly broad and potent mAbs. An Ab-based vaccine aiming at protecting against the diversity of HIV-1 circulating strains would certainly need to elicit such broad and potent neutralizing responses (Jon Cohen, 2013).

III. Broad HIV neutralization (BN)

A. BN characteristics

1. Evaluation of breadth

The breadth of an antibody or a serum is evaluated by its capacity to neutralize diverse viruses on panels composed of HIV-1 strains of diverse clades, geographical origins, tiers, infection time-points, etc. Several panels have been designed in order to better evaluate Ab breadth across the field (Simek et al., 2009) (Seaman et al., 2010) (deCamp et al., 2014). In particular panels comprising a relatively low number of viruses have been characterized to be predictive of the breadth on large panels (see part Materials and Methods). To be noted, “heterologous” refers to viral strains circulating worldwide, by opposition with “autologous” which defines virions relative to the particular infected individual being studied. The increasing resistance to NABs at the population level (Stefic et al., 2019) likely provides the need of a review of the panels currently used for the breadth evaluation.

2. Frequency of BN serum activity

As viral panels and diverse neutralization definitions tend to vary from one study to another, the estimation of the frequency of broad neutralization among the population of HIV infected individuals is not totally straightforward. One team designed a 5-virus panel (5vP) to screen the neutralizing activity of 1234 serum samples and defined the elite neutralizing activity as the ability to neutralize, on average, more than one pseudovirus at an ID50 titer greater than 1/300 within a clade group and across at least four clade groups. Using this definition, they identified 1% elite neutralizers in their cohort (Simek et al., 2009). Another team used a 10vP to screen 70 plasmas for neutralizing activity but the ID50 threshold used to define neutralization was decreased to a 1/45 dilution. Using this definition, they showed that 23% sera were able to neutralize more than 80% of the viruses tested (Gray et al., 2009). In parallel, the sera of 103 HIV-1-infected subjects were tested against a 20vP and 20% of them neutralized more than 75% of the viruses at an ID50 greater than 1/100 (Doria-Rose et al., 2010). Four years later, 205 HIV-1 sera from HIV donors were tested against a 219vP with an ID50 threshold for neutralization defined at 1/33.1: approximately 50% of sera neutralized more than 50% of viruses from this large panel (Peter Hraber et al., 2014). Our team later used a 6vP adapted from Simek et al. to screen neutralizing activity among 439 individuals and showed that 11% of them could neutralize more than 50% of the panel and 2% more than 80% (Landais et al., 2016).

The percentage of viruses neutralized among a given panel varies of course according to where the threshold has been placed to define a serum as potent. That being said, the overall frequency of heterologous broad neutralizing activity among the population is estimated between 10 to 20%. To be noted, BN activity can also be observed in infants, with a similar frequency than in adults (Goo et al., 2014).

3. BN activity kinetics

The acquisition of neutralization breadth and potency typically takes several years (Burton & Mascola, 2015). Within the large longitudinal Sub-Saharan HIV primary infection cohort studied by our team, for instance, high neutralization scores were reached 3.5 years after the infection on average, and either plateaued or decreased afterward. Another study mentioned an average of 2.5 years, with the earliest cases appearing at 1 year post-infection (Mikell et al., 2011). The number of HIV-1 infected individuals who develop BN activity after 4 years post-infection seems paltry (Landais et al., 2016). Interestingly, heterologous neutralizing activity in infants appeared to reach adult-like levels in a much shorter time - only a year post-infection - likely due to very rapid SMH, exposure to high antigenic load and/or intact immune system (Goo et al., 2014)(Ditse et al., 2018).

4. Associated clinical correlates

Several studies focused on the features associated with the development of BN and highlighted determinants associated with either the host, the virus or the disease. Among the latter category of determinants, one correlation that has often been observed associated with the development of BN is a high viral load (Van Gils et al., 2009) (Sather et al., 2009) (Piantadosi et al., 2009) (Doria-Rose et al., 2010) (Landais et al., 2016) (Rusert et al., 2016). Indeed, it may be that a high level of constant antigenic stimulation could favor the activation of a large number of B cells and consequently increase the stochastic probability of activating a B cell bearing a BN specificity. Moreover, a greater Env diversity likely comes with the high viral load, potentially favoring the elicitation of Abs able to recognize a larger Env number (to be noted *env* diversity was associated with BN responses while *gag* diversity was not (Piantadosi et al., 2009)). Additionally, it was shown that dual infection resulted in enhanced breadth (Powell et al., 2010) and elite controllers – with low viral load – rarely develop BNabs (Doria-Rose et al., 2010). Paradoxically, even though Env diversity appears as favoring BNabs elicitation, the opposite relation might be true too, as BNab responses could be the cause Env diversity in exerting a selective pressure on autologous viruses. Nonetheless, high viral levels are associated with - but not sufficient for - BN activity (Moore et al., 2015).

PD-1+CXCR3–CXCR5+ Memory Tfh cells levels correlate with BN activity as well. This goes with the fact that BNabs are often highly mutated (detailed below) and Tfh cells play a role in SMH acquisition in GCs (Locci et al., 2013).

The total levels of plasma IgG and of anti-gp120 and anti-gp41 binding IgGs are also correlated to the BN activity, as well as low levels of CD4+ T cells. This appears to be explained by the high viral loads found in the individuals with BN responses, as explained above (Landais et al., 2016).

Another notable correlate that was found is the length of untreated infection (Rusert et al., 2016) which appears linked to the viral load as well, as the antiretroviral therapy diminishes the viral load below detectable levels.

Concerning the host, no influence of gender, age, geographical origin (Landais et al., 2016) nor route of exposure or race/ethnicity (Doria-Rose et al., 2010) was found associated to BNAb development. Notwithstanding an observation was made that black individuals showed significantly higher rates of BNAb responses than white individuals (Rusert et al., 2016).

Albeit with a relatively low significance, HLA-A*03(-) genotype also correlated with BN activity (Landais et al., 2016).

The question of whether the virus itself could contain specific intrinsic features that enabled BNabs elicitation was raised too, as it is already known that disease progression and viral transmission are affected by particular virus factors (Fraser et al., 2014)(Claiborne et al., 2015). In this instance, an infection by a subtype-C HIV-1 appeared somehow correlated with the development of BNabs (Landais et al., 2016). This virus-subtype association was even extended to the specificity of the BN responses: higher frequencies of CD4-bs BNabs were observed in infection with subtype B viruses and higher frequencies of V2-glycan-specific BNabs in infection with non–subtype B viruses (Rusert et al., 2016). Also, shorter V1 loops, lower level overall of glycosylation, and the absence of a specific glycan at 332 (which forms part of a major BNAb supersite ; see below) on Env were associated with increased breadth (van den Kerkhof et al., 2013). Another study however added that longer V1V2 loops were associated with more potent BNAb responses (P. Hraber et al., 2014).

B. Broadly Neutralizing Antibodies

1. Conserved target regions on Env

As mentioned earlier, BNAb epitopes are relatively conserved among the diverse circulating HIV-1 strains Env. Six epitopes for broad neutralization have been listed so far (Fig III-1): the CD4-bs, the base of the gp120 V3 loop, the apex formed by the V1 and V2 gp120 loops, the interface between gp41 and gp120, the membrane proximal external region (MPER) and, more recently, the central region of the outer domain of gp120 named the “silent face”.

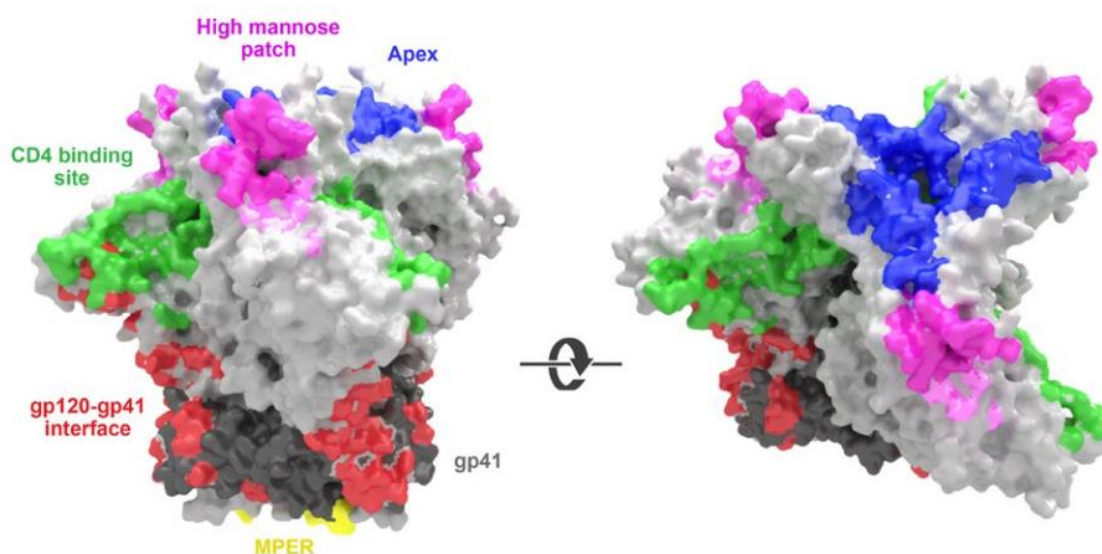


Figure III-1: Epitope regions targeted by HIV BNABs. Model based on the fully glycosylated BG505 SOSIP.664 trimer (PDB: 4ZMJ). The gp120 and gp41 subunits are colored light gray and dark grey respectively. The five BNAb epitope regions are labeled as follows: the apex site is colored purple, the high-mannose patch is colored magenta, the CD4-bs is colored green, the gp120-gp41 region is colored red, and MPER is colored yellow (McCoy & Burton, 2017).

- **CD4-bs**

The CD4-bs is straddled between the outer and the inner domain of gp120, connected by a bridging sheet (Fig III-2). Binding to the immunoglobulin-like amino-terminal domain of CD4 induces gp120 conformational changes and allows subsequent interaction with a co-receptor (CCR5 or CXCR4).

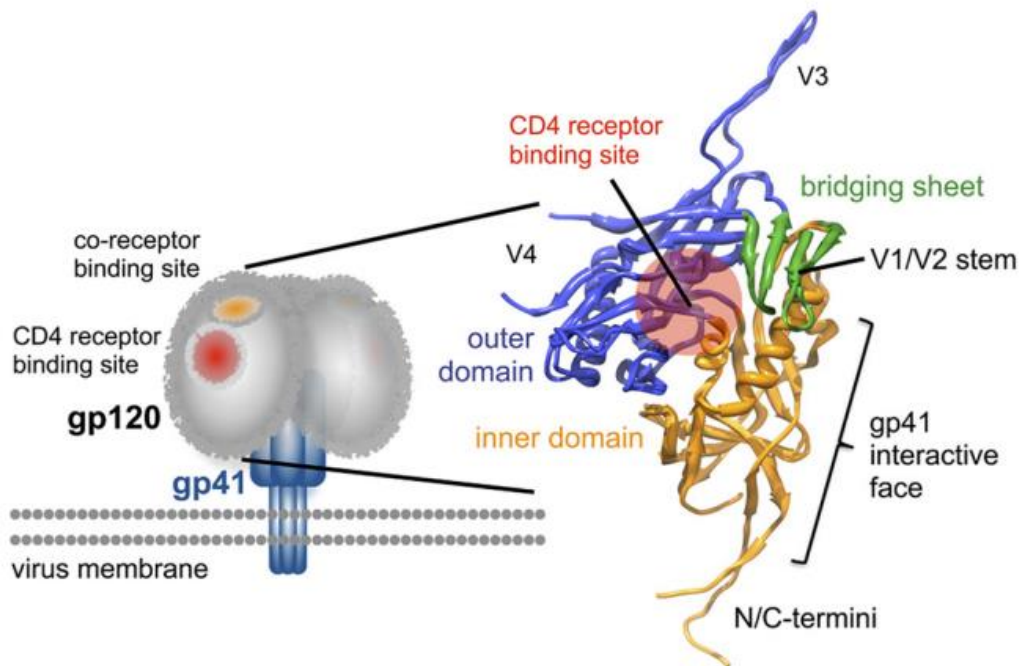


Figure III-2: gp120 core structure. Crystal structure of gp120 from the gp120-sCD4- 48D Fab complex (PDB 3JWD) superimposed with the gp120 core containing an intact V3 loop (gp120-sCD4-X5; PDB 2B4C) reveals the organization of gp120 in terms of inner (orange) and outer (blue) domains. The 4-stranded β -sheet subdomain termed the bridging sheet (green) is composed of two strands from the outer domain and the V1/V2 stem from the inner domain. The position of the CD4-bs on gp120 is indicated by the red circle. gp120 is believed to interact with gp41 primarily through interactions involving the inner domain and N-/C-terminal extensions. From Guttman et al., 2012.

CD4-bs BNAs usually display the best combination of breadth and potency (Table III-1), making them favorite candidates for vaccine strategies. Among individuals able to develop BNAs, the frequency of the CD4-bs specificity seems relatively rare, estimated at around 5% (Landais et al., 2016). These Abs also appear to take longer than any other specificity to develop (5 years) and display, on average, the highest number of SMH (30%) among all BNAs (Sok & Burton, 2018). CD4-bs BNAs can be classified in several groups based on their mode of recognition.

The first group is the one of the “CD4-mimic Abs” that recognize the CD4-bs in a CD4 manner, thanks to the common usage of VH1-2 or VH1-46 with a long CDRH2 and a short CDRL3, which mimic the terminal Ig-like domain of CD4. The most potent BNAs among this group are the VRC01-class BNAs, and particularly N6, that evolved to avoid the problematic glycans of the region, and N49P7 which, unlike its Ab relatives, recognizes particular residues of the inner domain of gp120 (Table III-1). VRC01-class BNAs likely go through a long and complex affinity maturation process, making them thus difficult to elicit by vaccination (X. Wu et al., 2015). The PCIN63 lineage appears however as a promising exception: this is a lineage of VRC01-class Abs with similar high breadth and potency than its relatives but 2 to 3 fold less mutated (Umotoy et al., 2019).

The second group of CD4-bs Abs contains the « loop dependent Abs » which paratope relies on the CDRH3, like b12 and CH103 (Table III-1), that uses diverse VH genes.

Ab	Ic ₅₀ geo mean (µg/ml)	Coverage (nb of viruses tested)	HC v gene	CDRH3 length (aa)	Lc v gene	CdrL3 length (aa)	V _H J + V _H K _H J or V _H J (% nt)	Insertions (+) or deletions (-)	Refs
N49-P7	0.100	100 % (117)	HV1-2*02	21	LV2-11*01	5	19	CDRL1 (-6 aa)	Sajadi et al., 2018
N6	0.058	98 % (327)	HV1-2*02	15	KV1-33*01	5	25		Huang et al., 2016
IOMA	2.320	50 % (118)	HV1-2*02	19	LV2-23*02	8	10		Gristick et al., 2016
CH235	5.927	19 % (176)	HV1-46*01	15	KV3-15*01	8	6		Gao et al., 2014
CH235.12	0.650	90 % (199)	HV1-46*01	15	KV3-15*01	8	19		Bonsignori et al., 2016
B12	2.204	47 % (200)	HV1-3*01	20	KV3-20*01	9	12		Burton DR & Barbas CF, 1994
VRC01	0.329	91 % (196)	HV1-2*02	14	KV3-20*01	5	23	CDRL1 (-3 aa)	Wu et al., 2010
3BNC117	0.097	85 % (198)	HV1-2*01	12	KV1-33*01	5	19	H-FR3 (+4 aa), CDRL1 (-4 aa)	Scheid et al., 2011
CH103	0.699	85 % (150)	HV4-61*08	15	LV3-1*01	1	15	CDRL1 (-3 aa)	Liao et al., 2013

Table III-1: Breadth, potency and selected characteristics of human CD4-bs-BNABs (non exhaustive list). The first-generation BNABs (isolated before 2009) are in grey. Neutralization breadth and potency from the database CATNAP or the published manuscript (ref right column). Geometric mean neutralization IC₅₀ was calculated by exclusion of resistant viruses. Neutralization breadth was calculated on the basis of a neutralization IC₅₀ cut-off between 10 µg/mL and 50 µg/mL. Overall nucleotide mutation (% nt) was calculated as the mutation frequency given the genes encoding the heavy-chain variable (V) region and joining (J) region (HC V gene), as well as those encoding the light chain V and J region (LC V gene). Adapted from Sok & Burton, 2018.

- **Apex V1/V2 Loops**

The Env apex is constituted by the gp120 V1 and V2 loops that assemble into a five stranded β -barrel structure (named A, B, C, C', and D). All 3 gp120 V1V2 join together. They constitute the most diverse epitope in sequence and are covered by a dense array of N-glycosylations. The apex shields the co-receptor binding site and partially occludes the third variable loop region (V3), which both get exposed upon binding to CD4 (Hessell et al., 2019).

The frequency of apex-targeting BNABs within a cohort of neutralizers was estimated to reach approximately 14% by Landais et al., 2016. Those Abs usually have long CDRH3 regions (>24 residues) (with exceptions such as VRC38) in a hairpin or hammerhead conformation, allowing them to slip between the glycans, and an anionic tip to interact with one of the β -strand of the apex (Sok & Burton, 2018). As mentioned before, they represent the most potent BNABs but usually have moderate breadth (exemple Ab VRC26, Table III-2). They also show heterogeneity in the rates of somatic mutation and length of affinity maturation process.

The V2q epitope family contains the BNABs that target the apex with a quaternary Env preference, even though PG9 and PG16 can bind to some gp120 monomers. They both recognize a region in the C strand of V1V2 and bind to two N-linked glycans (N160 and N156 or N137) (McLellan et al., 2011).

The V2qt epitope family include trimer-specific BNABs such as PGT145 and PGDM1400, that have long and straight CDRH3, which hairpin tip reaches the central opening of the trimer apex (Hessell et al., 2019) (Duerr & Gorny, 2019).

Ab	Ic ₅₀ geo mean (µg/ml)	Coverage (nb of viruses tested)	HC v gene	CDRH3 length (aa)	Lc v gene	CDRL3 length (aa)	V _{hHj} + V _{khj} or V _{Hj} (% nt)	Insertions (+) or deletions (-)	Refs
VRC26 .25	0.002	59% (174)	HV3-30*03	38	LV1-51*02	12	10		Doria-Rose et al., 2016
PCT64 - 24E	0.911	33% (115)	HV3-15*01	25	KV3-20*01	8	8		Landais et al., 2017
VRC38 .01	0.361	31% (210)	HV3-13*01	18	KV2-28*01	10	13		Cale et al., 2017
PG9	0.118	84% (200)	HV3-33*05	30	LV2-14*01	10	11		Walker et al., 2009
PG16	0.150	73% (162)	HV3-33*05	30	LV2-14*01	10	?	?	Walker et al., 2009
PGDM 1400	0.017	81% (194)	HV1-8*01	34	KV2-14*01	9	18		Sok et al., 2014
CH01	1.007	52% (195)	HV3-20*01	26	KV3-20*01	9	13		Bonsignori et al., 2012
PGT145	0.290	48% (162)	HV1-8*01	33	KV2-28*01 or 2D-28*01	9	?	?	Walker et al., 2011

Table III-2: Breadth, potency and selected characteristics of human Apex-BNABs (non exhaustive list). Neutralization breadth and potency from the database CATNAP or the published manuscript (ref right column). Geometric mean neutralization IC₅₀ was calculated by exclusion of resistant viruses. Neutralization breadth was calculated on the basis of a neutralization IC₅₀ cut-off between 10 µg/mL and 50 µg/mL. Overall nucleotide mutation (% nt) was calculated as the mutation frequency given the genes encoding the heavy-chain variable (V) region and joining (J) region (HC V gene), as well as those encoding the light chain V and J region (LC V gene). Adapted from Sok & Burton, 2018.

- **Base of the V3 loop / HM Patch**

The V3 loop, formed by the bridge between cysteins 296 and 331 (Fig II-9) is highly immunogenic and elicit a strong humoral response, albeit relatively poorly neutralizing. As mentioned before, the V1/V2 apex hides the upper part of the V3 loop. The base of the loop however is structurally exposed although extremely glycosylated. BNABs responses against this area are common.

The V3-base epitope the most targeted by sera with broad neutralizing activity is the High Mannose patch, centered on the N332 glycan. Thirty-eight% of HIV-1 infected neutralizers show BN activity against this epitope (Landais et al., 2016), suggesting lesser structural constraints in comparison to other epitopes, as HM Patch BNABs show high flexibility in their binding modes, notably in term of glycan requirements, and angles of approach (L. Kong et al., 2013).

The PGT families (121 to 124, 125 to 128 and 135 to 143) notably relies on the patch made of the 6 conserved residues N295, N301, N332, N339, N385, and N392 (Fig III-3), with a preferential interaction with the central N332 glycan. However, some BNABs can use alternate promiscuous N-linked glycans in the absence of the N332 and consequently neutralize a substantial number of viruses lacking the N332 site (Sok, Doores, et al., 2014). They sometimes can recognize the N332 glycan when it has shifted to N334.

BNABs targeting the HM patch have learned to either accommodate, bind or avoid the glycans of the region. For instance PGT121 and PGT123 interact with N197 while PGT124 avoids it (Garces et al., 2015).

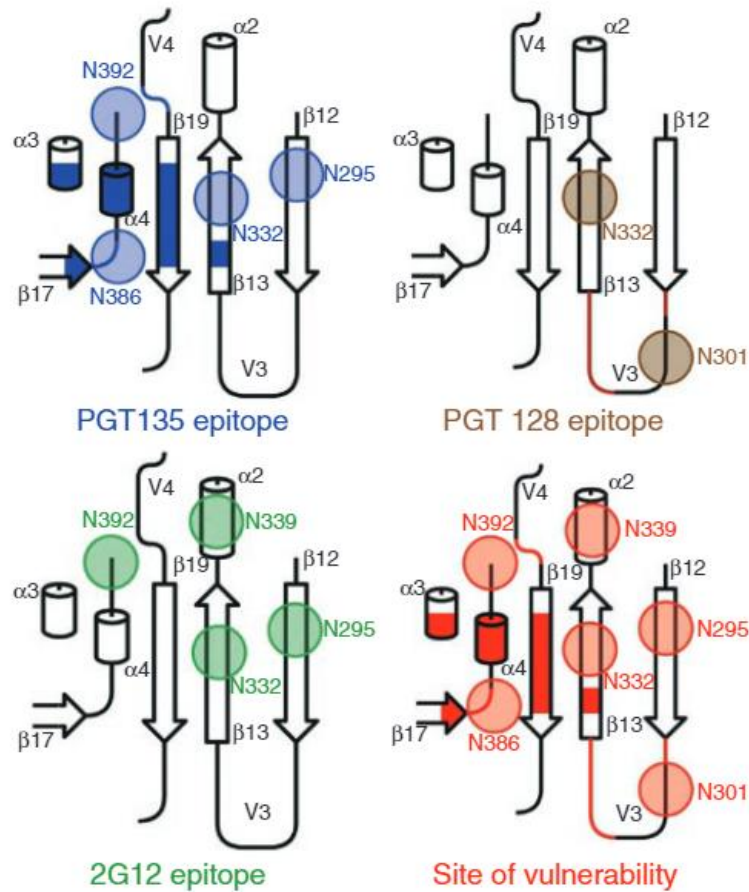


Figure III-3: The epitopes of PGT 135, PGT 128 and 2G12, shown in topology representations in which cylinders are α -helices, arrows are β -strands and lines are loops. N-linked-glycan sites are circled. The structures of 2G12 (PDB 1OP5) and PGT 128 (PDB 3TYG) were obtained from the Protein Data Bank. From L. Kong et al., 2013.

Another epitope is made by the HM Patch glycans and the ³²⁴GDIR³²⁷ peptide stretch at the base of the gp120 V3 loop. This motif is part of the CCR5 co-receptor binding site and camouflaged by the HM patch glycans (Sok, Pauthner, et al., 2016). This combined epitope is targeted by PGDMs families that show great breadth (Table III-3).

Gene usage among the V3 targeting BNABs is various, although VH4 is frequently reported (Table III-3). Notably, VH4-34 is used by PGDM21, PCDN-33A, the lineages of PC76 (MacLeod et al., 2016) and PC39 (*unpublished*). VH4-34 is known to be associated with autoreactivity, which implication in BNAB elicitation is currently studied by our team. Associated VK or VL are different except for donors PC76 and PC39, which nonetheless have different ancestors and maturation pathways.

Most V3-base BNABs show great affinity maturation, with overall nucleotide mutation in HC and KC/IC V genes ranging roughly from 10 to 18% (Table III-3). Another shared feature are the long CDRH3 loops. To be noted, long CDRH3 loops serve to penetrate the glycan shield and reach residues underneath (L. Kong et al., 2013).

V3-base BNABs are particularly interesting for vaccine strategies. First, because of protective properties in animal models, as observed with PGT121 for instance (Moldt et al., 2012). Second, because of promising elicibility, as PGT121 lineage could be successfully induced in knockin mice with B-cell precursors for PGT121-family BNABs (Steichen et al., 2016). A more attractive target would be BG18, best combination of breadth and potency among the V3-base BNABs, with no indels but numerous SMH though a large fraction of them are not require for the neutralizing activity (W.R. Schief, unpublished observations). Of note, indels and high SMH are major barriers for vaccine design, making the targeted Ab more complicated to elicit. PC76 lineage is interesting too, with lower mutations and no indels (MacLeod et al., 2016).

Ab	Ic ₅₀ geo mean (µg/ml)	Coverage (nb of viruses tested)	HC v gene	CDRH3 length (aa)	Lc v gene	CDRL3 length (aa)	V _H J + V _K HJ or V _H J (% nt)	Insertions (+) or deletions (-)	Refs
BG18	0.032	62% (119)	HV4-4*02	23	LV3-25*03	11	18		Freund et al., 2017
DH270.1	0.510	42% (179)	HV1-2*02	20	LV2-23*02	10	11		Bonsignori et al., 2017
DH270.6	0.151	57% (179)	HV1-2*02	20	LV2-23*02	10	11		Bonsignori et al., 2017
PGDM 12	0.134	57% (111)	HV3-11*03	21	KV2-24*01	9	16	CDRH1 (-2 aa)	Sok et al., 2016
VRC41 .01	0.275	53% (107)	HV4-39*07	21	KV3-20*01	9	16		Munir Alam et al., 2017
PGDM 21	0.139	50% (111)	HV4-34*08	20	KV3-20*01	9	18	CDRH2 (+4 aa)	Sok et al., 2016
PCDN-33A	0.410	49% (125)	HV4-34*01	22	KV3-20*01	8	11		MacLeod et al., 2016
VRC29 .03	1.276	28% (179)	HV4-59*08	20	KV3-20*01	9	12	CDRH2 (+6 aa)	Longo et al., 2016
PGT121	0.048	66% (200)	HV4-59*01	26	LV3-21*01	12	17	L-FR3 (+3 aa), L-FR1 (-7 aa)	Walker et al., 2011

10-1074	0.039	68% (200)	HV4- 59*01	26	LV3- 21*01	12	NA	L-FR3 (+3 aa), L-FR1 (- 4 aa)	Mouquet et al., 2012
2G12	2.380	20% (162)	HV3- 21*01	16	KV1- 5*03	9	?	?	Trkola et al., 1996
PGT135	0.170	20% (162)	HV4- 39*07	20	KV3- 15*01	9	?	?	Walker et al., 2011
PGT128	0.002	44% (162)	VH4- 39*07	21	LV2- 8*01	10	?	?	Walker et al., 2011

Table III-3: Breadth, potency and selected characteristics of human V3-base-BNABs (non exhaustive list). The first-generation BNABs (isolated before 2009) are in grey. Neutralization breadth and potency from the database CATNAP or the published manuscript (ref right column). Geometric mean neutralization IC50 was calculated by exclusion of resistant viruses. Neutralization breadth was calculated on the basis of a neutralization IC50 cut-off between 10 µg/mL and 50 µg/mL. Overall nucleotide mutation (% nt) was calculated as the mutation frequency given the genes encoding the heavy-chain variable (V) region and joining (J) region (HC V gene), as well as those encoding the light chain V and J region (LC V gene). Adapted from Sok & Burton, 2018 and Burton & Hangartner, 2016.

- **Gp120/gp41 interface**

BNAbs targeting the interface between gp120 and gp41 (Table III-4) get contacts with residues from both subunits – notably the fusion peptide (FP) of gp41 (VRC34) – and glycans of the area (N88, N611...). Because of their strict recognition of native conformation, these Abs can be used for Env recombinant purification, antigenicity evaluation or stabilization (example with PGT151 than only recognize fully cleaved trimers).

Interface BNAbs seem to be elicited in 12% of the individuals able to develop BNAbs (Landais et al., 2016).

Ab	Ic ₅₀ geo mean (µg/ml)	Coverage (nb of viruses tested)	HC v gene	CDRH3 length (aa)	Lc v gene	CDRL3 length (aa)	V _H J + V _K J or V _H J (% nt)	Insertions (+) or deletions (-)	Refs
VRC34 .01	0.310	49% (179)	HV1-2*02	15	KV1-9*01	9	11		Kong et al., 2016
ACS202	0.140	44% (81)	HV3-30*03	24	KV1-33*01	9	15	CDRH2 (+1 aa)	Van Gils et al., 2016
PGT151	0.023	72% (200)	HV3-30*03	28	KV2D-29*02	9	16		Falkowska et al., 2014
35O22	0.151	56% (200)	HV1-18*03	16	LV2-14*02	10	22	H-FR3 (+8 aa)	Huang et al., 2014
8ANC195	1.115	66% (200)	HV1-3*03	22	KV1-5*03	9	21	CDRH1 (+1 aa) H-FR3 (+4 aa) CDRH2 (-2 aa) HCDRL1 (+1 aa)	Scheid et al., 2011

Table III-4: Breadth, potency and selected characteristics of human Interface-BNAbs (non exhaustive list). Neutralization breadth and potency from the database CATNAP or the published manuscript (ref right column). Geometric mean neutralization IC₅₀ was calculated by exclusion of resistant viruses. Neutralization breadth was calculated on the basis of a neutralization IC₅₀ cut-off between 10 µg/mL and 50 µg/mL. Overall nucleotide mutation (% nt) was calculated as the mutation frequency given the genes encoding the heavy-chain variable (V) region and joining (J) region (HC V gene), as well as those encoding the light chain V and J region (LC V gene). Adapted from Sok & Burton, 2018.

- **MPER**

The membrane proximal external region (MPER) belongs to gp41 and is a small helical linear motif (19 amino-acids approximately) that precedes the transmembrane (TM) region. It presents a high sequence conservation among HIV-1 strains (Salzwedel et al., 1999). The MPER has an important role in the conformational changes that occur during the membrane fusion. It is poorly accessible by BNABs on native Env (pre-fusion state) but gets exposed following binding to CD4 and CCR5/CXCR4 (intermediate state) (Chen et al., 2014). The estimation of frequency of MPER-BNABs elicitation is unclear, as some studies assert it is high (27% (Huang et al., 2012)) and others low (2.5% (Landais et al., 2016)). BNABs targeting the MPER usually display impressive breadth, but often moderate potency (Sok & Burton, 2018 ; Table III-5). They also often tend to be autoreactive (directed against self-components) and/or polyreactive (binding non-specifically to a number of structurally unrelated targets), likely because they were selected and matured to interact with the lipid membranes nearby. To this same aim, MPER BNABs also have long HCDR3 loops with hydrophobic character, shown to be necessary for neutralization (J.-P. Julien et al., 2010). These latter features might make most of BNABs from this class difficult to elicit by vaccination.

BNAB 10E8, however, is an interesting vaccine candidate, because of its high breadth and potency (Table III-5) and its absence of autoreactivity (Huang et al., 2012). Besides, the presence of 10E8 in a 4-antibodies cocktail provided significantly better neutralization *in vitro* (Wagh et al., 2016). 10E8 recognizes the same epitope than 4E10 and DH511 but through a different angle of approach (Bonsignori et al., 2017).

Ab	Ic ₅₀ geo mean (µg/ml)	Coverage (nb of viruses tested)	HC v gene	CDRH3 length (aa)	Lc v gene	CDRL3 length (aa)	V _{H1J} + V _{KHJ} or V _{HLJ} (% nt)	Insertions (+) or deletions (-)	Refs
DH511 .11P	0.674	99% (180)	HV3- 15*01	23	KV1- 39*01	11	15		Williams et al., 2017
2F5	2.027	55% (36*)	HV2- 5*02	24	KV1- 13*02	9	?	?	Purtscher et al., 1994
4E10	1.765	98% (200)	HV1- 69*17	20	KV3- 20*01	9	10		Purtscher et al., 1994
10E8	0.299	98% (199)	HV3- 15*05	22	LV3- 19*01	12	17		Huang et al., 2012
LN01	1.100 (median)	92% (118)	HV4- 39*07	20	KV1- 39*01	9	?	?	Pinto et al., 2019

Table III-5: Breadth, potency and selected characteristics of human MPER-BNABs (non exhaustive list). The first-generation BNABs (isolated before 2009) are in grey. Neutralization breadth and potency from the database CATNAP or the published manuscript (ref right column). Geometric mean neutralization IC₅₀ was calculated by exclusion of resistant viruses. Neutralization breadth was calculated on the basis of a neutralization IC₅₀ cut-off between 10 µg/mL and 50 µg/mL. *The 36vP was used in Landais et al, PLOS Pathogen, 2016, and is not exactly predictive of the neutralization breadth on larger panels. Overall nucleotide mutation (% nt) was calculated as the mutation frequency given the genes encoding the heavy-chain variable (V) region and joining (J) region (HC V gene), as well as those encoding the light chain V and J region (LC V gene). Adapted from Sok & Burton, 2018 and Burton & Hangartner, 2016.

- **Silent Face**

The silent face is located on the outer domain of gp120 (Fig III-4) and was originally considered as resistant to BNABs recognition - hence its designation (Wyatt R et al., 1998). It is the most glycosylated part of Env.

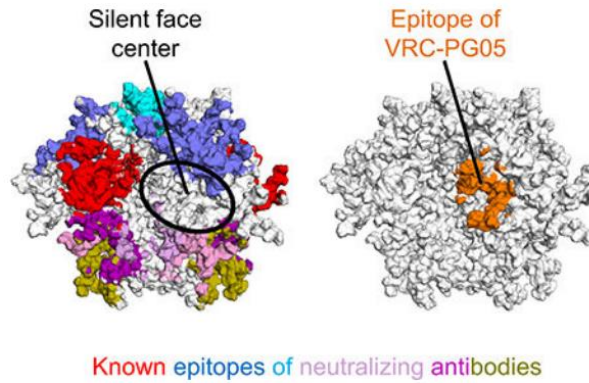


Figure III-4: Localization of the center of the silent face of HIV-1 and the precise epitope of VRC-PG05. Zhou et al, *Immunity*, 2018.

No antibody was reported to recognize this silent face until 2018, with the identification of VRC-PG05 (Table III-5) targeting the N295, N448 and N262 glycans, between the HM Patch and the gp120/gp41 interface. The following year were identified SF12 and related Abs, targeting the same glycans, but apparently in very different ways (Schoofs et al., 2019).

Considering the properties of these BNABs, the silent face might be a very interesting vaccine target too. It is likely that a lot of silent face-BNABs were missed because of the design of the isolation strategies used in most studies, making the informations on this part of Env limited (yet).

Ab	Ic ₅₀ geo mean (µg/ml)	Coverage (nb of viruses tested)	HC v gene	CDRH3 length (aa)	Lc v gene	CDRL3 length (aa)	V _H Hj + V _K Hj or V _H Ij (% nt)	Insertions (+) or deletions (-)	Refs
VRC-PG05	0.800	27% (208)	HV3-7*01	17	KV4-1*01	8	9		Zhou et al., 2018
SF12	0.200	62% (119)	HV4-59*01	23	KV3-20*01	6	?	?	Schoofs et al., 2019

Table III-5: Breadth, potency and selected characteristics of human Silent Face-BNABs (non exhaustive list). Adapted from Sok & Burton, 2018.

A large number of BNABs, any epitope combined, were compared for their breadth and potency, as shown Fig III-5. To be noted, different panels of viruses have been used among the BNABs studies, so comparisons of breadth are approximate. Some of those isolated BNABs were tested for prophylaxis and therapeutics, as developed below.

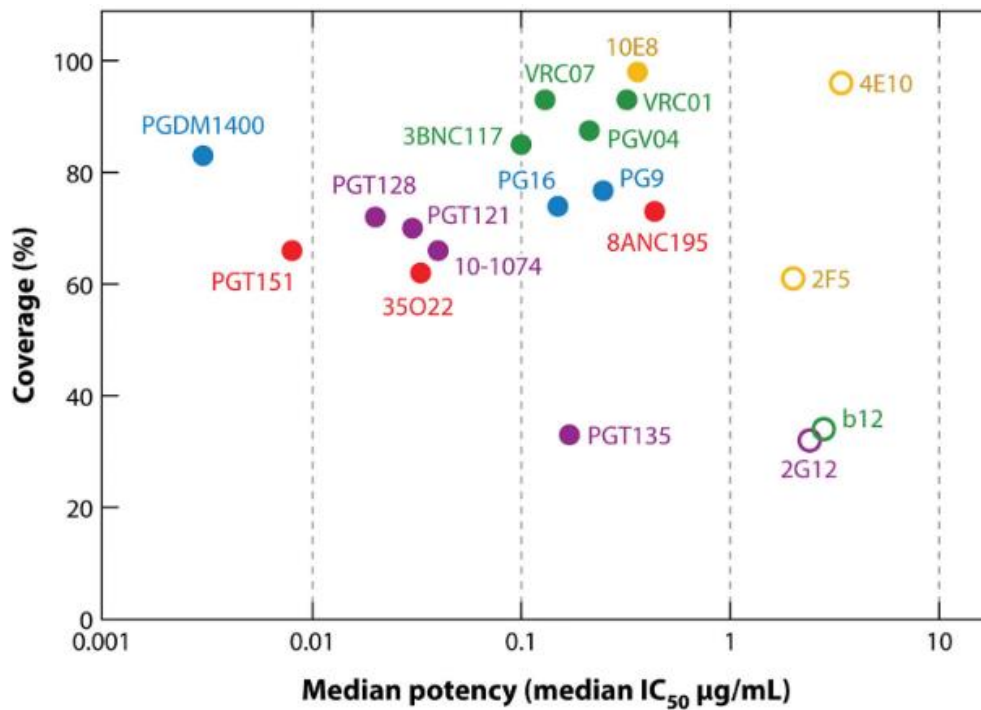


Figure III-5: Breadth and potency of BNABs. The percentage of large panels of isolates neutralized at an IC₅₀ < 50 µg/mL (coverage) in pseudovirus assays plotted against the median inhibitory concentration (IC₅₀ in µg/mL) for all neutralization-sensitive viruses. First-generation antibodies are indicated by unfilled circles and second-generation antibodies by filled circles. The epitope specificity is indicated by color, with antibody specific for the CD4-bs depicted in green, apex-specific antibodies in blue, antibodies binding to the high-mannose patch in purple, antibodies binding to the gp120-gp41 interface in red, and MPER-specific antibodies in light brown. From Hangartner and Burton, *Annu Rev Immunol*, 2016.

2. BNABs isolation

Numerous methods were employed to isolate HIV-1 BNABs (McCoy & Burton, 2017). The first attempts were made with phage display: briefly, libraries of Abs made from HIV infected donors were expressed on phages which were tested through cycles of panning for their binding to immobilized antigens (ELISA) to enrich in phages binding with high affinity. This is how the first generation BNAB b12 was isolated for instance (Burton DR & Barbas CF, 1994). Another approach that was used early on was based on B-cell immortalization (via EBV immortalization or hybridoma electrofusion) with which B-cells bearing the Abs at their surface can be cultured and screened for antigen-specificity and neutralizing activity (Buchacher et al., 1994) (Corti D & Lanzavecchia A, 2014). The other first generation BNABs Abs 2F5, 4E10 and 2G12 were obtained through EBV immortalization. Two main issues can explain why only few BNABs were isolated early on: the lack of proper donor (the ones used at the time were not elite neutralizers) and the use of screening methods based essentially on monomeric gp120 and laboratory isolates.

Starting from the late 2000s appeared single B-cell-based approaches, thanks in particular to new methods allowing amplification and cloning of Ig genes from single cells. Such methods coupled with a better selection of donors to isolate Abs from, which was finally possible with the development of pseudo-virus-based neutralization assays and of large panels of primary isolates, led to the isolation of a still increasing number of second generation BNABs. Broadly, 2 approaches are currently used for BNAB isolation: single B cell culture followed by a functional/neutralization assay, and single B cell sort with specific baits. Both approaches start with memory B cells from Peripheral Blood Mononuclear Cells (PBMC) samples.

In the first approach, memory B cells (most usually IgG-expressing) are distributed at 1 to 4 cell per well in 384-well plates and activated by a cocktail of cytokines and feeder cells expressing CD40 ligand (example of a protocol described in Huang et al., 2013). The activation leads B-cell to secrete IgGs that accumulate in the supernatant over the next 7-15 days. Of note some laboratories still use in addition methods based on improved EBV immortalization protocols (Corti D & Lanzavecchia A, 2014). Supernatants from the activated B cells can then be functionally screened for their properties of binding (to gp120 or recombinant trimer, by ELISA) or most interestingly neutralization (using usually a pseudovirus assay and a small panel of heterologous viruses). This is how 10E8 (Huang et al., 2012), PG9, PG16 (Walker et al., 2009), PGT145 (L. M. Walker et al., 2011) and PCT64 lineage (Landais et al., 2017) Abs were isolated.

In the second approach, memory B-cells are directly sorted by FACS based on their ability to specifically bind Env baits, in the form for example of mgp120 (as done for the PCDN lineage, MacLeod et al., 2016) or recombinant Env trimers (see part IV-A-4) (as for isolation of PGDM1400 members, Sok, Gils, et al., 2014)).

Following both approaches RNAs of the selected B-cells are extracted to go through single cell RT-PCR and PCR amplification of the variable part of the Ig genes, that are then cloned in vectors, for expression usually as an IgG1. Abs are produced by transfection of mammalian cells (usually HEK293) for further characterization, as described in Materials and Methods.

Having the informations of the functional specific activity of the Abs is the major advantage of the activation approach. This strategy usually results in low number of Ab selected candidates with high chances of having NAbs. However the whole protocol is quite labor-intensive and more costly than the specific sort. This latter approach offers an easy pre-selection of Abs with interesting binding properties, though the baits have to be chosen very carefully, based on a proper prior mapping of the neutralizing activity of the plasma. This strategy is less labor-intensive and overall cheaper but can nevertheless result in more Ab candidates and PCR to run with lesser chances of getting Abs with neutralizing activity.

3. Use of BNABs in the clinic

- **BNABs for prevention of HIV infection**

The ability of NABs to confer protection against viral challenge has been demonstrated in animal models (macaques mainly), notably with early isolated BNABs such as b12 (directed against the CD4-bs), 2G12 (V3 HM Patch), 2F5 and 4E10 (MPER) (Baba et al., 2000) (Hessell, Rakasz, et al., 2009) (Hessell, Poignard, et al., 2009) (Hessell et al., 2010). Animal protection was confirmed with second-generation BNABs, that could be used at lower concentration: PGT121, PGT126 (HM Patch) and 3BNC117 (CD4-bs) (Moldt et al., 2012) (Moldt et al., 2016) (Julg, Sok, et al., 2017). Importantly, the potency *in vitro* translated into protection at low plasma concentration *in vivo*, potentially achievable through vaccination.

While studies have demonstrated that Abs can protect in the macaque models, leading to sterilizing immunity, it was not known until recently whether NABs can in fact protect humans and if so, at which titer. Indeed, the macaque model may in particular underestimate the abilities of NABs to protect as high viral doses need to be used for challenge experiments, in order to have all control animals infected. Recently, two phase IIb efficacy trials were launched to evaluate the protective efficacy of NABs in humans, using VRC01 (a CD4-bs-targeting BNAB, as described below). First, the proper tolerability and safety of the administration of multiple doses of VRC01 was verified (HVTN104, Mayer et al., 2017). The two Antibody Mediated Prevention (APM) studies concerned at-risk cisgender men and transgender persons from Americas and Europe (HVTN704 / HPTN085, NCT02716675), and at-risk women from subsaharian Africa (HVTN703 / HPTN081, NCT02568215) (4625 participants total). The long-awaited data were recently analyzed, yielding mixed results. Overall, there was no prevention of HIV acquisition in the VRC01-treated group. However, VRC01 could protect against acquisition of VRC01-sensitive viruses, which was to be expected, though it appears that the protection concerned only the most sensitive viruses, with an IC80 lower than 1µg/ml, which was somehow disappointing (Corey et al., 2021).

Nevertheless, those recent results show that Nabs can protect in humans, and support the necessity of using combinations of BNABs to protect against such viral diversity. This is reminiscent of experiments in the macaque model with PGT121 (HM Patch BNAB) and PGDM1400 (V2 apex) which on their own both failed to protect against a viral challenge by two SHIV in monkeys but their combination provided 100% protection (Julg, Liu, et al., 2017). To predict the best BNABs combinations that could be used *in vivo*, a mathematical model was notably developed based on their breadth and potency *in vitro* and identified triple and quadruple combinations of BNABs significantly more effective than the best double combinations (Wagh et al., 2016). Finally, in the wake, safety and tolerance of the combination of 3BNC117 (CD4-bs-targeting BNAB) and 10-1074 (HM Patch) administrated in humans have been confirmed (NCT02824536), likely leading to the forthcoming evaluation of the prophylaxis ability of this BNABs combination against HIV-1 infection (Cohen et al., 2019).

- **BNAbs as treatment of HIV infection**

BNAbs have now been tested in a number of studies for their ability to treat established infection in the macaque model. For example, PGT121 (targeting the V3 HM Patch) temporarily suppressed plasma viremia in chronically SHIV infected rhesus monkeys. Viremia eventually rebound when serum mAb titers decreased (Barouch, Whitney, et al., 2013). PGT121 administered in combination with half-life-improved N6 (CD4-bs Ab) also suppressed plasma viral loads in all chronically SHIV-infected macaques. Viral rebound was also observed with the decline of the Ab plasma concentrations (Julg, Pegu, et al., 2017). Although viral escape is detected in some studies, in particular with monotherapy, this is not always the case, notably when using Ab combinations. As an example, a combination of anti-V3 HM patch and anti-CD4-bs BNABs – 10-1074 and 3BNC117 – was tested for therapeutic effects in macaques SHIV-infected. A first co-administration rapidly suppressed plasma viremia and following rebound as Ab concentrations declined, a second administration controlled the viral rebound showing the absence of escape (Shingai et al., 2013).

Interestingly, it appears that treatments by BNABs can in some cases offer a prolonged viremia control, in particular as compared to cART, through a stimulation of specific CD8+ immunity in rhesus macaques (Nishimura et al., 2017). This vaccine-like effect (reviewed in Naranjo-Gomez & Pelegrin, 2019) could lead to a way to obtain a functional HIV cure in the future. In addition, in a shock and kill approach, combining BNABs with innate immunity stimulation could also represent a potential strategy to eliminate the viral reservoir, as suggested by Borducchi et al., 2018. Indeed, a viral reservoir is formed early in the infection, making the HIV-infection impossible to cure entirely using traditional cART.

Several BNABs are now being tested in humans for their ability to control HIV infection and to impact the viral reservoir (reviewed Walker & Burton, 2018).

Passive administration of BNABs thus may have a bright future in therapeutic approaches. However, their relatively short half-life would impose regular administration. To overcome that problem, 2 aminoacid mutations “LS” have been designed to engineer a longer half-live through enhanced binding to FcRN (Zalevsky et al., 2010). VRC01-LS administration in humans notably showed good safety, tolerability and a 4-fold greater half-life compared to WT VRC01 (Gaudinski MR et al., 2018).

Nonetheless, the cost of mAb production remains very high, which may be an important hurdle for future therapeutic usages.

C. Development of BNABs during natural infection

1. BNABs and HIV-1 co-evolution

The development of BNABs in an infected individual occurs in the context of a co-evolution of Ab responses and HIV-1. Such process has been described in several detailed longitudinal studies, characterizing BNAB lineages targeting the MPER (L. T. D. Williams et al., 2017) (Krebs et al., 2019), the CD4-bs (Bonsignori et al., 2016)(Gao et al., 2014) (H. X. Liao et al., 2013) (Umotoy et al., 2019), the V1/V2 apex (Doria-Rose et al., 2014) (Landais et al., 2017), the V3 HM Patch (Bonsignori et al., 2017) (MacLeod et al., 2016), the silent face (Zhou et al., 2018) or cooperation of lineages directed to different epitopes (Wibmer et al., 2013).

The question those studies aimed to answer is: how does the antigenic stimulation exerted by the autologous viral population result in the elicitation of a broad Ab neutralizing heterologous activity?

All these studies started with the isolation of BNABs, using the methods described above, from longitudinal samples of individuals selected for their broad spectrum neutralization. Corresponding Abs genes were amplified and cloned for Ab production. Abs were further characterized through neutralization assays against panels of heterologous viruses but also in a neutralization matrix of Abs and autologous viruses isolated at different time points. The studies resulted in the identification of common steps in the interplay between the processes of maturation of Ab lineage and viral evolution (Doria-Rose & Landais, 2019).

In most cases, the primary activation of a B cell with a potential to evolve into a BNAB lineage appears to result in a rapid expansion and diversification of the B lymphocytes population (Fig III-6). The majority of early B cells of the lineage bear Abs that neutralize autologous viruses and do not display broad heterologous neutralizing activity. The autologous neutralizing response exerts a selective pressure on the viral population. Viral escape mutants are thus selected in response to the arising neutralizing activity (Richman et al., 2003). At this stage, the B cell evolution presents a « contraction phase », likely due to the rapid viral escape that tends to prevent rapid selection of B cells.

Further, the B cell lineages eventually follow a multi-limb evolution as shown by phylogenetic trees built from the longitudinal sequencing by NGS of B cells of the lineage (example in donor PC76 (MacLeod et al., 2016)). The data shows that some limbs stop evolving, corresponding to « dead-ends » likely because of a failure of recognition of the emerging viral variants by these B cells (Bhiman et al., 2015). Other limbs however keep evolving, accumulating SMH and continuously adapting to the new viral variants. Importantly, this continued maturation is not necessarily associated with breadth acquisition (Sok et al., 2013) (Sok, Gils, et al., 2014), corresponding to « off-track » limbs (Fig III-6). In some limbs however, the successive viral and Ab selection steps lead the Ab maturation toward the ability to recognize not only autologous but also a large number of heterologous viruses, through the acquired

capacity to focus on a highly conserved epitope. These “on-track” limbs (Fig III-6) are those which maturation mechanisms interest us the most, notably for vaccine design. Sometimes, several limbs evolve in parallel in different ways to recognize the same epitope (example with donor PC76 (MacLeod et al., 2016)).

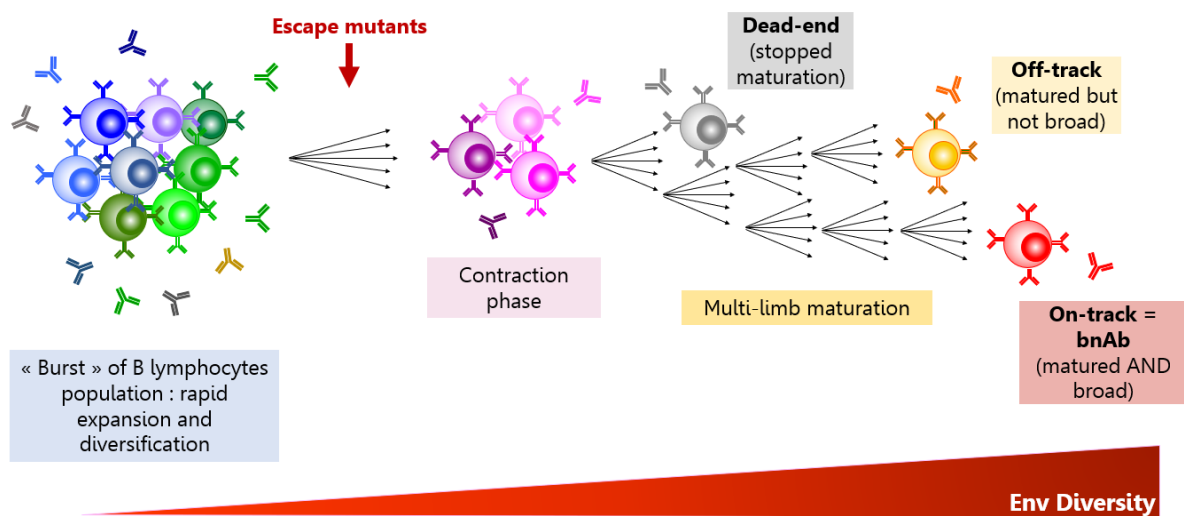


Figure III-6: BNAbs evolution. During infection, both the virus and the antibodies evolve rapidly through relatively random genetic variation. Adapted from Landais and Moore, *Retrovirology*, 2018.

Focusing on viral sequences reveals that an intense viral diversification occurs in parallel (Bhiman et al., 2015). Mutations at certain positions of Env cannot be selected as negatively impacting necessary functions required for the virus to replicate, the corresponding regions therefore remained conserved despite the pressure of selection of NABs. This might help with breadth development by constantly presenting the same conserved epitopes in the context of rapidly evolving highly diverse Env population, over a long period to the humoral system. Hence it appears that the NABs selection triggers a selection of viral variants and that those escape variants conversely select Abs capable of putting further pressure in a virtuous circle. The identification of the precise Env that stimulated BNAbs lineages throughout infection via such longitudinal studies is very useful for immunogen design as we will explain later (Moore et al., 2015). This picture of single BNAbs lineage-autologous virus interplay is certainly quite over-simplified as many NAb lineages, most of them not broad, are certainly developing in parallel, in a very dynamic manner. In this regard, the viral population may explore escape mutations from some NAb lineages that actually make them more sensitive to other circulating NABs, preventing the selection of these particular mutations and limiting the escape landscape. On the other hand, escape mutations from one neutralizing lineage may lead to the emergence of a novel epitope that will be able to elicit a new potentially neutralizing Ab lineage. This latter type of Ab cooperation has been described as “helper lineages” and was observed for CH235 (CD4bs) that selected escape viruses with mutations in the loop D region of gp120 Env that rendered loop D mutant viruses more sensitive to a second lineage CH103 (Gao et al., 2014), or DH270 (V3 HM Patch) that can neutralize variants that were previously selected by two non BNAbs lineages (Bonsignori et al., 2017).

Finally, the tremendous genetic Env diversity usually takes precedence over NAb maturation and in most Nab lineages described to date, total viral escape was demonstrated, i.e. the Nab can no longer neutralize the autologous viral population. This phenomenon is however not a show stopper for vaccine design aiming at eliciting BNABs, as HIV vaccine would preferentially lead to sterilizing immunity, as seen in the macaque model, and therefore HIV would be stopped before any chances of replicating and selecting escape mutations.

2. Important highlights from BNABs lineage the longitudinal studies

The observation and comparison of longitudinal studies of co-evolution Env-Ab have been essential to identify the chronological steps described above and remain crucial to the feeding of the lineage-based vaccine approaches we think will be successful at eliciting BNABs (see part IV).

- **Unmutated Common Ancestor (UCA) identification**

These studies have notably looked into the B cell at the origin of the BNAB lineages. Because of unequal availabilities of very early samples in the longitudinal cohort studied, it has often been difficult to figure the Unmutated Common Ancestor (UCA) Ab and some studies have only reported inferred sequences. The development of Next-Generation Sequencing (NGS) methods for exploring B-cell repertoires has helped in the Ab UCA identification (ex for HC-UCA of donor PC63 (Umotoy et al., 2019)). Identifying the Env that has first activated the lineage has been even harder. Of note, the eliciting Env is not necessarily the one of transmitted/founder virus but of a variant arising weeks/months later, or in some cases of a superinfecting virus (Doria-Rose et al., 2016). Importantly, it has been shown that the primary BCR/UCA binds the initial antigen with no significant or a very low affinity (ex Landais et al., 2017).

- **Role of SMHs**

BNAB lineage studies also have helped clarify the importance of SMHs. They showed that early Abs with low levels of SHM usually are relatively narrow in their neutralization spectrum, and that breadth is acquired through the accumulation of SHMs. SMHs lead to epitope focusing, improved shape complementarity, increased buried surface area at the interface with the antigen, conformational reorganization and paratope stabilization (Landais & Moore, 2018). However, lineage studies have also helped demonstrating that a high level of SHM is not always required for broad neutralization. The first isolated BNABs indeed had a high level of SMH, which was then thought to be a necessary feature for having a broad and potent neutralizing activity, raising concerns about the easiness of BNABs elicitation in a vaccine context. However, some potent BNABs recently isolated with relatively low level of SMH (as in the PCIN63 lineage (Umotoy et al., 2019)) show that relatively few mutations can be

enough to gain breadth. Studying the elicitation and evolution of BNAb lineages notably permits the identification of the SMH that are relevant or not to the BN activity and can inform about “shorter ways” to elicit BN activity. For instance, VRC01 present numerous SMH that are actually non relevant for its heterologous neutralizing activity (X. Wu et al., 2015) in comparison to donor PC63’s VRC01-like BNABs that display comparable neutralizing breadth and potency but are associated with a much smaller number of SMHs (Umotoy et al., 2019), thus highlighting a maturation guiding path that might be favored.

- **Number of lineages involved in serum BN**

Most of the time, the neutralizing activity of a serum is explained by one lineage: this is the case of donors PC63 for instance (Umotoy et al., 2019). However there can be several lineages implicated in the breadth, either targeting one common epitope, like the MPER (Krebs et al., 2019), the CD4-bs (Gao et al., 2014) or the HM patch (MacLeod et al., 2016) or even displaying distinct specificities : V1/V2 apex and CD4-bs (Bonsignori et al., 2012 and Wibmer et al., 2013).

4. Potential constraints to the development of BNABs

If neutralizing activity is observed in HIV-1 infected individuals, breadth however is rare. Why do BN responses develop in 10 to 20% of infected individuals only? Here follows a non exhaustive overview of the issues that may thwart BNABs development, whether coming from the humoral immune system or from HIV-1.

- **Low number of Env trimers at the surface of virions**

The number of spikes at the surface of HIV is limited, estimated as being only 6 to 20 Env (average ~14), as mentioned previously (P. Zhu et al., 2003) (P. Zhu et al., 2006). By comparison, the virus responsible for the COVID-19 pandemic, SARS-CoV-2, that has an equivalent size (100 versus 120 nm roughly for HIV), bears around 24 +/- 9 spikes (Ke et al., 2020). It has been suggested that HIV-1 may have evolved toward this low number of Env at the cost of a better infectivity in order to avoid bivalent binding by Abs (J. S. Klein & Bjorkman, 2010). The distance between the Env trimers may prevent the cross-linking necessary for some B lymphocytes to get activated. However, it allows all angles of approaches for the Abs.

- **High variability of Env**

The extreme sequence variability of Env mentioned earlier is obviously an hindrance to BNAb elicitation, especially because its high tolerance to sequences changes without loss of function facilitates rapid viral escape (Klasse et al., 2020).

- **Conformational flexibility of Env, hidden epitopes**

The conformation variability adds to the one of the sequence and consequently increases Ab recognition difficulties by constant changes in the Ab-target exposure. Env is indeed extremely labile and displays diverse degrees of openness, as explained previously with the notion of tiers. For the most compact closed tier 3 conformation, some Abs can only recognize the open conformation (example: b12, F105). Nonetheless, some Abs conversely favor the close conformation (examples: VRC01, PG16, PGT128, PGT145, and 2G12) (Burton & Hangartner, 2016).

Furthermore, the conserved epitopes targeted by BNABs often have poorly accessible locations on the quaternary structure: for instance the MPER is partially buried in the membrane and the V2 loop shields both the CD4bs (implicated in the CD4-receptor binding) and the V3 loop (implicated in the co-receptor binding) (Burton & Hangartner, 2016).

In the end, HIV best hiding trick might be the glycan shield. The dense array of glycans that cover all Env prevent the exposure of most of the conserved vulnerable sites (Klasse et al., 2020). For instance, N-glycans at position 276 and 197 were shown to restrict Ab access to the CD4bs (Jardine et al., 2013 and Crooks et al., 2015). Besides, the addition or loss of glycosylations sites is another way of HIV to disturb antibody recognition (Wei X et al., 2003) (Garces et al., Cell, 2014).

- **Potential scarcity of B lymphocytes susceptible to mature into BNAB lineages**

It is very likely that the naïve B lymphocytes susceptible to mature toward BNABs production are rare in the naïve repertoire and/or difficult to activate and/or guide toward “on-track” maturation, although this may be very dependent on the BNAB class. A comforting study showed that there were no significant differences in germline IGHV repertoires between individuals who do and do not develop BNABs (Scheepers et al., 2015), thus the naïve repertoire might not be a limit to BNAB elicitation. In parallel, similar maturation pathways were observed among individuals, for instance concerning BNABs lineages targeting the apex (Gorman et al., 2016) or the CD4bs (Zhou et al., 2013), whether it would start from identical VH usage or not, hence strengthening the probability of feasibility of eliciting such BNAB classes in naïve individuals.

- **Immunodominance of non-BNAb epitopes**

As mentioned above, some epitopes, like the V3 loop, are immunodominant, meaning the elicitation of Abs targeting them will be favored in spite of other Abs that may eventually have a better potential to evolve to BNABs. Moreover, several maturing lineages that target the same epitope might also enter in competition. One with a better affinity might then progress further in its maturation in contrast to another that could have initial low binding but higher probability to gain breadth and potency (Xiao et al., 2009). This competition can also occur between Abs of a same lineage.

- **Potential autoreactivity and polyreactivity**

The particular autoreactive and/or polyreactive features of MPER-targeting BNABs have been mentioned previously. Even if these properties are more frequent with anti-MPER BNABs, they are not exclusive to this specificity. Protein microarrays experiments assessing the binding to >9,400 human proteins showed that BNABs are significantly more poly- and autoreactive than NNABs (Liu et al., 2015). Reasons might be that BNABs sometime need to accommodate interactions with self components such as MPER-BNABs that have to deal with the lipid membrane, or mimic host components such as anti-CD4bs-BNABs which VH imitate the Ig-like domain of CD4. Alternatively, preponderant auto- and poly-reactivity may also reflect the reduction of peripheral immune tolerance often seen in HIV-infected individuals (Vega & Espinoza, 2018).

The elaboration of a BNAB-eliciting vaccine therefore may have, for certain specificities, to ensure that an healthy immune systems could tolerate some auto- or poly-reactivity and not halt the maturation of Abs with such properties but also prevent the emergence of a potential autoimmune disease that might be associated with such elicitation.

- **High level of SMHs and indels**

BNABs often have high mutation rate, from 10% to 25%, due to repetitive cycles of SMH in the GC throughout years of infection. Number of Abs that reach such mutation rate is very low, explaining why, if this is indeed a necessary feature to BNABs, they would be so rare. This is an important issue in terms of vaccination, because even numerous immunizations might fail to lead such SMH levels. However, as discussed before, the level of SHM required for breadth may have been overestimated. A second issue is that some SMH necessary for BN activity may be pretty improbable, because in “cold spots” for AID (Bonsignori et al., 2017).

Similarly, indels (insertions and deletions) are more frequently observed among BNABs than in memory B lymphocytes of healthy donors. For some BNABs like VRC01 and CH31 (CD4bs), it was shown

that indels are strictly necessary to their acquisition of BN activity, which may be an important hurdle for vaccine design.

- **High HCDR3 length**

Human CDRH3 are usually 10 to 14 amino acid long (T. Te Wu et al., 1993). BNAbs often have particularly long CDRH3 (>20 residues), as Abs PG9, PG16, 2F5, PGT121, PGT151. Notably, VRC26 even reaches a length of 38 amino acids. This particular feature can allow Abs to sneak through the glycan shield in order to get to the hidden Env residues as this is the case for PGT135 (L. Kong et al., 2013). *A contrario*, BNAbs CDRL3 can be shorter than usual. In a naive B repertoire, λ -CDRL3 lengths range between 8 and 13 amino acids while κ -CDRL3 range between 8 and 11 (Sok & Burton, 2018). However, the VRC01 BNAbs-class is characterized by a short κ -CDRL3 of 5 residues, allowing an adaptation to the N-glycan at position 276 (Zhou et al., 2013).

CDR lengths come from the VDJ recombination rather than indels (Briney et al., 2012), thus illustrating a restriction in the genes usage and likely explaining BNABs rarity also.

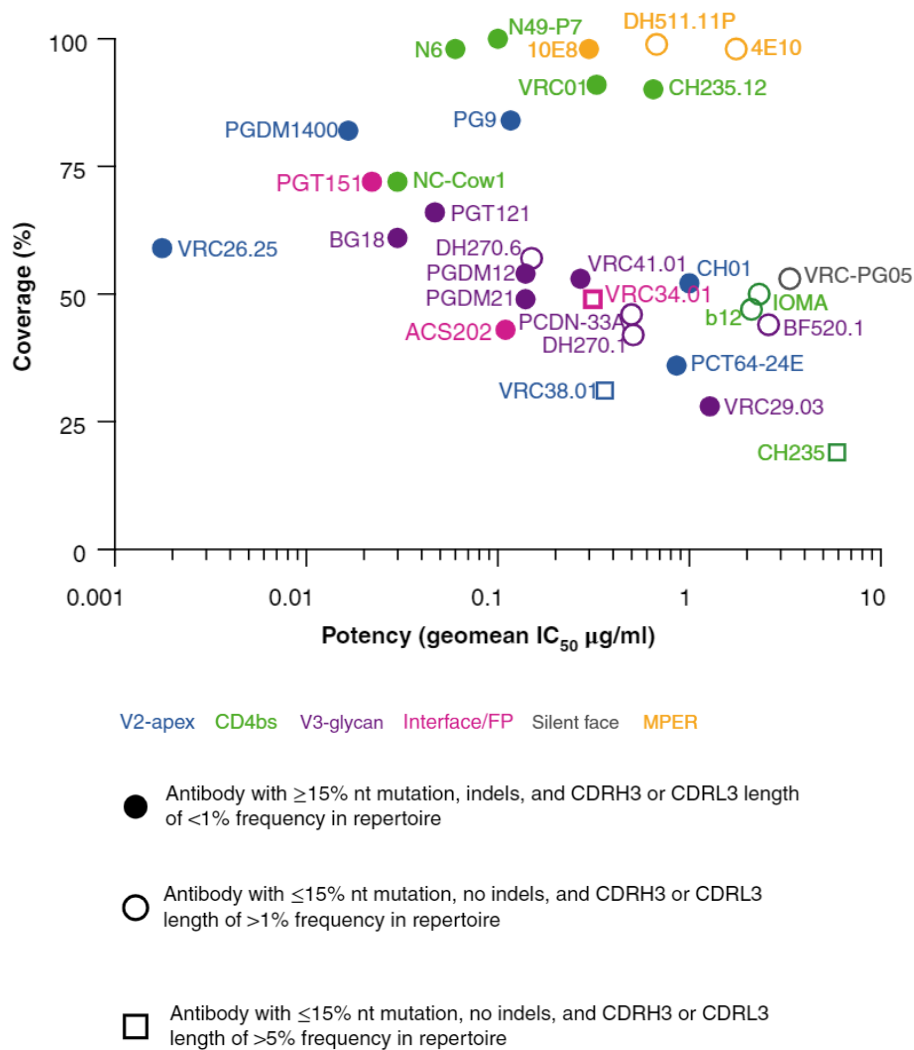


Figure Fig III-7: Neutralization coverage of large panels of global isolates by BNAbs as a function of neutralization potency. Neutralization was measured through the use of pseudoviruses in a TZMbl assay and is presented as potency (geometric mean (geomean) IC₅₀, in µg/ml) of second-generation BNAbs (bold) and first-generation BNAbs (less intense), grouped by epitope targeted (colors in bottom). Symbols (bottom) indicate 'elicibility'. From Sok and Burton, 2018.

All-in-all the SMH rates, the presence of indels and the CDRH3 or CDRL3 frequency in the repertoire together can help defining the elicibility of an Ab (Sok & Burton, 2018) (Fig III-7) :

- the least favorably elicited would be those with ≥15% overall nucleotide mutation in the HC and IC combined, indels or CDR3 length frequencies (CDRH3 or CDRL3) <1% that of the Ab repertoire
- the realistic Abs are those with ≤15% overall nucleotide mutation in the HC and IC combined, no indels and CDR3 length frequencies >1% that of the Ab repertoire
- the very realistic Abs are those with ≤15% overall nucleotide mutation in the HC and IC combined, no indels and CDR3 length frequencies >5% that of the Ab repertoire (only VRC34.01, VRC38.01 and CH235 are in this category, Fig III-7).

IV. Strategies for eliciting BNABs

Facing those numerous barriers that may preclude elicitation of BNABs, what are the vaccine strategy(ies) that could achieve broad neutralization in HIV naïve individuals?

Vaccine approaches can be roughly classified into 4 main groups (reviewed in [Callaway, 2020](#)):

- the virus vaccines, based on the virus itself, in either attenuated or inactivated form, that still bear the viral surface proteins,
- the nucleic acid vaccines, RNA or DNA-based, aiming at encoding *in situ* the antigen(s) against which an immune response is sought (notably recently pushed at the forefront with Moderna and Pfizer SARS-CoV2 vaccines),
- the viral vector vaccines, similar to DNA-vaccines with the difference that the protein genes of the pathogen to vaccine against are carried by non pathogenic viruses, replicative or not (as the AstraZeneca SARS-CoV2 vaccine),
- the protein-based vaccines, corresponding to the direct administration of full or fragments of viral proteins, either directly in recombinant soluble form, or exposed on Virus-Like Particles (VLP).

Numerous HIV-1 vaccines strategies were attempted using all the approaches cited above during the past 4 decades. However, we will only further focus on the protein-based vaccines here, which are notably effective at eliciting Abs, and have been largely studied in the HIV vaccine field for BNAB elicitation.

A. What immunogen(s) for an HIV protein-based vaccine?

There is notably a very important notion to keep in mind when it comes to immunization: a molecule that shows a proper antigenicity, i.e. that is able to be recognized by given BNABs, is not necessarily a good immunogen, meaning it might not be able to induce the corresponding Abs. While all immunogenic substances are antigenic, not all antigenic substances are immunogenic ([Ilinskaya & Dobrovol'skaia, 2016](#)).

1. gp120/gp160

Early protein-based HIV vaccines were designed based on recombinant monomeric gp120 or gp160 (the precursor uncleaved version of Env). It was quickly shown that purified or recombinant Env were safe and immunogenic, and had the ability to induce NAbs in non-human primates (NHP) (Arthur et al., 1987), opening the door to the first clinical trials. VaxSyn[®], based on gp160 expressed in a baculovirus system (Dolin R et al., 1991), launched a ballet of more than 35 phase I trials evaluating diverse formulations (Belshe et al., 1993)(Graham et al., 1996). Globally, the trials showed proper safety and antigenicity of the vaccines, which were able to elicit binding and sometimes NAbs.

From 1998 to 2003, the VaxGen company led the two first efficacy trials based on bivalent gp120 preparations:

- VAX003, which evaluated AIDSVAX[®] B/B in 5417 volunteers (mostly men-who-have-sex-with-men) in North America (Flynn NM et al., 2005) (NCT00006327)
- VAX004, which evaluated AIDSVAX[®] B/E in 2545 volunteer drug users in Thailand (Pitisuttithum et al., 2006) (NCT00002441)

Both trials however resulted in no protection against HIV, strongly eroding the “B cell strategy” enthusiasm.

If AIDSVAX[®] B/E gave disappointing results in VAX004 trial, its administration in combination with ALVAC[®], a canarypox vector, with aluminium as adjuvant, provided unexpectedly good results. The RV144 trial tested a prime-boost combination of both vaccines among 16 402 adults in Thailand from 2003 to 2009 (NCT00223080). All participants were recruited as HIV-1 naive and tested for seropositivity every 6 months for 3 years. The trial finally demonstrated 60% protective efficacy against HIV infection at 12 months and 31.2% at 3.5 years although the analysis of the results has been controverted (Desrosiers, 2017). The study of the immune correlates associated with protection indicated that anti-V1/V2 Abs may have played a crucial role (Haynes et al., 2012), not for their neutralizing capacity though but possibly through ADCC mediation (Montefiori et al., 2012) (Wren & Kent, 2011). Albeit modest and controversial, RV144 results were promising enough to push another similar clinical trial in South Africa: HTVN 702. This trial tested a prime-boost administration of the same canarypox vector ALVAC[®] and a subtype C recombinant gp120 (corresponding to the local subtype), the aluminium adjuvant being replaced by MF59, and an additional boost scheduled at 12 months (NCT02968849). HTVN 702 was however stopped in february 2020, after more than 5400 inclusions, because the intermediate analysis showed that the vaccine combination was ineffective in preventing HIV transmission (UNAIDS) (Cohen J, 2020). Nonetheless the results motivated a large number of studies of the T effector responses, that were suggested to be sufficient to protect against HIV-1 infection. The latter hypothesis may however be ruled out soon because of a recent study showing that effector function does not contribute to protection from virus challenge by PGT121 in NHP (Hangartner Science Translational Medicine, 2021).

2. Small peptides

To prevent the elicitation of non-desired Abs against highly immunogenic but non-neutralizing Env epitopes, it was suggested to administrate the epitope to be targeted only, in a peptide format.

Great optimism was notably placed in the V3 loop of gp120, rapidly appointed as the “Principal Neutralizing Determinant” (Profy et al., 1990). The abolishment of HIV infectivity through the co-inoculation of HIV with an anti-V3 mAb in chimpanzees (Emini et al., 1990) as well as the induction of NAbs thanks to the immunization of guinea pigs with V3 peptides (Javaherian et al., 1990) initiated few clinical trials (Gorse et al., 1996) (Toledo et al., 2001). However, if V3 had a great antigenicity, the mAbs it could elicit were essentially non-neutralizing or very restricted in breadth. Indeed it was shown afterward that the V3 is the principal neutralizing determinant for laboratory T-cell-line-adapted HIV-1 strains but not for primary viruses freshly isolated from infected individuals, highlighting the implication of other Abs against other epitopes on V3 for the neutralization of those refractory strains (Spence et al., 1998). The great antigenicity notably concerned the tip of the V3 loop. Only one V3 apex Ab was considered as a potential target for vaccine, 447-52D (Stanfield et al., 2004), though because of the low accessibility of this area on most V3 of primary isolates, eliciting 447-52D-like Ab neutralizing responses will likely be difficult (Chakraborty et al., 2006).

In parallel, the isolation of the first-generation BNABs allowed the better identification of Env supersites: the CD4-bs (Ab b12), the MPER (Abs 4E10 and 2F5) and a glycan epitope on the base of V3 (Ab 2G12) (Burton & Hangartner, 2016). The linear epitopes (MPER and V3) were tested for small peptides-based strategies.

The MPER of gp41 subunit was indeed an interesting target, because of its high conservation among HIV-1 strains. Nonetheless, although the MPER BNABs bind to very limited linear sequence of gp41 (ELDKWA) none of the numerous strategies attempted to induce MPER NABs was convincingly successful at eliciting tier 1 and 2 neutralizing activity (reviewed in Caillat et al., 2020). Indeed the peptides used did elicit Abs but none were neutralizing. Reasons for the failure may be: i) the polyreactive character of such BNAB exclude them to go through maturation process, ii) the epitope is included in a lipid environment, creating high structural constraints for Ab access.

Concerning the V3-base supersite, despite extensive immunizations attempted, none resulted in BNABs elicitation (Kwong & Mascola, 2018). Glycans components of the epitope might be the reason of the difficulty.

With the second wave of BNABs isolation, new epitopes were identified like the FP. The latter has thus been used in different approaches to elicit BNABs. Successive immunization of FP coupled to carrier proteins and BG505 SOSIP (detailed below) displayed the induction of heterologous tier-2 Nabs in mice and, to a lesser extent, in guinea pigs and macaques (Xu et al., 2018). Hence, this peptide approach is likely the most promising so far.

Thanks to the second wave of BNAB isolation still, other epitopes, or rather BNABs modes of

recognition, were better characterized, like the V3-base. Thus recently, a synthetic V3-glycopeptide (“Man9-V3”) encompassing two key features of the V3 region recognized by V3-glycan BNABs – the conserved GDIR motif and the N332 glycan – was designed. The BNAB DH270.6 (Table III-3) was shown to bind this V3 glycopeptide in an intact HIV-1 Env trimer binding manner, maybe opening a door to use it as a vaccine immunogen (Fera et al., 2018).

Nevertheless, soluble peptide approaches do not consider the conformation of the epitope they aim to present.

3. Scaffolds

A first solution to overcome this problem of conformation might be the scaffolds: these are proteins designed to hold peptide stretches in a proper spatial arrangement (Burton, 2010)(Azoitei et al., 2012).

As an example, V3-cholera toxin scaffolds were designed to display V3 epitopes shared by the majority of global HIV-1 isolates. These immunogens were then used as boosts in rabbits immunizations, following a priming with codon-optimized clade C gp120 DNA (S. Zolla-Pazner et al., 2011). This resulted in a successful elicitation of NABs, toward tier 1 and 2 pseudoviruses with notable level of breadth.

Similar constructs were done with the MPER, with diverse types of scaffolds tested: a membrane-anchored fusion intermediate conformation of gp41 (Lai et al., 2014), the E2 protein of *Geobacillus stearothermophilus* that self-assembles into 60-mer particles, thus exposing 60 copies of the fused target (Krebs et al., 2014) or the recombinant HIV-1 transmembrane domain (Oakes et al., 2018) for instance. The immunization of guinea pigs with the first construct did result in Ab elicitation, although with modest breadth and potency. The immunization of rabbits with the second construct in conjunction with DNA encoding full-length gp160 resulted in NABs elicitation, though the latter were mainly directed against the immunodominant V3 loop. Consequently, V2 and V3 coding sequences were removed from the gp160 DNA for the second vaccination, and all rabbits still had Nabs, likely focused on MPER, and more broad (albeit breadth was moderate). The third construct has not been tested in animal immunization but the combination of *in vitro* assays and molecular dynamics simulations could allow the identification of the atomistic structures that positively impacts MPER epitope exposure. A more recent work succeeded to reconstitute a full-length Env clone into a nanodisc complexed with a MPER, exposing the quaternary epitope of 10E8, for which the introduction of stabilizing mutations might allow its use in future immunization studies (Rantalainen et al., 2020).

Scaffolds were also tested for V1/V2 loops forming the Env apex. One study evaluated the ability to induce Abs in rabbits of diverse constructs of 6 V1V2 sequences (from 3 different clades) crafted on 9 different scaffolds (the core variant of the B1 domain of streptococcal protein G, the typhoid toxin subunit B, the signaling protein GlnK1 of *Methanococcus jannaschii*, the chromo domain of the human transcription factor MRG15...) administered with gp120 DNA in various immunization regimens (Susan

Zolla-Pazner et al., 2016). Results showed that the animal polyclonal Ab responses were focused on the V1V2 apex, broad in binding activity to gp120s and V1V2 regions of strains from different clades, including V2 Abs with specificities similar to those found in HIV-infected individuals, and remained detectable >1 year after the last boosting dose. Also the sera from rabbits that had received V1V2-scaffold immunogens displayed Ab-dependent cellular phagocytosis whereas sera from rabbits receiving only gp120 did not. No neutralizing activity was observed. A similar study followed, in which glycosylated V1V2 domains were crafted onto five different multimeric scaffold proteins (identical to those used for the rabbit immunization right above) and tested for their immunogenicity in macaques, still administered with gp120 DNA (Hessell et al., 2019). The animals developed high titers of plasma and mucosal Abs directed against structurally distinct V1V2 epitopes, however they were moderately neutralizing but showed ADCC activity and phagocytosis, even 1 or 2 years after the immunizations occurred.

The scaffold constructions showed great ability to induce Ab responses oriented toward particular epitopes, though the neutralizing activities of these Abs have so far only been shown to be moderate. Scaffolds-Env thus remain interesting immunogen candidates in the HIV-1 vaccine course for their ability to present epitope in a desired conformation but likely require further optimization.

4. Trimeric Env

Otherwise full stabilized native Env recombinant trimer would present the targeted epitopes in the same conformation than those found on a real viral particle and not expose non-desired epitopes that are hidden in the real Env context (as opposed to monomeric gp120). Tremendous efforts were hence provided to produce recombinant Env trimers as close as possible to the native one found at the surface of HIV-1 (Sanders & Moore, 2017).

- **SOSIP trimers**

The main platform to date is called SOSIP : “SOS” refers to the disulfide bond that stabilizes gp41 and gp120 subunits and “IP” to the mutation of the Isoleucine 559 to Proline that maintains the gp41ecto components in a pre-fusion form for trimerization improvement (Beddows et al., 2005). This stabilization in a pre-fusion conformation reduces the exposure of non-neutralizing epitopes, as in the native Env trimer (Escolano et al., 2017). The best characterized SOSIP is the BG505_SOSIP, a subtype A tier 2 transmitted/founder virus isolated from a 6-weeks old HIV-infected infant who developed BNabs within 2 years post-infection (Sanders et al., 2013) (Do Kwon et al., 2015) (Fig VI-1). The stability of SOSIP trimers continued to be optimized in the past years (Rutten et al., 2018). Importantly, if recombinant SOSIPs show remarkable structural similarity to their surface Env counterparts, subtle differences in glycosylation can be observed, impacting antigenicity (Torrents de la Peña et al., 2019).

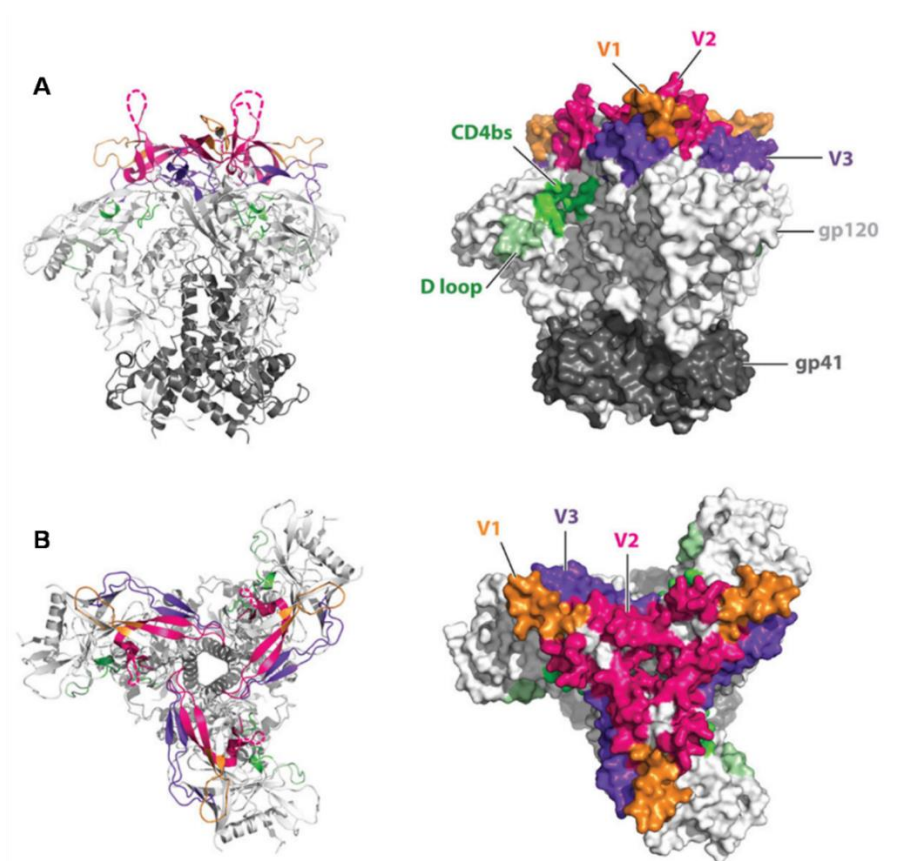


Figure VI-1: Crystal structure of the HIV strain BG505 expressed as SOSIP.664 near-native trimers (Kwon et al, *Nat Struct Mol Biol*, 2015). The location of some of the most important Env trimer domains is indicated as viewed (a) from the side or (b) from the top. The gp120 subunits form the blades of a propeller-like structure, whereas the gp41 subunit forms a central stalk and a membrane-proximal, pedestal-like structure. As predicted from other experimental data, hypervariable loops V1 through V3 are located at the apex of the trimer, with hypervariable loop V3 being partially buried under hypervariable loops V1 and V2. These loops are also in proximity to the CD4-bs that is recessed left in the cleft formed by two propeller blades. Loops that did not resolve in the structure are indicated by a dashed line. From Burton and Hangartner, *Annu Rev Immunol*, 2016.

SOSIP trimers have now been used in a number of immunization studies. In mice, single immunization by BG505 SOSIP.664 elicited Env-binding Abs, high-quality T follicular helper (Tfh) cell and germinal center (GC) responses but no BG505.T332N NAb (Hu et al., 2015). To be noted, the Thr naturally found in BG505 Env sequence at position 332 is replaced by an Asn in the SOSIP construct to recreate the N-glycosylation site at this position, known to be crucial for the binding of the majority of HM-Patch-targeting BNabs. In guinea pigs and rabbits however, Tier-2 autologous virus NAb could be elicited (Feng et al., 2016) (Sanders et al., 2015) (Voss et al., 2017). In cows, a single BG505 SOSIP.664 immunization resulted in rapid elicitation of broad and potent serum antibody responses in all 4 animals (Sok et al., 2017). However, cow Abs have longer CDRH3 than humans, and that specific feature explains why eliciting BNabs with long HCDR3s is easier in this model (the virus did not evolve to escape cow Abs). In macaques, the most appropriate pre-clinical model for HIV vaccine studies, 3 successive BG505 SOSIP.664 immunizations were tested, and reproducible and strong tier 2 NAb responses were observed (Pauthner et al., 2017). Following those results, a Phase I clinical trial named W001 started in the end of 2018 with the aim of evaluating the tolerability and immunogenicity of a

single administration of BG505 SOSIP.664 in 60 healthy HIV naive participants (trial identification: NCT03699241). Results are expected this year 2021.

- **NFL trimers**

Another platform to make native-like Env trimers is the Native Flexible Linker (NFL), in which trimers are stabilized by a flexible glycine/serine linker (G4S) that replace the gp120/gp41 cleavage site, making cleavage-independent Env mimics called NFL trimers (Sharma et al., 2015) (Fig IV-2). The NFL construct has been further improved to ensure a proper stabilization for B or C subtype trimers, by the addition of an interprotomer disulfide bond I201C-A433C (CC) and several Trimer-Derived (TD) residues mutations (Guenaga et al., 2016). ELISAs showed that NFL-CC-TD trimers were efficiently recognized by BNABs while not by NNABs.

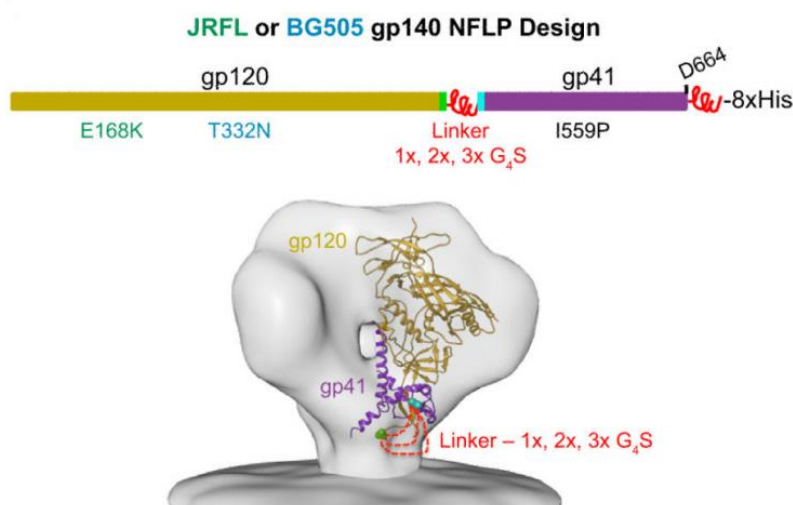


Figure IV-2: NFL Trimer Design and Linker Length Screening. Top, schematic representing the NFL trimer design. Gp140 refer to the gp160 precursor removed from the C-term and the transmembrane domain of gp41, thereby to the extracellular part of Env only. The NFL glycoprotein trimers contain a N-terminal CD5 leader sequence followed by gp120 covalently linked to gp41 by one, two, or three copies of G4S flexible peptide linkers. The C terminus of gp41 has one copy of the G4S linker followed by a His8 tag. Both proteins were designed with and without the I559P substitution. JRFL gp140 NFLs possess an additional substitution of E168K for PG9/PG16 recognition, while BG505 gp140 NFLs have a T332N mutation for the N332 glycan supersite. Bottom, model of gp120 (in golden rod with C terminus in green) linked to gp41 (in purple with N terminus in teal) by different linker lengths (red dashed lines) in the context of the viral spike; one protomer is shown for clarity. From Sharma et al, Cell Reports, 2015.

The NFL trimers were also evaluated in animal models. The immunization of guinea pigs with cross-linked BG505 and NFL trimers also shown autologous tier-2 neutralization responses (Feng et al., 2016). To be noted, cross-linking, here done with glutaraldehyde, increases overall thermostability and maintenance of well-ordered trimer integrity. Interestingly, CD4-bs BNABs could be elicited in rabbits by prime:boosting approaches using heterologous NFL trimers coupled to liposome (BG505, JRFL and 16055 NFL) (Dubrovskaya et al., 2019). BG505 NFL appears to elicit similar responses in macaques as BG505 SOSIP, although slower (Pauthner et al., 2017). No immunization with NFL trimers has been performed in human yet.

- **UFO trimers**

To avoid recombinant trimer fusion intermediate states that happen sometimes with the NFLs, the linkers were lengthened and the bend connecting the HR1 helix to the fusion peptide redesigned in order to stabilize the gp41 (L. Kong et al., 2016). These recombinant Env spike are named the uncleaved prefusion-optimized (UFO) trimers.

Immunization with UFO trimers also induced autologous tier-2 neutralization responses in rabbits, although these tended to decrease after the third immunization (Aldon et al., 2018). The immunization of rabbits with UFOs having a BG505-gp41 ectodomain and exposed on ferritin nanoparticles also elicited autologous NAb responses, as well as heterologous ones, though very limited (He et al., 2018). Some UFOs are currently used in an experimental phase I clinical trial: prime/boost combinations of UFO trimers are compared with SOSIP constructs (BG505 or mosaic gp140) in regard to B and T cells responses elicitation and breadth of elicited Nabs (NCT03816137). Results should also be published anytime soon.

5. Minimizing off-target epitopes on trimers

The recombinant stabilized Env trimers appear for the most to expose BNAb epitopes in the optimal native-like conformation, however they still expose relatively highly immunogenic, isolate-specific epitopes, that may interfere in the BNAb elicitation process (Sanders et al., 2015). Among these epitopes that elicit non-broad Abs can be cited for instance the V3 loop, which is largely immunodominant. Strategies to minimize the access of those off-target epitopes were developed in an attempt to decrease elicitation of non-desired NNAb and guide the immunogenicity more specifically toward BNAb elicitation (McGuire et al., 2014).

- **Trimer stabilization**

A first method to stabilize the recombinant Env trimers and reduce the binding of NNAb can be the chemical crosslinking, as successfully done in Schiffner et al., 2016.

Another way is to stabilize with Abs. A study immunized guinea pigs with the BG505 SOSIP stabilized by the Fab of PGT145, a BNAb directed at the V1/V2 apex region, which binding hinders the V3 loop exposure (Cheng et al., 2016). Decreased V3 reactivity was demonstrated, however it did not impact antigenicity, leading to comparable autologous and heterologous neutralization as following immunization in absence of PGT145-stabilization. Similar results were observed in rabbits (De Taeye et al., 2015) and macaques (Pauthner et al., 2017) immunized with SOSIP trimers stabilized by specific residue mutations.

- **Disruption of immunodominant epitopes**

Even with further alteration of the exposure of the V3 non neutralizing epitope (through glycans insertion (Ringe et al., 2017) or hydrophobic mutation introduction (De Taeye et al., 2018) (Kulp et al., 2017)) the heterologous tier-2 responses were not improved. This result was suggested to be due to the exposure of other immunodominant NNABs epitopes (Pauthner et al., 2017), such as the base of the trimer, usually embedded in the membrane in the context of the full virion. Guided by computational and structure-guided design, both V3 and base epitopes were modified, respectively by glycan masking and single-residue mutagenesis-scanning affecting the binding energies (Kulp et al., 2017). Those modified trimers successfully showed an altered access to NNABs *in vitro*, though the impact of modifications remain to be evaluated in immunization studies.

- **Utilizing glycan holes**

As said previously, some conserved BNAbs epitopes are hidden by N-glycans ; their access would thus only be allowed by the formation of local holes in the glycan shield (McCoy et al., 2016).

For instance, one BNAbs epitope is constituted by residue 241 that occupies a hole in the glycan defenses of the BG505 isolate. The immunogenic nature of such breaches in the glycan shield should be further exploited in HIV vaccine strategies aiming at eliciting BNABs (McCoy et al., 2016).

The study of VRC01 and PGT121 elicitation, directed at the CD4-bs and the HM Patch respectively, have also highlighted the role of such glycan hole for their elicitation. Indeed VRC01 have probably matured because of the stimulation of an Env with a rare glycosylation pattern missing the glycans N276 and N460 (McGuire et al., 2013) while PGT121-like BNABs might have first recognized Env lacking N137 and then were selected to accommodate with (Garces et al., 2015). To be noted, glycan holes are often immunodominant. So the idea is to use trimers with glycans specifically removed as a starting point of immunization and then restore them gradually (Zhou et al., 2017).

In vivo application of the strategy shows that creating holes in the glycan shield can indeed redirect the Nab response to newly unmasked epitopes (Ringe et al., 2019). In this instance, the CD4-bs-BNABs elicited in rabbits by heterologous NFL trimers - liposome prime/boosting were obtained thanks to N-glycans removal around the CD4-bs and restoration in the boost doses (Dubrovskaya et al., 2019). Moreover, the immunization of rabbits with Env trimers presenting a glycan hole at the V2 apex gave promising potent autologous tier 2 neutralizing responses targeting basic residues in the core epitope for V2-apex BNABs, and presenting high potential of affinity maturation toward broad HIV neutralization (Voss et al., 2017).

B. Lineage-based vaccine approaches

A vaccine strategy that would re-create the co-evolution that happened between the autologous viruses and the humoral response in elite neutralizers might be efficient at eliciting BNABs. The principle would be to reproduce the BNAB development history from the activation of the right initial B-cell and its driving towards affinity maturation thanks to the sequentially evolved Env variants (Gruell & Klein, 2014) (Fig IV-3).

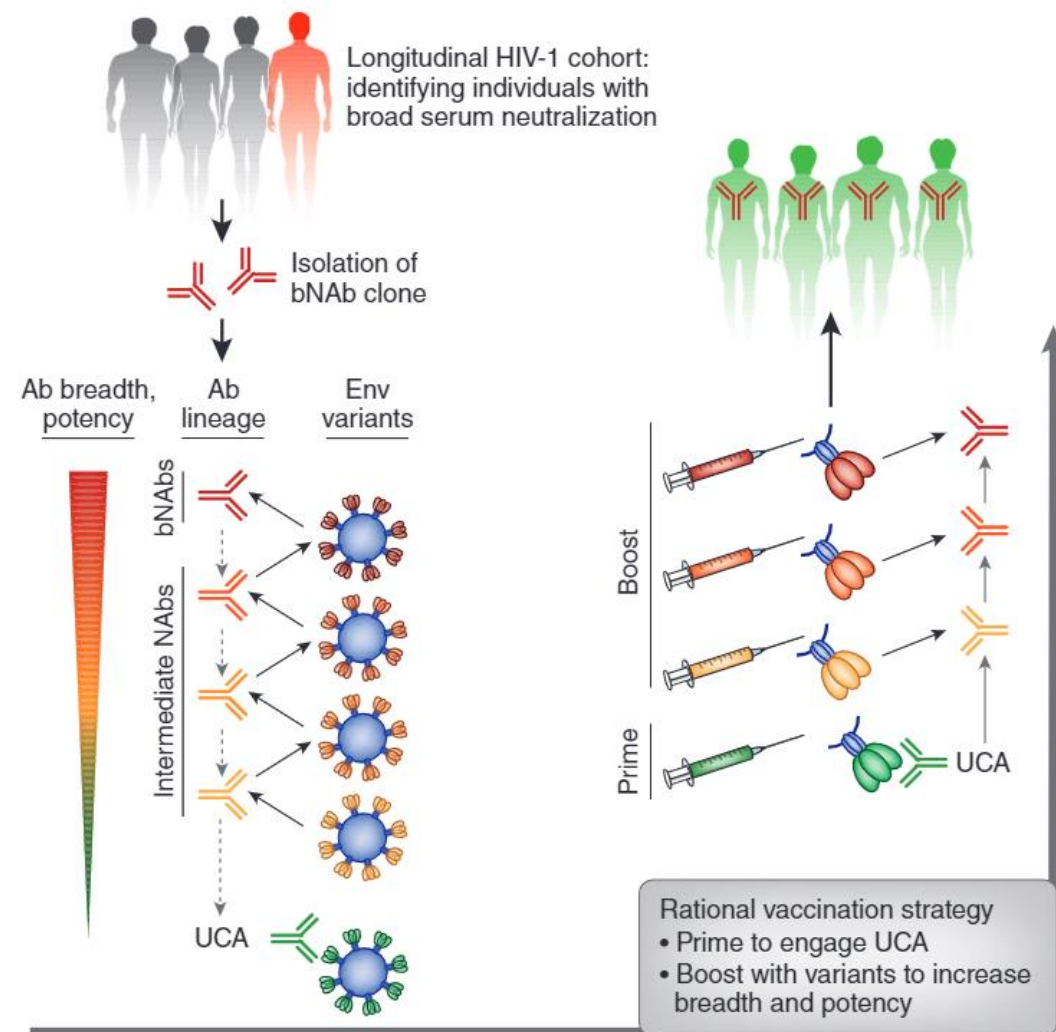


Figure IV-3: Deciphering BNAB development in an HIV-1-infected subject to guide vaccine strategies. Individuals with acute HIV-1 infection are identified and followed up for the development of broad neutralizing serum activity. Ab cloning, next-generation sequencing and computational analysis of longitudinal samples can be used to reconstruct the development of a BNAB clone, identifying intermediate NABs and the unmutated common ancestor (UCA). Information on the corresponding evolution of HIV-1 Env could then be applied in reverse to vaccination strategies: HIV-1 Env variants are used to engage the UCA, initiating B cell maturation, and to steer antibody development toward the desired bNAB. Gruell & Klein, 2014.

1. Targeting the germline

The crucial starting step of triggering a BNAbs B cell lineage is the activation of the precise potentially rare naive B-cell(s) at the origin of the BNAbs lineage(s) of interest. As an example, naive B cell frequency for VRC01-class BNAbs is estimated at 1 per 400 000 B cells (Abbott et al., 2018). As mentioned previously, identifying the precursors is usually a first uneasy challenge. Activating them is a second challenge, likely because of their weak binding with the viral variant that initiated their activation in a first place. Diverse antigens were designed and tested for their ability to specifically bind identified or inferred germline B cells specific of various B cell lineages (Van Schooten & Van Gils, 2018).

The VRC01-class BNAbs has been the most exploited germline-targeting vaccine strategies. The 426c gp140 TM4DV1-3 protein (McGuire et al., 2013) and the engineered outer domain germline-targeting (eOD-GT6) gp120 multimerized on nanoparticles (J. Jardine et al., 2013) were both designed based on mgp120 with deletions of glycan sites and/or variable loops. *In vitro*, they successfully specifically bind germline VRC01-class B cells, recognizable by their VH1-2 gene usage, short CDRL1 and 3 and particular mutations favoring the binding to gp120. The last version eOD-GT8 60mer (60 subunit self-assembling nanoparticle (J. G. Jardine et al., 2015) was tested *in vivo* in transgenic mice with human Ig loci. Twenty-nine% immunized mice produced a VRC01-class memory response, while the average frequency of VRC01-class precursor was estimated of one per mouse. Thus priming appears to success even when only very few precursor cells are present (Sok, Briney, et al., 2016). Knowing that the frequency in human of VRC01-class naive B cell precursors is calculated as 1 in 400,000 naive B cells (Jardine JG et al., 2016), those results demonstrate the feasibility of using germline targeting immunogens to prime specific and rare target B cells. However, due to the lack of the glycan N276 of eOD-GT8, the presentation of the epitope only allowed the elicitation of a small subset of potential VRC01-class antibody progenitors (Escolano et al., 2017).

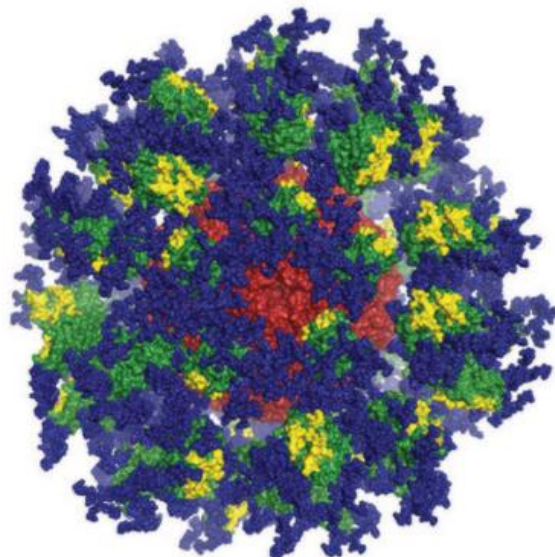


Figure IV-4: Computer image of the eOD-GT8 immune-stimulating protein. eOD-GT8 60mer is a self-assembling nanoparticle of engineered HIV Envelope (Env) proteins linked to a spherical protein structure. Image courtesy of J. Jardine, S. Menis, and W. Schief of Scripps Research and IAVI.

On the occasion of the last HIV Research For Prevention (HIVR4P) meeting that took place beginning of February 2021, W. Schief, PhD, announced the very encouraging results of IAVI G001, a phase 1 clinical trial aiming at evaluating in a first human trial the ability of eOD-GT8 60mer to specifically stimulate B-cells with the potential to develop into VRC01-type BNABs secreting cells, among millions of B cells. Forty-eight healthy adult volunteers received either a placebo or two doses of the eOD-GT8 60mer. No safety concerns arose among the participants and 97% of the vaccinated developed detectable VRC01-class IgG B cells, at a frequency high enough to consider boosting. This proof of principle provides hope that germline-targeting strategies could be successful, and may be also extendable to other pathogens like influenza, dengue, Zika or malaria.

In parallel, other immunogens were designed to specifically activate precursors of other lineages, like apex or HM Patch Ab lineages. Env trimers with chimeric V1V2s displayed interactions with inferred ancestor and intermediate V1/V2 BNABs (Gorman et al., 2016). The synthetic V3-glycopeptide (“Man9-V3”) is thought to be usable to prime precursors of V3-glycan B-cell lineages (Fera et al., 2018). Moreover, RC1, a modified BG505 SOSIP.664 successfully elicited in mice, rabbits and macaques, Abs which resemble precursors of BNABs directed at the V3-HM patch (Escolano et al., 2019). SOSIP trimers were also modified to bind some inferred germline precursors from several BNABs classes at the same time. BG505 SOSIP.664 was engineered to engage germline precursors of BNABs that target either the trimer apex and the CD4-bs *in vivo* (Medina-Ramírez et al., 2017). Other design features were brought to BG505 SOSIP (T332N) which could activate PGT121 inferred-germline B cells *ex vivo* when multimerized on liposomes, and primed PGT121-like responses in PGT121 inferred-germline knockin mice (Steichen et al., 2016). This immunogen could likely be improved to elicit additional BNAB lineages, toward the HM Patch or other epitopes.

Recently, an ultra-deep human antibody sequencing database was used to set up a method for the identification of a diverse set of potential antibody precursors for given BNABs with dominant HCDR3 contacts (as observed for most BNABs). From these informations, and using Ab BG18 as a proof of concept, HIV envelope trimer-based immunogens were developed to prime responses from rare BNAB-precursor B cells in a mouse model. Such guiding the germline-targeting approach based on B cell repertoire could also be applied to most HCDR3-dominant antibodies, also not only in the HIV context (Steichen et al., 2019).

2. Guiding the maturation

Once the proper precursor B cell is activated, its maturation has to be driven toward breadth and potency.

Administrating the Env of autologous viruses that participated in the elicitation of BNABs in a real infection was suggested to shepherd maturation toward BN.

This strategy was attempted separately of the germline-targeting starting step on the base of the Env-CH103 co-evolution data. CH103 is a CD4-bs BNAB isolated from an African donor identified as CH505, able to neutralize 85% of HIV-1 isolates and displaying low level of SMH (H. X. Liao et al., 2013) (Table III-2). In the study of W. B. Williams et al., 2017, 4 sequential gp120 of autologous CH505 Env were administrated in rhesus macaques, inducing antibodies with CH103 epitope overlap but poor neutralization breadth, likely due to an incomplete activation of lineage-specific BNAB clones and the unavailability of a suitable IGLV to pair with the IGHV4-J for neutralizing activity. Then 8 rhesus macaques were immunized with sequential CH505 Env gp140C trimers primed with CH505 Transmitted/Founder gp145 DNA (electroporation delivery). One only developed autologous and various heterologous Tier-2 Nabs. This animal also surprisingly developed V1V2-glycan Abs with some breadth. Albeit small, those results led to the phase I clinical trial HVTN 115 (NCT03220724), in which the tolerability and immunogenicity of 4 sequential CH505 gp120 administrations (TF and weeks 53, 78 and 100) are evaluated in 107 humans. Results will be release by 2022.

Similar lineage-based immunizations were conceptualized for V1/V2 BNABs, notably based on the deciphering of the co-evolution process of the 33 mAbs of the VRC26 family from donor CAP256 (Doria-Rose et al., 2014) (Bhiman et al., 2015).

Another donor, CAP257, has also been highly inspiring, as the longitudinal study of his sera samples displayed 3 distinct waves of neutralization, directed against the V2 region, the CD4-bs and an undefined quaternary epitope, respectively (Wibmer et al., 2013). Gp160 DNA and gp140 trimeric Env proteins representing CAP257 envelopes were co-administrated in rabbits and macaques and successfully elicited Tier 1A, 1B, and Tier 2 heterologous NABs. The maximum neutralizing antibody titers were obtained with 3 immunizations and additional boosts did not further improve NAB elicitation (Malherbe et al., 2020).

C. Additional insights to assist the vaccine strategy

1. Heterologous prime-boost

In parallel to the immunogens themselves, the administration strategies have been greatly studied as well. The usual homologous prime/boost strategies, in which the same immunogen is used for both the prime and boost regimens (Kardani et al., 2016), have not been effective for eliciting NABs against HIV-1. Heterologous prime/boost, in which different immunogens for the priming and the boosting(s) are used, appeared as a better option (Haynes et al., 2012) (Excler & Kim, 2019). First, it was shown that the use of different delivery vehicles can limit the inhibitory effects caused by vector-specific immune responses (Brown et al., 2010). In addition, in the context of lineage-based HIV vaccine, triggering naive B cell may require different antigens to those driving affinity maturation (Sattentau, 2014). For instance, a clade C trimer boosted BNAbs responses that had been primed by earlier immunizations with clade A and B trimers (Torrents de la Peña et al., 2018).

Several antigen presentation (peptide, scaffold, trimer...) could even be combined in the same vaccine strategy. We notably mentioned earlier the use of gp160 DNA in conjunction with protein-scaffolds or FP coupled to carrier proteins administrated in combination with BG505 SOSIP.

2. Delivery mode

Therefore the use of multiple Env antigens might be necessary to elicit neutralizing breadth, but whether they should be administrated individually, sequentially or in cocktail has been a relevant matter. Some *in silico* models of affinity maturation suggested that BNAbs elicitation would be favored by sequential immunization rather than simultaneous administration (S. Wang et al., 2015). *In vivo*, rabbits were immunized with trivalent and tetravalent combinations of SOSIP trimers from clades A, B, and C, delivered simultaneously or sequentially. The results were that NAb responses against each immunogens were reduced when they were delivered in combinations compared to when delivered alone (probably because of immunogen interference) (Torrents de la Peña et al., 2018).

Moreover, slow delivery immunization should also be seriously considered, as it results in more robust T follicular helper (Tfh) cell responses and GC B cells with improved Env-binding in rhesus monkeys (Cirelli et al., 2019).

3. Using the cooperation of T helper lymphocytes

The relatively high level of SMH usually necessary for BNAbs activity has been mentioned before. SMH happen in GC and cannot occur without Tfh cells (see II-B-6). Importantly, in the context of HIV, the B cells that have the potential to become BNAbs are very rare and have low affinity for their initial antigen.

Thus, chances of the B cells with neutralizing specificities to get Tfh help might get diluted by the B cells with non-neutralizing specificities that compete. Notably, epitopes are recognized in a hierarchical manner (Angeletti & Yewdell, 2018) which is potentially not in favor of the epitopes of the BNAb that are often not immunodominant. Increasing the help of Tfh cells - by including T-cell epitopes and adjuvants - may allow for a greater number of B-cell specificities to undergo increased SHM, thus maximizing the chance of maturing a B-cell lineages with neutralizing capacities (Havenar-Daughton et al., 2017)(Sattentau, 2014).

4. Consensus and mosaic Env

Numerous ideas to overcome viral diversity were brought to the HIV vaccine field.

Among them was the usage of consensus Env immunogens, designed to present minimized genetic distance with all HIV-1 circulating stains. However the group M consensus Env Con-S induced low-levels of heterologous Nabs in both guinea pigs (H.-X. Liao et al., 2013) and macaques (Hulot et al., 2015).

As an alternative, mosaic antigens were designed to present a high number of different BNAb epitopes in one single antigen, in order to optimize coverage of HIV-1 diversity. They were first polyvalent, made with the most immunogenic features of the total virion (*gag*, *pol* and *env*)(Barouch, 2010), and then focused on Env (mosaic Env). In guinea pigs, the comparison of a monovalent clade C gp140 with a tetravalent regimen consisting of 4 clade C gp140s and a tetravalent regimen consisting of clade A, B, C, and mosaic gp140s showed that the two last prime/boost regimens could elicit great waves of mAbs directed against the V2 loop but only weak tier 2 NAb responses (Bricault et al., 2018). In rhesus monkeys, the protective efficacies of Adenovirus/poxvirus and adenovirus/adenovirus vector-based vaccines expressing HIV-1 mosaic Env, Gag, and Pol were evaluated against SHIV challenges. Substantial protection could be observed, correlated with low level of NABs but also Env-specific binding and NNAb-dependent cellular phagocytosis (Barouch, Stephenson, et al., 2013). The APPROACH clinical study (NCT02315703) consequently evaluated the safety and immunogenicity in 393 participants of Ad26 vectors expressing mosaic Env/Gag/Pol antigens and boosting with Ad26 or MVA vectors expressing these antigens with or without aluminum adjuvanted clade C Env gp140 protein. Safety and tolerability were favorable. The mosaic Ad26 prime/Ad26+gp140 boost vaccine was the most immunogenic: Env specific binding antibody responses, Ab-dependent cellular phagocytosis responses, and T-cell responses elicitation (Barouch et al., 2018). No tier-2 NABs were elicited though. Another on-going phase IIb clinical trial concerns an Ad26 vector encoding for diverse mosaic HIV-1 genes (*gag*, *pol*, gp140 clade C *envs*) delivered in prime-boost combinations with aluminium adjuvant. This trial named HPX2008/HVTN705 or Imbokodo trial (NCT03060629) started in 2017 and concerns women of five southern African countries. Same immunogens are parallelly tested in a phase III clinical trial in Men who have sex with Men from both Americas and Europe since 2019

under the name of the MOSAICO trial (HVTN706, NCT03964415). Both trials should be ending by 2022 and 2023 respectively (NIH News Release, July 2019).

5. Antigen presentations

We focused on the type of protein that might be able to participate in BNAb elicitation, though they can be given to the immune system in different formats.

Protein-immunogen may be carried by nanoparticles for instance, like are the eOD-GT immunogens. Nanoparticles allow multimerization, which offers several advantages : i) B cell activation improvement by BCR cross-linking increase, ii) better avidity, iii) more efficient trafficking to GC by components of the immune system, iv) hiding of the immunodominant trimer-base epitope and v) repetitive display can break immunological tolerance checks that block the maturation of some DNAb lineages (Moral-Sánchez & Sliopen, 2019). Nanoparticles can be lipid-based or fusion-protein based.

The vaccine base-protein(s) can also be administrated in a nucleic acid format (DNA or RNA vaccine) for an *in situ* encoding. In this instance, partnership between IAVI, Scripps Research and the company Moderna (recently pushed under the spotlights for its vaccine against SARS-CoV2) was established to develop and test mRNA-based vaccines that would encode for the eOD germline-targeting antigen in order to stimulate specific naïve B cells. A phase 1 trial might start at spring 2021.

6. Adjuvants

Vaccine efficiency also relies on adjuvants that act on : recruitment of immune cells, enhancement of antigen uptake and presentation, and promoting antigen transport to draining lymph nodes... (Awate et al., 2013) which thus have to be seriously taken into account in the vaccine formulation.

7. Problematic of the animal model

Different animal models have been used for Immunogenicity studies: mice, guinea pigs, hamsters, rabbits, cows, lamas, macaques, but their immune systems are not perfect mimics of those of humans, macaques being the closest. As an example, mice do not have Abs with very long HCDR3 loops in their repertoire. Therefore it not possible to elicit in this model BNABs with long HCDR3s. On the other hand, cows have the ability to make Abs with extralong HCDR3s that average ~26 amino acids in length with 10–15% of the repertoire that can even reach 70 amino acids (Sok et al., 2017) making it easy to elicit BNABs in this model.

Furthermore, more sophisticated models such as knock-in mice may also have issues. For example, the frequency of CD4bs epitope-specific B cell precursors in the germline HC VRC01 Ig-knockin mouse

models that express the pre-rearranged V(D)J encoding inferred germline genes was estimated 400-fold higher than those predicted for humans (Andrabi et al., 2018).

As a final example, the STEP trial was launched in humans based on convincing macaque model results. However, the recombinant adenoviruses expressing HIV Gag, Pol, and Nef proteins failed to prevent infection in humans (Buchbinder et al., 2008), which might be due to the selection for particular MHC alleles in the monkey model (Walker & Burton, 2008).

Hence, all immunization results obtain in animal models have to be considered with a pinch of salt, as they can hardly be extrapolated to humans most of the time, reminiscent of a citation of Girard & Plotkin, 2012: « mice lie and monkeys exaggerate ».

V. PhD project

A. Context

We explained above how an HIV vaccine could be based on series of immunogens that would: i) prime specific naive B cell(s), and ii) guide the maturation toward broad neutralization. Such approaches need an in-dept understanding of the co-evolution between Abs and viral variants, to feed a proper immunogen design to activate the right B cell(s) and keep its(their) Ab maturation “on track” (fig III-6).

Our team has been crucially involved in the isolation of BNABs (Walker et al., 2009) (L. M. Walker et al., 2011) and in several longitudinal studies of BNAB development (MacLeod et al., 2016) (Landais et al., 2017) (Umotoy et al., 2019), thanks especially to access to samples from Protocol C, the largest longitudinal cohort of primary infection (see Material and Methods and Landais et al., 2016). Those co-evolution studies followed the same global pipeline (Fig V-1): BNAB isolation from longitudinal PBMCs samples (by functional screening of single B-cell micro-culture and/or antigen-specific cell sorting), in parallel to the sequencing of the viral Env variants from the corresponding longitudinal plasma samples. NGS sequencing of the memory B-cell repertoire is also done and the data are used to re-construct the Ab phylogenies over the course of infection and BNAB maturation. Detailed functional and structural evaluations are done *in vitro* with the cloned Abs and Env variants, in order to retrace as much as possible the virus-Ab interactions and their interplay from early elicitation to BN activity acquisition.

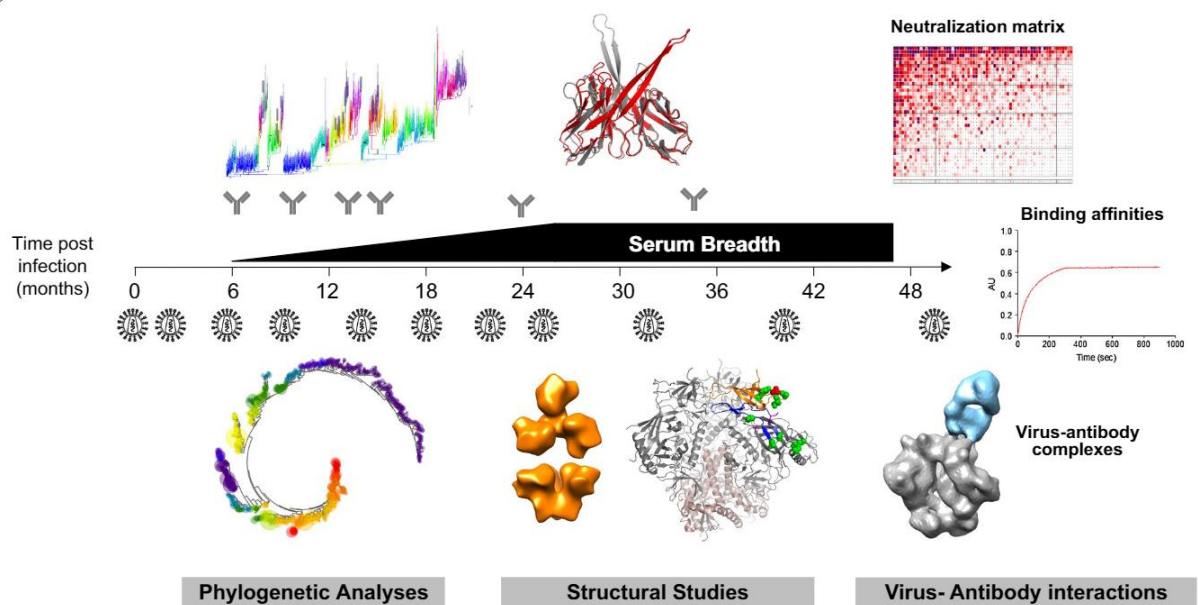


Figure V-1: HIV and BNAB co-evolution studies. From Landais and Moore, *Retrovirology*, 2018.

Longitudinal studies about BNAb development are still not many and yet extremely valuable for HIV vaccine design, by providing key understandings about BNAb elicitation. Despite the recent studies, our knowledge of the process of bnAb elicitation during natural infection remains somehow limited. There is a need to characterize more lineages corresponding to the different broad specificities. For instance, studying additional broad neutralizers will help understand whether and how certain specificities may be more amenable to elicitation through vaccination and should be favored in vaccine approaches. It is also important to study BNAb developmental pathways in various individuals sharing the same broad specificity as similarities between donors in Env evolution or in the nature of the Env triggering the broad lineage could strongly suggest a path for immunogen design.

B. Thesis subject

My PhD project aims thus to continue the work started on the Protocol C, i.e. the deep characterization of BNAb development as it occurred in elite neutralizers. The course of the project (schematized Fig V-2) follows the same global pipeline used for the previous studies of our laboratory.

A first part of my PhD focused on donor PC02, the fourth best neutralizer of the protocol C cohort, in particular in attempting to map the Ab serum specificity responsible of the remarkable breadth of this elite neutralizer. Because the primary mapping of the serum specificity that was aimed to inform a sorting strategy could not precisely identify the epitope his BNAb target, the work done within this PhD was essentially epitope mapping. Despite the usage of diverse methods for an in-dept mapping of PC02 serum specificity, the epitope(s) remains currently unknown. Finally, another sorting strategy – epitope agnostic - was designed, but applied to a second donor.

A second part is then about donor PC94, second best neutralizer of the protocol C, from whom a former PhD student – Claire Rousset – isolated 27 NAb of 2 different lineages targeting the HM patch, but that were not recapitulating the entire serum BN. The second objective of my PhD project was hence to isolate the other BNAb that might participate to PC94 serum neutralizing activity. Ab isolation strategy was modified compared to Claire Rousset's work and resulted in the production of 36 additional Abs among which 16 showed neutralizing activity (albeit with very various breadths and potencies). Ten of them belong to one lineage Claire Rousset could highlight before and 2 others, from new lineages, showed particularly interesting neutralizing activity, because of better potency and/or completion of the spectrum of neutralization.

Finally, PC02 project remained at the early isolation step and PC94's stopped at the BNAb identification and beginning of further characterization. The difficulties of the BNAb isolation and pros and cons of the diverse strategies will notably be discussed in this manuscript.

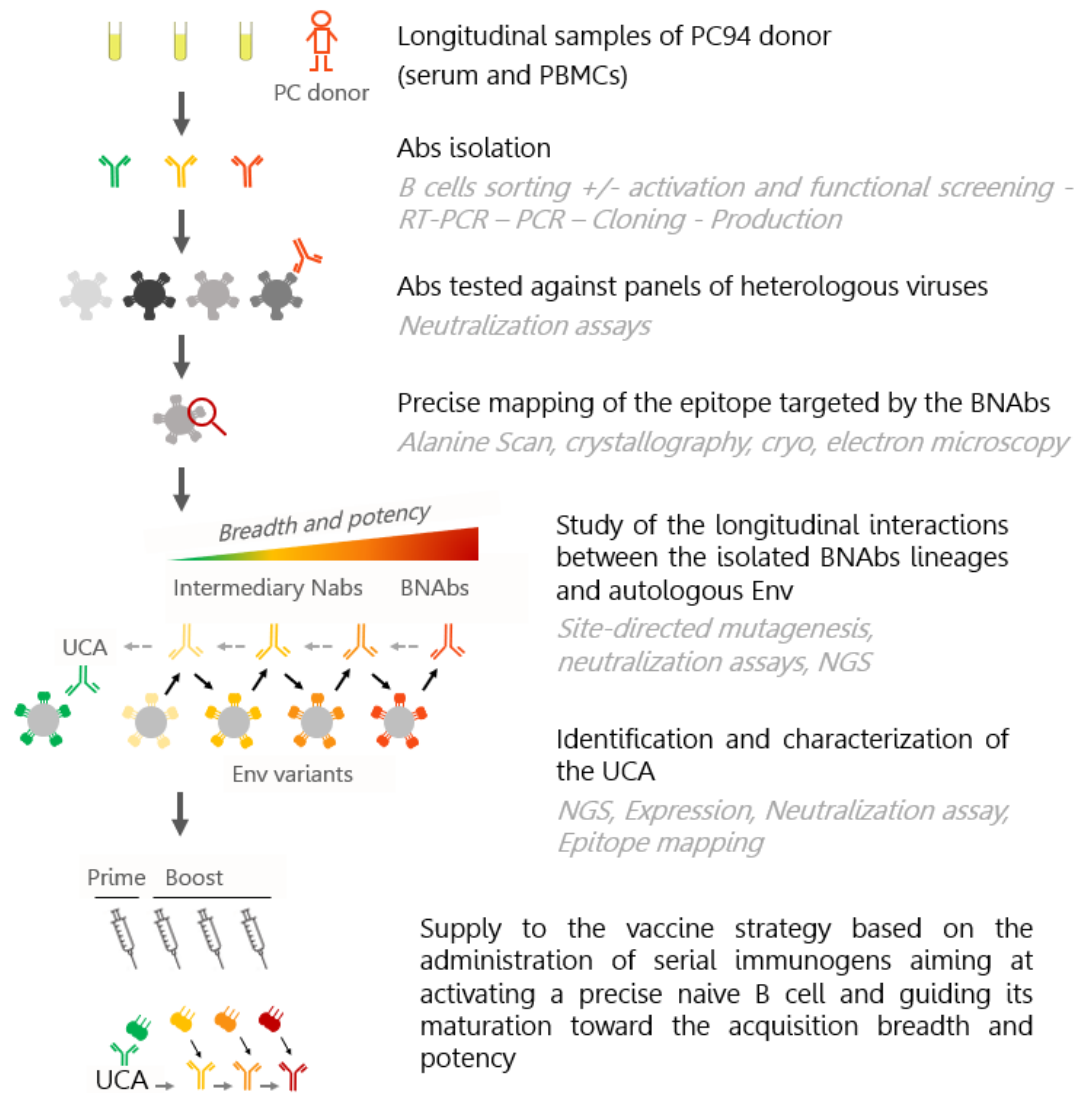
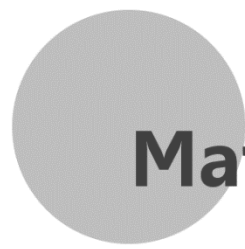


Fig V-2: Global project pipeline. Four main steps constitute such longitudinal studies : i) isolation of Abs, ii) characterization of the isolated BNABs in regard to their potential BN, iii) deciphering of the co-evolution mechanisms between the isolated BNABs and autologous Env and iv) identification of the UCA at the origin of the BNAB lineage(s). Method employed for each step are indicated in grey.



Materials and methods

A. The IAVI Protocol C

The protocol C cohort of International AIDS Vaccine Initiative (IAVI) has been described by Landais et al., 2016. Briefly, the protocol C cohort is derived from an incidence cohort in which naïve participants from nine sites in Sub-Saharan Africa were regularly monitored for HIV seroconversion between 2006 and 2011. Any certified seroconversion led to the consented inclusion of the newly infected individual into the protocol C cohort. A total of 613 participants was thus enrolled, all within the early steps of their infection (82 days in average following their assumed exposure). Four hundred thirty-nine (72%) of them – older than 18 and ART naïve – were followed every three to six months for more than a year, thus allowing the longitudinal collection of plasma and peripheral blood mononuclear cells (PBMCs) samples. Each inclusion and sampling were collected following written consent and the protocol was approved by the appropriate ethical committees. The visits, scheduled and coded based on the number of estimated months post-infection (mpi), consisted in the collection of the blood samples and data such as: HIV risk behavior, demographics, symptom-directed examinations (comorbidities and opportunistic infections), CD4 T cell count and viral load. Enrollment closed in 2011 but the longitudinal follow-up is still on-going. The study of 2220 samples allowed the identification of plasmas with a broad neutralizing activity (BNa) and the study of the clinical correlates associated with this BNa (Landais et al., 2016). The neutralizing activity was initially screened by Monogram Biosciences on a 6-virus panel (6vP), predictive of the neutralization on larger panels (Simek et al., 2009; see part C.). A plasma was defined as neutralizing if able to neutralize 50% of viral infectivity at a dilution $\geq 1/100$. A neutralization score for the 6 virus panel was calculated using the formula $\text{Score} = \text{Average}(\log_3(\text{dilution}/100)+1)$. A score of 1 was predictive of neutralization of 50% of viruses on a larger panel. The study of clinical correlates linked to the development of BNa revealed no influence of gender, age or geographical origin, but correlations were found with high viral load, low CD4 T cell counts, subtype-C infection and HLA-A*03(-) genotype (Landais et al., 2016). Samples of the top 42 best neutralizers were studied in regard to the broadly neutralizing epitope targeted on HIV Env. Few donors were selected for further deciphering of the longitudinal process of development of BNabs in co-evolution with autologous HIV-1 Env variants. The development of BNabs lineages was then described in donors PC76 and PC39 (lineage targeting the N332 region) (MacLeod et al., 2016; unpublished), donor PC64 (lineage targeting the Env apex) (Landais et al., 2017) and donor PC63 (lineage targeting the CD4-bs) (Umotoy et al., 2019).

B. Mammalian cell culture

Four cell lines were used during the project. They are all maintained at 37°C in a 5% CO₂ atmosphere.

HEK293T are adherent cells and come from a transformed kidney embryonic human line. They are cultured in 10% FBS (Foetal Bovine Serum – VWR #97068-086) PSG (Penicilline-Streptomycin-Glutamine - Gibco™ #10378016) NaPyr DMEM (Gibco™ #21969035), aka complete DMEM or DMEMc. For each passage, media is aspirated from the flask and the adherent cells are incubated 2 minutes (min) at 37°C with 0.25% EDTA Trypsine (Gibco™ #LS25200072), leading to their detachment. Detachment is stopped by dilution of the Trypsin solution volume in 3 volumes of DMEMc. A small amount of cells is harvested for counting while the rest is centrifuged 5 min at 1200 rpm before elimination of the supernatant. Cell viability and count are evaluated using trypan blue while cells are being centrifuged. The cell pellet is then resuspended in DMEMc at a concentration of 5*10⁶ cells/mL. Five million cells are further distributed in new 225 cm² flask with 50 mL fresh media. They usually reach their confluence in 3 to 4 days. When keeping HEK293T cells in culture, we do not exceed 30 passages. These cells are mainly used for pseudovirus production and sometimes Ab production. They are of human origin and therefore proteins post-translational modifications – most notably glycosylation – are similar to those happening in humans *in vivo*.

HEK293S cells were genetically modified to delete the gene encoding the N-acetylglucosaminyltransferase I (GnTI). Their culture is done with the same protocol as for HEK293T cells. They are used for production of pseudoviruses with no complex and hybrid glycans.

TZMbl cells derive from HeLa CXCR-4+ cells and express CD4, CCR5 and a luciferase reporter gene under the control of an HIV TAT-dependent promoter. Those cells are also adherent and cultured in a manner identical to the HEK293T. Highly susceptible to HIV infection, TZMbl cells are used for neutralization assays.

HEK293F is a cell line derived from the HEK293T cells which has adapted to being cultured in suspension, requiring agitation (120 rpm). The culture media is the protein-free and antibiotic-free 293 Freestyle media (Gibco™ #12338018). Cells are initially diluted at 0.5*10⁶ cells/mL and usually rediluted after 3 to 4 days, to not exceed 5*10⁶ cells/mL. They are used for trimer and antibody production, because of their high-yield production with adequate post-translational modifications. Transfections are only carried if the cell viability is above 95%.

C. The panels of HIV-1 strains

In order to evaluate the breadth of the sera or mAbs that are studied, four panels of HIV-1 subtypes, predictive of the neutralization on larger panels, have been used during this project (Table VI-1). The smaller one, the 6 virus panel (6vP), constituted by [Simek et al., 2009](#), was used to identify the neutralizers among the protocol C cohort in first intention, as mentioned above. The 12vP, also called the Montefiori panel ([deCamp et al., 2014](#)), is probably the panel that has been the most frequently used throughout this work, because of its adequate representation of HIV-1 spectrum breadth combined with a moderately low number of strains facilitating its manipulation. The 37vP was constituted “in-house” to confirm the neutralizing activity of the Protocol C sera observed with the 6vP on a medium-sized panel ([Landais et al., 2016](#)). The 37vP actually includes the 6vP. To be noted, this panel might be biased in term of worldwide HIV-1 breadth representation because it contains a high proportion of clade B viruses (22/37) while the most common circulating clade is known to be the C’s (46% of global infections ; [Gartner et al., 2020](#)).

Finally, the 121vP, adapted from the 109vP of [Seaman et al., 2010](#), is the largest panel available in the laboratory to assess the breadth of a neutralizing serum or mAb against a large panel. It notably includes some strains of the 12vP and the 37vP.

The nucleotides positions of each Env variant are numbered by alignment with the strain HxB2, a clade B HIV-1 ([Ratner et al., 1985](#)).

6vP		
Strain	Clade	Tier
94UG103	A	2
92TH021	AE	2
92BR020	B	2
JRCSF	B	2
93IN905	C	1B
IAVic22	C	1B

12vP		
Strain	Clade	Tier
398F1	A	2
246F1	AC	2
CNE55	AE	2
CNE8	AE	2 or 3
Bjox 2000	AG	2
CH119	AG	2
TRO.11	B	2
X2278	B	2
CE0217	C	?
CE1176	C	2
25710	C	1B or 2
X1632	G	2

37vP		
Strain	Clade	Tier
92RW020	A	2
BG505	A	2
KNH1144	A	3
94UG103	A	2
92TH021	AE	2
HXB2	B	1B
MN	B	1A
SF162	B	1A
SS1196	B	1B
BaL	B	1B
DH12	B	2
TRO.11	B	2
89.6	B	2
92BR020	B	2
6535.3	B	1B
JRFL	B	1B
SC422661.8	B	2
JRCSF	B	2
REJO	B	2
ADA	B	2
RHPA4	B	3
QH0692.42	B	3
PVO.4	B	2
WITO	B	2
TRJO	B	3
YU2	B	3
CAAN5342.A2	B	2
93IN905	C	1B
IAVIC22	C	1B
Du156.12	C	2
CAP210.2.00.E8	C	2
ZM249M.PL1	C	2
Du422.1	C	2
CAP45.2.00.G3	C	2
Du172.17	C	2
ZM214M.PL15	C	2
ZM135M.PL10a	C	2

105vP							
NIH	Strain	Clade	Tier	NIH	Strain	Clade	Tier
1	6535.3	B	1B	64	0260.v5.c36	A	?
2	QH0692.42	B	3	65	191955_A11	A	2
3	SC422661.8	B	2	66	191084 B7-19	A	2
4	PVO.4	B	2	67	900455_A3_4	A	2
5	TRO.11	B	2	68	620345.c01	AE	2
6	AC100.29	B	2	69	C1080.c03	AE	2
7	CAAN5342.A2	B	2	70	R2184_c04	AE	2
8	WEAU_d15_410_5017	B	2	71	R1166.c01	AE	2
9	1006_11_C3_1601	B	2	72	R3265.c06	AE	2
10	1054_07_TC4_1499	B	2	73	C3347.c11	AE	2
11	1056_10_TA11_1826	B	1B	74	C4118.c09	AE	2
12	1012_11_TC21_3257	B	1B	75	CNE8	AE	2 or 3
13	6240_08_TA5_4622	B	2	76	CNE5	AE	2
14	6244_13_B5_4576	B	2	77	BJOX009000.02.4	AE	2
15	62357_14_D3_4589	B	2	78	BJOX015000.11.5	AE	2
16	SC05_8C11_2344	B	2	79	BJOX010000.06.2	AE	2
17	Du156.12	C	2	80	BJOX025000.01.1	AE	2
18	Du172.17	C	2	81	BJOX028000.10.3	AE	2
19	Du422.1	C	2	82	X1193_c1	G	2
20	ZM197M.PB7	C	1B	83	P0402_c2_11	G	2
21	ZM214M.PL15	C	2	84	X1254_c3	G	2
22	ZM233M.PB6	C	2	85	X2088_c9	G	2
23	ZM249M.PL1	C	2	86	X2131_C1_B5	G	2
24	ZM53M.PB12	C	2	87	P1981_C5_3	G	2
25	ZM109F.PB4	C	1B	88	X1632_S2_B10	G	2
26	ZM135M.PL10a	C	2	89	3016.v5.c45	D	2
27	CAP45.2.00.G3	C	2	90	A07412M1.vrc12	D	2
28	CAP210.2.00.E8	C	2	91	231965.c01	D	2
29	HIV-001428-2.42	C	2	92	231966.c02	D	2
30	HIV-0013095-2.11	C	2	93	191821_E6_1	D	2
31	HIV-16055-2.3	C	2	94	3817.v2.c59	CD	2
32	HIV-16845-2.22	C	2	95	6480.v4.c25	CD	2
33	Ce1086_B2	C	2	96	6952.v1.c20	CD	2
34	Ce0393_C3	C	2	97	6811.v7.c18	CD	2
35	Ce1176_A3	C	2	98	89-F1_2_25	CD	2
36	Ce2010_F5	C	2	99	3301.v1.c24	AC	2
37	Ce0682_E4	C	2	100	6041.v3.c23	AC	2
38	Ce1172_H1	C	2	101	6540.v4.c1	AC	2
39	Ce2060_G9	C	2	102	6545.v4.c1	AC	?
40	Ce703010054_2A2	C	2	103	0815.v3.c3	ACD	2
41	BF1266.431a	C	2	104	3103.v3.c10	ACD	2
43	249M B10	C	2	105	?	?	?
44	ZM247v1(Rev-)	C	2	106	T250-4	AG	2
45	7030102001E5(Rev-)	C	2	107	T251-18	AG	2 or 3
46	1394C9G1(Rev-)	C	2	108	235-47	AG	2
47	Ce704809221_1B3	C	2	109	?	?	?
48	CNE19	BC	2	110	?	?	?
49	CNE20	BC	2	111	Ce1176_B5	?	?
50	CNE21	BC	2	112	Ce2010_C1	?	?
51	CNE17	BC	2	113	ZM249M.C1 or B4	?	?
52	CNE30	BC	2	114	T257-31	AG	?
53	CNE52	BC	2	115	928-28	AG	2
54	CNE53	BC	2	116	263-8	AG	2
55	CNE58	BC	2	117	T278-50	AG	2 or 3
56	Q168ENVa2	A	2	118	T255-34	AG	2
57	MS208.A1	A	1 or 2	119	T211-9	AG	?
58	Q23.17	A	1B	120	THRO4156.18	B	2
59	Q461.e2	A	2	?	RHPA4259.7	B	2
60	Q769.d22	A	2	?	REJO4541.67	B	2
61	Q259.d2.17	A	?	?	TRJO4551.58	B	3
62	Q842.d12	A	2	?	WITO4160.33	B	2
63	0330.v4.c3	A					

Table VI-1: The main three virus panels used throughout this PhD project.

D. Mapping strategies

Site-Directed Mutagenesis (Soluble or Pseudoviral Env)

The site directed mutagenesis consists in the introduction of a mutation at a specific position of a plasmid DNA. This is done via Polymerase Chain Reaction (PCR) with specific primers. The design of these is done according to certain guidelines: one primer for one amino acid change ; the number of nucleotides to mutate has to be as small as possible ; the mutated nucleotides are to be roughly localized in the middle of the primer, however the 3' end can be extended ; the primer length goes from 23 to 35 nucleotides ; the percentage of G and C is comprised between 35 and 60% ; the primer T_m between 50 and 62°C ; ideally one G or C are located at the 3' end ; and it is better to avoid 3 identical bases in a row in the sequence.

The PCR is performed with: the DNA template (100ng), the primers pair bearing the mutation of interest (0.25 μM each), 1/50 DMSO and 1X Phusion High-Fidelity PCR Master Mix containing a *Pyrococcus furiosus*-like DNA Polymerase and dNTPs in a reaction buffer with MgCl₂ (Thermo Scientific™ # F532S). The cycle is composed by a first step at 98°C for 1 min and a second step repeated 25 times: 30 sec 98°C + 1 min 30sec 60°C + 3 min 72°C. Those three steps correspond to the denaturation of the DNA, the annealing of the primer and the elongation, respectively. To be noted, the annealing temperature usually has to be adapted depending on the primers characteristics : it is usually set up as the average of both primers T_m minus 3°C. A third step of final elongation is done at 72°C for 5 min. Following amplification, half of the PCR product is digested by DpnI (2h, 37°C)(NEB #R0176L) in order to get rid of the original template DNA. The other half is used as a control. Both DNAs are transformed in competent *E.coli* DH5α (Invitrogen™ #18263012) via heatshock: 30 min incubation on ice, 42°C dry bath for 45 sec and 2 min on ice again. Bacteria are let to recover during 1 hour at 37°C in 150 μL of LB. Cultures are spread on antibiotic-LB-agar plates to develop overnight at 37°C. This step allows the selection of the transformed bacteria: only those which integrated the plasmid form colonies.

DNA amplification and purification

DNA is amplified from transformation plates, from which colonies are picked up and inoculated in 5 mL LB with antibiotic. All experiments of this project requiring bacteria used DH5α *E.coli*. The number of colonies picked up depends on the context: 1 colony for regular DNA amplification, 2 colonies if the transformation follows an Ab cloning (see below), 3 colonies if it follows a site-directed mutagenesis (see above). Indeed the two last cases require some screening. The extraction is made thanks to a QIAprep® Spin Miniprep Kit (QIAGEN #27106). Briefly, 5 mL of bacterial culture is centrifuged (4000 rpm, 10 min, room temperature (RT)). Supernatant is discarded in order to resuspend the pelleted bacterial cells in 250 μL of resuspension buffer (P1: glucose, Tris, Cl, EDTA and RNase). The suspension

is incubated 4 min with 250 μ L of lysis buffer (P2: SDS + NaOH). Three hundred fifty μ L of a third buffer are used to stop the lysis reaction and precipitate proteins (N3: potassium acetate, glacial acetic acid). A centrifugation (10 min, 13 000 rpm, RT) allows the separation of cell debris from DNA in suspension. Supernatant is centrifuged again (1 min, 13 000 rpm, RT) through a filtering column which captures the DNA via electrostatic interactions. The column is washed twice by 1 min centrifugation with 750 μ L of ethanol 70%, in order to get rid of non-specific interactions with genomic DNA. Plasmid DNA is then eluted twice with 20 μ L of water. Finally, the product is sequenced to check the presence of the mutation.

For larger amplification - when the DNA sequence is known to be correct – a maxi-preparation of the DNA is made, using the HiSpeed® Plasmid Maxi Kit (QIAGEN #12663), which protocol is close to the one of the miniprep. The initial bacterial culture of 5 mL is inoculated in a bigger one of 200 mL for an overnight amplification. The 200 mL culture is first centrifuged (15 min, 6000g, 4°C). Pellet is resuspended in 10 mL of buffer P1, before the addition of 10 mL of lysis buffer P2. After a 4 min-incubation, 10 mL of precipitating buffer P3 are added. The precipitate is then transferred into a column and incubated during 10 min, to allow the separation between cell debris (up) and the DNA in solution (down). In parallel, a HiSpeed tip column is equilibrated with 10 mL of buffer QBT. Cell lysate is filtered and added to the HiSpeed tip column, enabling plasmid DNA to bind to silica inside. A wash is done (60 mL buffer QC) before the elution (15 mL buffer QF). DNA is then precipitated by incubation with 10.5 mL isopropanol during 5 minutes. The mixture is filtered through a precipitator which captures the plasmid DNA. It is washed by 2 mL of ethanol and DNA eluted twice with 750 μ L of water. The final extracted DNA is sequenced to check whether an unexpected mutation may have appeared.

Sequencing

Sequencing is made by Genewiz, with a Sanger method, from samples of 15 μ L of DNA concentrated at 100 ng/ μ L and primers previously designed *in silico*.

Pseudovirus production and titration

Pseudoviruses are generated by co-transfection of HEK293T cells with: the *PSG3* vector containing the whole genome of the HIV-1 with exception of the *env* gene (made inactive by the insertion of a stop codon upstream), and a plasmid containing the missing *env* gene of choice. Following transfection, the viral machinery ensures the release of virions that incorporate the Env protein produced thanks to the corresponding plasmid. Nevertheless, those virions - called pseudoviruses - carry an HIV-1 genome without the *env* gene, leading to the production of naked virions by the infected cells (Fig. VI-2). Viral particles produced are thus not able to infect new cells after this single cycle of replication. To be transfected, HEK293T cells are prepared in 2 mL of DMEMc at $0.5 \cdot 10^6$ cells/wells and incubated at

37°C 24h to adhere to the bottom of the well again, and reach the appropriate confluence (~70%). In 100 µL of OptiMEM (Gibco™ #11058021), 4 µg of *PSG3* vector are mixed with 2 µg of *env* plasmid. This mixture is filtered through 0.2 µm. In a second tube with another 100 µL of OptiMEM, 18 µL of HelixIN® (Ozbioscience # HX11000) are added (Table VI-3). Tube A (DNAs) is transferred into tube B (transfection reagent) and both are mixed by gentle pipetting, before a 25-min incubation at RT. The mix is added to the cells, which also receive 200 µL of HIB 100X boost before a 72h incubation at 37°C. Supernatants containing the produced pseudoviruses are harvested, centrifuged briefly to remove any remaining cell, aliquoted and stored at -80°C.

Before use, supernatants are tittered in order to evaluate the global amount of pseudovirions that were produced. The titration requires TZMbl cells that are prepared at $0,3 \cdot 10^6$ cells/mL in DMEMc. Fifty µL of cell preparation is distributed/well in 96-well (96-w) white plates (Greiner #675083) for a 24h-incubation at 37°C. The next day, a pre-mix is prepared: 60 µL of each pseudovirus are introduced in the first line of a 96 wells round bottom plate, while the rest of the plate is filled with 30 µL of DMEMc. Then, 30 µL of the first line (pure virus) are transferred to the next line, mixed with the media, and harvested again to be mixed with the next line, making serial 1/2 dilutions until the 7th line. The last line does not contain any virus. To mimic the virus-Ab incubation step that is part of the neutralization assay, pre-mixes are incubated 1h at 37°C. 25 µL are then transferred onto TZMbl cells, which media was previously aspirated. Cells are refeeded with 75 µL of DMEMc after a day. After 72h of contact between the cells and the pseudoviruses, supernatant is aspirated, and potentially infected TZMbl cells are lysed with 45 µL of 1X lysis buffer (Ozbioscience # LUC1000) for 15 to 60 min. Then 30 µL of luciferase substrate (Ozbioscience #LUC1000) is added and the Relative Luciferase Activity (RLA) is measured with a luminometer (TECAN Spark® 10M). The final aim is to determine the appropriate dilution of pseudovirus to obtain about 200 000 RLU per well. Indeed, we aim to obtain an average RLU in virus positive control wells 10 fold greater than the average RLU of cell control wells (no virus) (Sarzotti-Kelsoe et al., 2014), which is usually around 2500 on our reader with our luciferin.

Pseudovirus production with glycan alterations

To evaluate the N-glycan dependency of a neutralizing serum or a mAb, the neutralizing activity against WT pseudoviruses (with complete N-glycans shield) is compared to the one against pseudovirus with altered N-glycans. To this aim, pseudoviruses are produced under conditions where N-glycans biosynthesis is stopped or trimmed at different steps. A pseudovirus production in DMEMc containing 25 µM of kifunensin (mannosidase I inhibitor ; Sigma-Aldrich # k1140) blocks the trimming of $\text{Man}_9\text{GlcNac}_2$ and possibly $\text{Man}_8\text{GlcNac}_2$ N-glycans. The use of 20 µM of swainsonine (α -mannosidase II inhibitor ; Sigma-Aldrich # S9263) in the DMEMc during virus production prevents the addition of complex glycans, allowing the addition of some hybrid glycans. Production in HEK293S (N-acetylglucosamine transferase I (GnT I) deficient cell line) inhibits the process of $\text{Man}_5\text{GlcNac}_2$ N-glycans formation (Fig. VI-1) leading to the absence of hybrid and complex glycans. Otherwise, the

transfection protocol remains strictly identical to the one of the classical pseudovirion production (right above).

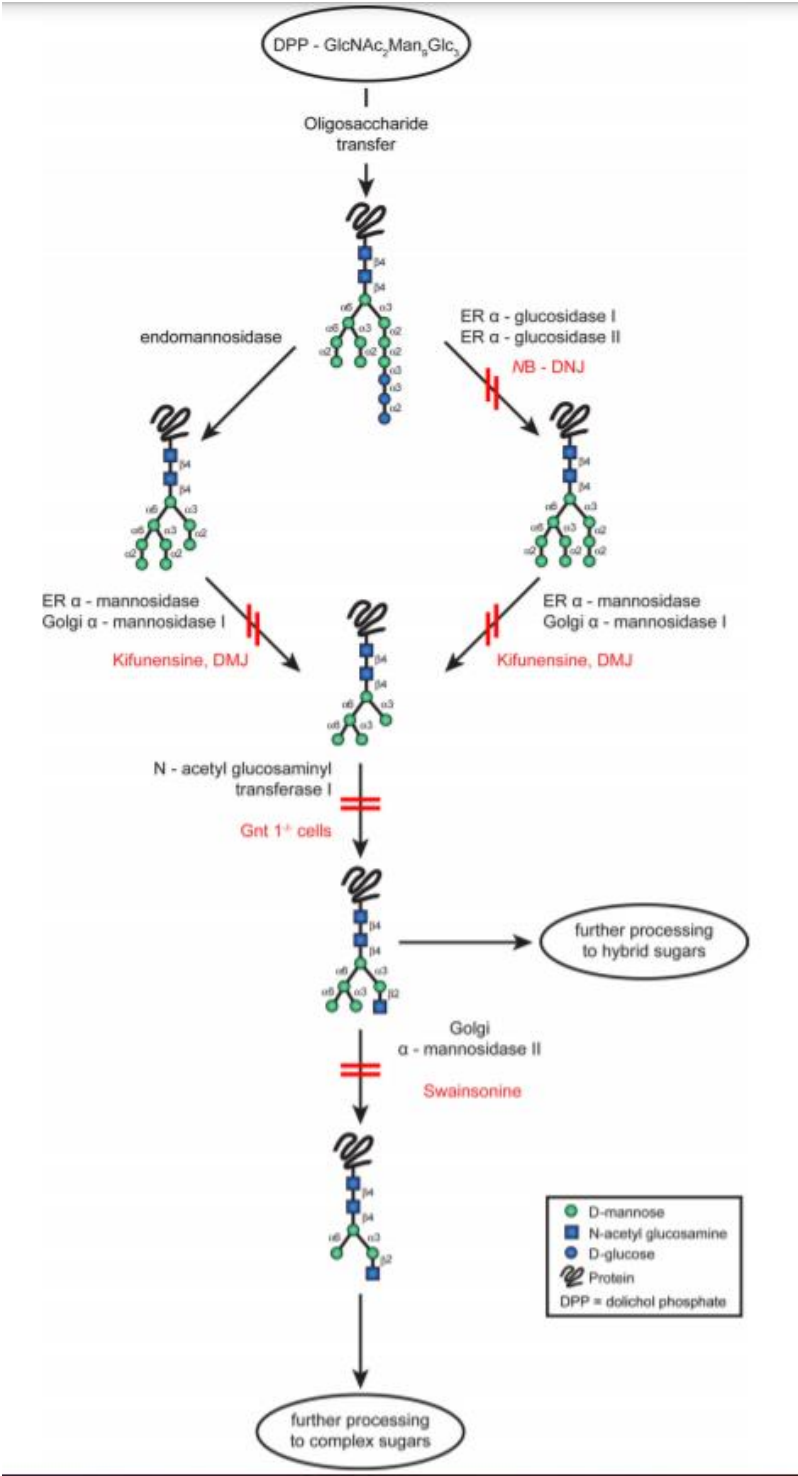


Figure VI-1: N-linked glycosylation pathway to show the formation of high-mannose, complex, and hybrid glycans. Glycosidase inhibitors (red) are shown underneath the enzyme they inhibit. Doores and Burton, JVI, 2010

Pseudovirus neutralization assay (serum)

Serial 5-fold dilutions of serum usually starting from 1/50 in DMEMc are incubated with pseudoviruses for 1h at 37°C before the mix is added to TZMbl cells prepared the day before in 96-w white plates (Greiner #675083) (Fig. VI-2). Plates are incubated for 24h at 37°C, protected from evaporation, then 75 µL of DMEMc is added to cells, and the incubation is pursued for another 48h. Media in each well is then aspirated and replaced by 45 µL of cell lysis buffer that acts for 1h under agitation. Thirty µL of luciferase substrate is then added (Ozbioscience # LUC1000) and luminescence is measured. Neutralizing activity of the serum against the pseudoviruses is defined by the reduction of virus infectivity *in vitro*, calculated by the percentage of neutralization obtained with the following formula: $[(\text{RLU when no serum} - \text{RLU when diluted serum}) / \text{RLU when no serum}] * 100$. The slope of the neutralization curves obtained is used to determine the IC50 of the serum, i.e. the dilution corresponding to 50% of neutralization.

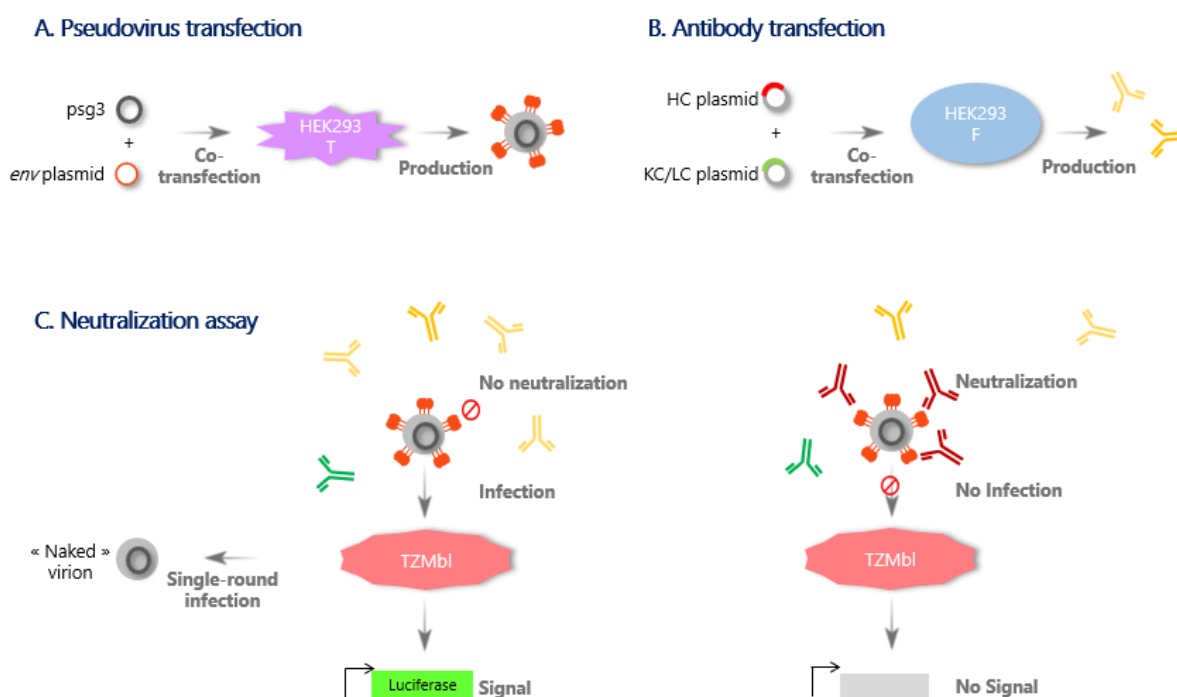


Figure VI-2: Synthetic scheme of the set-up of a neutralization assay. A. Pseudovirus transfection process. B. Antibody transfection process. C. Functioning of the neutralization assay.

Serum depletion of specific Abs

The depletion assay consists in the removal of some particular Abs from a polyclonal serum. This is done by incubation of the serum with beads coated with a protein specifically targeted by the Abs to be removed, and separation of the beads-mAb complex with the rest of the serum.

Beads have to be coated upstream of the depletion assay. Twenty five mgs (250 μ L) of magnetic beads (Dynabeads MyOne Tosylactivated – Invitrogen #65501) are transferred into a new 2-mL tube. A second tube is prepared in parallel, for the coating of an irrelevant protein, to be used as a negative control. The tubes are placed against magnets which retain the beads and supernatants are aspirated. Beads are washed three times with \sim 500 μ L of Coating Buffer (0.1M Na Borate pH9.5 + 1M $(\text{NH}_4)_2\text{SO}_4$). Beads are resuspended in 317 μ L of the same buffer. Hundred μ L at 10 mg/mL of mgp120 are added to the first tube (final concentration 4mg protein per mL of beads) while Bovine Serum Albumine (BSA – Sigma Aldrich #A7906) is concentrated at 3% in the control tube. Two hundred and eight μ L of 3M $(\text{NH}_4)_2\text{SO}_4$ are added in both, and the mixture is incubated 24h at 37°C, in a rocking wheel. The coating is followed by a blocking step. Beads are placed against magnets for supernatant aspiration. Then, after removing the magnet, 500 μ L of Blocking Buffer (1X PBS pH7.4 + 0.5% BSA + 0.05%Tween20) is added for another 24h-incubation at 37°C on a rocking wheel. Three successive washes follow, with \sim 500 μ L Washing Buffer (1X PBS pH7.4 + 0.1% BSA + 0.05%Tween20), and beads are finally conserved at 4°C in 400 μ L of storage buffer (1X PBS pH7.4 + 0.1% BSA + 0.05%Tween20 + 0.02%NaAzide).

A typical depletion assay starts with 4 washes with 1 mL of DMEMc, still using the magnets. The fourth wash is done for 30 min at RT, rocking. Beads are next resuspended in 460 μ L DMEMc, to which are added 40 μ L of serum (1/12.5 dilution) and incubated for 30 to 60 min, RT, rocking, again. Supernatant is collected at this step: it corresponds to the Depleted Fraction (DF) 1. Three successive washes with \sim 500 μ L 1X PBS + 0.5M NaCl follow. Three elutions of the mAb that have potentially bound to the coated beads are done with 500 μ L of 100mM Glycine HCl pH2.7. They are immediately neutralized with 31 μ L/well 1M of Tris pH9. Pooled together, those three volumes constitute the Eluted Fraction (EF) 1a. Three additional elutions follow, with 500 μ L/tube 100mM Glycine HCl pH2.2, neutralized with 45 μ L/well 1M of Tris pH9.0. They constitute the EF1b. EF1a and EF1b fractions that are later pooled together to form EF1. Coated beads are washed three times with 1 mL 1X PBS and resuspended in 1 mL DMEMc, for a 30 min incubation, RT, rocking. Supernatant is then aspirated and replaced by the DF, which is incubated again on the beads for 30 min, at RT, rocking, before being harvested. Beads are eluted in the same manner than previously. The process is repeated three times in total, for a final collection of: EF1, EF2, EF3 and DF3.

The success of the depletion is checked by ELISA (Enzyme-Linked ImmunoSorbent Assay) on those four fractions in parallel to a full fraction of serum and known binding mAbs. Briefly, the same mgp120s that were chosen for the depletion are coated on a 96-w plate (Nunc® #P7491) at 2 μ g/mL, 50 μ L/well, in 1X PBS, overnight, at 4°C. Plates are then blocked by 1h incubation at RT with 100 μ L/well 1X PBS-BSA 3%. Serial dilutions of mAbs or serum are prepared in the same time: mAbs starting at 10 μ g/mL,

serum at 1:100, and EF and DF fractions at 1:0, in 1X PBS-BSA1%. Three washes of the plate are done with 120 μ L/well of 1X PBS-0.05% Tween20. The serial dilutions of serum/ mAb/EF/DF are distributed in the plate, 100 μ L/well, and incubated for 1h at RT. Five washes are done and 50 μ L of Alkaline-Phosphatase conjugated Goat anti-Hu F(ab) IgG ([Jackson Immuno #109 055 097](#)) diluted at 1/1000 in 1X PBS-BSA1% are added in each well for another 1h incubation at RT. Plates are washed again, three times. Finally, the signal is detected with 50 μ L/well of PNPP reagent ([Interchim #UP664791](#)) and read at 405nm after 15 to 45 min incubation.

Depleted fractions are then used in neutralization assay to highlight the importance of the removed Ab fraction in the neutralizing activity of the serum.

Neutralization competition assay with soluble Env

The competition assay is based on the neutralization assay as described above. In order to potentially compete the neutralizing activity of specific Abs in the serum, a primary 1h-incubation step of the neutralizing serum or mAb with the soluble competitive protein (concentrated from 20 to 200 μ g/mL) is added before the serum/mAb-virus incubation step.

Competition assays usually provide results that are less clear to interpret than neutralization assays with serum depleted from specific Abs, but they require smaller amounts of trimers and are less work-intensive.

E. Env protein production and purification

Antibody column

Env proteins are purified on mAb columns. These Ab-affinity-columns are made using 2.5 g CNBr Sepharose beads (GE Healthcare #GE17-0430-01) activated by incubation in 15 mL of 1mM HCl at 4°C for 30 min. The hydrated beads are washed with 400 mL 1mM HCl, flowing through a sintered glass filtered (Sigma-Aldrich # Z546593). In parallel, 20 mg of mAb are buffer exchanged and concentrated to obtain 10 mL of mAb at 2 mg/mL in coupling buffer (100mM NaHCO₃ + 0.5M NaCl, pH8). Once dried, the beads are quickly harvested and added to the Ab solution, for an incubation overnight at 4°C with agitation.

Following the overnight binding step, beads are centrifugated for 1 min at 2000 rpm. The concentration of the remaining coupling buffer is measured to ensure that mAbs are properly bound to the beads in the pellet. The beads are washed 3 times with 15 mL of coupling buffer, before being blocked for their binding to the mAbs, by an incubation in 15 mL of blocking buffer (1M Tris-Base, pH9, in coupling buffer) for 2h at RT.

Three successive high-low pH are done to remove any IgG that might not have bound. The high-pH buffer is 100mM NaAc pH4 while the low-pH buffer is the coupling buffer. Finally, mAb-beads are resuspended in 1X PBS and poured into a glass column (BioRad #7372507).

Ab columns are regenerated after use by three successive high-low pH washes and a final large 1X PBS wash.

Trimer mimics production

Env trimers mimicking the native functional spike at the viral surface are in particular used to sort specific B cells. To be noted, we produce trimers based on 2 different platforms, SOSIP and NFL: SOSIP are trimers stabilized with mutations that cross-link the cleaved gp120 and gp41 through a disulfide bond (Sanders et al., 2013) while NFL refers to a Native Flexible Linker that stabilizes the trimer without any cleavage (Guenaga et al., 2016).

The trimers are produced in HEK293F cells, typically in 1L culture at 1.2×10^6 cells/mL. The transfection is done the following way: 5 min incubation of two tubes A and B containing respectively: 20 mL of OptiMEM media (Gibco™ #11058021) and 500 µg DNA (350 µg trimer DNA and 150 µg furin DNA if the trimer is a SOSIP) in A and 20 mL of OptiMEM media and 1 mL of Fectin transfection reagent (Invitrogen™ #12347500) in B (Table VI-3). Tube A is transferred into B. The mix goes through a 25 min incubation at RT before being poured on the cells. After 5 days at 37°C, the cells are centrifugated (4000 rpm, 25 min, RT), the supernatant harvested and filtered (0.22 µm) and a protease inhibitors cocktail (Roche #5056489001) is added to the filtrate. Then the filtrate goes through the affinity column overnight, by slow dripping. A lectin column (GE Healthcare #GE17-0444-01) is usually used for

the NFL constructs while a PGT145 mAb column is used for the SOSIP constructs. PGT145 is a mAb targeting the V1/V2 apex, which was chosen for the affinity column because it selects only closed trimeric conformations. The protocol for making Ab columns is detailed above. Columns are washed with at least 20 column-volume (CV) of 1X PBS. The lectin column is also washed with >10 CV of 1X PBS-NaCl 0.5M. The elution is done with 10 mL of 1M α -mannose-D-methylpyranoside for the lectin column and 3M MgCl₂ for the Ab column. The eluate is immediately buffer exchanged against 1X PBS. Then, it goes through a F105-column to carry out a negative selection of trimers that have an open conformation (F105 is a mAb that does not bind to the native trimeric Env but only to open conformations and msp120). The fraction that comes out of the column is concentrated, measured, and biotinylated ([Avidity Kit #NC9204985](#)) overnight at 4°C. A size-exclusion column finally enables the selection of trimers, which are buffer-exchanged against 1X TBS, aliquoted and frozen. Biotinylation and antigenicity are checked before use (Biotinylation test and ELISA).

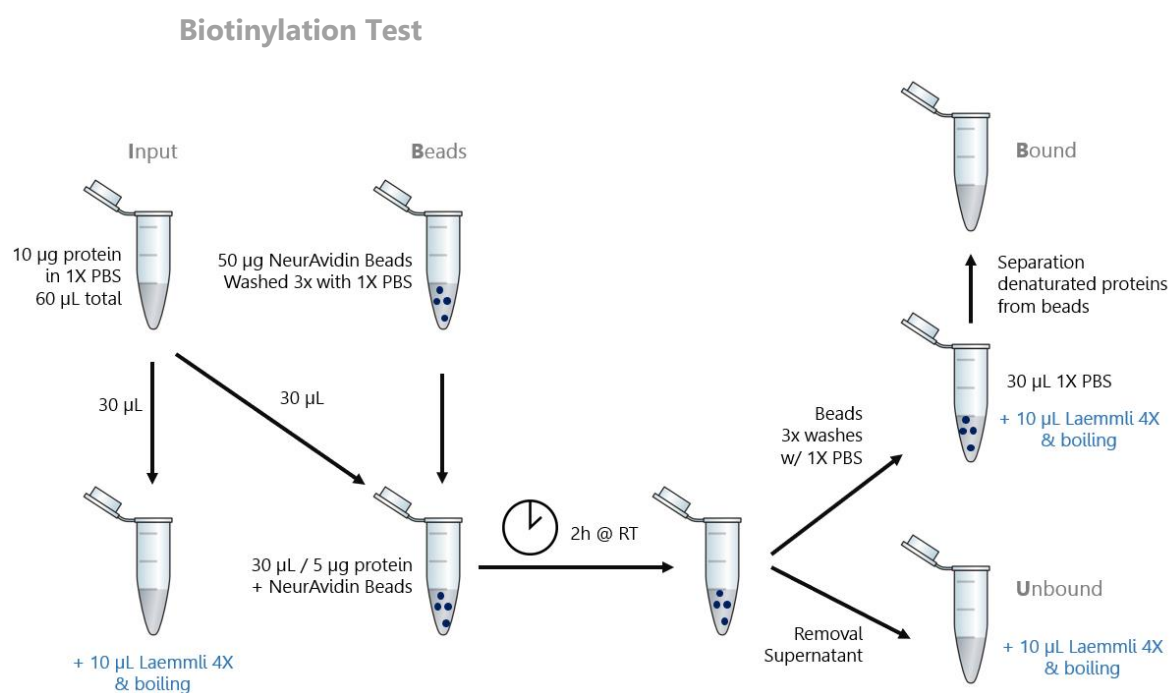


Figure VI-3: Scheme of the biotinylation test process. All denaturated fractions (Input, Unbound and Bound) are then ran in an acrylamid gel.

The proper biotinylation of the trimer is checked by a quick test based on Neuravidin Agarose ([Thermo Scientific #29200](#)). If properly biotinylated, the Env mimics can bind to the resin with which they are incubated (Fig VI-3). The percentage of biotinylated trimers is evaluated by the observation of the gel profiles of the Bound and Unbound fractions compared with the Input fraction.

ELISA (Enzyme-Linked Immunosorbant Assay)

An ELISA is done in order to verify the proper antigenicity of the purified trimers. 96-wells (96w) plates (Nunc® #P7491) are coated with streptavidin (2 µg/mL in 1X PBS ; Sigma-Aldrich #189730) overnight at 4°C. They are washed 3 times with 1X PBS-Tween 20 0.05%, before being blocked with 1X PBS-BSA 3% for 1h at RT (Sigma-Aldrich #a7906). After 3 new washes, the trimer is incubated at 2 µg/mL in 1X PBS-BSA 1%, 1h at RT. The washes are repeated, and serially-diluted mAbs are added. They are prepared by ¼ serial dilution in 1X PBS-BSA 1%, with a starting concentration of 10 to 40 µg/mL, and incubated for 1h at RT. Three washes are done again, before the addition of the secondary Ab: a goat alkaline-phosphatase conjugated anti-human Fc Ab (Jackson Immuno #109 055 097), diluted at 1/1000 in 1X PBS-BSA 1%, that is also incubated 1h at RT. Washes are repeated once again before addition of the PNPP reagent (Interchim #UP664791). Plates are read for their absorbance at 405nm. The binding results are compared with the neutralization results, as listed in the database CatNap from Los Alamos National Laboratory (<https://www.hiv.lanl.gov/components/sequence/HIV/neutralization/main.comp>).

F. B-cell sorting

Single B-cell specific isolation

B-cells of interest are sorted in a specific manner using a [BD FACSAria™ Fusion III Cell Sorter](#). The biotinylated protein-baits are initially bound to different streptavidins conjugated with diverse fluorochromes, for 30 min on ice (panel of streptavidins: [SA-AF647 ThermoFisher #S11223](#) ; [SA-AF488 ThermoFisher #S21374](#) ; [SA-BV421 BD Biosciences #563259](#) ; [SA-PE ThermoFisher # 12-4317-87](#) ; [SA-BUV395 BD #564176](#) ; [SA-PerCP-Cy5.5 ThermoFisher #45-4317-82](#)). The bait amount is calculated for a final staining concentration of 200nM (dilution in 1X PBS) and the bait:streptavidin ratio is 1:2 for Env trimers and 1:4 for mgp120. Those baits-streptavidin-fluorochromes conjugates and additional fluorochromes conjugated-antibodies able to recognize specific cell-surface antigens (used to identify memory B cells) are added at a 1:100 dilution and incubated with recently thawed PBMCs for 25 to 30 min on ice in the dark in FACS buffer (1X PBS + 1% FBS + 25 mM Hepes + 1 mM EDTA). To be noted, baits that are planned to be used for a negative selection are incubated before positive selection baits. Another staining is done, with 1:300 Live-Dead marker ([Invitrogen™ # L34966](#)) for 15 min on ice in the dark. The conjugated Abs are all supplied by BD Biosciences and enable the selection of: living cells (Live-Dead marker), various lymphocyte populations (negative [CD3-APC-Cy7 #560176](#), [CD8-APC-Cy7 #560273](#) and [CD14-APC-Cy7 #561384](#)) ; B lymphocytes ([CD19-PE-CF594 #562321](#) positive) ; memory B-lymphocytes (negative [CD20-PE-CF594 #562295](#)) ; IgG producer ([IgM-BV605 #562977](#) and [IgD-BV786 #740997](#) negative). Stained cells are kept on ice and preferentially in the dark until the sort.

Several checks are done prior to the sort : the machine is extensively cleaned (or even sterilized if the sort is followed by a cellular activation and culture), in both the fluidic and the optic parts ; the precision of the drop stream is adjusted thanks to fluorescent beads ([BD #345249](#)) ; and the compensations of the fluorescences of both conjugated-streptavidins and Abs are done with neutral beads staining ([Spherotech #TP-30-5](#); [BD #51-90-9001291](#)). Lymphocytes B of interest are selected depending on their binding or not to the chosen baits and sorted at one cell per well, with recording of the index sorting.

B-cell activation

Sorted B-cells are then cultured for activation: each cell is dropped in a well of a 384-w plate (Corning® #3701) containing 65 µL of freshly prepared activation media, which is composed of: 11.8×10^3 NIH 3T3-msCD40L feeder cells (Sigma Aldrich # 93061524 ; Kershaw et al., 2001), 50 ng/mL IL-2 (Roche #11147528001), 50 ng/µL IL-21 (Invitrogen PCH0215) and 0.1 ng/mL of mouse Ab anti-Human IgG in IMDM media (Jackson Immuno #109-006-127) completed with 10% FBS and mycoplasma antibiotic (MycoZap™ – Lonza # LT07-918). The plates that have received the B-cells are then incubated for 14 days, during which lymphocytes will get activated and release IgGs. This protocol is derived Huang et al., 2013.

G. Functional screening

Harvesting of B-cell supernatants

After the 14 days of activation, plates are centrifuged at 1200 rpm for 5 min. Supernatants potentially containing the soluble IgG are transferred into new 384-w plates (Corning® #3964) while the B-cells centrifuged to the bottom go through the RNA extraction and purification described below.

RNA extraction

This step uses the TurboCapture® 384 mRNA kit (QIAGEN #72271). Briefly, in an area that has been extensively decontaminated with anti-RNase, 20 µL of buffer TLC-2% β-mercaptoethanol are added into each well of the harvested culture plates and pipetted up and down to lyse the B cells. All lysis are transferred into 384-w plates with V-shaped bottoms containing immobilized oligo-dT which hybridize with the recently released mRNA (TurboCapture®). The process continues in those specific wells for 1h at RT, with agitation. mRNA being captured, the supernatants are eliminated by overthrowing and plates are dried by upside-down tapping. Wells are washed 3 successive times with 30 µL TCW buffer. The elution is done using 20 µL of TCE buffer, which action is improved by a 5-min incubation at 65°C (a thermocycler is used at this step). Plates are cooled down to RT and spined down briefly. Eluates are transferred into new 384-w plates from the kit and kept at -80°C.

Micro-ELISA

The activation of B-cells is checked by ELISA by measurement of the concentration of IgG in cultured B cell supernatants. Transparent 384-w plates (Corning® #3700) are coated overnight at 4°C with 10 µL at 2 µg/mL of Mouse or Goat anti- human IgG (Fc) in 1X PBS. A blocking step follows with a 1h-incubation at RT of 20 µL/well of 1X PBS-BSA 3%. Wells are washed 3 times with 50 µL of 1X PBS-Tween20 0.05% and dried with great care. Five µL of each IgG-supernatant incubates for 1h at RT. Wells are washed 3 times again and dried before the addition of the Alkaline Phosphatase-conjugated secondary Ab Goat anti-Human IgG (Fab) (Jackson Immuno #109 055 097), 10 µL at 1/5000 in 1X PBS-BSA 1%. Incubation is done for 1h at RT and is followed by 3 successive washes and meticulous drying again. Substrate is added at a volume of 20 µL per well and plates are kept 30 to 45 min in the dark before absorbance is measured at 405 nm.

Micro-Neutralization assay

The supernatants of the activated B-cells are tested for functional activity in a micro-neutralization assay. In a 384-w white plate (Corning® #3570), 10 µL of each supernatant are mixed with 10 µL of high-titer pseudovirus solution (>600 000 RLA) for an incubation of 45 min at 37°C. At the same time, TZMbl cells are detached, counted, diluted at 0.3×10^6 /mL in DMEMc and then distributed at 10 µL per well (3 000 cells per well total). The mix incubates for 2 days at 37°C. Then supernatants are harvested and 30 µL of lysis buffer containing luciferase substrate (Promega Bright-Glo™ Luciferase Assay System # E2610) are added in each well for immediate reading. Values are compared with those of negative control wells containing pseudovirus and cells only (RLU corresponding to 0% neutralization) to identify wells with neutralizing activity.

RNA from wells selected through this screening will be transferred in 96-w plates and go through the following Ab gene recovery and cloning pipeline.

H. Ab gene recovery, cloning and production

This pipeline, originally inspired by the work of [Tiller et al., 2008](#), has been highly improved in the past decade, notably by our collaborators of the Neutralizing Antibody Center in Scripps Research CA. The most recent version of the pipeline is probably the one described in [Rogers et al., 2020](#).

RT-PCR

Retro-transcription is done with the entire RNA extraction volume obtained with the use of the TurboCapture® 384 mRNA kit.

cDNAs are synthesized in 96w plates with a reaction volume of 25 µL per well, with the SSIV enzyme ([ThermoFisher #18090200](#)) and its buffer, dNTPs ([ThermoFisher #10297117](#)) and random hexamers ([ThermoFisher #SO142](#)). The thermocycle program is : 10 min at 42°C + 10 min at 25°C + 60 min at 50°C + 5 min 94°C + hold at 4°C. The newly synthesized cDNA are kept at +4°C, or -20°C for longer conservation, before being proceeded into PCR1.

PCR 1 and 2 for sequencing (PCR 2.seq)

Both reactions are nested-PCRs aimed at amplifying the cDNA obtained at the previous step (Fig VI-4). The PCR1 is operated with the polymerase enzyme HotStar ([HotStarTaq Master Mix Kit – QIAGEN #203445](#)), dNTP and the primers set described in [Table VI-2](#) used at 0.5 µM. The thermocycler program is : 94°C 5 min + (94°C 30sec + 58°C 30sec + 72°C 1 min) x50 + 72°C 5 min + hold 4°C.

The PCR 2.seq protocol is similar than the PCR1, only the primer sets are different ([Table VI-2](#)). PCR efficiency is assessed using 96-w E-gels ([ThermoFisher #G720802](#)).

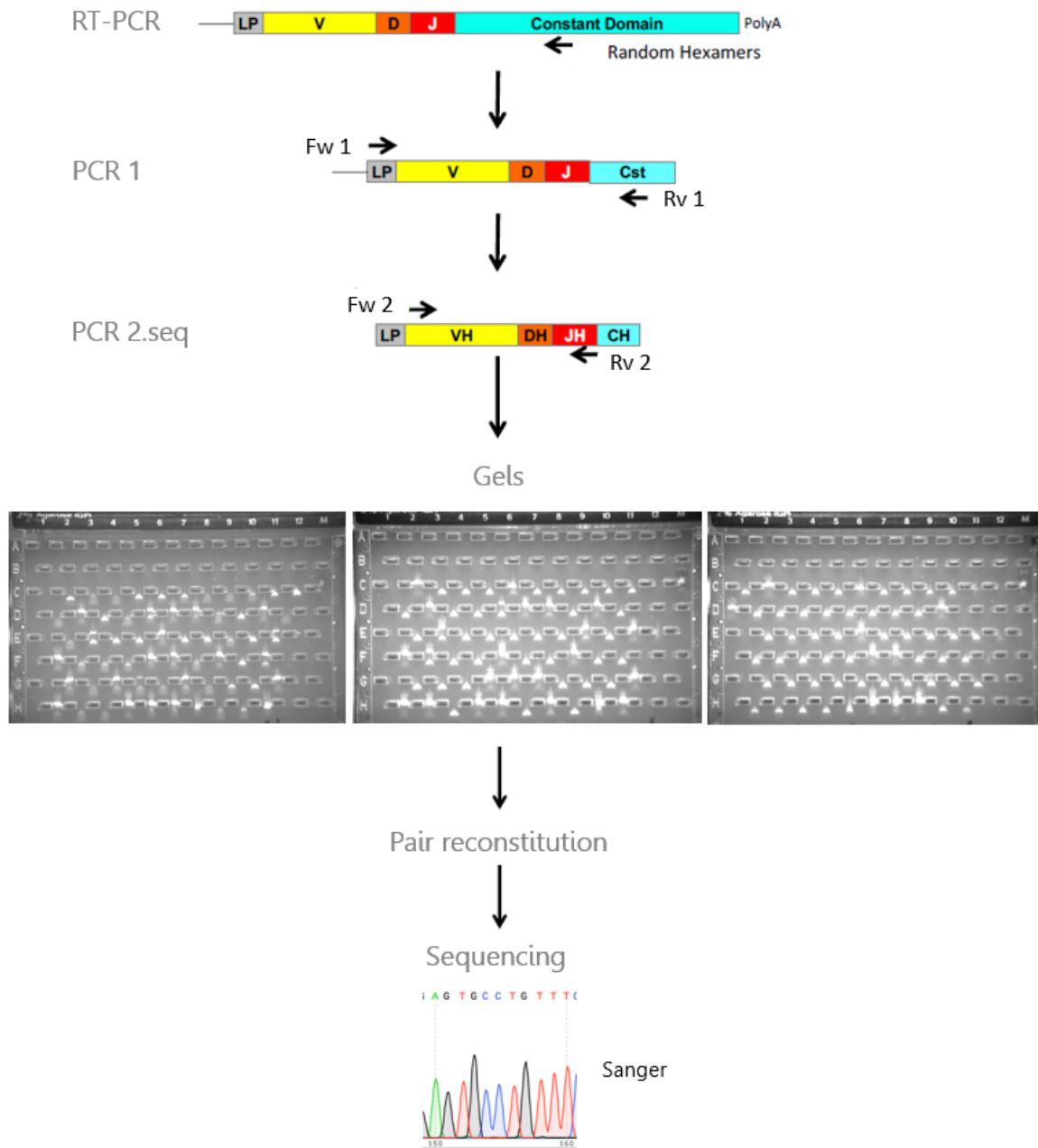


Figure VI-4: Principle of the nested PCRs for Ig HC, KC or KC gene recovery. Inspired from an illustration of Elise Landais, PhD, from IAVI San Diego (2019).

Sequencing

Ig genes sequences are identified by i) plate-sequencing operated by Genewiz with the Sanger method - from the primers described in Table VI-1 - and ii) analysis by the ImMunoGeneTics (IMGT) database : http://www.imgt.org/IMGT_vquest/vquest.

Step	Chain & Sens	Primer Name	Seq 5' --> 3'	
RT-PCR	H K and L Rv	Random Hexamers		
PCR 1	H Fw	5' L-VH 1	ACAGGTGCCCACTCCCAGGTGCAG	
		5' L-VH 3	AAGGTGTCCAGTGTGARGTGCAG	
		5' L-VH 4/6	CCCAGATGGGTCTGTCCCAGGTGCAG	
		5' L-VH 5	CAAGGAGTCTGTCCGAGGTGCAG	
	H Rv	3' CH1	GGAAGGTGTGCACGCCGCTGGTC	
	K Fw	5' LVK 1/2	ATGAGGSTCCCYGCTCAGCTGCTGG	
		5' LVK 3	CTCTTCTCCTGCTACTCTGGCTCCCAG	
		5' LVK 4	ATTTCTCTGTTGCTCTGGATCTCTG	
	K Rv	3' Cκ 543	GTTTCTCGTAGTCTGCTTTGCTCA	
	L Fw	5' L Vλ 1	GGTCCTGGGCCAGTCTGTGCTG	
		5' L Vλ 2	GGTCCTGGGCCAGTCTGCCCTG	
		5' L Vλ 3	GCTCTGTGACCTCCTATGAGCTG	
		5' L Vλ 4/5	GGTCTCTCTCSCAGCYTGTGCTG	
		5' L Vλ 6	GTTCTTGGGCCAATTTTATGCTG	
		5' L Vλ 7	GGTCCAATTCYAGGCTGTGGTG	
		5' L Vλ 8	GAGTGGATTCTCAGACTGTGGTG	
		L Rv	3' Cλ	CACCAGTGTGGCCTTGTGGCTTG
	PCR 2.seq	H Fw	5'Agel VH1/5	CTGCAACCGGTGTACATTCCGAGGTGCAGCTGGTGCAG
			5'Agel VH3	CTGCAACCGGTGTACATTCTGAGGTGCAGCTGGTGGAG
5'Agel VH4			CTGCAACCGGTGTACATTCCAGGTGCAGCTGCAGGAG	
5'Agel VH3-23			CTGCAACCGGTGTACATTCTGAGGTGCAGCTGTTGGAG	
5'Agel VH4-34			CTGCAACCGGTGTACATTCCAGGTGCAGCTACAGCAGTG	
H Rv		3' IgG (internal)	GTTCCGGGAAGTAGTCCTTGAC	
K Fw		5' Pan VK	ATGACCCAGWCTCCABYCWCCCTG	
K Rv		3' CK 494	GTGCTGTCCTTGCTGTCCTGCT	
L Fw		5' Agel VL 1	CTGCTACCGGTTCTGGGCCAGTCTGTGCTGACKCAG	
		5' Agel VL 2	CTGCTACCGGTTCTGGGCCAGTCTGCCCTGACTCAG	
		5' Agel VL 3	CTGCTACCGGTTCTGTGACCTCCTATGAGCTGACWCAG	
		5' Agel VL 4/5	CTGCTACCGGTTCTCTCSCAGCYTGTGCTGACTCA	
		5' Agel VL 6	CTGCTACCGGTTCTTGGGCCAATTTTATGCTGACTCAG	
		5' Agel VL 7/8	CTGCTACCGGTTCCAATTCYAGRCTGTGGTGACYCAG	
L Rv	3' XhoI CL	CTCCTCACTCGAGGGYGGGAACAGAGTG		
Sequencing for gene identification (post PCR2.seq)	H Rv	PCR2clon_HC_Rv	GAAGTAGTCCTTGACCAG	
	K Rv	PCR2clon_KC_Rv	TAGAAGTTATTCAGCAGGCAC	
	L Rv	PCR2seq_LC_Rv	GGGYGGGAACAGAGT	
Sequencing for gene insertion checking (post PCR2.clon and cloning)	H Rv	PCR2clon_HC_Rv	GAAGTAGTCCTTGACCAG	
	K Rv	PCR2clon_KC_Rv	TAGAAGTTATTCAGCAGGCAC	
	L Rv	PCR2clon_LC_Rv	TGTGATGCTATTGCTTTATTGTA	

Table VI-2: Synthesis of the primers used for the Ab gene recovery pipeline. They have all been synthesized by the company Genewiz, and purified with a method based on HPCL. Primers alignments maps can be found in Annex III.

Preparation of cloning vectors

Cloning is done using a seamless restriction-independent method based on homologous recombination.

Opening of the HC expression vector is done by double digestion with the XhoI and NheI restriction enzymes (NEB #R0146 ; NEB #R3131), the LC vector is opened using AgeI and XhoI (NEB #R3552 ; NEB #R0146) and the KC vector using AgeI and BsiWI (NEB #R3552 ; NEB #R3553). All digestions are done in CutSmart Buffer (NEB #B7204) at 37°C for 2 hours. Enzymes are inactivated by a 15-min incubation at 65°C and mixes are cooled down on ice for 5 min. Digested vectors are dephosphorylated by the Antarctic enzyme (NEB #M0289) in its appropriate buffer for 1h at 37°C. The phosphatase is inactivated as well before the samples are run on a 1% agarose gel and purified (QIAquick Gel Extraction Kit from QIAGEN #28706).

PCR 2 for cloning (PCR 2.clon)

PCR 2.clon is done from PCR1 products, with a single pair of primers, specific of the gene identified by the sequencing post-PCR2.seq, but modified to include additional nucleotides. Those inserted sequences at both extremities overlap with the chain expression vector (Fig VI-5). Primers are used at 0.5 µM for the PCR reaction. The enzyme used is the Phusion (Thermo Scientific™ # F532S). PCR 2.clon products are purified thanks to E-gel™ CloneWell II (Invitrogen # G661818).

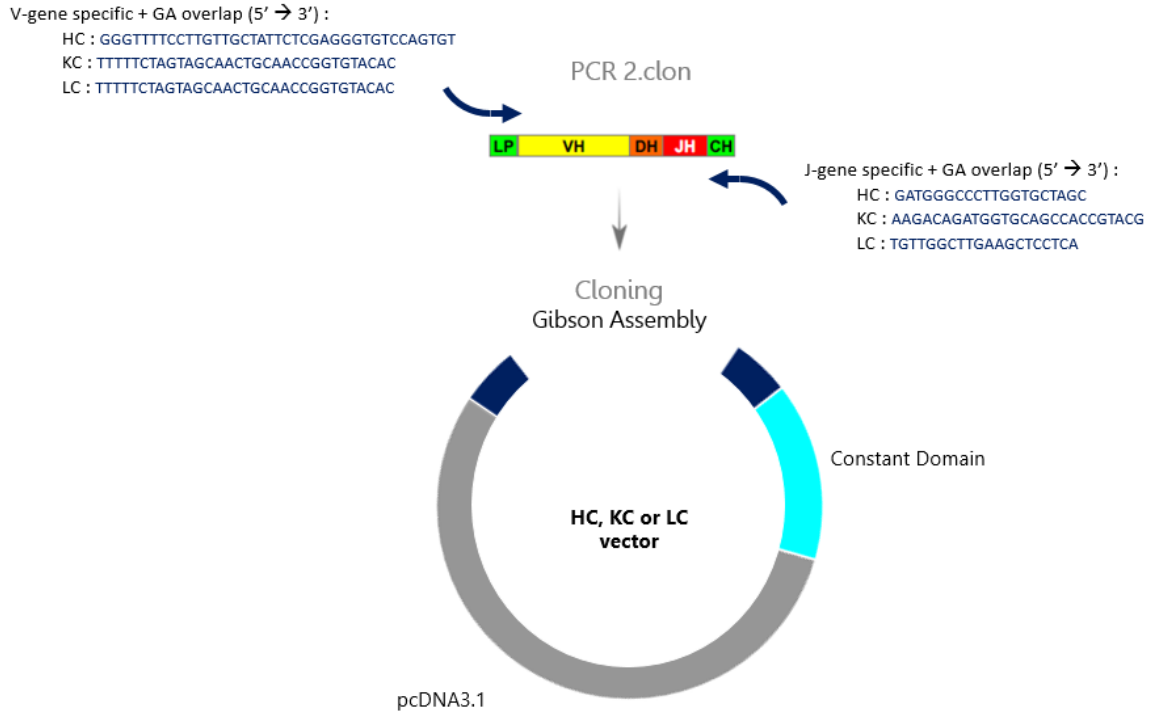


Figure VI-5: Concept of the PCR2.clon.

Cloning

- **Gibson Assembly kit**

The cloning of the insert obtained from PCR2.clon in the open vector (mammalian expression vector with HC, KC or LC constant part) is done by mixing 15ng of insert with 50ng of cut vector (molar ratio 3:1). Gibson Assembly Mastermix ([NEB # E2611L](#)) is added and the insertion happens in 1 hour at 50°C. Three µL of recombination product is added to 25 µL of competent *E.coli* DH5α for transformation. Resulting colonies are screened by amplification, extraction and sequencing, to ensure the proper insertion of the Ig variable part into the Ig expression vector.

- **T4 ligase kit**

The chain sequences that could not be properly cloned with the Gibson Assembly were cloned by ligation. The insert with the 5' and 3' overlap from the PCR2.clon first goes through a double-digestion step (as a reminder: XhoI and NheI for HC, AgeI and BsiWI for KC and AgeI and XhoI for LC) in order to ensure a perfect match with sticky ends for both the vector and the insert. Then 15ng of insert are mixed with 50ng of cut vector before the addition of the T4 ligase buffer and enzyme ([ThermoFisher #E10011](#)). Ligation happens in 30 min at 22°C and the whole product is transformed in *E.coli* DH5α. Resulting colonies are screened as above.

All antibodies are reconstituted as IgG1 ([Doria-Rose et al., 2014](#)). Successful cloned plasmids are further amplified for transfection.

Ab production for high yield and purity

Abs are produced in HEK293F cells after co-transfection with both plasmid constructs coding for heavy and light chains (Fig. VI-2). 50 mL of 293F cells culture at 1.2×10^6 cells/mL are prepared ; the dilution has to be made with at least 50% fresh 293 Freestyle media (Gibco™ #12338018). Fifteen µg of heavy chain DNA and 15 µg of light chain DNA are mixed in 1 mL of OptiMEM media (Gibco™ 11058021) in a first tube (A), and filtered in 0.2 µm (Millipore # UFC30GV0S), 5 min at 12 000 rpm, in order to ensure the sterility of the mix. In a second tube (B), 50 µL of 293Fectin™ (Invitrogen™ 12347500) are diluted in 1 mL of OptiMEM media and incubated 5 min at RT (Table VI-3). Tube A content is transferred into tube B, and the mix incubates during 25 minutes at RT, before being added onto the HEK293F cells. Cells are cultured for 3 to 4 days, at 37°C, with agitation. Then, the cell culture is centrifuged for 10 min at 2500 rpm. Pelleted cells are trashed while the supernatants containing the Abs are filtered through 0.2 µm. The Ab purification is done on a Poly-prep column (BioRad #7372507) containing sepharose beads recovered of protein A (GE Healthcare # GE17-1279-03), which binds specifically to the Ab Fc part. The column is equilibrated with 1X PBS before the loading of the supernatant, which go through the column by gravity flow. Successive washes are made: 1X PBS, 1X PBS + 0.5 M NaCl, 1X PBS again. The elution is made with 15 mL of glycine 0.1M at pH2.5, neutralized with 4.5 mL of Tris 1M at pH9 directly after. Eluate is buffer-exchanged against 1X PBS thanks to centrifugal filter (Amicon® #UFC903008) with 3 to 4 successive centrifugations (15 min, 4200 rpm). The Ab solution is concentrated and filtered through 0.22 µm. Ab concentration is measured by absorbance at 280nm via nanodrop.

Ab production for quick screening

Abs can also be produced in HEK293T cells, with a protocol similar to the pseudovirus production, for a rapid preliminary screening. Cells are prepared the day before the transfection in 6-w plates (Costar® #3516), 2 mL per well at 0.5×10^6 cells/mL. For the transfection : 50 µL of OptiMEM transfection media are added in two 1.5 mL tubes A and B. In tube A are added 2 µg of each plasmid encoding both heavy and light chains of the Ab to produce. In tube B are added 10 µL of HelixIN transfection reagent. Both tubes incubate for 5 min at RT before tube A is transferred into tube B, which incubates another 25 min at RT before being added to the HEK293T cells (Table VI-3). Production is boosted by addition of 200 µL of 100X HIB boost right after the transfection mix. Supernatant containing the newly produced Abs are harvested 3 to 4 days after the transfection.

The production is checked by ELISA (see part D.) with a first step of coating of 2 µg/mL of goat anti-Human Fc IgG. The supernatants can be used pure in a neutralization assay.

Protein to produce	Volume & Cells to transfect	Volume OptiMEM each tube	Volume plasmid DNA 1 in tube A	Volume plasmid DNA 2 in tube A	Volume transfection reagent in tube B
<i>Env trimer</i>	500 mL HEK293F	10 mL	250 µg NFL-DNA or 175 µg SOSIP-DNA	75µg Furin DNA if SOSIP	500 µL 293Fectin
<i>Env trimer</i>	1L HEK293F	20 mL	500 µg NFL-DNA or 350 µg SOSIP-DNA	150 µg Furin DNA if SOSIP	1 mL 293Fectin
<i>Pseudovirus</i>	2 mL/ 2 million HEK293T or S	100 µL	4 µg PSG3 vector	2 µg env plasmid	18 µL HelixIN
<i>Pseudovirus</i>	10 mL/ 8 million HEK293T or S	400 µL	20 µg PSG3 vector	10 µg env plasmid	100 µL HelixIN
<i>mAb</i>	50 mL HEK293F	1 mL	15 µg HC plasmid	15 µg KC or LC plasmid	50 µL Hype293
<i>mAb</i>	100 mL HEK293F	2 mL	25 µg HC plasmid	25 µg KC or LC plasmid	100 µL Hype 293
<i>mAb</i>	2 mL/ 8 million HEK293T	100 µL	3 µg HC plasmid	3 µg KC or LC plasmid	18 µL HelixIN

Table VI-3: Summary of the most commonly used transfection protocols during this project.


I. Ab characterization

ELISA

Protocol for this ELISA is extremely similar to the one used for trimer characterization (part D.), except the primary step of coating is done with mgp120 or goat anti-human IgG (Fc)(Jackson Immuno # 109-001-008), also concentrated at 2 µg/mL.

Neutralization assay (mAb)

Serial 5-fold dilutions of Abs starting from 100 µg/mL (screened Abs and their mutants) or 10 µg/mL (known bNAbs) are let in contact with the pseudoviruses for 1h at 37°C before the mix is added to TZMbl cells prepared the previous day in 96-w white plates (Greiner #675083). Plates are incubated 24h at 37°C, protected from evaporation, then cells are fed again with 75 µL of DMEMc, and incubated for another 48h. Finally, medium in each well is aspirated and replaced by 45 µL of 1X cell lysis buffer (Ozbioscience # LUC1000) which is left to act during 15 to 60 min under agitation. As in the titration protocol, 30 µL of luciferin substrate are then added and RLU is measured instantly by a luminometer. Neutralizing activity of the Abs is defined by the reduction of virus infectivity *in vitro*, calculated as a percentage of neutralization obtained with the following formula: $[(\text{RLU when no Abs} - \text{RLU when diluted Abs}) / \text{RLU when no Abs}] * 100$. The slope of the neutralization curve obtained is used to determine the IC50 of the antibody, i.e. the concentration required to reach 50% of neutralization.



Results and discussion

I. Donor PC02

A. Preliminary Data

1. PC02, 4th best neutralizer of the Protocol C cohort

PC02 is a male individual infected by a clade A1 HIV-1, whose longitudinal serum samples displayed high levels of neutralization breadth, as evaluated through the measurement of a neutralization score (Landais et al., 2016).

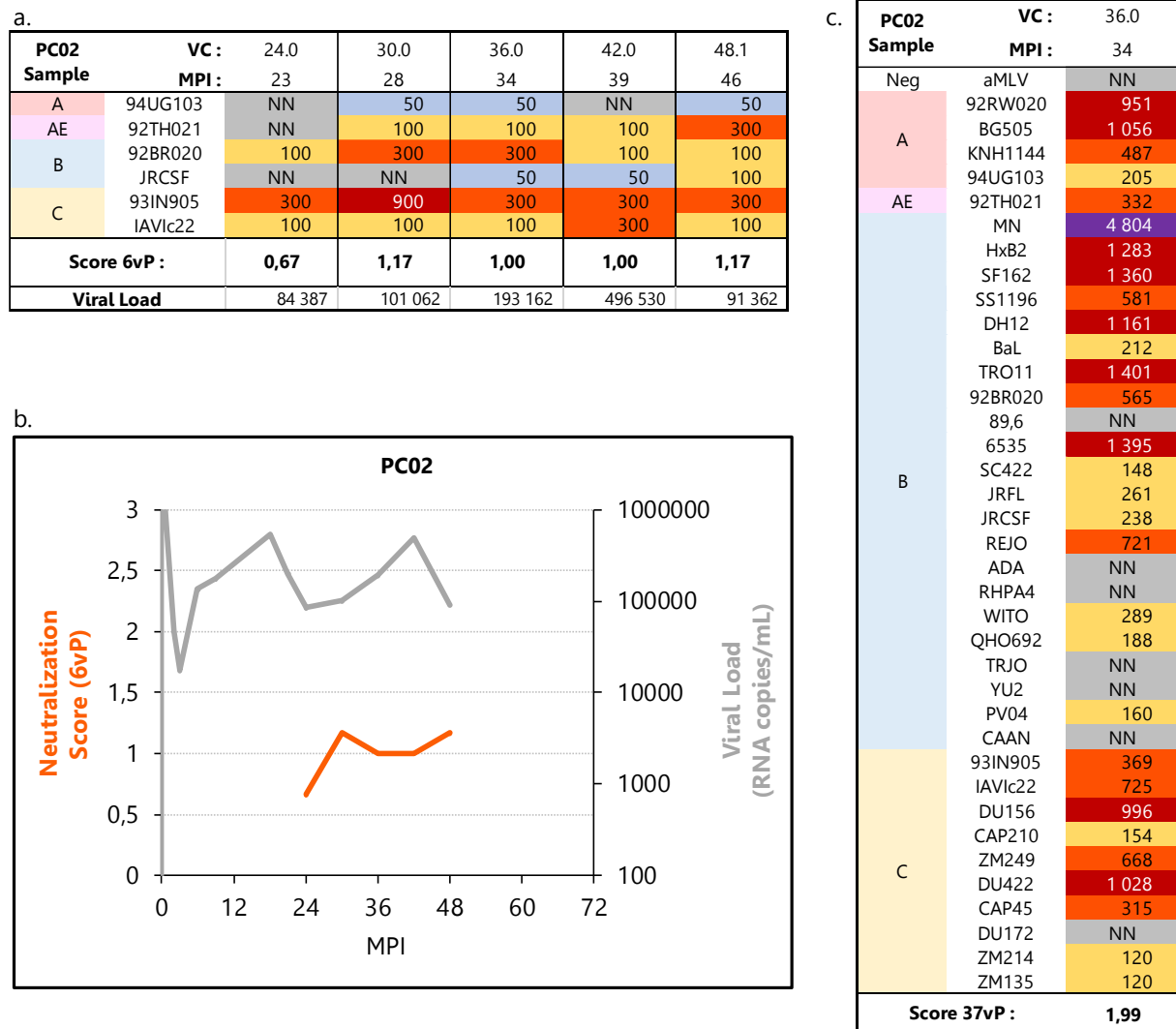


Figure VII-1: PC02 is an elite neutralizer. a : neutralization ID50 and scores of five time-point PC02 serum samples against the 6vP. b : longitudinal evaluation of the 6vP neutralization score and viral load. c : neutralization ID50 and score of v36 PC02 serum sample (34 MPI) against the 37vP. VC = Visit Code ; MPI = months post infection. Data from Landais et al, PLOS Pathogens, 2016.

This score of neutralization, as first described by Simek et al., 2009, gives information on both the breadth (number of virus neutralized) and potency (ID50) of a serum (see Materials and methods), it is calculated from results of neutralization on a small virus panel in order to evaluate potential overall breadth and potency. A score of 1 on a specific 6-virus panel (6vP, see Materials and methods) predicts for instance a neutralization of more than 50% of viruses among larger panels at an ID50 greater than 1/100. In the evaluation of donors from the Protocol C cohort, 2 panels of viruses were used to calculate a neutralization score: the 6vP and the 37vP (Landais et al., 2016).

The PC02 serum was first tested against the 6vp at 23 months post-infection (MPI), giving a neutralization score of 0.67. At 28 MPI, the score reached 1.17 (Fig. VII-1, a), slightly decreasing to 1 at 36 and 42 MPI, before reaching 1.17 again at 48 MPI. Against the 37vP, the 34 MPI serum gave a score of 1.99, with 30/37 virus neutralized, corresponding to a breadth of 81% (Fig. VII-1, c). On this panel, the 34 MPI sample appeared to neutralize the different clades in an identical manner. Eventually, the donor PC02 was found to be the 4th best neutralizer of the protocol C cohort, making him a candidate of great interest for the study of the mechanisms of development of neutralizing breadth and potency.

2. Preliminary mapping of PC02 BNAb specificity(ies)

Several Protocol C donors BNAb lineages were successfully isolated thanks to a specific strategy based on a differential sorting (MacLeod et al., 2016) (Umotoy et al., 2019). The principle is to specifically sort B cells that bind to wild-type (WT) probe(s) - Env trimer mimics or monomeric gp120 - but not to corresponding knocked-out (KO) probe(s) mutated in the specific epitope-binding region (Fig. VII-2). The application of such strategy accordingly requires beforehand the identification of the precise Env epitope targeted by the BNAb in the serum. In a view to use the differential sorting approach to isolate PC02 BNAb, the Ab specificities involved in the breadth and potency of the donor serum were mapped (Table VII-1).

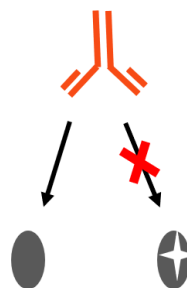


Figure VII-2: Principle of the epitope-based sorting strategy. Mgp120 probes WT (left) and mutated (right).

Antibody specificities associated with the plasma broad neutralization were subsequently evaluated (Landais et al., 2016).

Donor	VC	gp120 adsorption	CD4 binding site		gp41-MPER	Glycan-dependent epitopes					Dominant Specificity	
			b6 effect	RSC3 compet.	MPER peptide compet.	Kifunensine effect	N160 Glycan	166 169 171	N332 Glycan	Other Supersite PNGs		gp120/gp41 Interface residues
PC002	36	-	-	NB	-	+	-	-	+/-	+/-	+/-	Quaternary Kif+

Table VII-1: Specificities mediating neutralization breadth and potency of donor PC02 serum. Symbols recapitulate the strength of the phenotypes tested using the different approaches detailed below. Absent (-), very weak (+/-), weak (+), moderate (++) , strong (+++), phenotype was attributed based on i) the median fold or average percent decrease in ID50 and ii) the fraction of viruses which neutralization ID50 was decreased <2 fold, <10 <50 fold or <20%, <40%, <60%, <80%. NB= not binding. A dominant specificity was attributed based on results from all these experiments. From Landais et al, PLOS Pathogens, 2016.

To start with, the 34 MPI PC02 plasma (identified as “visit code” v36) was incubated with monomeric recombinant gp120 (mrgp120) coated on beads in order to remove all mrgp120-directed Abs (the absence of binding was verified in ELISA). The adsorbed fraction was then tested for neutralization on a 16vP of cross-clades HIV-1. Almost complete neutralizing activity was retained by the v36 PC02 serum after depletion on mrgp120. In order to verify that the results were not biased by the viral strain of the mrgp120 used (i.e. in case the broad specificity would not bind to this particular strain), a total of five different strains were used for adsorption (Table VII-1). Overall, the data suggested the presence of quaternary and/or gp41-specific BNABs in this sample.

The second option – the presence of gp41-MPER specific BNABs – was evaluated through measuring the neutralizing activity of the PC02 serum against HIV-2 chimeric pseudoviruses bearing either the full or partial HIV-1 MPER. While the HIV-2 WT could not be neutralized by the PC02 plasma, as expected, the chimera bearing the complete HIV-1 MPER region of HIV-1 YU2, named as HIV-2 C1, could be neutralized with an ID50 of 1/219 (Fig S9 Landais et al., 2016). As neutralization of the HIV-2 chimera is highly sensitive, in order to verify the presence of a broad MPER specific neutralizing activity, a competition with an MPER peptide was performed. This latter experiment did not show a reduction of the neutralizing activity of PC02 plasma against HIV-2 C1 (Table VII-1), and the presence of BNABs targeting the MPER was thus not confirmed.

Further, the presence of CD4 binding site (CD4bs) specific BNABs in the PC02 plasma was assessed, using a Resurfaced Stabilized Core 3 (RSC3), a probe selective for VRC01-like CD4bs BNABs, and its KO mutant which has a decreased CD4bs BNABs binding capacity. PC02 plasma had no reactivity against RSC3 nor KO-RSC3 (Table VII-1), leading to the conclusion of the absence of VRC01-like Abs. It was however suggested that some CD4bs BNABs may not bind this probe. Therefore, a plasma rgp120 adsorption in the presence of the non-broadly neutralizing Ab b6 at saturating concentrations was performed. The adsorption on rgp120 of the broad plasma neutralizing activity of PC02 was not significantly inhibited by the presence of b6 (Table VII-1). Thereby the presence of BNABs targeting the CD4bs was not confirmed.

The next query concerned the N-glycans dependency of the PC02 plasma neutralizing activity. Notably, PG9-like BNABs recognizing quaternary epitope at the Env V2 apex region require the N-linked glycosylation site at residue 160 while another class of BNABs recognizes the high-mannose (HM) patch centered around the N332 glycan in the V3 region. Those BNABs are sensitive to the pseudovirus treatment by the glycosidase inhibitor kifunensine. The PC02 plasma showed sensitivity to the kifunensin treatment, however it did not appear to require the N160 nor any of the N332 supersite glycans for its broad neutralizing activity. Overall the data suggest an epitope made of Man₅GlcNac₂, hybrid or complex glycans (because their absence affect PC02 serum neutralizing activity) but does not seem located at the usual glycosylated sites, aka the apex or the HP patch.

Finally, mutations of residues at the gp120/gp41 interface area, known to abrogate neutralization by quaternary BNABs such as PGT151, did not lead to any decrease in PC02 serum neutralizing activity. Hence, the presence of BNABs directed at the interface could not be confirmed either.

To conclude, the preliminary mapping done by [Landais et al., 2016](#) could not precisely identify the preferential binding region for PC02 BNABs, which was thus defined as an undefined quaternary epitope sensitive to kifunensin treatment (that prevent the formation Man₅GlcNac₂, hybrid or complex glycans, see Materials and methods) (Table VII-1).

B. Further mapping of PC02 BNAbs specificities

1. Mapping potential BNAbs specificities targeting the gp41/gp120 interface

As the preliminary mapping of the PC02 serum suggested the presence of BNAbs directed at a quaternary epitope on Env, we first studied epitopes corresponding to known quaternary BNAbs, at the gp120/gp41 interface and trimer apex (Table III-4 and III-2). To start, a survey of papers describing interface-targeting BNAbs was done, in order to list all Env residues implicated in the contact or binding involved in BNAbs interactions with the interface region of Env. The residues that appeared the most frequently were selected and those for which HIV-1 Env sequences mutated at those positions were available in the laboratory were transfected to produce and test the corresponding mutant pseudoviruses in a neutralization assay, as compared to pseudoviruses bearing WT gp120 (Table VII-2). Using this method, a strong decrease in serum ID50 is expected when a significant fraction of the serum neutralization is due to Nabs sensitive to the corresponding amino acid change. To be noted, residue positions cited throughout this manuscript correspond to the HxB2 numbering.

Pseudovirus		Fold Decrease ID50		
Strain	Mutation	Mean	+/-	SD
JRCSF	E83A	1,44		
JRCSF	E87A	0,80	+/-	0,22
JRCSF	N88A	0,61		
JRCSF	T90A	0,49	+/-	0,28
JRCSF	N241A	1,50		
JRCSF	V506A	0,02	+/-	0,02
JRCSF	A512W	0,69	+/-	0,18
BG505	A512W	1,26		
JRCSF	V513W	0,17	+/-	0,09
JRCSF	G514W	0,79	+/-	0,11
JRCSF	I515W	1,33	+/-	0,64
JRCSF	G516W	0,86	+/-	0,26
JRCSF	F517W	0,88	+/-	0,20
JRCSF	L518W	0,68	+/-	0,24
JRCSF	L520W	0,63	+/-	0,12
JRCSF	G521W	0,29	+/-	0,07
JRCSF	G524W	0,38	+/-	0,14
JRCSF	A525W	0,37	+/-	0,03
JRCSF	S528W	0,63	+/-	0,11
JRCSF	A533W	0,10	+/-	0,04
JRCSF	T536W	0,00	+/-	0,00
JRCSF	L537W	0,46	+/-	0,17
JRCSF	T538W	0,46	+/-	0,04
JRCSF	Q540W	0,49	+/-	0,06
JRCSF	N611A	2,43		
92BR020	N611A	1,47		
BG505	N611A	1,44		
BG505	N611A-N637A	0,30		
JRCSF	N616A	1,63	+/-	0,23
JRCSF	S618A	1,74		
BG505	N625A	0,40		
BG505	E647A	0,36		

Impact of the mutation on the neutralizing activity

Ratio ID50 WT / ID50 mutant

	<0,5	gain
	0,5-2	neutral
	2-20	loss
	>20	severe loss

Table VII-2: Fold Decrease in ID50 due to the mutation at the interface : mean and standard deviation (if experiment repeated). All experiments were done on PC02 serum v48.1 (46 MPI) and ID50 calculated with Excel.

As shown in Table VII-2, none of the mutation tested appeared to have any significant impact on neutralization by the PC02 serum. Only the removal of the N-glycan at position 611 led to a slight decrease in ID50, although this effect was seen with JRCSF but not 92BR020 nor BG505, somehow excluding an involvement of this change in the binding of PC02 BNABs. Overall, the results suggested that the BNABs involved in the PC02 serum breadth did not correspond to known specificities directed at the gp120/gp41 interface. We therefore shifted our attention towards the apex region of Env, made of the V1/V2 loops.

2. Mapping potential BNABs targeting the apex

The same procedure was applied to the Env apex area, i.e. listing of the Env residue implicated in the binding of previously described apex-targeting BNABs, production of pseudovirus bearing Env with changes at those positions and testing in neutralization assays in comparison to pseudoviruses with WT Env.

Pseudovirus		Fold Decrease ID50		
Strain	Mutation	Mean	+/-	SD
JRCSF	K121A	0,49		
JRCSF	V127A	0,04	+/-	0,01
JRCSF	N160A	0,09		
BG505	N160K	1,94		
JRCSF	T162A	0,11	+/-	0,15
BG505	T162A	0,28		
JRCSF	L165A	0,27		
BG505	L165K	0,17		
JRCSF	R166A	0,37	+/-	0,08
JRCSF	D167A	0,19	+/-	0,03
BG505	D167A	0,55		
JRCSF	K168A	0,44	+/-	0,18
JRCSF	K169A	0,32	+/-	0,37
BG505	K169A	0,83		
BG505	Q170A	0,92		
BG505	K171A	1,14		
BG505	V172E	1,55		
JRCSF	Y173A	0,06	+/-	0,02
BG505	N234A	0,60		
BG505	S241N	0,43		
JRCSF	P299A	0,87		
JRCSF	K305A	0,04		
JRCSF	I307A	0,01	+/-	0,01
JRCSF	I309A	0,01	+/-	0,00
JRCSF	F317A	0,01		
JRCSF	N392A	0,38	+/-	0,19
JRCSF	I420A	0,04	+/-	0,06
JRCSF	I423A	0,01	+/-	0,01
JRCSF	I424A	0,02	+/-	0,01

Impact of the mutation on the neutralizing activity

Ratio ID50 WT / ID50 mutant

	<0,5	gain
	0,5-2	neutral
	2-20	loss
	>20	severe loss

Table VII-3: Fold Decrease in ID50 due to the mutation at the apex: mean and standard deviation (if experiment repeated). All experiments were done on PC02 serum v48.1 (46 MPI) and ID50 calculated with Excel.

All tested pseudoviruses with Env changes at the apex appeared to be neutralized equally to WT Env pseudoviruses (Table VII-3), thus somehow eliminating the hypothesis of the apex being the preferential epitope for PC02 BNABs (at least based on the mutations tested, affecting known apex BNABs, and not impacting viral entry).

Those results led us to wonder whether the neutralizing activity of PC02 BNABs would be prevented by parasitic binding of non-neutralizing Abs (NNABs) that would outcompete for the binding of the same epitope. In order to dismiss this assumption, the serum was first specifically depleted of Abs directed against mgp120 (JRFL strain; Fig. VII-3, a) and the adsorbed fraction was tested in a neutralization assay in parallel to the serum full fraction.

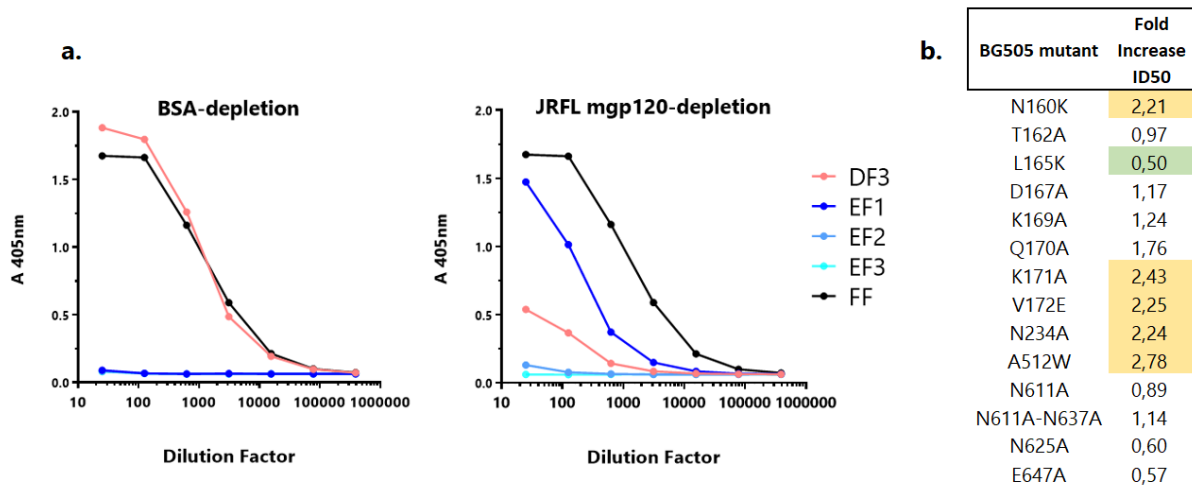


Figure VII-3: a. Indirect ELISA confirming the depletion of the serum from the mgp120-specific Abs. The protein coated initially is JRFL mgp120, the same as that of the depletion. BSA = Bovine Serum Albumine (negative control), DF = Depleted Fraction, EF = Elution Fraction, FF = Full Fraction. b. Fold Increase in serum ID50 due to the mgp120 depletion (ID50 Depleted Fraction / ID50 Full Fraction) for the neutralization of several BG505 mutants. All experiments were done with PC02 serum v48.1 (46 MPI) and ID50 calculated with Excel.

The neutralizing activity of the mgp120-specific-Abs depleted fraction was not significantly different than that of the full serum fraction, as shown Fig. VII-3, b. Hence, the hypothesis of the presence of NNABs preventing the action of the BNABs in the assay against apex mutants was rejected.

3. Studying the implication of glycans in recognition by PC02 BNAbs specificities

Preliminary mapping data about the glycans dependency of PC02 BNAbs, as shown above, indicated an effect of kifunensin treatment of Env on PC02 serum neutralizing activity. As the kifunensin drug prevents the action of mannosidase I, it interferes at a very early stage on N-glycan synthesis. Hence, we wanted to better define which type of N-glycans, among the different forms produced during the N-glycosylation process, may be targeted by PC02 BNAbs. A panel of six pseudoviruses was produced in three different conditions:

- inhibition of α -mannosidase I by kifunensin treatment that block the trimming of $\text{Man}_9\text{GlcNac}_2$ and possibly $\text{Man}_8\text{GlcNac}_2$ N-glycans
- N-acetylglucosamine transferase I exemption by production in HEK293S which inhibits the process of $\text{Man}_5\text{GlcNac}_2$ N-glycans formation leading to the absence of hybrid and complex glycans
- α -mannosidase II inhibition by the swainsonin treatment that still allows the addition of some hybrid glycans but prevents the addition of complex glycans (see Materials and Methods, Fig VI-1).

Neutralization assays of the N-glycans-altered pseudoviruses by PC02 serum were done in parallel to the corresponding WT pseudoviruses. Only the kifunensin treatment appeared to have a relatively large negative effect on the PC02 serum neutralizing activity, although not on all viruses (Table VII-4). The production in HEK293S (no hybrid nor complex glycans) affected only the neutralization of DH12. Together those results suggest there may be a specific role for the trimmed $\text{Man}_5\text{GlcNac}_2$ glycans to the neutralizing activity of PC02, but not for larger glycan trees appearing earlier ($\text{Man}_8\text{GlcNac}_2$ and $\text{Man}_9\text{GlcNac}_2$) or later (hybrids and complexes) in the N-glycosylation process. This experiment could only confirm what had been shown by Landais *et al* (Landais *et al.*, 2016).

Pseudovirus			Fold Decrease ID50
Strain	Clade	Modification	
92RW020	A		0,24
92TH021	AE		108,16
DH12	B	Δ α -mannosidase I (kifunensine ttt)	249,94
REJO	B		24,92
DU156	C		0,17
93IN905	C		4,21
92RW020	A	Δ N-acetylglucosamine transferase I (HEK293S prod)	0,25
92RW020	A		0,14
92TH021	AE		0,26
DH12	B		87,05
REJO	B		0,43
92RW020	A	Δ α -mannosidase II (Swainsonine ttt)	0,32
92TH021	AE		0,16
DH12	B		11,88
REJO	B		0,94
DU156	C		0,91
DU156	C		0,01
93IN905	C		1,35

Impact of the mutation on the neutralizing activity
Ratio ID50 WT / ID50 mutant

	<0,5	gain
	0,5-2	neutral
	2-20	loss
	>20	severe loss

Table VII-4: Fold Decrease in ID50 due to glycans modifications of Env. All experiments were done once, on PC02 serum v48.1 (46 MPI) and ID50 calculated with Excel.

4. Mapping potential BNABs targeting the MPER

We next turned towards the Membrane-Proximal External Region (MPER) of Env as a potential BNAB epitope candidate. As explained previously, the absence of impact on serum neutralization of the depletion of Abs binding to mgp120 (preliminary data) could actually be explained by the presence of Abs directed at gp41, in particular at the MPER. Although as described above the initial screening did not suggest a broad anti-MPER activity (Landais et al., 2016), we decided to more definitely eliminate the possibility that some MPER Abs may be involved in the PC02 serum neutralization. We firstly re-assessed on a greater number of time-points whether the PC02 sera had some neutralizing activity against the HIV-2 chimer bearing the HIV-1 MPER region (Yu2-C1) (Fig VII-6, a). Notably, the neutralizing activity was greater with later time-point serum samples, increasing from v30 to v48, as shown Fig VII-6, b (right panel), suggesting that MPER specific BNABs may have emerged over time.

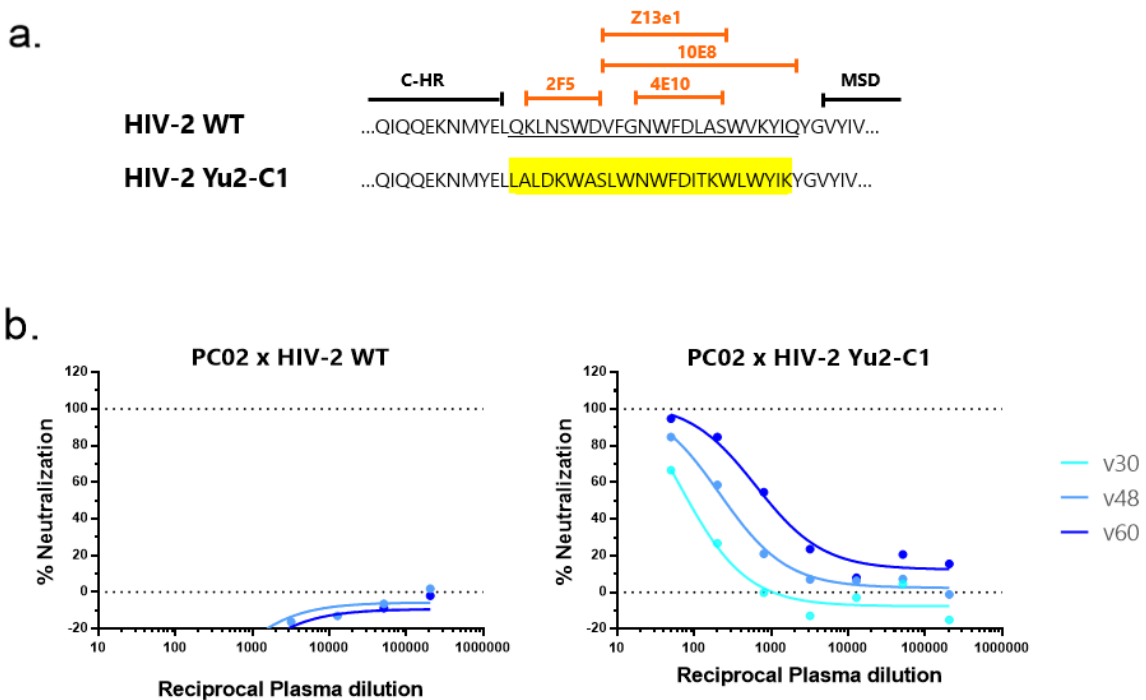


Figure VII-6: Neutralization assays of HIV-2 WT and HIV-2 Yu2-C1 MPER chimera. a. Amino acid sequence of HIV-2 WT and MPER-chimer. The HIV-1 Yu2 MPER region (yellow highlight) was inserted in HIV-2 7312 sequence, replacing the HIV-2 MPER amino acid sequence (underlined). C-HR = C-terminal Heptad Repeat, MSD = Membrane Spanning Domain. HIV-1 MPER targeting BNABs precise epitopes are indicated above in orange. b. Neutralization assays of three time points PC02 sera samples (v30, v48 and v60) against the HIV-2 WT (left panel) and MPER-chimer (right panel).

The MPER chimera assay is highly sensitive and the presence of potent neutralizing Abs targeting the MPER has to be confirmed through neutralization competition assays in presence of a soluble MPER peptide, as explained previously. We notably wanted to re-assess the competition effect of an MPER

peptide on the v60 later time-point serum sample, which showed a stronger neutralizing activity against the HIV-2 chimer than the v36 sample tested by Landais et al. (Landais et al., 2016). The competing peptide, furnished by C. Caillat, PhD, corresponded to the following aminoacid sequence: KWASLWNWFNITNWLWYIK. The capacity of the MPER peptide to compete was first tested with control BNABs 4E10 and 10E8, both directed against the MPER region. As shown in figure VII-7, a, those two mAbs lost their ability to neutralize HIV-1 REJO (clade C strain) and the HIV-2 chimer (yellow and orange curves) in the presence of the MPER peptide. However, when done on the v60 PC02 serum, the experiment did not show a loss of neutralizing activity in the presence of the peptide (Fig. VII-7, b). To be noted, the peptide we used is shorter than the entire MPER region and lacks a few aminoacids, among them some part of the epitope of the BNAB 2F5. Consequently, we could not totally rule out that the PC02 serum may contain some 2F5-like BNABs. As in any case, an MPER BNAB specificity could not explained the early breadth of the PC02 serum, we decided not to focus on this epitope and pursue mapping.

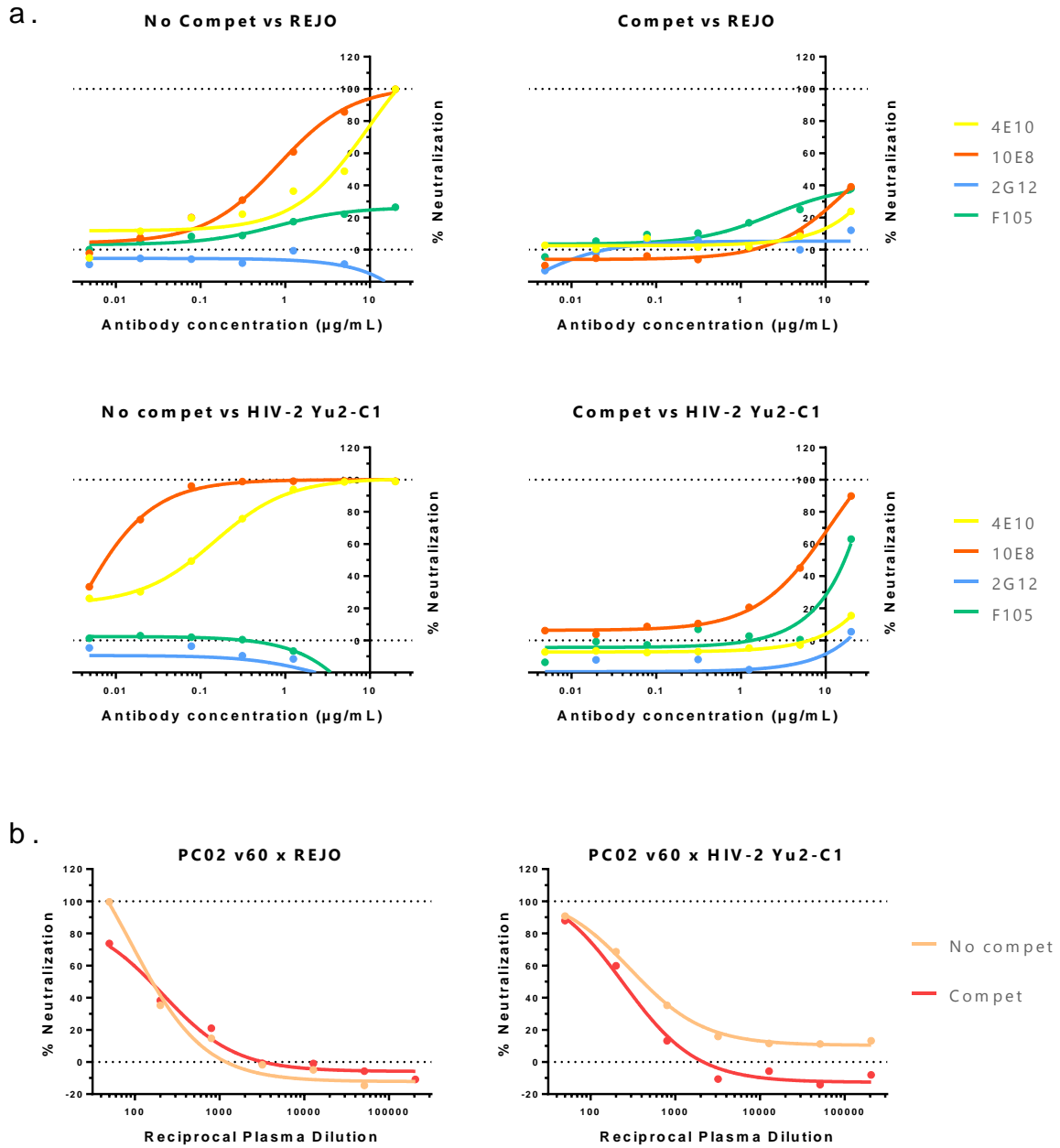


Figure VII-7: Competitive activity of the MPER soluble peptide (KWASLWNWFNITNWLWYIK), used at 100µg/mL. *a.* Competition assay of REJO (top panels) and HIV-2 MPER chimer (bottom panels) neutralizations by control MPER BNABs: 4E10 and 10E8. F105 is a Nab targeting open-conformation Env only and serves as a control of the proper Env conformation. 2G12 is a BNABs targeting the HM patch and serves as a negative control for MPER binding. *b.* Competition assay of REJO (left panel) and HIV-2 MPER chimer (right panel) neutralizations by v60 PC02 serum.

5. Agnostic mapping of PC02 serum broad neutralizing activity through EM

As the first mapping results did not provide clear insights about the epitope(s) targeted by the PC02 BNABs, our collaborators of the Ward Lab at the Scripps Research in California used a complementary mapping method as described in [Bianchi et al., 2018](#).

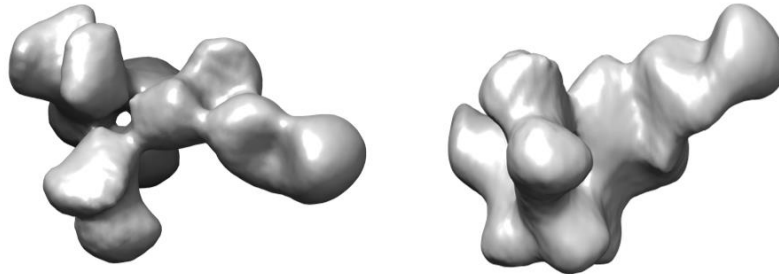


Figure VII-4: 3D reconstructions of PC02 purified IgGs from 60 MPI serum sample in complex with the trimeric SOSIP construct of the TRO11 strain. View from the apex (left) and the side (right). EM and reconstitution performed by Dr C. Cottrell, 2019.

The Ward Lab performed electron microscopy (EM) on purified Fabs from serum IgGs, in complex with a recombinant Env SOSIP trimer. Three dimension reconstructions provided a picture of the localization of Fabs bound to Env. Following incubation of TRO11 SOSIP trimers with, PC02 purified Fabs were showed to bind in a large part to the high-mannose patch area (Fig VII-4) which was consequently re-considered and explored with greater attention.

6. Mapping potential BNAbs specificities targeting the HM Patch

First, a functional mapping was done with all available Env mutants of the N332 region, similarly to what had been done for the gp120/gp41 interface and apex areas. Several time-point samples were tested this time, as well as a larger diversity of viral strains. However, none of the tested Env mutants-bearing pseudoviruses showed any decrease in neutralization by the serum (Table VII-5). A decrease of neutralization was only observed with the mutation N332A in the BG505 strain for v30 serum, but was not seen with later time-point sera samples.

Visit Code Serum	Pseudovirus			Fold Decrease ID50
	Strain	Clade	Mutation	
V48.1	92BR020	B	N295A	1,06
V60.1	92BR020	B	N295A	1,46
V30.0	BG505	A	N332A	8,57
V48.1	BG505	A	N332A	0,48
V60.1	BG505	A	N332A	0,96
V48.1	BG505	A	N332A	0,36
V60.1	BG505	A	N332A	0,39
V48.1	398F1	A	N332A	0,56
V60.1	398F1	A	N332A	1,20
V48.1	Bjox2000	AG	N332A	0,35
V60.1	Bjox2000	AG	N332A	0,28
V48.1	CH119	AG	N332A	<0,002
V60.1	CH119	AG	N332A	>0,132
V48.1	JRFL	B	N332A	<0,001
V60.1	JRFL	B	N332A	<0,001
V48.1	TRO11	B	N332A	0,27
V60.1	TRO11	B	N332A	1,66
V48.1	WITO	B	N332A	1,06
V60.1	WITO	B	N332A	1,55
V48.1	X2278	B	N332A	0,28
V60.1	X2278	B	N332A	0,77
V48.1	6535	B	N332A	0,56
V60.1	6535	B	N332A	1,51
V48.1	CE0217	C	N332A	0,73
V60.1	CE0217	C	N332A	1,09
V48.1	CE1176	C	N332A	0,60
V60.1	CE1176	C	N332A	0,25
V60.1	25710	C	N332A	0,98
V48.1	92RW020	A	N332A - N295A	0,26
V60.1	92RW020	A	N332A - N295A	0,17
V48.1	92BR020	B	N332A - N301A	0,38
V60.1	92BR020	B	N332A - N301A	0,62
V48.1	BaL	B	N332A - N301A	0,23
V60.1	BaL	B	N332A - N301A	0,05
V48.1	JRCSF	B	N332A - N301A	0,02
V60.1	JRCSF	B	N332A - N301A	0,01
V48.1	92BR020	B	N332A - N392A	0,47
V60.1	92BR020	B	N332A - N392A	0,25
V48.1	JRCSF	B	N332A - N392A	0,05
V60.1	JRCSF	B	N332A - N392A	0,05
V60.1	IAVIC22	C	N332A - N392A	<0,57

Impact of the mutation on the neutralizing activity

Ratio ID50 WT / ID50 mutant

	<0,5	gain
	0,5-2	neutral
	2-20	loss
	>20	severe loss

Table VII-5: Fold Decrease in ID50 due to mutations in the high-mannose patch of Env. All experiments were done once and ID50 calculated with GraphPad Prism.

To definitely eliminate the high-mannose patch as a potential epitope for PC02 BNABs, and check for a strain specific effect, five more double mutants were produced using the TRO11 strain, the SOSIP construct of which was used in the EM mapping experiment. Indeed, if almost all BNABs directed against the high mannose patch interact directly and preferentially to N332, the single removal of the corresponding glycan does not systematically lead to a strong and homogenous neutralization inhibition effect. This is explained by the ability of most HM Patch targeting BNABs to also promiscuously recognize other glycans than the one at N332. The knock-out of this latter can thus be by-passed thanks to secondary interactions of the BNABs with glycans adjacent in the structure (Sok, Doores, et al., 2014). Therefore we inserted double mutations targeting various adjacent glycans in the TRO11 Env and tested the corresponding pseudovirus in neutralization assays. None of the mutations tested led to a decrease in the neutralizing activity of PC02 serum, neither from v48.1 nor v60 samples (Fig VII-5, bottom panels).

In consequence, our experiments could not confirm the presence of a N332 region-specific neutralizing activity neither for the various strains tested nor specifically for the strain used on the EM serum cartography. The assumption that the HM patch could be the preferential target of PC02 serial BNABs was then set aside.

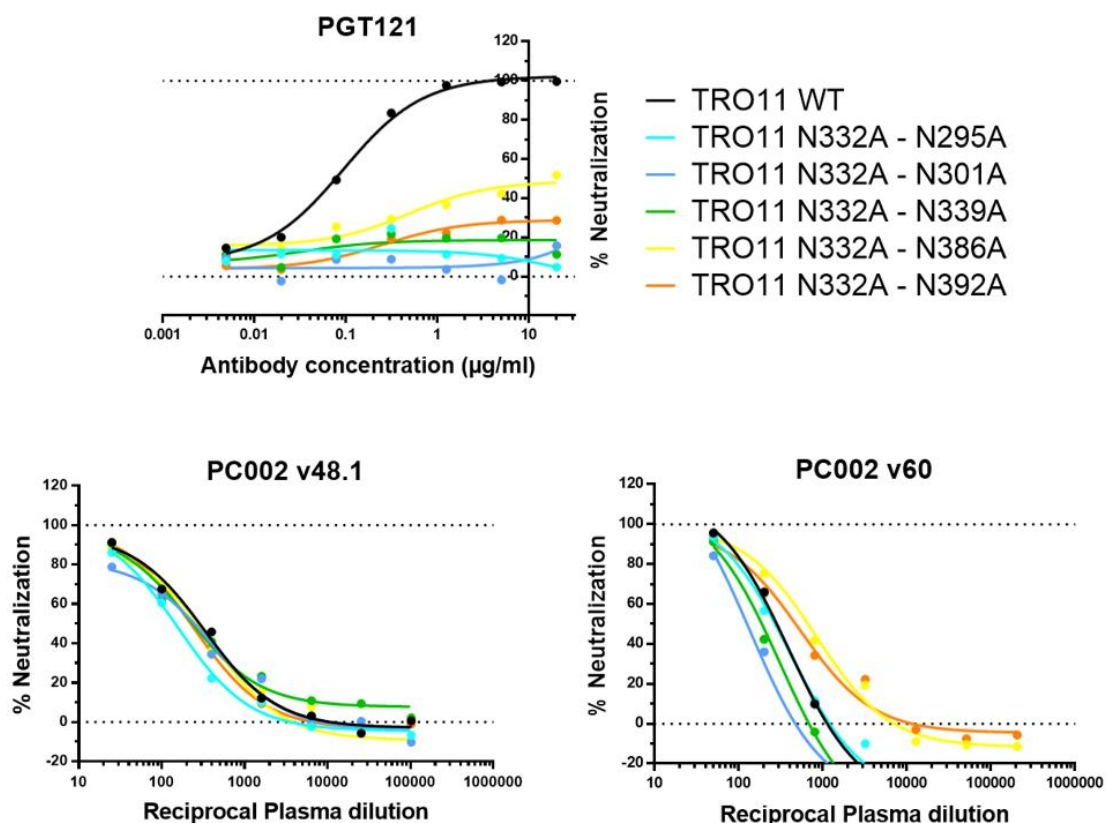


Figure VII-5: Functional mapping (neutralization assay) of PC02 v48.1 and V60 serum against V3 glycans double-mutants of TRO11. PGT121 (upper panel) is a high-mannose patch targeting BNAB and serves as a positive control of the presence of the mutations.

7. Mapping PC02 serum BNAB specificities: conclusions / summary

In conclusion, despite the multiple mapping strategies employed, the epitope of the putative PC02 BNABs could not be precisely defined, preventing thus the realization of a differential B-cells sorting and leading us to redesign sorting strategies.

C. Choice of a BNAb isolation strategy for PC02: epitope-agnostic and trimer-based B cell sort

The absence of knowledge of the BNAb specificity offers 2 alternatives: sort all memory B-cells without selection and activate them for functional screening or a trimer-based sort.

We decided to sort using Env trimers, which, as explained above, expose (or are supposed to) all BNAb epitopes similarly to native trimers. Therefore, sorting with those trimers should allow for isolation of BNABs. Furthermore, as epitopes from NNABs are not exposed, theoretically no Ab that is not neutralizing should be selected, limiting the amount of work downstream of the sort (Fig VII-6). The choice of this sorting strategy for PC02 BNABs is also supported by the demonstration that the latter may have a preference for quaternary epitope(s) (Table VII-1).

We started to produce recombinant Env for which we had SOSIP or NFL sequences and tested their ability to bind BNABs in order to find the best candidate and use a single trimer bait for the B cells sorting.

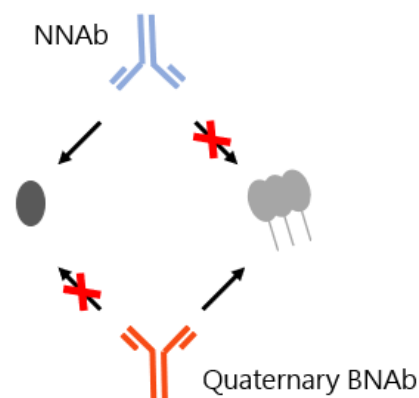


Figure VII-6: Principle of the trimer-based sorting strategy. Mgp120 (left) and trimeric Env (right) WT probes. NNAB = non-neutralizing Ab.

1. Production of Env trimers and test in PC02 serum neutralization competition assays

Recombinant trimers expression vectors and purification protocols were given by our collaborators at Scripps Research CA who developed the platforms to properly stabilize the Env protein in a native conformation, NFL or SOSIP constructs, from R. Wyatt and A. Ward laboratories respectively.

The ability of those trimer candidates to bind BNABs was tested in neutralization competition assays with (i) control BNABs (Annexe Table X-1) and (ii) PC02 serum (Table VII-6). Briefly, mAbs or serum were incubated with the soluble trimer candidate prior to a classic neutralization assay (see Material and Methods). Binding of the soluble trimer to NABs prior to virus-Ab interaction would prevent the neutralizing activity of the latter. Demonstrating competition of a given trimer for the neutralization by PC02 serum of a panel of viruses would suggest that the trimer does bind the NABs responsible of the serum breadth and can therefore be used as a bait to fish the corresponding potential BNABs. To be noted, all recombinant trimers were produced with the mutation D368R that abolishes binding to CD4. This was done to prevent trimer interaction to target cell CD4 during the experiment, which

would have inhibited viral entry and been confused with neutralization, which we precisely aimed to evaluate. We used 3 BNabs (PG123, 145 and 151) as controls to verify the ability of the tested trimers to compete for neutralization (Annexe Table X-1).

The first competition assay was done with three soluble trimers - BG505_D368R_G508A_NFL, BG505_D368R_NFL and SC422_D368R_NFL - using small panels of HIV-1 strains and the control BNabs (Annexe Table X-1). To be noted, a G508A change in the Glycine-Serine Linker of BG505_D368R_NFL was noticed, as compared to the published sequence, and reverted, even though it did not seem to impact the conformation of the trimer as probed with Abs (ELISA Annexe Fig X-1, orange pads). Competition effects were observed when the competitive trimers were used at concentrations above 25 µg/mL. Except for the neutralization of BG505 when competitors were used at 25 µg/mL, this effect was always observed for the neutralizing activity of the Ab PG123 (directed against the HM patch) that was tested with the three competitors. No competition was observed with PGT151 (interface), though this Ab was only tested in competition with BG505_D368R_NFL at low concentration of 25 µg/mL, and for the neutralization of three pseudoviruses. PGT145 (apex) neutralizing activity could be competed only when the soluble trimers tested (in this case BG505_D368R_G508A_NFL and SC422_D368R_NFL) were at a 50 µg/mL concentration (no data at 25 µg/mL).

We then extended the competition experiments to a larger panel of soluble trimer mimics (BG505_D368R_NFL, SC422_D368R_NFL, 25710_D368R_SOSIP, 398F1_D368R_SOSIP and TRO11_D368R_SOSIP; Annexe Fig X-1, green pads) was tested in competition assay with concentrations ranging from 33.3 to 200 µg/mL, for neutralization on larger pseudovirus panels, by PC02 serum (Table VII-6). Less competitive activity was observed with the polyclonal serum than with mAbs. First, soluble trimeric Env had to be used at higher concentrations to display any competitive activity. Then, trimers that showed competition for monoclonal BNAbs activity were not necessarily competing serum neutralization, even at higher concentrations, as shown with SC422_D368R_NFL, for instance. Finally, a trimer used at a certain concentration could enter in competition for the neutralization of one pseudovirus but not another, with no obvious clade tendency, suggesting the trimer competitor retains only a small fraction of the Nabs of interest in the serum.

Competitor Trimer			Pseudovirus		Fold Decrease ID50					
Strain	Platform	Concentration (µg/mL)	Strain	Clade	Mean	+/-	SD			
SC422	NFL	40	BG505	A	1,38					
			92TH021	AE	0,63					
			TRO11	B	0,80					
			DH12	B	0,84					
			92BR020	B	0,49					
			SC422	B	0,10					
			25710	C	0,00					
			CAP45	C	0,36					
			IAVlc22	C	0,04					
		50	BG505	A	1,01					
			92RW020	A	1,34					
			92TH021	AE	~ 1,00					
			TRO11	B	~ 1,00					
			JRCSF	B	1,54					
			DH12	B	0,93					
			SC422	B	0,86					
			93IN905	C	0,94					
			IAVlc22	C	0,96	+/-	0,06			
25710	SOSIP	40	BG505	A	0,68					
			92TH021	AE	2,26					
			TRO11	B	1,02					
			DH12	B	1,52					
			92BR020	B	3,41					
			SC422	B	>14					
			25710	C	1,85					
			CAP45	C	0,94					
			IAVlc22	C	0,94					
		50	BG505	A	1,55					
			92TH021	AE	~ 1,00					
			TRO11	B	0,88					
			IAVlc22	C	~ 1,00					
			398F1	SOSIP	40	BG505	A	2,16		
						92TH021	AE	1,93		
						TRO11	B	1,92		
						DH12	B	2,00		
						92BR020	B	2,46		
25710	C	2,91								
CAP45	C	1,42								
TRO11	SOSIP	33,3				92TH021	AE	~ 1,00		
						TRO11	B	1,43		
			IAVlc22	C	~ 1,00					
		40	BG505	A	0,72	+/-	0,00			
			92TH021	AE	3,92	+/-	1,80			
			TRO11	B	2,10	+/-	1,16			
			CAP45	C	0,58	+/-	0,22			
			IAVlc22	C	0,89					
			25710	C	1,70	+/-	0,19			
		BG505	NFL	40	BG505	A	3,76			
					92TH021	AE	1,34			
					TRO11	B	1,45			
					DH12	B	2,19			
					92BR020	B	2,54			
					SC422	B	1,73			
					25710	C	1,46			
					IAVlc22	C	0,73			
					CAP45	C	19,24			
50	BG505			A	~ 1,00					
	92TH021			AE	~ 1,00					
	TRO11			B	1,25					
	IAVlc22			C	~ 1,00					
	92RW020			A	1,56					
	BG505			A	3,63					
	JRCSF			B	2,13					
	DH12			B	2,25					
	SC422			B	1,53					
IAVlc22	C	0,37								
CAP45	C	10,56	+/-	2,23						
200	NFL	100	92RW020	A	2,63					
			BG505	A	4,62					
			KNH1144	A	0,69					
			92TH021	AE	6,34					
			JRCSF	B	1,53					
			DH12	B	1,63					
			SC422	B	3,28					
			BaL	B	1,06					
			JRFL	B	0,98					
		REJO	B	1,26						
		200	WITO	B	6,43					
			92BR020	B	4,30					
			IAVlc22	C	1,75					
			CAP45	C	8,24					
			ZM249	C	1,91					
			92RW020	A	3,22					
			BG505	A	4,47					
			JRCSF	B	1,12					
DH12	B		4,41							
SC422	B	1,95								
IAVlc22	C	2,52								

Table VII-6: Mean and standard deviation (if experiment repeated) of fold decrease in ID50 due to binding competition of D368R soluble trimer mimic in a neutralization assay. All experiments were done on PC02 serum v48.1 (46 MPI).

Impact of the mutation on the neutralizing activity

Ratio ID50 WT / ID50 competition

- <0,5 gain
- 0,5-2 neutral
- 2-20 loss
- >20 severe loss

So competitive activity could be observed for serum neutralization, but the interpretation of the results was more difficult than for the competitions by mAbs. Therefore, none of the trimers produced and tested emerged as a possibility of being used as a unique bait able to capture all PC02 BNABs.

An hypothesis for this weak competition would be that the soluble trimers used might not be proper mimics of the Envs that are found on the surface of HIV-1 virions. This is notably supported by the fact that both conformational BNABs PGT145 and 151 were less or not competed by the trimers than was PGT123 (directed on the glycans of mgp120) (Annexe Table X-1). The quality of the trimers had been tested prior to the competition assays in EM, showing homogeneity as expected. Following the somehow disappointing competition results we further tested the trimers in ELISA to probe antigenicity with a panel of Abs. We thus noticed that some batches of recombinant trimers presented a large fraction of Env in open conformation as shown by the binding of the F105 mAb in ELISA, Annexe Fig X-1, grey pads). This is thus likely that a large proportion of them was not in a proper close conformation, explaining their low ability to catch the quaternary BNABs in the serum.

In order to eliminate open forms of Env in the preparations, we decided to change the trimer purification method, originally based on lectin affinity, was thus changed to be more conformation-specific. The new method was based on a double purification step with successive Ab-columns, with one containing a conformational BNAB PGT145 used for the capture of the soluble trimers in the appropriate close conformation only while the other bearing the F105 Ab served to negatively select the open-conformation trimers that might remain (see Materials and Methods). The antigenicity of the newly purified Envs was also systematically checked by the evaluation of the binding of known conformational BNABs (ELISA).

All in all, soluble trimers were still defined as suitable baits for B cell sorting in Peripheral Blood Mononuclear Cells (PBMC) samples, if properly purified and characterized.

2. Production of a panel of trimers

With the design of new SOSIP constructs corresponding notably to the Env of the HIV-1 strains of the panel designed by Montefiori and colleagues (12vP, predictive of breadth on larger panels, see Material and methods), and in the view of our previous results, we decided to improve our trimer-based sorting strategy with the idea of using several Env-baits for which the corresponding strains would be neutralized by the PC02 serum. The B cells able to bind the most number of trimers among the defined panel of baits would be selected (Fig VII-7).

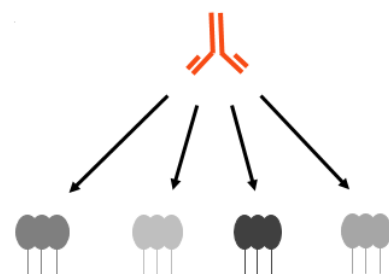


Figure VII-7: Scheme of the principle of the trimer-panel-based sorting strategy.

We expected that basing the B cell selection on their ability to bind several heterologous trimers would increase the chances of isolating Nabs with breadth.

We thus run PC02 sera from different time points against this virus panel in order to choose the most appropriate baits (Table VII-7). From the neutralization results, 5 SOSIPs (CE0217, CNE8, TRO11, 25710 and BG505-T332N) and 1 NFL (JRFL) trimers were chosen and further produced for PC02 BNABs sorting, using the Ab-column purification method (Annexe Fig X-1, yellow pads).

	Bjox 2000	CE0217	CE1176	CH119	CNE55	CNE8	TRO11	X1632	X2278	246F3	25710	BG505 T332N	JRFL
	AG	C	C	AG	AE	AE	B	G	B	AC	C	A	B
V00.1	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
V02.0	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
V03.0	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
V06.0	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
V09.0	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
V21.0	NN	NN	NN	NN	NN	>200	>50	NN	NN	NN	NN	NN	NN
V24.0	62	NN	NN	NN	NN	>200	>50	NN	NN	>200	NN	>50	NN
V30.0	83	148	37	25	NN	206	>200	NN	NN	NN	NN	>200	79
V42.0	99	255	130	75	>50	>50	714	NN	>50	NN	>200	281	206
V48.1	182	2 302	298	125	331	810	1 110	67	402	NN	742	630	368
V60.0	225	1 292	355	187	370	1 271	31 486	158	372	>50	4 102	1 494	410

Table VII-7: Neutralizing ID50 of PC02 longitudinal sera against the pseudoviruses for which SOSIP or NFL constructs were available in our laboratory. Neutralization assays were done on 11 time-points post-infection PC02 plasma samples (lines) against 13 candidate strains (columns). NN = Non neutralizing. ID50 were estimated by GraphPad Prism.

D. PC02: what have we learned?

The work on donor PC02 started with a substantial mapping research on the BNABs preferential epitope on Env.

1. Epitope mapping : limitations, bias and potential improvement

As the preliminary mapping suggested the presence of BNABs targeting a quaternary epitope, the gp120/gp41 interface and apex regions were explored in a first place. We used a strategy based on point mutations at Env residues of interest and testing of the mutant pseudovirus in neutralization assay by PC02 plasma. As explained above, no particular residue mutation could be identified as significantly impacting PC02 broad neutralization. However, the number of mutations we tested was relatively moderate and the number of strains in which they were inserted limited (2 to 3). The absence of a consistent pattern of sensitivity to a mutation across various viruses suggests that the BNABs potentially present in the PC02 serum are not sensitive to any of the mutations tested, that abolish or strongly decrease the binding of known BNABs to the apex and interface. This may be due to the fact that the putative PC02 BNABs either do not recognize any of the corresponding tested or recognize similar regions/epitopes but through a different recognition mode not impacted by the mutations that were tested. In the future more mutations corresponding to newly isolated BNABs to these regions could be further explored. Finally, it must also be said that the number of mutations in our panel was limited and we may just have missed to test the right one(s).

The HM patch was studied with the same method, but more extensively (more residues mutated and more strains), in particular in view of the results of the Ward lab. No residue could be highlighted either though, contrasting with the EM result. Further discussions with Dr Ward led to the conclusion that the EM reconstitution they obtained from PC02 samples could also be interpreted as binding to the apex, as they could sometimes observe somehow similar profiles with some anti-apex Abs. This data could fit with the hypothesis that the apex mapping we performed might have been too limited, both in terms of number of mutations and number of strains tested. Of note the presence of a BNAB specificity directed at the apex is also supported by the sensitivity of the PC02 serum neutralizing activity to kifunensin treatment, similar to PG9 and PG16.

The preliminary mapping results showing an absence of impact on the PC02 serum neutralizing activity of Ab adsorption on monomeric gp120 could also be interpreted as suggesting the existence of BNABs directed at the MPER. Therefore, we also focused our studies on this region, using an HIV-2 chimeric pseudovirus bearing an HIV-1 MPER. As explained above the presence of potent anti-MPER neutralizing activity could not be confirmed through competition assays. However, the peptide we used did not include all possible neutralization epitopes, and we cannot rule out that Abs corresponding to the missing one 2F5 may be present. Further competition experiments using a longer

peptide should be done to exclude this possibility. Alternatively, adsorption experiments could be done on MPER peptides, similarly to experiments done on monomeric gp120. In addition, more sophisticated presentations of the MPER could be used in such experiments, such as the membrane anchored gp41 in a fusion intermediate conformation made in the Weissenhorn lab (Lai et al., 2014), or full-length MPER incorporated into lipid assemblies as described by Rantalainen, et al. (Rantalainen et al., 2020), although such experiments may be technically challenging.

In the future more explorations of the PC02 serum BN activity can be foreseen. Notably, mutations corresponding to the epitopes of newly isolated BNABs could be further tested. For example, with the silent face having been discovered as a BNAB epitope during my PhD, we could now contemplate changing the glycans of this area (N262, N448 and N295) to look for a potential impact on PC02 serum neutralizing activity (Zhou et al., 2018) (Schoofs et al., 2019). Most of the methods we used to map serum activity are limited as they tend to only characterize specificities directed against already known epitopes. However, it can be hypothesized that the BNAB activity of the PC02 serum is directed at a novel supersite. The EM approach we used in collaboration with the Ward lab was hoped to circumvent the issue. However as explained, the results we obtained coupling this method and mutagenesis, did not allow to clearly pin point a broad specificity responsible of the serum activity. Other methods may thus be better able to solve this epitope enigma : for example through the use of a library of pseudoviral Envs covalently labeled using Cys-reactive labels that masks each Cys on the surface (Datta et al., 2020) or via a technique developed in the Bloom lab called “mutational antigenic profiling” (Dingens et al., 2017).

Neutralization fingerprinting, as initially described in Georgiev et al., 2013 (and improved in Doria-Rose et al., 2017) could also bring more clarity on the nature of PC02 BNABs. In this method, an algorithm associates the neutralization profiles of well-characterized BNABs with that of the studied serum, against large panels of diverse strains, thus allowing the detection of potential NAb specificities in polyclonal samples.

In the end, it can also be that the PC02 serum breadth comes from various specificities, making the mapping difficult. Therefore, in order to understand the BNAB activity of PC02, and as our final goal was in any case to isolate BNAB lineages, we decided to consider strategies for isolating BNABs in an epitope-agnostic way.

2. Perspectives : isolating BNABs in the absence of known specificity, Env trimer-based sorting strategies

As explained before, sorting B cells with native-like Env trimers should lead to the essential isolation of NABs. Our study showing the inability of a single trimer to compete the neutralizing activity of the PC02 serum led us to consider a broader strategy based on a sort using a small panel of Env trimers. In

principle this strategy could be used for any donor, independently of the knowledge of the specificity targeted by the BNAb responses.

The choice of the trimers is based on the potent neutralization by the serum of the corresponding viruses. Six recombinant trimers were thus produced – 5 SOSIPs and 1 NFL – to be used as baits to isolate B cells carrying Abs able to bind as many as possible trimers of the panel.

Although this approach should theoretically be fairly efficient at mostly isolating Nabs with breadth, it is also known, as explained in introduction, that Env trimers are for the most not fully native-like and can somehow expose NN epitopes. Thus it possible that a fraction of Abs isolated through this strategy may not be of interest. Therefore, in order to optimize our BNAb agnostic isolation pipeline and gain in efficiency, we proposed to couple the sort with a B cells activation step. Briefly, sorted memory B cells can be dropped in a media containing feeder cells and a cocktail of cytokines inducing the activation, proliferation and differentiation of the memory B cell into plasmablasts, secreting Abs in the culture supernatants (Huang et al., 2013). Activation was notably prior used by our team in collaboration with Theraclone (L. M. Walker et al., 2009)(L. M. Walker et al., 2011)(Landais et al., 2017) and protocols have been developed in the laboratory since. We promoted cell activation because of two main advantages: a better gene recovery and the possibility of a functional screening. Indeed, the level of mRNA molecules coding for Abs is increased in plasmablasts as compared to resting memory B cells, which should largely improve Ig gene single cell PCR efficiency from a 25-30% yield of Ig recovery with non-activated single B cells to 90-95% recovery following B cell activation (data from our collaborators at Scripps). Second, supernatants containing IgGs can be used for functional assay (ELISA and micro-neutralization) and allow to focus the amplification on the most interesting Ab candidates. The combined strategy we proposed was thus thought to be highly time and cost-effective since both RT-PCR and PCR would be highly efficient and performed only on cells which known binding and neutralizing activity against targets of interest.

As in the laboratory we needed to complete the study of another Protocol C donor, PC94, as discussed below, we decided to inaugurate the use of this newly thought strategy for the isolation of BNABs from donor PC94.

II. Donor PC94

A. Preliminary data on donor PC94

1. PC94, the 2nd best Protocol C neutralizer

PC94 is a male individual infected by a clade D HIV-1. His longitudinal serum samples displayed high scores of neutralization as described in Landais et al. (Landais et al., 2016).

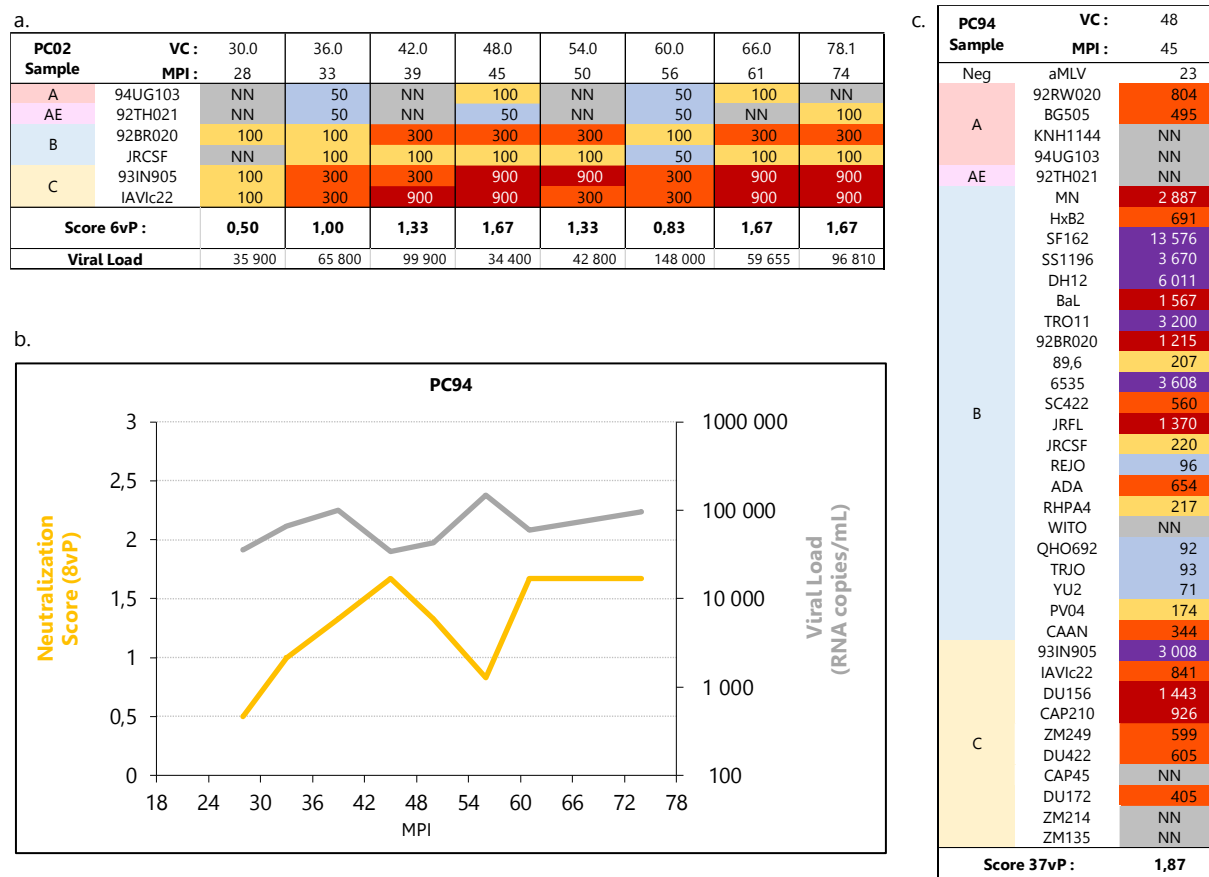


Figure VIII-1: PC94 is an elite neutralizer. a : Neutralization ID50 and scores of eight time-point PC94 serum samples against the 6vP. b : Longitudinal evaluation of The 6vP neutralization score and viral load. c : Neutralization ID50 and score of v48 PC94 serum sample (45 MPI) against the 37vP. VC = Visit Code ; MPI = months post infection. Data from Landais et al, PLOS Pathogens, 2016.

The score of PC94 samples as calculated against the 6vP started at 0.50 at 28 MPI to reach 1.67 at 45 MPI (Fig. VIII-1, a and b). A score decrease was observed between 45 and 61 MPI but it increased back to 1.67 at 61 and 74 MPI. To be noted, when viruses 92RW020 (clade A) and JRFL (clade B) were added to the 6vP, the score even reached 2.92 (for the 45 MPI sample, data not shown), albeit this 8vP has not been yet demonstrated to be predictive of the score on larger panels. The 45 MPI serum gave a score of 1.87 against the 37vP, associated to a 81% breadth (Fig VIII-1, c), and a breadth of 64% against was finally shown on a large 105vP (Landais et al., 2016, supp data).

2. The PC94 serum BNAB specificity is at least in part directed toward the HM Patch



Table VIII-1: PC94 serum mapping indicates a specificity toward the N332 region. a) Specificities mediating neutralization breadth and potency of donor PC94 serum (51 MPI). Symbols recapitulate the strength of the phenotypes tested using the different approaches detailed below. Absent (-), very weak (+/-), weak (+), moderate (++), strong (+++), phenotype was attributed based on i) the median fold or average percent decrease in ID50 and ii) the fraction of viruses which neutralization ID50 was decreased <2 fold, <10 <50 fold or <20%, <40%, <60%, <80%. Blank = not tested, NB: not binding, UD: Undefined. A dominant specificity was attributed based on results from all these experiments. From Landais et al, PLOS Pathogens, 2016. b) Mapping of the neutralizing activity against the HM patch N-glycans. Ratios of ID50 against WT virus / mutant virus by serum samples of 36, 48, 54 and 60 MPI. From Claire Rousset PhD manuscript, 2018.

The same preliminary mapping done to PC02 serum and described above was performed on PC94 by Landais et al. (Landais et al., 2016), showing a moderate serum adsorption on mgp120, suggesting the presence of BNABs targeting this antigen. The hypothesis was somehow confirmed by the global loss of neutralizing activity provoked by the removal of HM patch glycans through mutations of specific PNGS (Table VIII-2, a). The data were extended and results confirmed by Claire Rousset, a former PhD student in the laboratory (Table VIII-2, b). Indeed, in Table VIII-1 b we can observe that 5 viruses, 92RW020 (clade A), ADA, BaL, SF162 and JRCSF (B) were all less neutralized when mutated in the HM patch, with sometimes up to a 100 fold decrease in ID50 (as for the N332A-N301A double mutation in ADA and JRCSF). Other viruses, however, JRFL, 92BR020, TRO11, DH12 (clade B), IAVIc22 and DU156 (clade C), showed moderate or no decrease in neutralization as an impact of the mutations in the HM patch, even displaying sometimes an increased neutralization, mainly in the case of the single N332A mutants (red sticks), but the level of neutralization rarely increased more than 10 fold in ID50. The data thus demonstrated that part of the heterologous serum neutralization was directed against the HM patch centered on N332. Nevertheless, a significant fraction of the neutralizing activity appeared to be directed toward other neutralizing epitopes.

3. Two NABs lineages isolated from PC94 only partially explain the breadth and potency of the serum

Knowing that at least part of the PC94 serum BN specificity were directed toward the N332 region, a differential sorting was done on three PC94 samples time-points: 48, 54 and 60 MPI, by Claire Rousset, based on the selection of B cells bearing Abs able to bind two WT mgp120s (92BR020 and IAVIc22) but not a KO mgp120 (92BR020 N332A) (Table VIII-1, left column). The B cell sort was done at the IAVI HIV Neutralizing Ab Center with the help of Elise Landais, PhD. on a BD ARIA3. It resulted in the isolation of 523 memory B lymphocytes, 79% being specific of the glycan N332 (considering a fluorescence intensity ratio greater than 2 between mgp120 WT and N332A). Single B cell PCR amplification of Ig genes resulted in the recovery of 261 heavy and light IgG gene pairs: 151 with a Kappa IC and 110 with a Lambda IC. After sequencing, the observation of the VH gene usage among the 33 Abs that appeared to be most highly specific of N332 from the sorting data showed two main VH enrichments: IGHV1-18*01 (39%) and IGHV3-30*02 (42%). Two Ab lineages could then be identified and further studied, named A and B:

- lineage A, using Ig gene families IGHV1-18*01, IGHD3-3*01 or 02 and IGHJ6*02 associated with IGKV1-5*01 or 03 and IGKJ1*01, including 11 mAbs
- lineage B, using IGHV3-30*02, an uncertain IGHD segment and IGHJ4*01 or 02 associated with IGLV2-14*01 and IGLJ2*01, comprising 16 mAbs.

Twenty-seven mAbs from these 2 lineages were produced and tested against our laboratory 37vP, demonstrating heterologous neutralizing activity, although with diverse levels of breadth and potency

(data in Table VIII-9). Globally, lineage A contained more potent candidates than lineage B but Abs of lineage B could neutralize a greater number of pseudo viruses than lineage A. When tested in neutralization against a panel of longitudinal autologous PC94 viruses – isolated and cloned by Monogram Biosciences – the isolated NAbs revealed two distinct waves of neutralization throughout time. Indeed, lineage A Abs strongly neutralized 12 MPI viruses but only weakly 18 and 24 MPI autologous viruses, preceding a full viral escape occurring at 30 MPI. In parallel, Abs of lineage B did not neutralize 12 MPI viruses but neutralized the 18, 24 and 30 MPI viruses, albeit with lower IC50s than lineage A. Viral escape from lineage B neutralization happened around 54 MPI.

Even though some of the 27 isolated Abs showed relatively potent and broad heterologous neutralization, they were not able together to recapitulate the impressive neutralizing activity of the PC94 serum samples. For instance, the 45 MPI serum (visit code v48) had great neutralizing activity against viruses 89.6 (clade B) and CAP210 (C), with ID50 of 1/207 and 1/926 respectively (Landais et al., 2016), but none of the mAbs Claire Rousset isolated could neutralize these viruses. In parallel, other viruses were greatly neutralized by the serum while only moderately by even the best of the 27 NAbs. We therefore concluded that NAbs were missing to fully explain the serum neutralizing activity, whether belonging to the two identified lineages but potentially more mature, or part of other Ab family(ies), directed against the N332 region or other epitope(s). The second part of my Ph.D. project was thus focused on isolating the missing NAbs that could explain the great breadth and potency of the PC94 serum.

B. Isolation of new mAbs from PC94 in order to recapitulate the breadth of the serum neutralizing activity

The sorts were performed at Scripps Research where I had the chance to spend two 3-month stays during my PhD and benefit from the necessary equipment, notably a BD FACSAria™ III Cell sorter in an appropriate safety level hood, and the high expertise of our collaborators in B-cell isolation.

1. Four additional B cell sorts

The missing NABs could have been missed for several reasons with the sorting strategy that was first used. On the one hand, the differential sorting may have not been based on the most appropriate baits. Indeed, it was confirmed at posteriori that the PC94 serum was not much sensitive to the N332 mutation in the IAVIC22 and 92BR020 backbones, and better viral strains could have been selected to do the differential sorting. Nevertheless, it is interesting to note that despite this relatively poor choice, fairly broad and potent N332A-sensitive Nabs were still isolated. Furthermore, the use of a monomeric gp120 certainly excluded the possibility of isolating Nabs recognizing quaternary epitopes, although the initial mapping suggested that most of the PC94 serum neutralizing activity could be adsorbed on this antigen. On the other hand, as in any attempt to isolate BNABs through sorting, the final yield is only moderate and some interesting Abs that might have been properly selected during the sorting step itself may have been lost within the following molecular biology steps (no PCR amplification, unsuccessful cloning...).

To be more successful at isolating potential PC94 BNABs, we decided to take advantages of the sorting approaches designed following our work on donor PC02, as explained above. Baits were chosen based on the longitudinal neutralizing data of the PC94 sera against the 12vP described previously (Table VIII-2 and VIII-3).

	Bjox 2000	CE0217	CE1176	CH119	CNE55	CNE8	TRO11	X1632	X2278	246F3	25710	BG505 T332N	JRFL
	AG	C	C	AG	AE	AE	B	G	B	AC	C	A	B
V18.0	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
V24.0	NN	NN	NN	NN	NN	NN	227	NN	NN	NN	NN	NN	NN
V30.0	1 668	NN	NN	NN	NN	NN	>800	NN	NN	NN	NN	NN	285
V36.0	513	NN	208	1 599	NN	NN	1 115	NN	NN	NN	NN	>50	428
V42.0	1 247	NN	428	1 337	NN	>200	1 992	NN	>50	NN	NN	207	900
V48.0	3 044	NN	687	1 844	NN	>50	2 192	NN	>50	NN	NN	703	769
V54.0	1 489	NN	539	3 533	>3200	>50	1 099	NN	>50	NN	NN	476	773
V60.0	1 329	NN	528	534	>800	>800	908	NN	NN	NN	NN	417	1 195
V66.0	1 809	>3200	471	5 532	>800	>800	1 918	>50	>50	NN	NN	3 988	2 014
V78.1	810	17 236	475	2 097	>3200	>3200	3 691	>50	>50	NN	NN	1 748	876

Table VIII-2: Neutralizing ID50 of PC94 longitudinal sera against the pseudoviruses for which SOSIP or NFL constructs were available in our laboratory. Neutralization assays were done on 11 time-points post-infection PC02 plasma samples (lines) against 13 candidate strains (columns). NN = Non neutralizing. ID50 were estimated by GraphPad Prism.

Four successive additional sorts were then performed during 2 visits at the Scripps IAVI "Neutralizing Ab Center using PC94 PBMC longitudinal samples ranging from v30 to v78 (see Material and methods). We used strategies based on panel of trimers (experiments called "abc222a" and "abc233c" in Table VIII-3) and added in 2 sorts a N332 KO of 92BR020 rgp120 bait in order to get additional information on a potential N332 dependency (exp "abc233a and b") (Table VIII-3). Selected memory B cells were seeded at one cell per well in 384-well plates. Whereas the B cells sorted during the initial 2016 sort were all directly lysed to immediately go through the process of Ig gene amplification, those selected during the four 2020 sorts were activated through culture with CD40L-expressing feeder cells and a cocktail of cytokines in order to make them secrete IgGs (see Materials and methods). At the end of the 14-days activation, the plates were centrifugated to separate the B-cells from the supernatants. RNAs were extracted from the B-cells while supernatants were used for functional screening. First, the IgG level in each well was determined by ELISA, showing activation yields ranging from 10% to 29% (Table VIII-3). Then, all supernatants, with no regard to the IgG level, were tested in microneutralization assays, against a small 4vP. The latter was constituted by the pseudoviruses corresponding to the trimer baits used in the sorts. In order to select B cells with the most promising profiles for further processing, we took into consideration the index sorting data (when available) to evaluate the phenotypic characteristics of the B cell, the IgG level, and the results of the microneutralization assay. All those data were aligned to allow the selection of 179 wells with potential for BNAbs neutralization. Corresponding B-cells RNA were picked up for retro-transcription and PCR amplification of the IgG HC and LC variable part sequences.

2016 Sort (C. Rousset)				2020 Sorts (R. Rouzeau)											
<i>Experiment</i>				abc222a (1.13.20)		abc233a (3.6.20)		abc233c (3.9.20)							
<i>Sorting Strategy</i>	Epitope-specific (differential sorting)			Epitope-agnostic (based on panels of trimers)		Combination of both strategies		Combination of both strategies		Epitope-agnostic (based on panels of trimers)					
<i>Time point sample</i>	v48 - v54 - v60			v78		v54 - v60 - v66 - v78		v66		v30 - v36 - v42 - v48 - v60					
<i>Baits used</i>	3 mgp120: 92BR020_gp120_WT IAVic22_gp120_WT IAVic22_gp120_N332A (KO)			6 trimers: CE0217_SOSIP CNE8_SOSIP TRO11_SOSIP 25710_SOSIP (KO) BG505_T332N_SOSIP JRFL_NFL		2 mgp120 + 4 trimers: 92BR020_gp120_WT 92BR020_gp120_N332A (KO) BJOX2000_SOSIP TRO11_SOSIP BG505_T332N_SOSIP JRFL_NFL		2 mgp120 + 4 trimers: 92BR020_gp120_WT 92BR020_gp120_N332A (KO) BJOX2000_SOSIP TRO11_SOSIP BG505_T332N_SOSIP JRFL_NFL		4 trimers: BJOX2000_SOSIP TRO11_SOSIP BG505_T332N_SOSIP JRFL_NFL					
<i>Nb of sorted B cells</i>	523			1056		560		259		413					
<i>Process</i>	Direct lysis + whole amplification			B-cell activation + functional screening + selection before amplification											
<i>Nb of H genes recovered</i>	299		57%	109		10%	129		23%	74		29%	90		22%
<i>Nb of pairs recovered</i>	261		50%	5			60			31			83		
<i>Nb of selected pairs (based on the HV enrichment)</i>	27		10%	3		60%	10		17%	9		29%	29		35%

Table VIII-3: Synthesis of all sorts done for the isolation of PC94 BNABs. The baits which names appear in grey are those for which the index sorting is not known (no record for exp abc222a and non-functional streptavidins for exp abc233a and 233b).

2. Fifty-one Ab candidates retrieved following the sorts

In the end, 51 pairs (28,5% yield) of heavy and light chains genes were recovered. The index sorting, activation level and microneutralization data of the corresponding Abs were aligned as shown in Table VIII-4. Following genes sequencing, family assignments were done thanks to alignments with the IMGT database. Eighteen of the new IGHV isolated were part of the previously isolated lineage A (IGHV1-18*01, yellow boxes Table VIII-4) whereas one was part of the lineage B (IGHV3-30*02, blue box). Otherwise, the remaining IGHV genes were from diverse families, with a potential slight recurrence of IGHV4-34 and IGHV4-39.

All 102 variable sequences were processed as explained in Materials and methods in order to produce the corresponding IgGs. Finally, twenty-six pairs were cloned through usage of recombination and 10 using classical ligation by T4 ligase. Ten HC and 2 LC variable sequences could not be cloned, and 3 HC and 2 KC cloned inserts ended up corresponding to contaminating sequences.

3. Production of 33 mAbs

Because of this somewhat low cloning yield, only 36/51 (70,5%) pairs of HC and KC/LC genes could be transfected in HEK293T cells and 33 resulted in Ab production (91,7% efficiency) (as tested by ELISA, Table VIII-4).

Visit Code	Sort	Well	Timer	Index Sorting		Activation (ELISA)	Functional Screening				Well ID (96w plates)	Pair	Seq Heavy Chain		Seq Light Chain		Cloning	Prod In HEK293T (ELISA)		
				Monomer			BG-N	CNE8	DH12	JIRL			Neut Result /Atp	D gene	J gene	V gene			J gene	
V78	abc	P01-G16	nd	nd	nd	++	+	-	+	-	+	+	+	+	+	+	+	+	+	+
V78		P01-L07	nd	nd	nd	++	+	-	+	-	+	+	+	+	+	+	+	+	+	+
V78	222a	P02-L20	nd	nd	nd	++	+	-	+	-	+	+	+	+	+	+	+	+	+	+
V78		P01-C18	1/3	nmWT+ mMt-	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V78		P01-D20	3/3	nmWT- mMt-	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+
V66		P01-K13	3/3	nmWT+ mMt-	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V66		P01-K19	1/3	nmWT- mMt-	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V66	233a	P01-M16	1/3	nmWT- mMt-	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V66		P02-D18	0/3	nmWT+ mMt-	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V66		P02-H06	1/3	nmWT+ mMt-	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V54		P02-M19	2/3	nmWT- mMt-	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V54		P02-M20	2/3	nmWT- mMt-	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V66		P01-C10	2/2	nmWT+ mMt-	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V66		P01-C22	1/2	nmWT+ mMt-	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V66	abc	P01-D16	1/2	nmWT- mMt+	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+
V66		P01-G17	1/2	nmWT- mMt+	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V66		P01-H02	1/2	nmWT- mMt+	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V66	233b	P01-K22	1/2	nmWT- mMt+	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V66		P01-L14	1/2	nmWT- mMt-	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V66		P01-M11	0/2	nmWT+ mMt-	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V66		P01-N15	0/2	nmWT+ mMt-	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V48		P01-F14	2/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V48		P01-H04	2/4	nd	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+
V48		P01-H05	3/4	nd	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+
V48		P01-H14	2/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V48		P01-H17	2/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V48		P01-J10	2/4	nd	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+
V48		P01-K08	2/4	nd	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+
V42		P01-K09	1/4	nd	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+
V42		P01-L02	3/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V42		P01-L06	1/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V42		P01-L22	4/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V42		P01-M18	3/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V42		P01-M20	4/4	nd	+	-	+	-	+	-	+	+	+	+	+	+	+	+	+	+
V36	abc	P01-N11	3/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V36		P01-N12	4/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V36	233c	P01-N21	4/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V36		P02-C05	4/4	nd	++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V30		P02-D10	3/4	nd	++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V30		P02-D11	4/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V30		P02-D12	4/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V30		P02-E08	3/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V30		P02-E10	4/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V30		P02-E21	3/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V30		P02-G20	3/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V30		P02-G21	4/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V30		P02-H16	4/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V30		P02-H17	4/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
V30		P02-H20	3/4	nd	+++	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+

Table VIII-4: Screening data and production results of the 51 Abs selected for their potential of neutralization and for which pairs of heavy and light chains genes could be amplified. The complete left column indicates the visit code of the PBMC sample from which the cell was sorted. The sorts correspond of those described table VIII-3. The index sorting indicate the binding of the concerned cell to trimers and/or monomers (mWT = mgp120 WT while mMt = mgp120 mutant N332A). BG-N=BG505-T332N. The H, K and L families were assigned from the sequencing post PCR2.seq and alignment with the IMGT database. Yellow = lineage A, blue = lineage B.

C. Identification of Abs with potentially significant neutralizing activity

1. Eliminating mAbs with low BN potential

The 30 HEK293T supernatants containing mAbs were tested for their potential neutralizing activity against a home-made 6vP, comprising the 4 pseudoviruses used for the microneutralization assays and two additional clade C pseudoviruses, neutralized by the PC94 serum but by none of the Abs of lineages A or B isolated so far. Of note, DU172 often showed low titers, preventing the interpretation of the corresponding neutralization assays (empty boxes, Table VIII-5) and thus reducing the panel to 5 viruses in most cases.

For each supernatant that showed neutralizing activity, even if weak and only on one virus, the corresponding Ab was selected to be produced in HEK293F. To be noted, PcF07 was not tested in this 6vP screening because of a late cloning success but was still included in the selection for HEK293F production and further Ab characterization, as detailed below. In parallel, PcC11 did not show any neutralizing activity but has also been selected anyway, because of its belonging to lineage B.

Visit Code	Well ID (96w plates)	Pair	Screening 6vP					Selection for production in HEK293F	
			BG505-T332N	Bjox 2000	JRFL	TRO11	DU172		ZM249
v78	Pc-G06	HK	-	-	-	-	-	-	no
v78	Pc-G07	HL	-	-	-	-	-	-	no
v78	Pc-G11	HK	-	-	-	-	-	-	no
v78	Pa-B03	HK	-	-	-	-	-	-	no
v78	Pa-C04	HL	-	+/-	-	-	-	+	yes
v78	Pa-C07	HK	-	-	-	-	-	-	no
v66	Pa-D10	HK	+++	++	+	+/-	-	+	yes
v66	Pa-C11	HK	-	+/-	-	+	-	-	yes
v66	Pa-F07	HK	+	+++	+	+/-	-	-	yes
v60	Pa-F03	HK	-	-	-	-	-	-	no
v54	Pa-G08	HK	-	-	-	-	-	-	no
v54	Pa-G09	HK	-	-	-	-	-	-	no
v66	Pb-E05	HK	+	-	+/-	-	-	-	yes
v66	Pb-E02	HK	-	-	-	-	-	-	no
v66	Pb-E04	HL	+++	-	+++	+/-	-	-	yes
v66	Pb-F05	HK	+	-	-	-	-	++	yes
v66	Pb-F07	HK	-	-	-	-	-	-	no
v66	Pb-G08	HL	-	-	-	-	-	-	no
v48	Pc-C03	HK	-	-	-	-	-	-	no
v48	Pc-C06	HK	-	-	-	-	-	-	no
v48	Pc-C07	HK	-	-	-	-	-	-	no
v48	Pc-C11	HL	-	-	-	-	-	-	yes
v48	Pc-D06	HL	-	-	-	-	-	-	no
v42	Pc-E02	HK	-	+	+	+/-	-	-	yes
v42	Pc-E09	HK	-	+	-	-	-	-	yes
v42	Pc-F07	HK	-	-	-	-	-	-	yes
v36	Pc-F11	HK	-	+/-	-	-	-	-	yes
v36	Pc-G02	HK	-	-	-	-	-	-	no
v36	Pc-G05	HK	-	-	-	-	-	-	no
v36	Pb-D10	HK	+	++	+/-	+++	-	-	yes
v30	Pb-B08	HL	-	-	-	-	-	-	no
v30	Pb-C08	HK	-	+/-	-	-	-	-	yes
v30	Pb-C11	HK	+/-	-	-	-	-	-	yes
v30	Pb-D02	HK	-	++	-	-	-	-	yes

Table VIII-5: 6vP screening of the 33 HEK293T supernatants containing the Ab candidates. Yellow: lineage A while blue = lineage B. The number of '+' signs correlates with the intensity of the neutralizing activity observed.

2. Testing selected Ab candidates against the 37vP

The 16 selected Abs with potential neutralizing activity were then produced in HEK293F and purified, in order to more robustly evaluate their breadth and potency using our 37vP. The respective IC50s were calculated as reported in Table VIII-6. One mAb, PbF05, did not show any neutralizing activity against any of the 37vP members, while it appeared to barely neutralize BG505-T332N and moderately ZM249 in the previous screening (Table VIII-5). Abs PcF07 and PcF11 had very limited breadth and potency. The remaining 13 Abs however had notable spectra and were classified as potential BNAbs, although they displayed a range of breadth and potencies. These Abs globally showed similar neutralizing profiles in the fractions of virus they neutralized, with 2 exceptions: PbE04 had the best breadth among clade B viruses and neutralized some strains that other Abs could not or hardly (BaL, REJO, RHPA4, QH069 and PV04 for instance), and PaC04 which neutralizing activity, albeit with a low potency, appeared to better target clade C viruses in contrast to the other mAbs tested.

Clade	Virus	v30			v36			v42			v48			v66			v78
		PbC08	PbC11	PbD02	PbD10	PcF11	PcE02	PcE09	PcF07	PcC11	PacI1	PaD10	PaF07	PbE04	PbE05	PbF05	PaC04
Ctrl Neg	MLV	nd	nd	NN	NN	nd	NN	NN	nd	NN	NN	NN	NN	NN	NN	NN	NN
	92TH021	NN	NN	NN	NN	nd	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
A	92RW020	17,930	17,590	NN	3,972	57,580	0,025	97,990	NN	0,861	NN	NN	NN	NN	42,580	47,060	NN
	BG505	NN	17,860	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	0,029	NN	NN
	KNH	NN	NN	NN	NN	NN	NN	NN	7,726	NN	NN	NN	NN	NN	NN	NN	NN
	94UG103	NN	NN	NN	NN	10,310	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
	MN	NN	NN	NN	NN	NN	NN	NN	10,270	NN	NN	NN	NN	NN	NN	NN	NN
	HXB2	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
	SF162	0,037	20,070	0,013	0,009	32,250	0,024	0,027	NN	10,090	NN	NN	NN	NN	0,099	0,243	NN
	SS1196	0,449	0,247	0,034	0,051	NN	1,197	0,315	NN	40,450	0,605	1,334	0,066	0,152	19,630	NN	6,175
	DH12	0,097	NN	0,183	0,019	30,480	0,222	0,100	NN	NN	0,109	0,953	0,099	0,240	NN	NN	24,420
	Bal	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
TRO11	NN	NN	NN	0,063	NN	0,539	14,730	NN	NN	0,246	4,000	1,505	0,121	NN	NN	NN	NN
89.6	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
6535	5,623	8,373	0,959	0,275	41,930	0,227	0,337	NN	0,358	0,300	0,024	0,021	0,236	1,371	NN	NN	
92BR	NN	NN	46,290	0,057	NN	0,578	37,100	44,920	1,943	2,085	11,190	20,240	32,910	28,260	NN	NN	
SC422	NN	NN	NN	NN	NN	17,780	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
B	JRFL	3,840	NN	4,740	0,155	0,142	NN	NN	NN	19,130	0,094	0,405	0,068	0,926	NN	NN	44,900
JRC5F	NN	NN	26,610	2,355	NN	2,099	18,080	NN	NN	0,926	2,762	3,171	NN	NN	NN	NN	NN
REJO	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
ADA	10,530	NN	1,927	0,264	NN	27,590	3,252	NN	NN	0,413	8,604	2,739	3,982	0,064	NN	NN	0,342
RHPA	NN	31,110	NN	NN	NN	43,580	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
QH069	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
WTO	NN	17,190	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
TRJ0	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
PV04	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
VU2	NN	NN	NN	11,000	34,780	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
CAAN	NN	NN	1,032	0,977	NN	NN	NN	NN	NN	6,779	NN	15,100	NN	NN	NN	NN	NN
931N	14,400	NN	18,560	0,029	NN	0,140	9,615	NN	NN	7,197	NN	8,053	NN	33,110	NN	NN	NN
DU156	0,159	NN	0,010	0,010	NN	0,034	0,069	NN	NN	3,665	0,112	0,579	NN	1,475	NN	NN	0,220
IAMI c22	NN	NN	53,940	0,596	NN	2,774	NN	NN	NN	0,030	0,028	0,030	NN	0,062	NN	NN	NN
CAP210	NN	NN	NN	NN	NN	NN	NN	NN	NN	15,170	2,942	6,819	NN	13,610	NN	NN	19,290
ZM 249	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN
DU 422	NN	NN	20,420	NN	NN	12,420	7,398	NN	NN	NN	NN	NN	NN	NN	NN	NN	14,480
C	DU172	NN	nd	NN	NN	NN	NN	NN	NN	0,760	NN	NN	NN	NN	NN	NN	8,377
CAP45	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	49,590
ZM214	NN	NN	NN	NN	NN	21,240	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	17,730
ZM135	NN	nd	NN	NN	NN	13,200	NN	NN	NN	NN	32,340	NN	NN	>50	NN	NN	NN

Table VIII-6: IC50 of the 16 Ab candidates against the 37vP (in µg/mL). Those 16 Abs were those that showed a potential neutralizing activity against the 6vP and that were produced in HEK293F and purified. They are classified by chronological visit-codes of the PBMC samples from which they have been isolated. The underlined values indicate IC50 estimated from curves that do not reach 100% neutralization (unfinished curves or plateau). The curves of the % neutralization according to the Ab dilutions are in Annex D.

D. Characterization of the newly identified NABs

1. Thirteen NABs from 4 families

Lineage	mAb	Isolation Time point	Seq Heavy Chain					Seq Light Chain					
			V gene	D gene	J gene	CDRH3 length	Mutation HV (%nt)	Mutation HJ (%nt)	V gene	J gene	CDRI3 length	Mutation LV (%nt)	Mutation U (%nt)
Lineage A	PbC11	v30	HV1-18*04	HD3-3*02	HJ6*02	23	11,5	14,5	KV1-5*01 or 03	KJ1*01	10	9,7	13,2
	PbC08	v30	HV1-18*04	HD3-3*02	HJ6*02		11,5	14,5	KV1-5*01 or 03	KJ1*01	9	9,7	2,7
	PbD02	v30	HV1-18*01	HD1-1*01	HJ6*02		12,5	16,2	KV1-5*03	KJ1*01	9	10,4	0
	PbD10	v36	HV1-18*01	HD3-3*01	HJ6*02		11,8	9,7	KV1-5*03	KJ1*01	9	11,8	10,5
	PcE02	v42	HV1-18*01	HD3-3*01	HJ6*02		15,3	19,4	KV1-5*03	KJ1*01	10	11,8	2,7
	PcE09	v42	HV1-18*01	HD3-3*01	HJ6*02		15,3	17,7	KV1-5*03	KJ1*01			
	PaD10	v66	HV1-18*01	HD3-3*01	HJ6*02		16,6	19,4	KV1-5*03	KJ1*01	10	14,7	38,8
	PaC11	v66	HV1-18*01	HD3-3*01	HJ6*02		16,9	21	KV1-5*01 or 03	KJ1*01	9	15,4	21
	PaF07	v66	HV1-18*01	HD3-3*01	HJ6*02		17,4	19,4	KV1-5*03	KJ1*01	10	14,7	34,2
	PbE05	v66	HV1-18*01	HD3-3*01	HJ6*02		16,9	17,7	KV1-5*03	KJ1*01	10	15,4	5,3
Lineage B	PcC11	v48	HV3-30*02 or 5*02	HD5-12*01	HJ4*03	18	10,4	14,6	LV2-14*01 or *03 or *04	LJ2*01 or J3*01 or *02	10	29,1	13,9
Lineage C	PbE04	v66	HV4-39*07	HD2-21*01	HJ4*03	17	15,5	14,5	LV2-8*01	LJ1*01	11	8,4	15,8
Lineage D	PaC04	v78	HV5-51*03 or 04	HD3-10*01	HJ4*02	12	12,9	10,5	LV1-51*01	LJ2*01 or J3*01 or *02	10	8,1	4,7

Table VIII-7: NABs heavy (H) and light (L) sequences characteristics: V(D)J genes affiliation, CDR3 lengths (nt) and mutations (% nucleotides) from the IMGT database. Grouped by lineages and isolation time-point.

Ten of the 13 newly isolated NABs belonged to the lineage A previously identified in our laboratory by Claire Rousset through a first sort, as detailed previously in part II-A-3, and characterized by the gene usage combination IGHV1-18*01 – IGHD3-3*01 – IGHJ6*02 for the heavy chain and IHKV1-5*01 – IGKJ1*01 for the associated light kappa chain, and by a long CDRH3 of 23 nucleotides (Table VIII-7). The mutation rates for the V and J genes of both HC and LC were various, ranging from 0 to 38%, with more mutations observed for the Abs isolated on later time-points. Abs PbC11 and PbC08 were assigned to the IGHV1-18*04 gene usage, however IGHV1-18*01 and 04 differ from 1 nucleotide only, it is thus very likely that both Abs belong to lineage A but acquired the mutation in the VH gene that changed their sub-family assignment.

As mentioned previously, PcC11 was part of the lineage B described previously, that used IGHV3-30*02 – IGHD1-20*01 or 2-15*01 or 2-21*02 or 3-22*01 – J4*01, 02 or 03 for the heavy chain and V2-14*01 – J2*01 for the lambda light chain. IGHD gene of PcC11 was however attributed to D5-12*01 by IMGT, though given the small size and variability of the D segment, their attributions are rarely accurate. The PcC11 mAb appeared highly mutated, notably with 29% mutated nucleotides for the V sequence while the somatic variants isolated had rates around 9 to 12% (Claire Rousset, PhD manuscript).

The 2 additional mAbs isolated belonging to new families, PbE04 and PaC04, were respectively defined as part of lineages C and D. They had shorter CDRH3 length of 17 and 12 respectively and low to similar nucleotides mutation rates (10.5 to 15.5% for HC, 4.7 to 15.8% for IC) compared to the other PC94 Nabs (9.7 to 21% for HC, 0 to 38.8 for IC).

2. Neutralization of autologous viruses

We next wondered about the emergence and evolution in donor PC94 of the newly isolated Abs from lineages A and B, and of Abs from the 2 new lineages, relative to each other.

As discussed previously, lineage A members neutralized efficiently the 12-MPI autologous variants but started to lose activity against the 18 and 24-MPI viruses to finally face a total escape around 30 MPI. In contrast, while Abs of lineage B that were not able to neutralize the 12-MPI viruses, they had an activity against the 18, 24 and 30 MPI, which they lose around 48 MPI (data of Claire Rousset PhD thesis). To understand how our new Abs fitted in this picture, we selected four of them to be tested against the longitudinal panel of autologous PC94 pseudoviruses: PaD10 and PbD10 from lineage A, and the two others mAbs from the newly identified lineage C and D, Abs PbE04 and PaC04 respectively (Table VIII-8). Lineage A mAbs PbD10 and PaD10 neutralized viruses from 12 and 18 MPI, PaD10 remained active against 24 MPI viruses, but both mAbs completely lost neutralizing activities against the viruses after 30 MPI, replicating the neutralization pattern that had been found for lineage A Ab relatives. To be noted, PbD10, isolated from an earlier time-point PBMC sample (v36) was much less potent than the other lineage A Abs (10 to 100 fold). PbE04 from lineage C showed potent neutralizing activity against the autologous viruses over a large time period, from 12 to 54 MPI, even though the strong potency was lost at 42 MPI. Conversely, PaC04 from lineage D was barely neutralizing autologous variants, with a very weak activity against the 12 MPI viruses and, somehow surprisingly, a small rebound against viruses of 36 to 60 MPI.

The new lineage A NAbs we isolated seem thus to follow in time the activity of the other lineage A members, mainly targeting early viruses of the first-year post-infection. In parallel, the newly isolated PbE04 NAb appears to have kept its neutralizing activity during a long period of 2 years, before viral escape. Concerning PaC04, the fact that it has an heterologous neutralizing activity, albeit weak, but not much autologous is intriguing: indeed observing Abs able to neutralize autologous strains but not heterologous is comprehensible (“off-track” lineages), but the opposite scenario rises the question of “what did drive the maturation of such antibodies to recognize large panel of circulating HIV-1 variants but not so much the strains they have encountered?”. One hypothesis would be that PaC04 would neutralize autologous variants prior to 12 MPI, thus not observable in our data picture. Another hypothesis might be that, the autologous viruses tested here being just a very limited fraction of the whole population present in the infected donor at each time-point, the neutralizing profiles observed here are not properly illustrative of the Abs-autologous viruses interactions that actually occurred throughout time.

		Lineage A						B			C	D	
		v36	v48	v48	v48	v48	v60	v66	v60	v60	V48	v66	v78
		PbD10	7A	8A	9A	11A	10A	PaD10	8B	11B	12B	PbE04	PaC04
v12	v12-007	2,304	0,073	0,054	0,091	0,031	0,105	0,025	NN	NN	NN	0,612	11,820
	v12-043	1,315	0,120	0,072	0,119	0,050	0,119	0,022	NN	NN	16,244	0,665	>50
	v12-088	3,705	0,382	0,084	0,099	0,038	0,130	0,021	NN	NN	NN	1,489	>50
v18	v18-017	>50	0,823	NN	0,402	NN	6,760	1,421	6,961	5,402	12,086	3,750	NN
	v18-046	26,750	0,475	18,124	0,305	NN	34,763	0,346	10,399	5,625	8,417	1,978	NN
	v18-077	NN	NN	NN	NN	NN	NN	NN	0,516	0,563	6,639	0,990	NN
	v18-095	NN	1,517	NN	0,513	NN	NN	8,771	9,296	3,051	6,937	1,685	NN
v24	v24-025	NN	1,695	NN	0,276	NN	NN	2,716	2,564	1,534	3,186	0,952	>50
	v24-043	NN	NN	0,096	13,288	0,069	0,220	0,564	5,940	1,922	4,698	NN	NN
	v24-059	NN	NN	0,213	19,776	0,135	0,339	0,400	11,821	4,634	10,066	<5	NN
	v24-087	NN	1,402	NN	0,472	NN	NN	24,530	4,282	1,917	2,570	0,663	NN
v30	v30-033	NN	NN	NN	NN	NN	NN	NN	13,417	6,055	9,380	NN	NN
	v30-064	NN	NN	NN	NN	NN	NN	NN	4,134	1,787	2,901	4,126	NN
	v30-092	NN	NN	NN	NN	NN	NN	NN	3,912	1,818	3,964	3,838	NN
v36	v36-033	NN	NN	NN	NN	NN	NN	NN	NN	NN	NN	1,902	NN
	v36-076	NN	NN	NN	NN	NN	NN	NN	3,208	1,709	2,516	4,194	NN
	v36-102	NN	NN	NN	NN	NN	NN	NN	6,926	2,237	5,172	1,182	22,000
v42	v42-117	NN	nd	nd	nd	nd	nd	>50	nd	nd	nd	26,600	5,140
v48	v48-28	NN	NN	NN	NN	NN	NN	NN	NN	9,356	NN	90,610	14,740
v54	v54-025	NN	nd	nd	nd	nd	nd	NN	NN	NN	NN	50,380	28,890
	v54-093	NN	nd	nd	nd	nd	nd	NN	NN	NN	NN	NN	>50
v60	v60-037	NN	nd	nd	nd	nd	nd	NN	nd	nd	nd	NN	>50
	v60-142	NN	nd	nd	nd	nd	nd	NN	nd	nd	nd	NN	>50
v66	v66-029	NN	nd	nd	nd	nd	nd	NN	nd	nd	nd	NN	NN
	v66-029	NN	nd	nd	nd	nd	nd	NN	nd	nd	nd	NN	NN
	v66-045	NN	nd	nd	nd	nd	nd	NN	NN	NN	NN	NN	NN
v78	v78-040	NN	nd	nd	nd	nd	nd	NN	nd	nd	nd	NN	20,710
	v78-058	NN	nd	nd	nd	nd	nd	NN	NN	NN	NN	NN	NN

Table VIII-8: IC50 of 4 of the BNABs recently isolated (names in black) aligned with 5 members of lineage A and 3 of lineage B isolated prior this PhD project (names in grey), against the longitudinal autologous viruses of PC94, ordered by time-point post-infection. IC50 are in µg/mL. nd = no data.

3. Partial completion of PC94 serum neutralizing activity

In order to verify whether those 13 new NABs could help explain the impressive neutralizing activity of the PC94 serum, the IC50s against the 37vP of all NABs isolated from PC94 were measured and aligned with the serum ID50 against the corresponding viruses (Table VIII-9). Notably, 6 viruses efficiently neutralized by the PC94 serum – 89.6, CAP210, PV04, ZM249, DU172 and ZM135 – could not be neutralized by any of the NABs isolated at the occasion of the first sort (corresponding to those written in grey in Table VIII-9, data from Claire Rousset). Among the newly isolated mAbs (written in black), PbE04 (lineage C) potently neutralized PV04, PaC04 (lineage D) showed some neutralizing activity against ZM249 and DU172, and PcE02 (new member of lineage A) neutralized ZM135, although weakly too. The Abs responsible for the neutralization of 89.6 and CAP210 were however not retrieved. When focusing on the lineage A members, some of the newly isolated NABs demonstrated better neutralization than their relatives isolated earlier, bringing thus more concordance with the serum neutralization data: this is the case of PaD10 and PaF07 against 6535, PaC11 against JRCSF and DU422 and especially PbD10, that had the best IC50 against TRO11, 92BR020, ADA, CAAN, 93IN905, DU156 and IAVIc22, making it the best BNAB of lineage A.

E. Evaluation of our new isolation strategies

1. Sorting strategy

Regarding the final obtention of several NABs, of which the large majority belong to lineages that were previously isolated using the initial differential sorting strategy, our new sorting strategy based on the use of recombinant Env trimers appeared successful. Nonetheless, no exact same Ab could be isolated between the 2 series of sorts, despite the isolation from common time-points samples. This may be independent of the sorting strategy though, and may be due to the intrinsic rarity of the target B-cells or a loss of the candidates in the course of the recovery steps that follow the sort.

Nonetheless, for the Abs that reached the BN activity characterization step, the alignment of the binding profile to the trimeric Env baits with the IC50 against the corresponding pseudovirus revealed discordances between binding and neutralization (Table VIII-10). Indeed, half of the B-cells that bound to the trimers (either JRFL_NFL or TRO11_SOSIP) produced IgGs with no neutralizing activity against the corresponding pseudoviruses. Conversely, one third of the Abs that neutralized JRFL or TRO11 strains were retrieved from B-cells unable to bind the corresponding baits. To be noted, both recombinant Env showed similar levels of discordances, hence neither the platform neither the strain type could be particularly questioned. The seemingly only hypothesis for this phenomenon would be that a large fraction of the recombinant trimers, which antigenicity was checked prior the sort, lost stability during the sorting experiment and was in fact presented for some part in a non-native conformation for the B-cells to bind.

To conclude, the trimer-based sorting strategy we designed that appeared theoretically particularly effective was not as successful as expected. Fortunately, the selection we did was not only based on the binding profiles of the B cells but also on the activation and microneutralization results.

	JRFL		TRO11	
	NFL Index Sorting	IC50 in 37vP	SOSIP Index Sorting	IC50 in 37vP
PaC04	1	NN	0	NN
PaC11	0	19,130	1	0,246
PaD10	1	0,094	1	4,000
PaF07	0	0,405	0	1,505
PbC08	1	3,840	1	NN
PbC11	1	NN	1	NN
PbD02	1	4,740	1	NN
PbD10	1	0,155	1	0,063
PbE04	1	0,068	0	0,492
PbE05	1	0,926	0	NN
PbF05	1	NN	0	NN
PcC11	1	NN	0	NN
PcE02	0	0,142	0	0,539
PcE09	1	NN	1	14,730
PcF07	1	NN	1	NN
PcF11	1	NN	1	NN

Table VIII-10: Comparison of the binding to trimer Env baits during the sort and the neutralizing activities against the corresponding pseudovirus for each 16 Abs selected by the 6vP screening.

2. B-cell activation

The percentages of B-cell activation we obtained ranged from 10 to 29% between the 4 experiments. The publication from which our protocol of activation is inspired announced activation efficiencies of 50%, however they seem to have seeded 8 cells/well that were sorted from healthy donors samples (Huang et al., 2013). Two of my colleagues who have strong experience B-cell activation, Fangzhu Zhao and Axelle Amen, both PhD students, commonly obtain percentages of B cell activation of 45% and 60% respectively, for healthy donors B-cells seeded at 1 cell/well. We thus had disappointing activation rates, that might be explained by the fact we worked on HIV-infected individuals, whom B-cells are known to be more fragile and difficult to activate.

3. Relevance of the microneutralization assay

	Activation level	JRFL		TRO11	
		4vP μ neut	37vP neut	4vP μ neut	37vP neut
PaC04	+	+	NN	-	NN
PaC11	+++	+++	19,130	-	0,246
PaD10	+++	+++	0,094	+++	4,000
PaF07	+++	+++	0,405	++	1,505
PbC08	+++	-	3,840	-	NN
PbC11	+++	-	NN	-	NN
PbD02	++	-	4,740	-	NN
PbD10	++	+++	0,155	+++	0,063
PbE04	++	+	0,068	++	0,492
PbE05	+++	+++	0,926	+++	NN
PbF05	+++	+	NN	-	NN
PcC11	+++	-	NN	-	NN
PcE02	+++	+++	0,142	+++	0,539
PcE09	+++	-	NN	-	14,730
PcF07	-	-	NN	-	NN
PcF11	+++	-	NN	+	NN

Table VIII-11: Comparison of the neutralizing activities of each NABs between the 4vP (microneutralization by activation supernatants) and the 37vP (by purified mAbs). IC50 are in μ g/mL.

Results of the microneutralization assay done with the activation supernatants containing the secreted IgGs were aligned with the IC50s calculated against the 37vP for the Abs that went through all the selection pipeline (Table VIII-11), in order to better evaluate the value of the microneutralization assay as a BNABs selection tool. Like with the binding profiles, discordances, though less important, were observed between the neutralization levels obtained with the supernatants and the purified Abs, not always explained by low B-cell activation levels (low Ab concentration). For instance, a small amount of Abs showed low neutralizing activity in the microneutralization while none in the neutralization assay (PaC04 and PbF05 against JRFL or PcF11 against TRO11). Conversely, few mAbs showed no activity in the microneutralization while having decent IC50s (between 0,2 and 4,8 μ g/mL) in the neutralization assay (like PbC08 and PbC02 against JRFL and PaC11 against TRO11). Finally, apart from

the activity of PbE05 against TRO11, none of the Abs that showed moderate to strong neutralizing activity in the microneutralization were not neutralizing in the final assay, even if the neutralization intensities were sometimes quite different.

Microneutralization by the supernatants was then more predictive of the final neutralization evaluation than the index sorting data and appeared more reliable for the identification of NAbs, although the microneutralization results were not exactly transposable to the neutralizing profile of the purified Abs.

4. HC and IC pairs recovery depending on the activation state

Another of our interrogations was whether the activation state of the well would increase the chances of chains genes recovery or not. Indeed, we notably expected a high PCR efficiency following activation, as the levels of mRNA that serves as template for the first step of gene amplification should be highly increased. However, the total pair recovery was only 25%, while it is usually >50% when performing the lysis and RT-PCR directly after the B-cell isolation (example 64,7% in [Rogers et al., 2020](#) for the B-cells sorting of COVID+ patients).

		Activation level			
		High	Medium	None	
Chain gene recovery	Pairs	18%	4%	3%	25%
	Single	7%	9%	14%	30%
	None	2%	8%	35%	45%
		27%	21%	53%	100%

Table VIII-12: Analysis of the pairs recoveries according to the activation states. Based on the 174 candidates chosen among the sorts abc233a, b and c.

As shown in Table VIII-12, the chances of recovering a pair of heavy and light chains were much better when the concerned well was highly activated. Similarly, non-activated wells mainly resulted in no gene recovery at all, although some pairs could exceptionally be retrieved. Hence, the pair recovery chance was 67% when the cells were highly activated, 46% when they were moderately to highly activated, but sharply decreased to 3% if the cells were not activated. In the end, the non-activated candidates were most of the time lost, as we could not amplify Ig genes.

The activation might then have been the most limiting step in our process, as its low success made us lose numerous B-cells with potentially important BN activity.

To be noted, as the first results on experiment abc233a already displayed this tendency of no pair recovery in absence of activation, the activation state was taken into account in the selection of the B-cells isolated in abc233b and c.

5. Cloning and Ab production yields

The cloning step resulted in 87/102 (88,7%) variable genes Abs that could be inserted in expression vectors. Such imperfect cloning yields are common. As a matter of comparison, the bulk-transformed ligation product of Rogers et al., 2020 was estimated at >86% recovery of fully functional cloned genes. First rounds of our Ig gene cloning were done with the Gibson Assembly kit (recombination and gap completion) which was successful for 69/102 chains. The analysis of the failed clonings revealed that the Gibson Assembly method occasionally resulted in a shifting of the Ig ORF, leading to an aborted or impossible translation. Those candidates were then cloned thanks to a T4 ligase (enzymatic digestion and ligation), which allowed the recovery of 18 additional chains. Despite multiple efforts, a total of 15 chains could not be cloned: 8 for unknown reasons and 7 because contaminant sequences kept being inserted.

The next step of Ab production in HEK293T was also responsible for the loss of some candidates - 3/36 – that could not be produced even though the variable sequence insertions and ORFs appeared correct.

6. The 6vP screening

As done for the index sorting and the functional screening, the results of the quick 6vP screening of the supernatants containing the selected Ab were aligned with those of the final neutralization assay, to evaluate the relevance of the screening of supernatants that preceded larger scale production and characterization (Table VIII-13). Supernatants and purified Abs gave globally concordant neutralizing profiles, even though some supernatants gave a lower estimation of the corresponding Ab potency (ex PbC08 or PbD10 against JRFL or PbE04 against TRO11), not explainable by low production, as they all produced greatly (see last column Table VIII-4). The neutralization of ZM249 gave several important discordances, probably because of the low titers of the pseudovirus, preventing the accuracy of the estimation of the neutralizing activity.

Therefore in our experiments, no neutralization (-) observed with the supernatants corresponded to $IC_{50} > 14,7 \mu\text{g/mL}$, little neutralization (+/-) was equivalent to $IC_{50} > 0,4$, small neutralization (+) to $IC_{50} > 0,09$ and strong neutralization (+++) to $IC_{50} > 0,06 \mu\text{g/mL}$.

	JRFL		TRO11		DU172		ZM 249	
	6vP screen	37vP IC50	6vP screen	37vP IC50	6vP screen	37vP IC50	6vP screen	37vP IC50
Pa-C04	-	NN	-	NN		<u>8,377</u>	+	14,480
Pa-C11	-	<u>19,130</u>	+	0,246		NN	-	NN
Pa-D10	+	0,094	+/-	<u>4,000</u>		NN	+	NN
Pa-F07	+	0,405	+/-	<u>1,505</u>		NN	-	32,820
Pb-C08	-	<u>3,840</u>	-	NN		NN	-	NN
Pb-C11	-	NN	-	NN		nd	-	NN
Pb-D02	-	<u>4,740</u>	-	NN		NN	-	NN
Pb-D10	+/-	0,155	+++	0,063		NN	-	NN
Pb-E04	+++	0,068	+/-	0,492	-	NN	-	NN
Pb-E05	+/-	<u>0,926</u>	-	NN	-	NN	-	NN
Pb-F05	-	NN	-	NN		NN	++	NN
Pc-C11	-	NN	-	NN		NN	-	NN
Pc-E02	+	0,142	+/-	0,539	-	NN	-	NN
Pc-E09	-	NN	-	<u>14,730</u>	-	NN	-	NN
Pc-F11	-	NN	-	NN		NN	-	NN

Table VIII-13: Comparison of the neutralizing activities of each NAb between the 6vP-screening (by supernatants) and the 37vP (by purified mAbs). IC50 are in µg/mL.

F. PC94: discussion and conclusion

1. Synthesis of the results of PC94 BNabs isolation

PC94 being the 2nd best neutralizer of the Protocol C cohort that our team is studying, we aimed to isolate and characterized BNabs from this donor and to describe their development. As explained above, Claire Rousset, a former PhD student of the laboratory, previously isolated Abs from PC94's PBMCs using a differential sorting strategy based on preliminary knowledge of the serum specificity that appeared largely directed against the HM patch of gp120. Twenty-seven NAbs were recovered, belonging to 2 distinct families, named lineage A and B. Those Nabs could however not entirely recapitulate the PC94 serum neutralizing activity, suggesting that other NAbs may had been missed in the isolation process, from the same lineages or other(s), toward the same epitope or other(s).

Thereby we performed PC94 memory B-cells sorting again, mainly based on a new strategy designed following our work on the PC02 donor. The 4 sorts performed resulted in the isolation of 2288 memory B-cells that were activated, thanks to a cocktail of cytokines and feeder cells, to make them secrete Abs. Supernatants containing the secreted Abs were then harvested for screening tests. A total of 402 wells (17,6%) were shown to have been properly activated. The supernatants were then screened in a micro-neutralization assay against a 4vP. The alignments of the index sorting, the activation state and the microneutralization profile resulted in the selection of 179 candidates with potential neutralizing activity. The corresponding extracted RNAs went through RT-PCR and 2 successive PCRs to amplify the variable Ig genes (HC, KC and LC in parallel). Finally, 51 pairs of heavy and light chain pairs were recovered. Thirty-six could be cloned in expression vectors and transfected in mammalian cells for Ab production. Thirty-three were successfully produced and used in a screening neutralization assay against a 6vP. Sixteen were further chosen to be tested for their neutralizing activity against the 37vP. One Ab was not neutralizing and two very weakly and against very few pseudoviruses. The remaining 13 Abs were neutralizing, with various breadth and potencies. Ten of them belonged to the lineage A previously identified, with globally similar profiles as their relatives, though showing slightly better breadth and sometimes better potencies. One Ab was part of the lineage B but had very low breadth and potency. The 2 last Abs were from different families and showed specific profiles, with one displaying great BN activity against the viruses of clade B and the other more specifically directed against clade C viruses, although with lesser potency. They were affiliated to lineages named C and D respectively. Newly isolated lineage A NAbs had similar 23 aa-long CDRH3 as their relatives and HC SHM rates ranging from 11,5 to 19,4% nt. The lineage B NAb had also the same CDRH3 length of 18 aa as its relatives and an HC mutation rate similar to the less mutated NAbs of the same lineage. The 2 lineages C and D Abs had shorter CDRH3 loops of 17 and 12 aa, respectively, and HC mutation rates ranging from 14,5 to 15,5% nt for the lineage C Ab, and 10,5 to 12,9% nt for the lineage D Ab. The neutralizing activities of the 2 new lineage A and both lineage C and D Nabs were then evaluated against longitudinal autologous viruses. The early autologous activity of the lineage A members was

confirmed (12, 18 and 24 MPI), while the lineage C mAb potently neutralized most of the viruses until 36 MPI and the lineage D Ab had almost no activity against the autologous viruses. The comparison of the activity of the totality of the 40 isolated PC94 NAb against the 37vP with the one of the PC94 serum revealed that the newly isolated Abs were able to fill some “gaps” in the neutralizing spectrum, but that some viruses remained not or poorly neutralized by any Ab while being strongly neutralized by the PC94 serum. Overall, it appears that either Abs from the current lineages, in particular lineage A, that may recapitulate the breadth of the serum have still not been isolated or that PC94 may have developed various lineages, probably targeting several specificities, to reach his great BN.

2. PC94 Abs: Perspectives

The newly isolated NAb require further characterization. First they need to be evaluated against bigger panels of pseudoviruses, notably panels predictive of the breadth and potency against the largest panels, like the 105vP, to better estimate their BN activity.

Secondly, It would be interesting to identify their specificity, in particular those of lineages C and D. It is expected that the newly isolated NAb belonging to lineages A and B target the HM Patch like their relatives, but we are blind concerning the specificity of both PbE04 and PaC04. The only clue that may guide us is the comparison of the neutralization of BG505-T332N by the B cell supernatants in the 6vP-screening, with that of BG505 WT from the 37vP by the corresponding cloned and purified mAb (Table VIII-14). The data seem to confirm that some of the new lineage A mAbs, which showed activity in the 6vp screen, are N332 dependent, while Ab PbE04 from lineage C appears to be N332 independent.

		BG505-T332N	BG505 WT
Lineage A	PbC11	+/-	17,860
	PbC08	-	NN
	PbD02	-	NN
	PbD10	+	NN
	PcE02	-	NN
	PcE09	-	NN
	PaD10	+++	NN
	PaC11	-	NN
	PaF07	+	NN
	PbE05	+	NN
Lineage B	PcC11	-	NN
Lineage C	PbE04	+++	0,029
Lineage D	PaC04	-	NN

Table VIII-14 : alignment of the neutralizing activities against BG505-T332N (6vP-screening) with that of BG505 WT (37vP). IC50 are in µg/mL.


No indication on a potential N332 dependency can be taken for the Abs of lineages B and D as they did not neutralize neither BG505 WT nor BG505-T332N.

Data from the PhD manuscript of Claire Rousset however raised an interesting consideration. She evaluated the enrichment of the heavy chains she recovered at the end of the gene amplification step. Thirty-eight percent of the HV genes were from the HV5-51*03 family, although they were associated with at least 3 different HJ genes. This observation was not exploited further since Claire Rousset focused on the HV enrichment among the B-cells that showed N332 dependency in the index sorting, and as HV5-51*03 did not appear in this selection. Donor PC94 thus has an important proportion of his Env-specific B-cell repertoire that uses HV5-51*03, corresponding to Abs that are not N332 dependent. This leads us to hypothesize that PaC04, which uses HV5-51*03, may not be N332 dependent (although it may still target surrounding residues in the HM patch) and may have a number of relatives, potentially with broader and more potent neutralizing activity.

In any case, the epitope of the mAbs we have isolated should be precisely mapped, either via EM or eventually X-ray crystallography.

Finally, it would be of great interest to describe the evolutionary longitudinal pathway of each lineage, using specifically NGS approaches, as well as the interplay with autologous viruses.

Overall, the study of PC94 still needs to be completed to achieve the overarching goal of a deep understanding of the development of BN in this donor, that will eventually feed into rational design vaccine strategies.



General conclusion

This PhD project aimed thus to isolate and characterize the BNABs of two elite neutralizers, PC02 and PC94. All-in-all, the work performed on both donors, although informative in regard to breadth development, also illustrates how difficult BNAB isolation can be. Indeed, we did not succeed to characterize the BNABs specificities of PC02 on one hand, and to isolate BNABs recapitulating the remarkable PC94 breadth on the other hand, despite the use of state-of-the-art methods and tools.

One first hypothetical reason to the potential lack of some BNABs isolation might be technical. Our Ab isolation strategy was made of a succession of different steps, each of them not always optimal and associated with potential caveats. As a first step, the sorting with Env trimers was in itself imperfect. Indeed, it is likely for example from our results that the trimer baits are not entirely proper mimics of the native functional Env as can be found on the virion surface, allowing for the isolation of Abs with no or very narrow neutralizing activity. This is certainly of concern as the trimers were designed with BNAB elicitation in mind. Furthermore, each step of the Ab isolation pipeline had an imperfect yield. In consequence, the strategy we designed, combining bait-specific sorting and activation/functional screening, although theoretically powerful, may have led to a relatively poor yield by the end. As said in French « Le mieux est l'ennemi du bien ». We thus might have properly first selected B cells/Abs we were looking for but lost them by the end of the whole process comprising activation, screen, PCR amplification, cloning... In any case, we hope that the development, testing and trouble-shooting of the Ab isolation methods done in this PhD, will be beneficial to future studies HIV Abs but also for the isolation of mAbs to other pathogens, as currently in progress in our laboratory. In stepping back, maybe the current methods used for BNAB isolation are now at the edge of their capabilities, as limited by the PBMC sample size, proportion of activation that succeeds, the high work load and cost associated. Novel NGS approaches, such as 10X genomics, could constitute the best hope to provide a more complete picture of how neutralization arises in elite neutralizers, by both exploring the full B cell repertoire and enabling the production of the corresponding Abs thanks to conservation of the HC/IC pairing.

A second hypothetical reason for which we did not isolate BNABs recapitulating the breadth of donor PC94 might be that the very broad NAB lineage we were looking for does not exist, and the broad neutralization is made by the co-existence of several NAB lineages with mild breadth. In this regard, it is likely that the first described BNABs may have been isolated because selected from donors presenting an “easy scenario”: such as a clear single specificity explaining the whole serum breadth, or a high frequency of memory B cells bearing the BNAB specificity. In addition, successful isolations resulted in a published story, while the number of failed isolation attempts is unknown. Hence evaluating the proportion of individuals who develop BNABs fully explaining the breadth of their serum versus the role of technical issues and bias in BNAB isolation appears quite difficult. In a vaccination context, it may seem more appealing to design rational strategies to induce a single BNAB lineage (or rather x2 or x3 to optimize breadth coverage) than to have to spark multiple mild BN lineages. The germline-targeting and maturation driving approach susceptible to elicit BNABs has notably recently provided optimism by the successful activation of specific rare B cell precursor of VRC01-class BNABs

in humans. One small step for HIV vaccine, maybe a big step for mankind, though no proof has been made yet in human about the feasibility of maturation driving.

In conclusion, as an efficient HIV vaccine is still a scientific and public health Graal, eliciting BNABs is a high priority of the HIV vaccine field and explaining the mechanisms of development of such Abs remains essential. Why some infected individuals become neutralizers and others not is still mysterious, and the number of fully described BNAB lineages somehow limited. The latter, however, have provided crucial insights to innovative rational lineage-based vaccine approaches, potentially paving the way to novel vaccines for HIV and potentially against other pathogens.

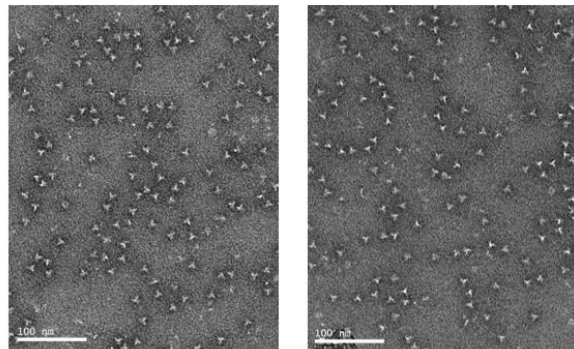
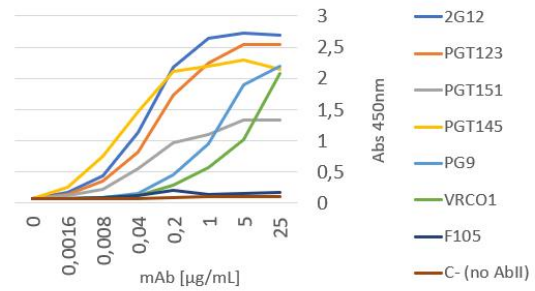


Annexes

A. Catalogue of all Env recombinant trimers produced

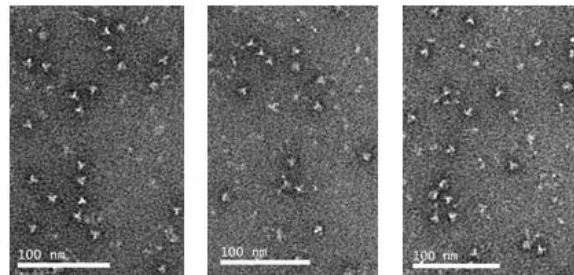
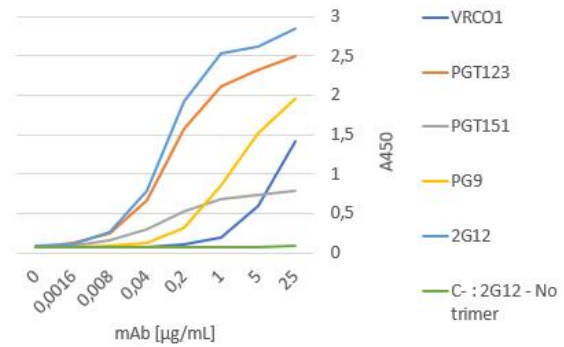
BG505_NFL_D368R_G508A

Clade	A
Tier	2
Tag	His
MW (kDa)	224
ϵ	1,4565
Date Production	20180118
Volume F	500 mL
Transfectant	Fectin
Affinity	Lectin
Negative selection	--
Biotinylation	--
Biotinylation Test	--
Final Yield	12 * 100 μ L * 1,95 mg/mL
Buffer	1X PBS
Comment	TD - CC, G508A
Conservation	-20°C



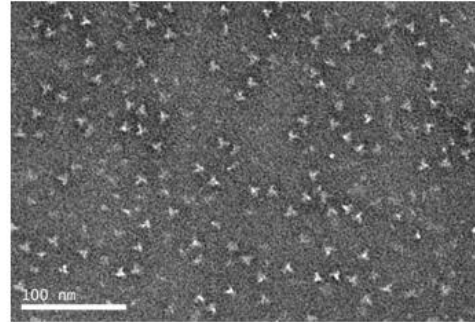
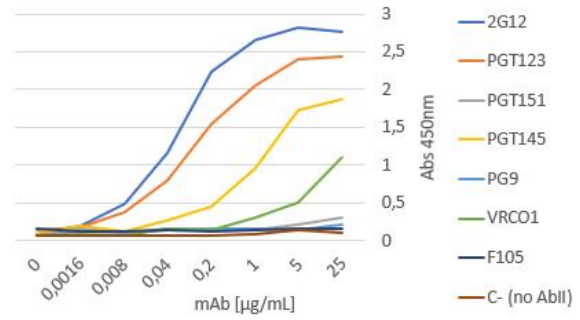
BG505_NFL_D368R

Clade	A
Tier	2
Tag	His
MW (kDa)	224
ϵ	1,4565
Date Production	20180817
Volume F	500 mL
Transfectant	Fectin
Affinity	Lectin
Negative selection	--
Biotinylation	--
Biotinylation Test	--
Final Yield	~ 20 * 100 μ L * 1,22 mg/mL
Buffer	1X PBS
Comment	TD - CC, Glycine linker restored
Conservation	-20°C



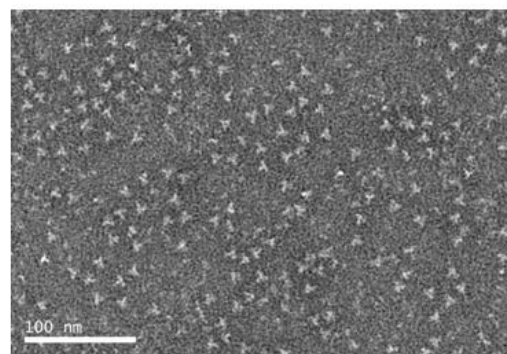
SC422_NFL_D368R

Clade	B
Tier	2
Tag	His
MW (kDa)	225
ϵ	1,6135
Date Production	20180126
Volume F	500 mL
Transfectant	Fectin
Affinity	Lectin
Negative selection	--
Biotinylation	--
Biotinylation Test	--
Final Yield	9 * 100 μ L * 0,38 mg/mL
Buffer	1X PBS
Comment	TD – CC, PG9 and PGT151 should bind
Conservation	-20°C



BG505_NFL_D368R

Clade	A
Tier	2
Tag	His
MW (kDa)	224
ϵ	1,4565
Date Production	20190122
Volume F	250 mL
Transfectant	Fectin
Affinity	Lectin
Negative selection	--
Biotinylation	--
Biotinylation Test	--
Final Yield	2,5 * 100 μ L * 0,64 mg/mL
Buffer	1X PBS
Comment	TD – CC
Conservation	-20°C



TRO11_SOSIP_D368R_p1

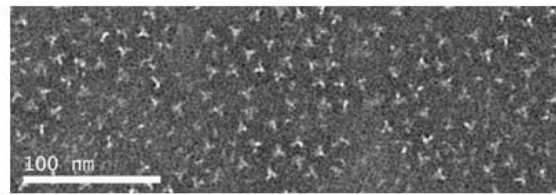
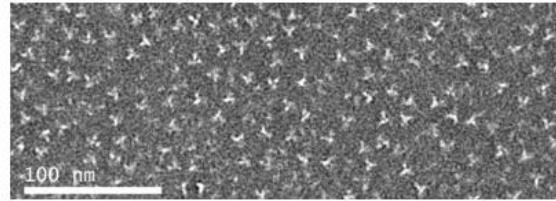
Clade	B
Tier	2
Tag	AVI
MW (kDa)	234
ϵ	1,448
Date Production	20190122
Volume F	250 mL
Transfectant	Fectin
Affinity	Lectin
Negative selection	--
Biotinylation	--
Biotinylation Test	--
Final Yield	2,5 * 100 μ L * 0,64 mg/mL
Buffer	1X PBS
Comment	--
Conservation	-20°C

TRO11_SOSIP_D368R_p2

Clade	B
Tier	2
Tag	AVI
MW (kDa)	234
ϵ	1,448
Date Production	20190122
Volume F	250 mL
Transfectant	Fectin
Affinity	Lectin
Negative selection	--
Biotinylation	--
Biotinylation Test	--
Final Yield	2,5 * 100 μ L * 1,38 mg/mL
Buffer	1X PBS
Comment	--
Conservation	-20°C

25710_SOSIP_D368R

Clade	B
Tier	1B or 2
Tag	AVI
MW (kDa)	229
ϵ	1,452
Date Production	20190123
Volume F	250 mL
Transfectant	Fectin
Affinity	Lectin
Negative selection	--
Biotinylation	--
Biotinylation Test	--
Final Yield	11 * 100 μ L * 1 mg/mL
Buffer	1X PBS
Comment	--
Conservation	-20°C

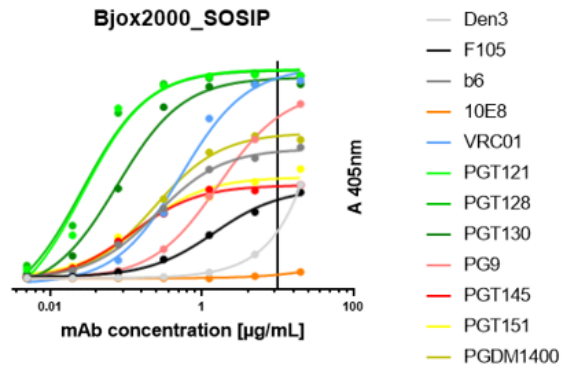


398F1_SOSIP_D368R

Clade	A
Tier	1
Tag	AVI
MW (kDa)	230
ϵ	1,545
Date Production	20190123
Volume F	250 mL
Transfectant	Fectin
Affinity	Lectin
Negative selection	--
Biotinylation	--
Biotinylation Test	--
Final Yield	2,5 * 100 μ L * 0,60 mg/mL
Buffer	1X PBS
Comment	--
Conservation	-20°C

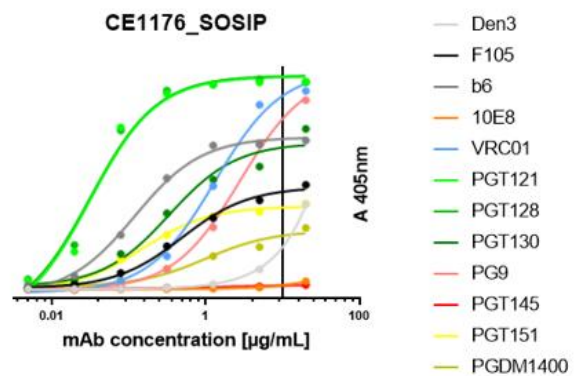
Bjox2000_SOSIP

Clade	AG
Tier	2
Tag	AVI
MW (kDa)	228
ϵ	1,537
Date Production	20190219
Volume F	2L
Transfectant	PEI 40K
Affinity	Lectin
Negative selection	--
Biotinylation	o/n 4°C
Biotinylation Test	OK
Final Yield	22 * 20 μ L * 0,94 mg/mL
Buffer	1X PBS
Comment	Open Conformation
Conservation	-80°C



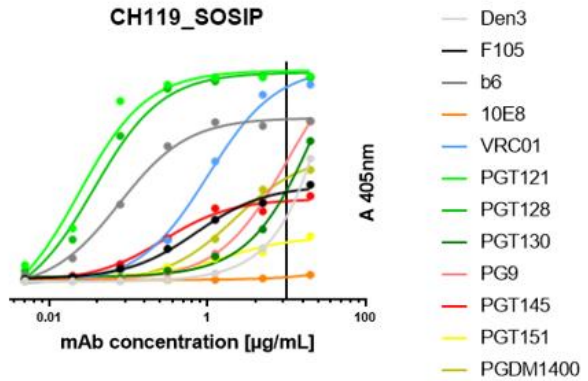
CE1176_SOSIP

Clade	C
Tier	2
Tag	AVI
MW (kDa)	229
ϵ	1,533
Date Production	20190308
Volume F	2L
Transfectant	PEI 40K
Affinity	Lectin
Negative selection	--
Biotinylation	o/n 4°C
Biotinylation Test	OK
Final Yield	37 * 20 μ L * 1 mg/mL
Buffer	1X PBS
Comment	Open Conformation
Conservation	-80°C



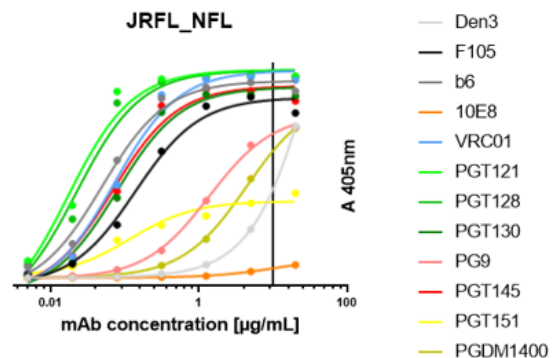
CH119_SOSIP

Clade	AG
Tier	2
Tag	AVI
MW (kDa)	232
ϵ	1,555
Date Production	20190219
Volume F	2L
Transfectant	PEI 40K
Affinity	Lectin
Negative selection	--
Biotinylation	o/n 4°C
Biotinylation Test	OK
Final Yield	26 * 20 μ L * 1 mg/mL
Buffer	1X PBS
Comment	Open Conformation
Conservation	-80°C



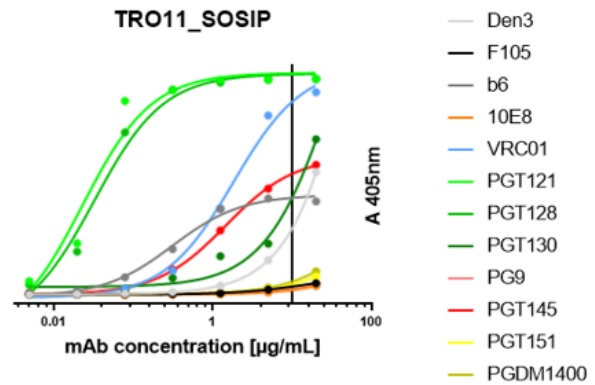
JRFL_NFL

Clade	B
Tier	3
Tag	AVI
MW (kDa)	229
ϵ	1,543
Date Production	20190228
Volume F	2L
Transfectant	PEI 40K
Affinity	Lectin
Negative selection	--
Biotinylation	o/n - 4°C
Biotinylation Test	OK
Final Yield	26 * 20 μ L * 1 mg/mL
Buffer	1X PBS
Comment	Open Conformation
Conservation	-80°C



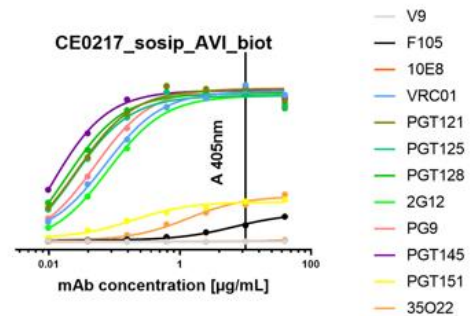
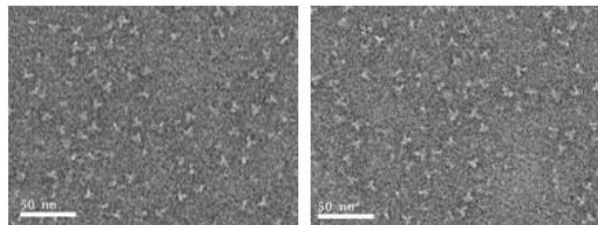
TRO11_SOSIP

Clade	B
Tier	2
Tag	AVI
MW (kDa)	234
ϵ	1,532
Date Production	20190305
Volume F	2L
Transfectant	PEI 40K
Affinity	Lectin
Negative selection	--
Biotinylation	o/n 4°C
Biotinylation Test	OK
Final Yield	44 * 20 μ L * 1,2 mg/mL
Buffer	1X PBS
Comment	Wrong profile
Conservation	-80°C



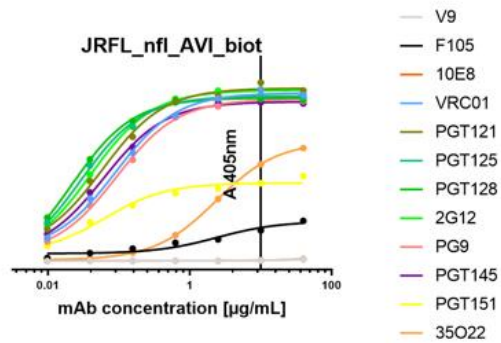
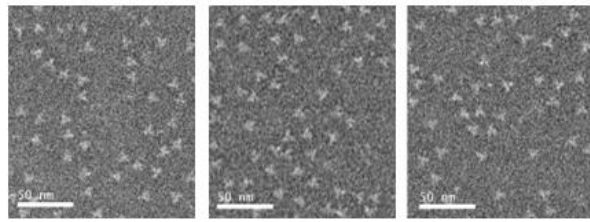
CE0217_SOSIP

Clade	C
Tier	?
Tag	AVI
MW (kDa)	229
ϵ	1,571
Date Production	20190910
Volume F	1L
Transfectant	Fectin
Affinity	Ab PGT 145
Negative selection	Ab F105
Biotinylation	4°C - o/n
Biotinylation Test	50%
Final Yield	8 * 10 μ L * 1,00 mg/mL
Buffer	1X TBS
Comment	--
Conservation	-80°C



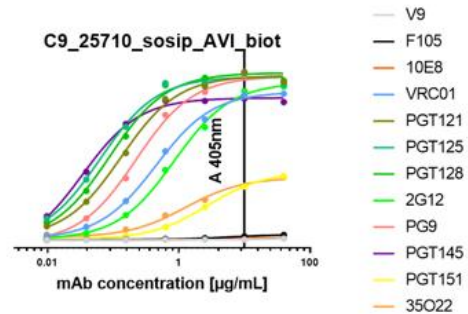
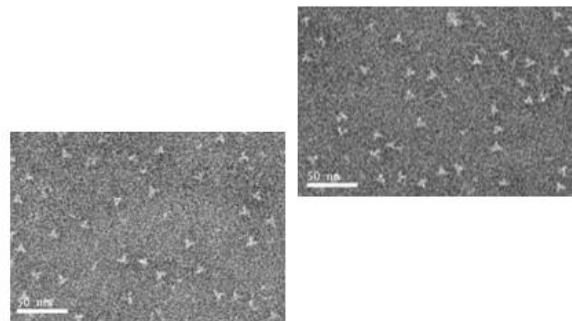
JRFL_NFL

Clade	B
Tier	3
Tag	AVI
MW (kDa)	229
ϵ	1,543
Date Production	20190914
Volume F	1L
Transfectant	Fectin
Affinity	Lectin
Negative selection	Ab F105
Biotinylation	RT – 2h
Biotinylation Test	50%
Final Yield	9 * 10 μ L * 1,00 mg/mL
Buffer	1X TBS
Comment	Sequencing showed T569G
Conservation	-80°C



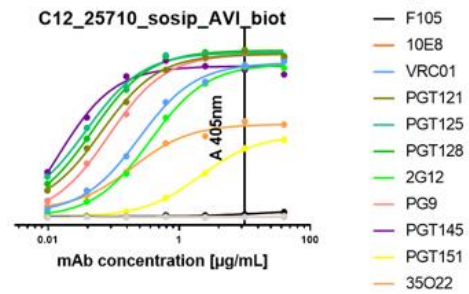
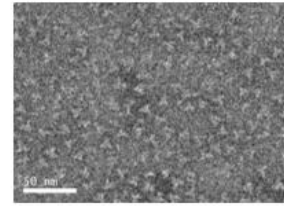
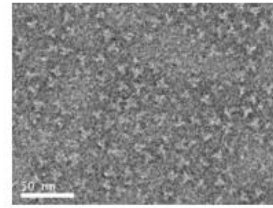
25710_SOSIP_p1

Clade	B
Tier	1B or 2
Tag	AVI
MW (kDa)	229
ϵ	1,533
Date Production	20190918
Volume F	500 mL
Transfectant	Fectin
Affinity	Ab PGT 145
Negative selection	--
Biotinylation	4°C – o/n
Biotinylation Test	75%
Final Yield	5 * 10 μ L * 0,65 mg/mL
Buffer	1X TBS
Comment	--
Conservation	-80°C



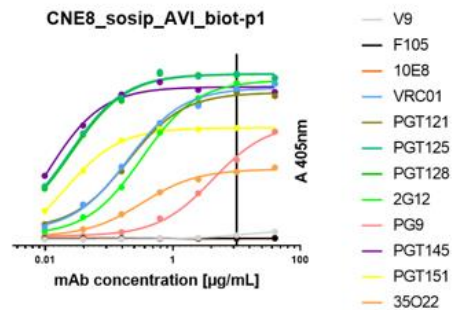
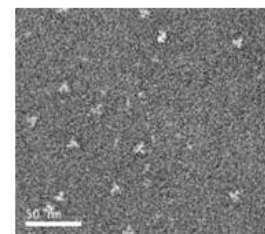
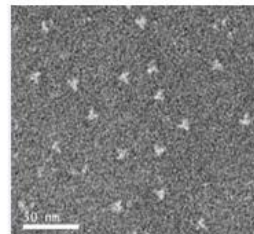
25710_SOSIP_p2

Clade	B
Tier	1B or 2
Tag	AVI
MW (kDa)	229
ϵ	1,533
Date Production	20190918
Volume F	500 mL
Transfectant	Fectin
Affinity	Ab PGT 145
Negative selection	--
Biotinylation	4°C – o/n
Biotinylation Test	75%
Final Yield	7 * 10 μ L * 1,00 mg/mL
Buffer	1X TBS
Comment	--
Conservation	-80°C



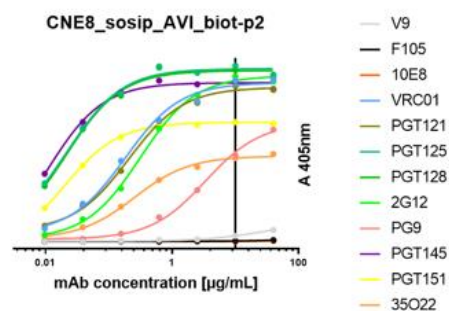
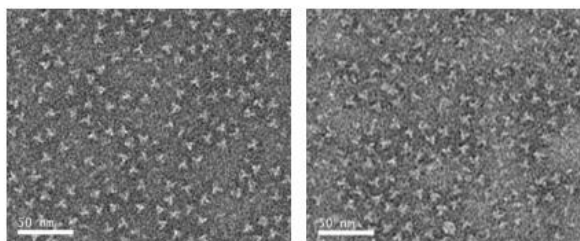
CNE8_SOSIP_p1

Clade	AE
Tier	2 or 3
Tag	AVI
MW (kDa)	224
ϵ	1,603
Date Production	20191003
Volume F	500 mL
Transfectant	Fectin
Affinity	Ab PGT145
Negative selection	Ab F105
Biotinylation	RT – 2h
Biotinylation Test	50%
Final Yield	12 * 10 μ L * 1,00 mg/mL
Buffer	1X TBS
Comment	--
Conservation	-80°C



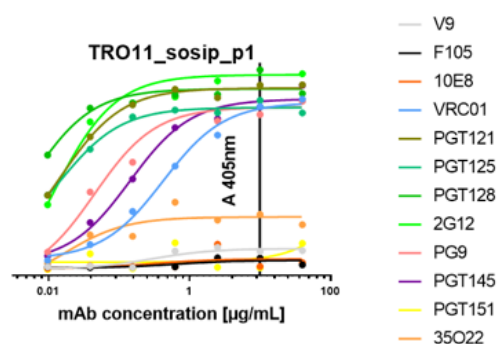
CNE8_SOSIP_p2

Clade	AE
Tier	2 or 3
Tag	AVI
MW (kDa)	224
ϵ	1,603
Date Production	20191003
Volume F	500 mL
Transfectant	Fectin
Affinity	Ab PGT145
Negative selection	Ab F105
Biotinylation	RT - 2h
Biotinylation Test	50%
Final Yield	3 * 20 μ L * 0,45 mg/mL
Buffer	1X TBS
Comment	--
Conservation	-80°C



TRO11_SOSIP_p1

Clade	B
Tier	2
Tag	AVI
MW (kDa)	234
ϵ	1,532
Date Production	20191217
Volume F	500 mL
Transfectant	Fectin
Affinity	Ab PGT 145
Negative selection	--
Biotinylation	RT - 2h30
Biotinylation Test	~70%
Final Yield	28 * 10 μ L * 1,00 mg/mL
Buffer	1X TBS
Comment	Eluted with H2O
Conservation	-80°C



TRO11_SOSIP_p2

Clade	B
Tier	2
Tag	AVI
MW (kDa)	234
ϵ	1,532
Date Production	20191217
Volume F	500 mL
Transfectant	Fectin
Affinity	Ab PGT 145
Negative selection	--
Biotinylation	RT - 2h30
Biotinylation Test	~70%
Final Yield	51 * 10 μ L * 1,00 mg/mL
Buffer	1X TBS
Comment	Eluted with H2O
Conservation	-80°C

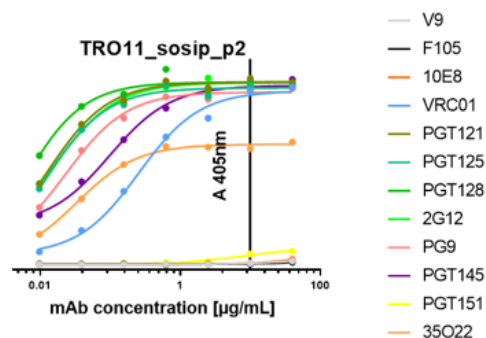


Figure X-1: Catalogue of the 21 soluble recombinant trimers produced during this PhD project : informations about the strains, the purification process, the yield and characterization (ELISA and/or EM). Antigenicity was compared with the neutralization data obtained from the database CatNap. EM negative staining was performed by Daphna Fenel, who used the EM facilities at the Grenoble Instruct-ERIC Center (ISBG ; UMS 3518 CNRS CEA-UGA-EMBL) with support from the French Infrastructure for Integrated Structural Biology (FRISBI ; ANR-10-INSB-05-02) and GRAL, a project of the University Grenoble Alpes graduate school (Ecoles Universitaires de Recherche) CBH-EUR-GS (ANR-17-EURE-0003) within the Grenoble Partnership for Structural Biology. The IBS Electron Microscope facility is supported by the Auvergne Rhône-Alpes Region, the Fonds Feder, the Fondation pour la Recherche Médicale and GIS-IBiSA.

B. Competition assays results with control BNABs

Competitor Trimer			Ctrl mAb		Pseudovirus		Fold Increase IC50			
Strain	Platform	Mutation	Concentration used (µg/mL)	Name	Epitope	Strain	Clade	Mean	+/-	SD
BG505	NFL	D368R - G508A	0,2	PGT145	Apex	BG505	A	0,91		
			1	PGT145	Apex	BG505	A	0,24		
			50	PGT145	Apex	BG505	A	1,01		
			50	PGT123	HM Patch	BG505	A	11,73		
			0,2	PGT145	Apex	KNH1144	A	0,71		
			1	PGT145	Apex	KNH1144	A	0,19		
			5	PGT145	Apex	KNH1144	A	0,37		
			0,2	PGT145	Apex	92TH021	AE	1,41		
			1	PGT145	Apex	92TH021	AE	0,29		
			5	PGT145	Apex	92TH021	AE	1,55		
		0,2	PGT145	Apex	DH12	B	1,72			
		1	PGT145	Apex	DH12	B	0,50			
		0,2	PGT145	Apex	REJO	B	2,47			
		1	PGT145	Apex	REJO	B	1,23			
		5	PGT145	Apex	REJO	B	2,85			
		0,2	PGT145	Apex	SC422	B	0,78			
		1	PGT145	Apex	SC422	B	0,07			
		5	PGT145	Apex	SC422	B	0,47			
		50	PGT145	Apex	SC422	B	24,19			
		50	PGT123	HM Patch	SC422	B	5,85			
0,2	PGT145	Apex	DU156	C	1,22					
1	PGT145	Apex	DU156	C	0,41					
D368R			25	PGT151	Interface	92RW020	A	0,87	+/-	0,89
			25	PGT123	HM Patch	92RW020	A	652,50		
			25	PGT123	HM Patch	BG505	A	1,27		
			25	PGT151	Interface	DH12	B	1,25		
			25	PGT123	HM Patch	DH12	B	287,35	+/-	0,88
			25	PGT151	Interface	JRCSF	B	1,62		
			25	PGT123	HM Patch	JRCSF	B	56,07		
25	PGT123	HM Patch	SC422	B	60,93	+/-	0,46			
25	PGT123	HM Patch	IAVIC22	C	355,50					
SC422	NFL	D368R	0,2	PGT145	Apex	BG505	A	0,72		
			1	PGT145	Apex	BG505	A	0,12		
			5	PGT145	Apex	BG505	A	0,15		
			25	PGT123	HM Patch	BG505	A	1,58		
			50	PGT123	HM Patch	BG505	A	3,26		
			0,2	PGT145	Apex	KNH1144	A	2,51		
			1	PGT145	Apex	KNH1144	A	0,45		
			5	PGT145	Apex	KNH1144	A	0,65		
			25	PGT123	HM Patch	92RW020	A	98,86		
			50	PGT123	HM Patch	92RW020	A	81,78		
		0,2	PGT145	Apex	92TH021	AE	0,51			
		1	PGT145	Apex	92TH021	AE	0,56			
		5	PGT145	Apex	92TH021	AE	0,70			
		0,2	PGT145	Apex	DH12	B	0,52			
		1	PGT145	Apex	DH12	B	0,40			
		5	PGT145	Apex	DH12	B	0,54			
		25	PGT123	HM Patch	DH12	B	45,53			
		50	PGT123	HM Patch	DH12	B	103,28			
		0,2	PGT145	Apex	REJO	B	0,88			
		1	PGT145	Apex	REJO	B	0,40			
5	PGT145	Apex	REJO	B	3,23					
0,2	PGT145	Apex	SC422	B	0,75					
1	PGT145	Apex	SC422	B	0,37					
5	PGT145	Apex	SC422	B	0,57					
25	PGT123	HM Patch	SC422	B	40,10					
50	PGT145	Apex	SC422	B	28,26					
50	PGT123	HM Patch	SC422	B	7,15					
25	PGT123	HM Patch	JRCSF	B	29,37					
50	PGT123	HM Patch	JRCSF	B	11,41					
0,2	PGT145	Apex	DU156	C	1,91	+/-	7,47			
1	PGT145	Apex	DU156	C	0,33					
5	PGT145	Apex	DU156	C	0,29					
25	PGT123	HM Patch	IAVIC22	C	76,50					

Impact of the competition on the neutralizing activity
Ratio IC50 Compet / IC50 No Compet

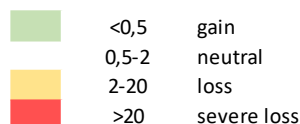
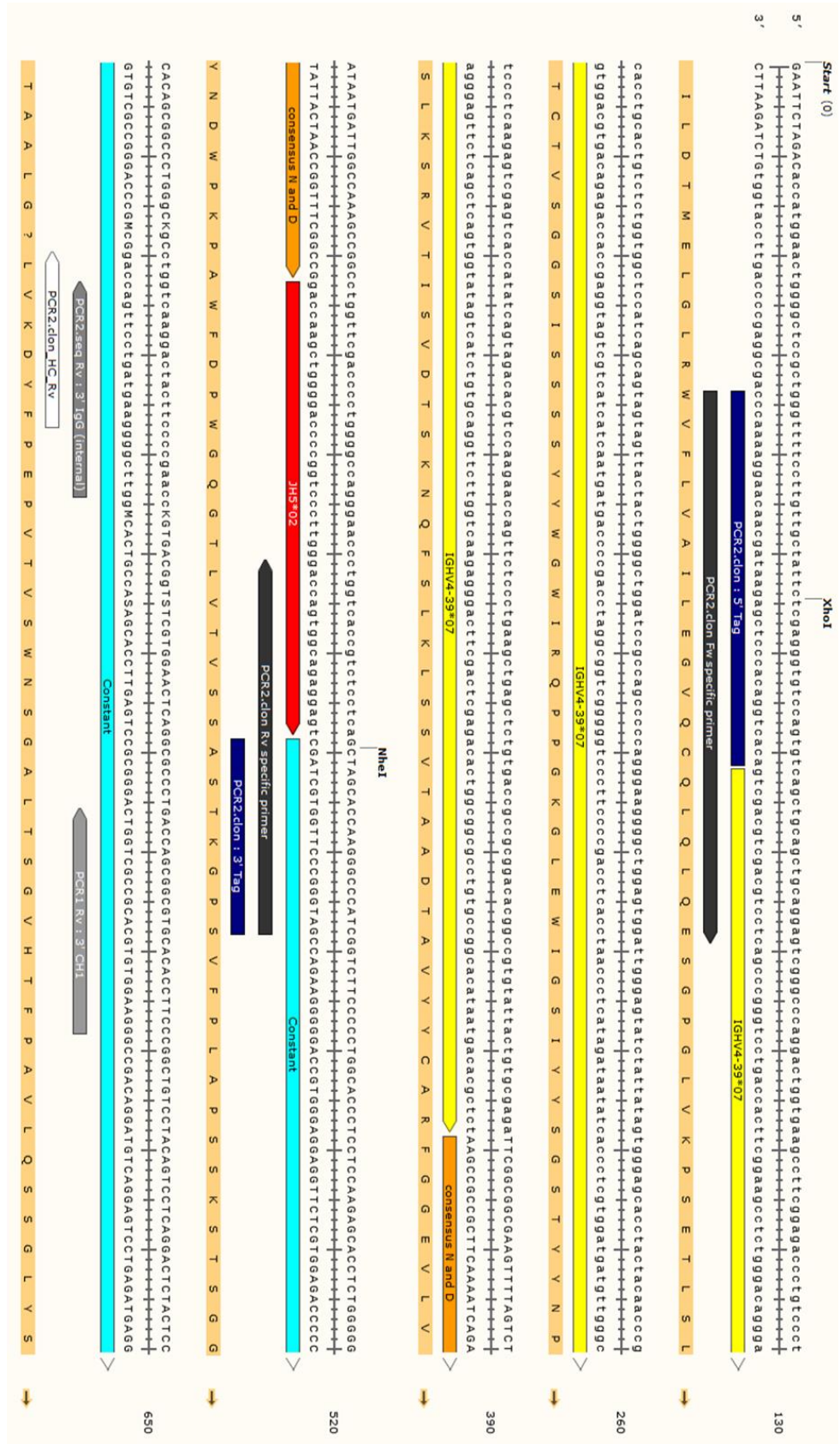


Table X-1: Mean and standard deviation (if experiment repeated) of fold increase in IC50 of monoclonal BNABs due to binding competition of soluble trimer mimics D368R in a neutralization assay.

C. Vector maps of heavy, light kappa and light lambda



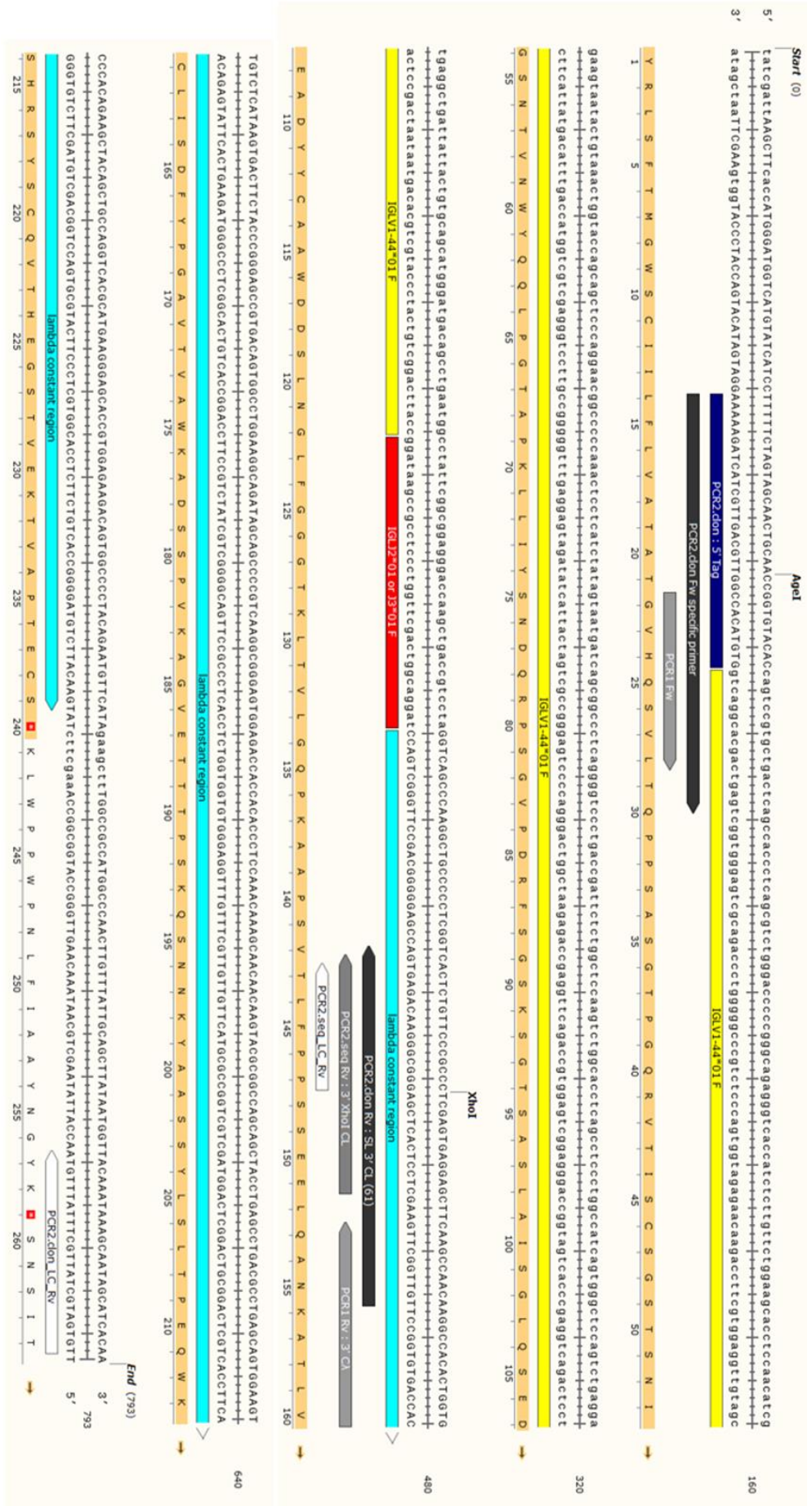
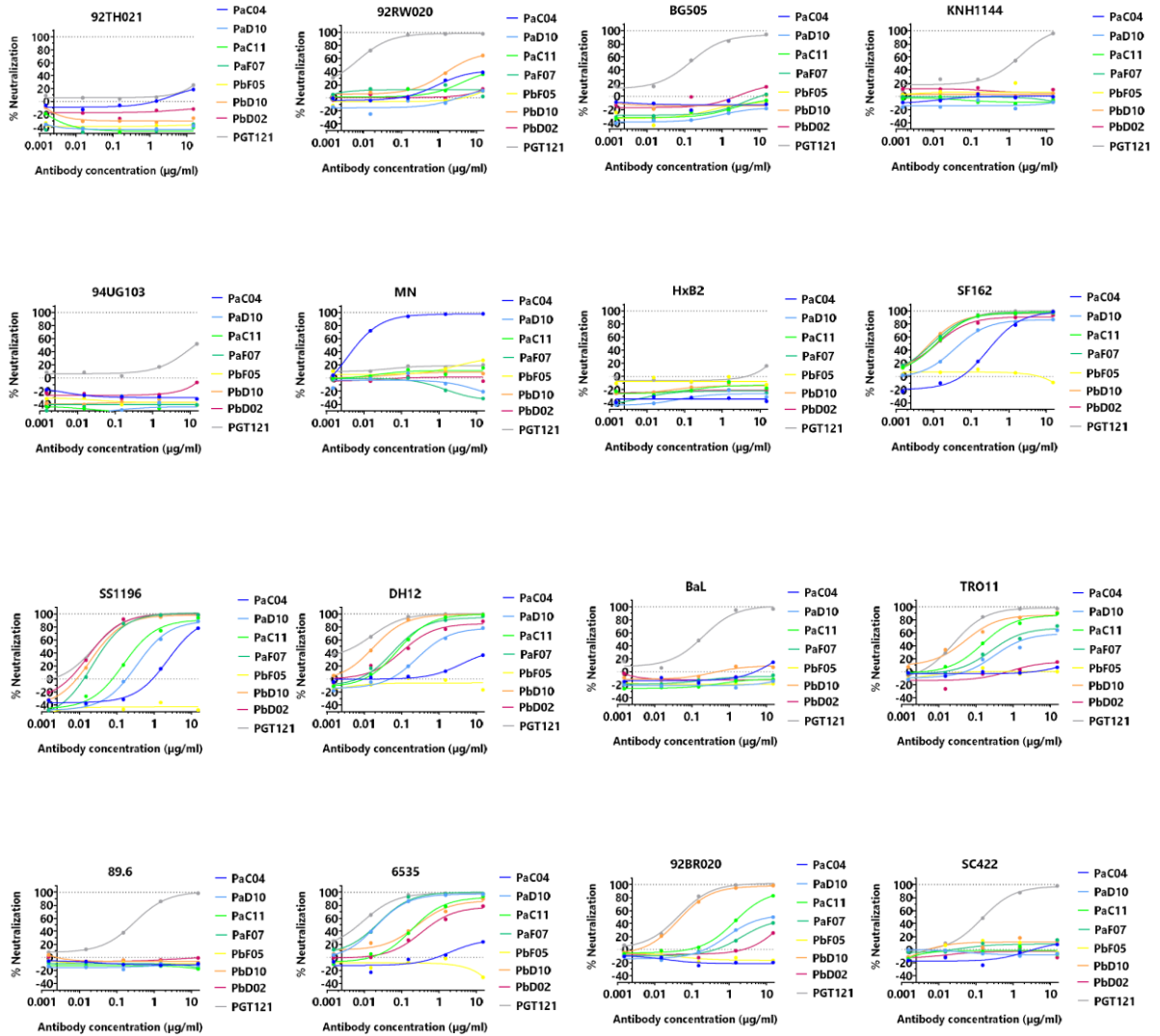
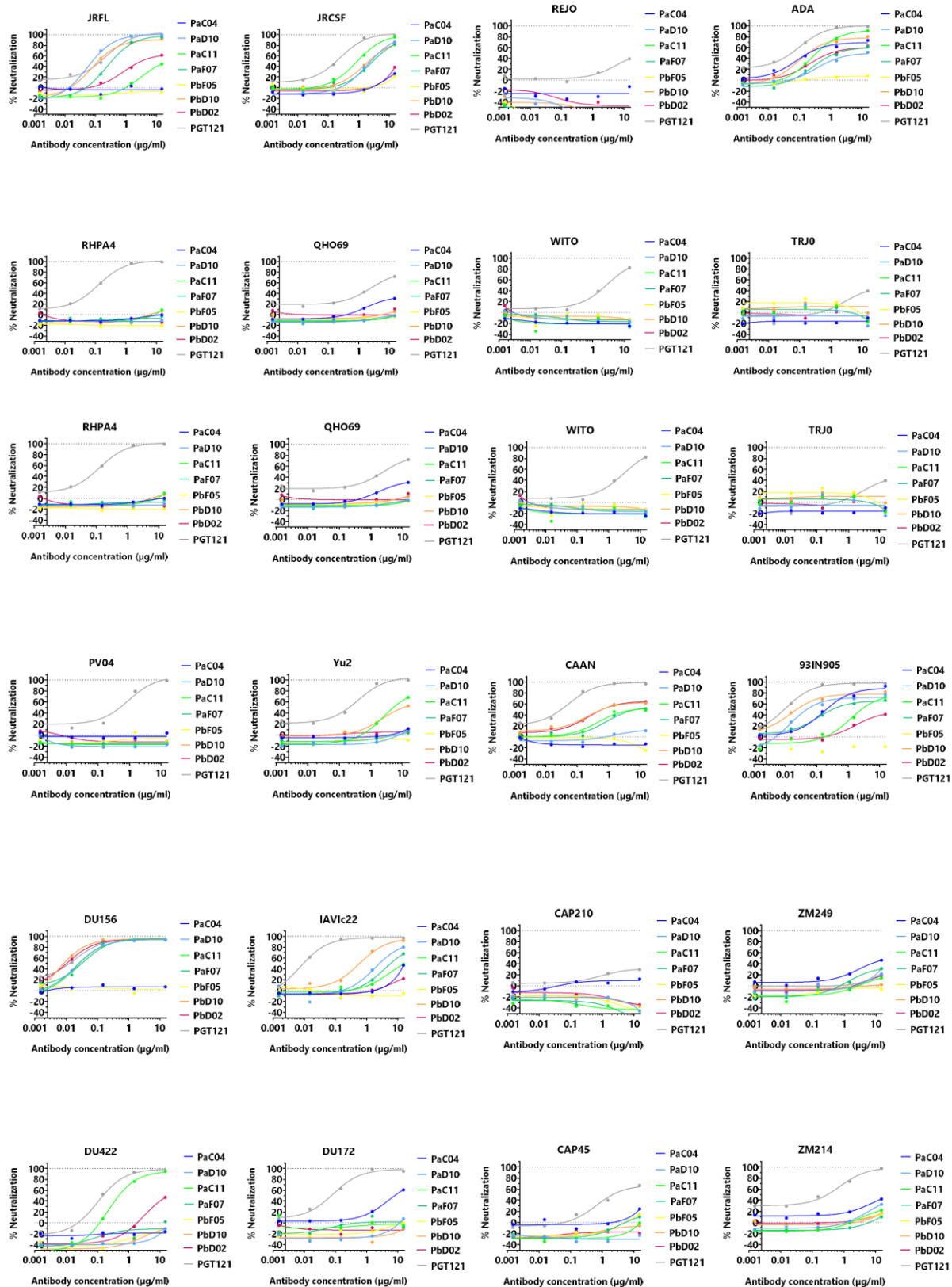
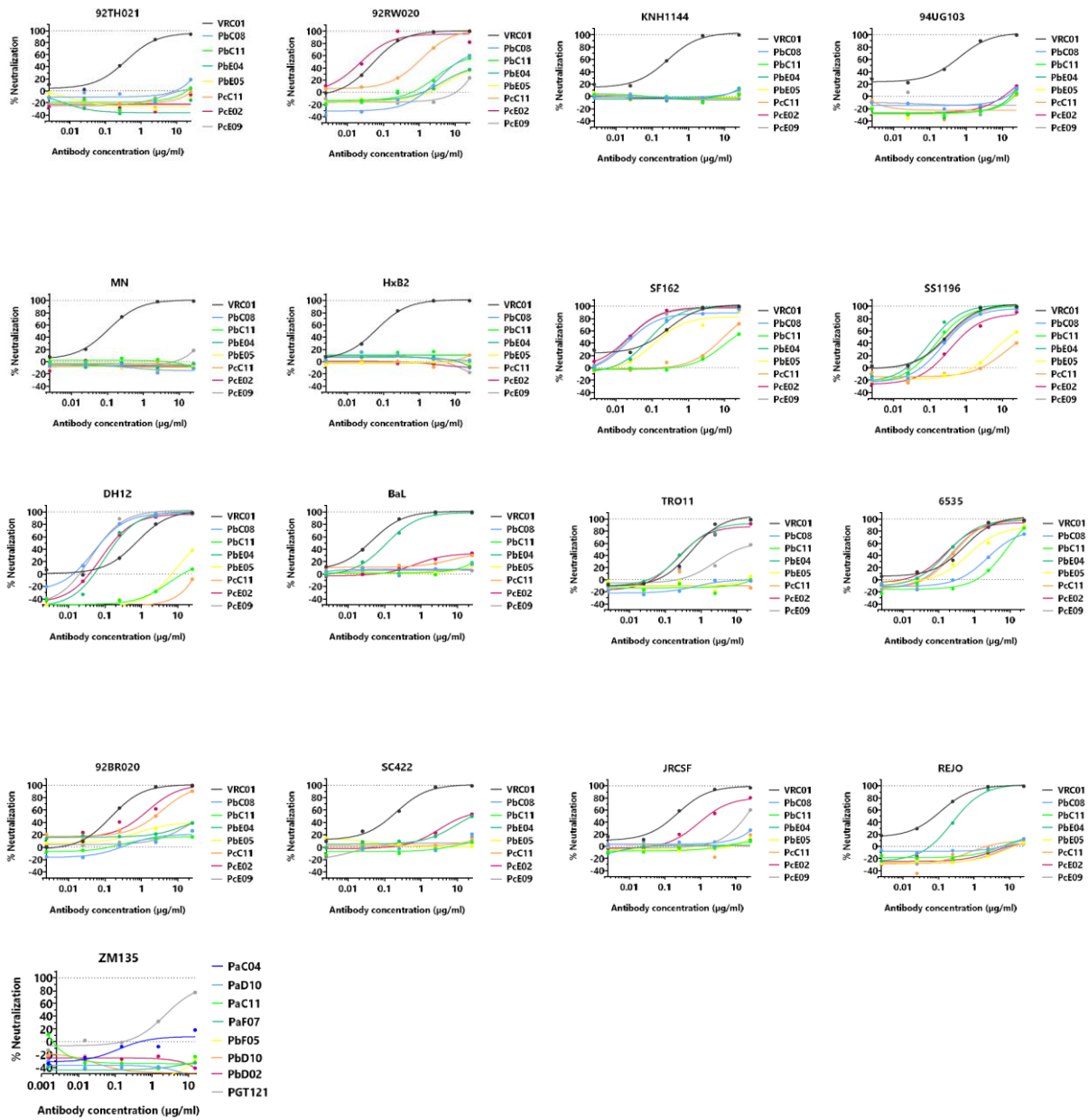


Figure X-4: Map of the Light Lambda Chain, variable and constant regions, and positioning of the primers used for the PCRs. From Snappgene Viewer software. The variable part is indicated in yellow and red for the V and J genes respectively. Here are the LC genes of the Den3 antibody that serve as example. The constant part is indicated in light blue. The Random Hexamers used for the RT-PCR are more downstream that this frame. Reverse primer of the PCR1 is in light grey and that of the PCR2.seq in medium grey. Forward primers do not match the vector frame but should appear nearby the 5' tag. Both forward and reverse primers of the PCR2.clon are in dark grey; they include the overlapping sequence inserted at extremity 5' is indicated in dark blue. All primers used for sequencing (post PCR2.seq or PCR2.clon) are in white. The vector contains the constant region.

D. Neutralization curves of the 16mAbs against the 37vP







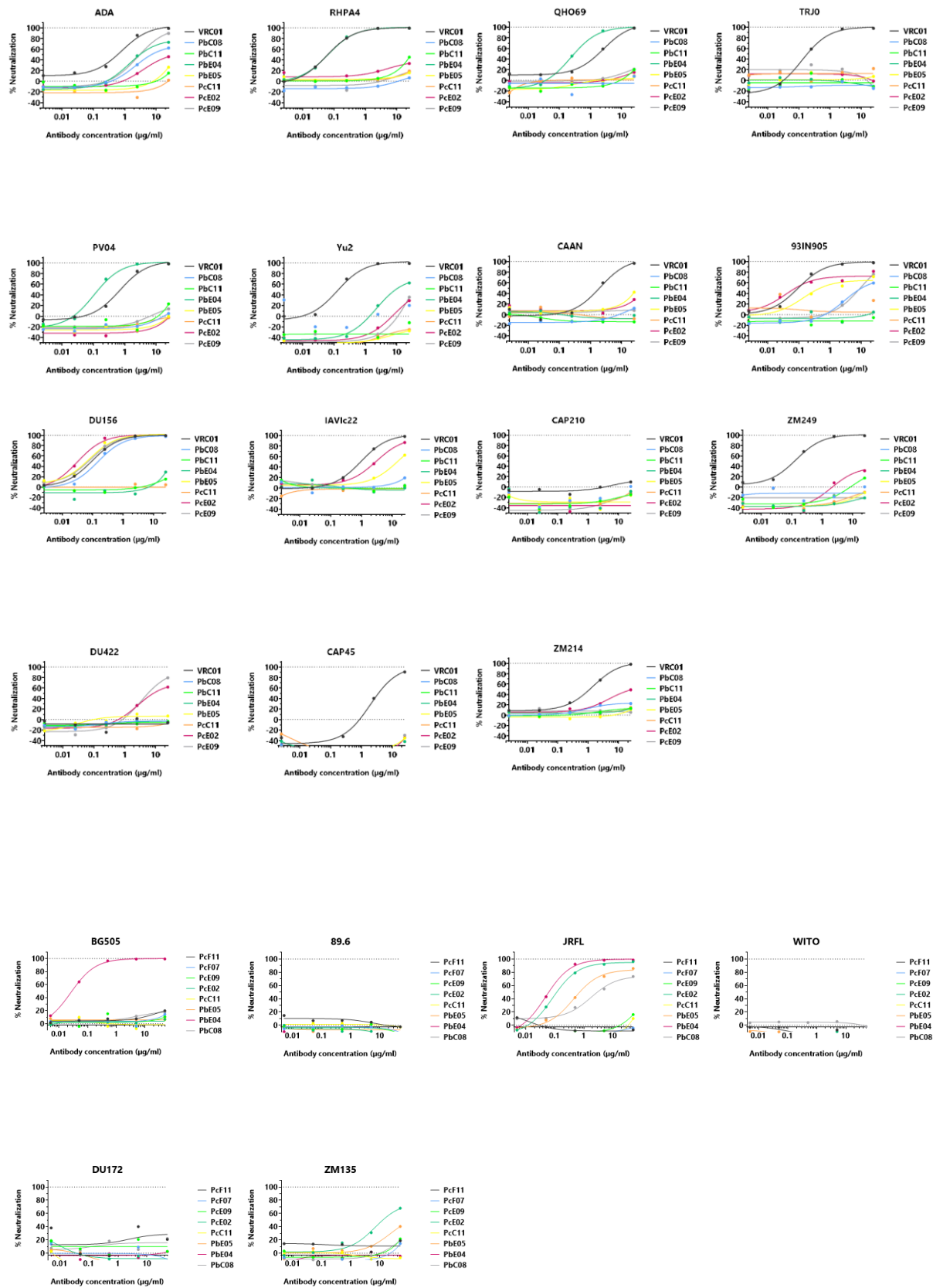


Figure X-5: Curves of the percentages of neutralization according to the mAb concentration ($\mu\text{g/ml}$) for all 16 selected mAbs isolated from PC94 tested in neutralization assay against the 37vP, from which IC50 were calculated (with GraphPad Prism).

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