

The Impact of Isotype and Structure on Antibody-HIV Interaction

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Declaration of Originality

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" Shoot for the moon. Even if you miss, you'll land among the stars. "

" Il faut toujours viser la lune, car même en cas d'échec, on atterrit dans les étoiles. "

Oscar Wilde

Abstract

Broadly neutralising antibodies that are capable of blocking HIV-1 infection at the mucosal portal of viral entry are probably the most researched types of antibodies. Both IgG and IgA antibodies are present in genital and rectal mucosal surfaces where HIV-1 infection is mainly acquired. While IgG only exists as a monomer, IgA is predominantly found as dimer in the mucosal compartment and as monomer in serum. Yet, very little is known about: (1) how envelope-specific IgG₁₋₄ and IgA₁₋₂ isotypes and structures influence antibody antiviral function, (2) which of these isotypes and structures provide the most effective mechanism to block HIV-1 infection and (3) how IgG and IgA may interact to prevent HIV-1 infection. This thesis seeks to test the hypothesis that, in addition to antibody epitope specificity, isotype and structure influence antibody ability to bind, capture and neutralise HIV-1 virions. Furthermore, it is proposed that the kinetic parameters of antibody-virus interaction have a critical impact on antiviral function. Finally, it is posited that competition between isotypes may cross-modulate their different antiviral functions.

The findings of this study indicate that IgG antibodies present greater ability to capture and neutralise HIV-1 particles than mIgA and dIgA antibodies targeting the same epitope and that this correlates with their faster association constant rates. Moreover, it was observed that dIgA antibodies displayed enhanced antiviral functions compared to their mIgA counterparts depending on the epitope specificity. Competitive assays also demonstrated that IgG and IgA antibodies of identical specificity competed for interaction with HIV-1 virions, potentially mitigating IgG effector functions.

Thus, this research suggests that antibody epitope specificity, isotype and structure all influence antiviral functions and indicates that the ability of an antibody-based vaccine to prevent HIV-1 infection may be critically dependent on the spectrum and proportions of induced isotypes and their relative expression within systemic and mucosal compartments.

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List of Abbreviations

Ab	Antibody
ADCC	Antibody-Dependent Cellular Cytotoxicity
ADCP	Antibody-Dependent Cellular Phagocytosis
ADCVI	Antibody-Dependent Cell-Mediated Viral Inhibition
ADE	Antibody-Dependent Enhancement
Ag	Antigen
AIDS	Acquired Immuno-Deficiency Syndrome
AMVA	Antibody-Mediated Viral Aggregation
APC	Antigen Presenting Cell
Arg	Arginine
AVCNI	Antibody Virion Capture Neutralisation Index
BCR	B Cell Receptor
BLI	Bio-Layer Interferometry
bNAb	Broadly Neutralising Antibody
C	Constant
CA / p24	Capsid
CADE	Complement-Mediated Antibody-Dependent Enhancement
CCR5	CC-chemokine receptor 5
CD	Cytoplasmic Domain
CDR	Complementary Determining Region
CHR	C-Heptad Repeat
CI	Confidence Intervals
CMI	Complement-Mediated Inactivation
CVM	Cervico-Vaginal Mucus
CRF	Circulating Recombinant Form
CSR	Class Switch Recombination
CTL	Cytotoxic T Lymphocyte
CXCR4	CXC-Chemokine Receptor 4
DC	Dendritic Cell
DC-SIGN	Dendritic Cell-specific ICAM3-Grabbing Non-Integrin
DEF	Dynamic Electrophoretic Fingerprint
DHFR	Di-HydroFolate Reductase
dIgA	dimeric Immunoglobulin A
EM	Electrophoretic Mobility
Env	Envelope glycoprotein
Fab	Fraction antigen-binding
Fc	Fraction constant
FcR	Fraction constant receptor
FDC	Follicular Dendritic Cell
GALT	Gut Associated Lymphoid Tissue

H	Heavy
HAART	Highly Active Antiretroviral Therapy
HCDR3	Heavy Chain Complementarity-Determining Region 3
HEPS	Highly Exposed Protected Seronegative
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
HSV	Herpes Simplex Virus
HTLV	Human T-Lymphotropic Virus
ICAM	Intercellular Cell Adhesion Molecule
Ig	Immunoglobulin
IN	Integrase
ITAM	Immuno-receptor Tyrosine-based Activation Motifs
KDa	Kilo Dalton
L	Light
LAV	Lymphadenopathy-Associated Virus
LC	Langerhans's Cell
LEDGF	Lens Epithelium-Derived Growth Factor
LFA	Lymphocyte Function-associated Antigen
LTR	Long Terminal Repeat
MA	Matrix
MALT	Mucosa-Associated Lymphoid Tissue
MCT	Multiple Comparison Test
MHC	Major Histocompatibility Complex
mIgA	monomeric Immunoglobulin A
MPER	Membrane Proximal External Region
NAb	Neutralising Antibody
NC	Nucleocapsid
NHEJ	Non-Homologous End-Joining
NHP	Non-Human Primate
NHR	N-Heptad Repeat
NK	Natural Killer
nNAb	non-Neutralising Antibody
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
PBS	Phosphate Buffered Saline
PEP	Post-Exposure Prophylaxis
Phe	Phenylalanine
PIC	Pre-Integration Complex
PLHIV	People Living with HIV
PR	Protease
PrEP	Pre-Exposure Prophylaxis

PRR	Pattern Recognition Receptor
P-TEFb	Positive Transcription Elongation Factor b
RNA	Ribonucleic Acid
RNase	Ribonuclease
RSV	Respiratory Syncytial Virus
RT	Reverse Transcriptase
RTC	Reverse Transcriptase Complex
SHIV	Simian Human Immunodeficiency Virus
SIV	Simian Immunodeficiency Virus
ssDNA	single-stranded DNA
STI	Sexually Transmitted Infections
SU / gp120	Envelope glycoprotein surface subunit
TAK	Tat-Associated Kinase
Tat	Trans-activator of transcription protein
TBS	Tris Buffered Saline
TCM	T cell Central Memory
TCR	T cell Receptor
TD	T cell-dependent
T _{FH}	T Follicular Helper cells
Th	T helper cells
TLR	Toll-Like Receptor
TM / gp41	Transmembrane subunit
TN	T cell CD4+ Naive
TNF	Tumour Necrosis Factor
TRIM5 α	Tripartite-motif-containing 5 α
tRNA	transfer Ribonucleic Acid
TTrM	Translational Tissue-Resident Memory
V	Variable
VCA	Viral Capture Assay
VLP	Virus-Like Particle
VSV	Vesicular Stomatitis Virus

Chapter 1

Introduction

1.1 Human Immunodeficiency Virus

1.1.1 HIV/AIDS discovery

The first clinical cases of the acquired immunodeficiency syndrome (AIDS) were reported in June 1981 by the Centres for Disease Control and Prevention (CDC) in the Morbidity and Mortality Weekly Report. American physicians described five cases of a rare lung infection, *Pneumocystis carinii* pneumonia among men who have sex with men (MSM) living in Los Angeles ^[1]. Additionally, Kaposi's sarcoma, a very aggressive form of skin cancer, was observed among MSM in San Francisco and New York; and by the end of the year, a total of 270 men were diagnosed with AIDS ^[2-4]. These patients presented a high susceptibility to opportunistic infections characterised by low CD4+ T cell count, a significant reduction of their immune responses to phytohemagglutinin (PHA) and no lymphocyte proliferative responses to soluble antigens ^[5]. The following year a newborn who received blood transfusions was reported with AIDS, as well as 22 other infants ^[6]. In 1983, Dr Françoise Barré-Sinoussi and Prof Luc Montagnier (France) isolated an RNA virus with an Mg²⁺ reverse transcriptase activity named lymphadenopathy-associated virus (LAV) from patients manifesting symptoms of AIDS ^[7]. In 1984, Dr Robert Gallo (USA), also identified, in healthy individuals and immune-depressed patients, a similar virus called human T-lymphotrophic virus, type III (HTLV-III) ^[8]. Subsequently, it was demonstrated that both viruses were variants of the same virus which was then named the human immunodeficiency virus of type I (HIV-1)^[9]. In 1985, a retrovirus related to HIV-1 called simian immunodeficiency virus (SIV_{mac}) was discovered in macaques ^[10]. Later, researchers found retroviruses in patients from West Africa presenting AIDS-like symptoms for which the serum reacted against SIV_{mac} but not with HIV-1 ^[11]. This newly described group of viruses were only observed in specific areas of sub-Saharan Africa and were named HIV-2 ^[12].

1.1.2 Routes of HIV transmission

In 1984, Vilmer et al. hypothesised that HIV could be transmitted by transfusion of blood products; they thus identified the exposure route presenting the highest risk of HIV transmission with over 90% of chance to be infected ^[13]. Contemporaneously, Levy et al.

suggested that HIV could be transmitted through sexual contact; hence this shed light on what is today, the most frequent route of transmission worldwide ^[4]. However, this risk is rather low and varies according to different factors including the viral load (VL) of the HIV-infected partner, co-infections with other sexual transmitted infections (STIs) and the type of mucosal surfaces involved ^[14-18]. Receptive anal intercourse represents the highest risk of transmission (up to 3.0%), followed by insertive penile-vaginal intercourse (up to 0.4%), while HIV transmission through oral sex is virtually non-existent (0–0.04%) ^[19, 20]. Furthermore, numerous epidemiological studies of HIV-serodiscordant heterosexual couples suggest that women are up to twice more likely to be infected by HIV-1 than men mainly due to biological reasons ^[19, 21]. The most commonly cited reasons include: a more exposed surface area in the female genital tract than in the male genital tract, greater concentrations of HIV in seminal fluids than in vaginal fluids, high levels of oestrogen and progesterone during pregnancy or from contraception that induce structural and immunological changes of the genital mucosa ^[22, 23], and higher risk of micro abrasion of the vaginal tissue during sexual intercourse ^[24]. Additionally, women are affected by gender inequality and gender-based violence which limit women's access to education, healthcare, resources and heightens their risk of contracting HIV ^[25, 26]. Other important risks of transmission are mother-to-child-transmission (MTCT) during pregnancy, delivery or breastfeeding; and needle-sharing during injection drug use (25% and 0.67% transmission-risks respectively) ^[13, 27]. Thus, HIV is transmitted from an infected individual to another person through specific bodily fluids, such as blood, breast milk, pre-seminal, semen, rectal and vaginal fluids; and biological and socio-economic factors modulates the risk of contracting HIV.

1.1.3 The origins of HIV

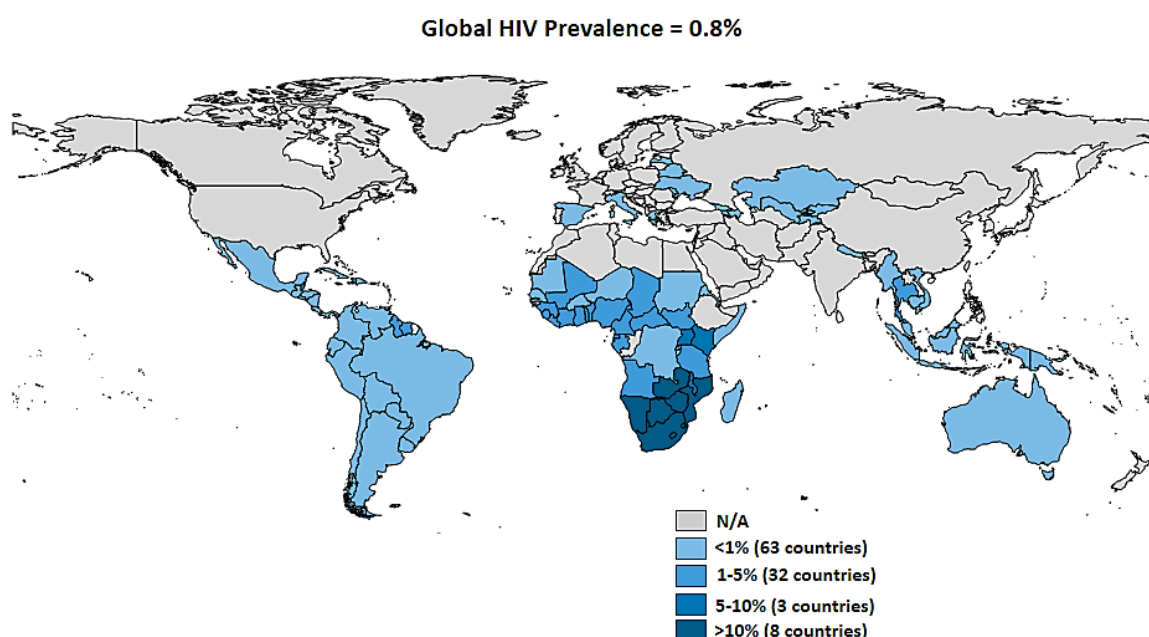
The origins of HIV have been rather controversial for several decades after its discovery. However, overwhelming amounts of evidence have recently shown that HIV-1 and HIV-2 originated from several independent zoonotic transmissions from species of African primates infected with the simian equivalent (SIV)^[1]. Indeed, researchers identified in *Pan troglodytes troglodytes* chimpanzees, the most closely related ancestor of HIV-1, SIV_{cpz}. HIV-2's ancestor, SIV_{smm} was found in sooty mangabeys ^[28]. In addition to these, many other species have been described as natural hosts of SIV including mandrills, African green

monkeys and baboons ^[29]. Nonetheless, the outbreak of HIV epidemic in humans and when, how and where it occurred, remain uncertain. In 1998, researchers reported the oldest ever known case of HIV-1 infection in an adult from Kinshasa, (The Democratic Republic of the Congo) and suggested that the virus was introduced in the human population at the beginning of the 20th century ^[30]. The common assumption is that HIV arose as a result of SIV adaptation following transmission of SIV from primates to humans. SIV is thought to have crossed to humans through exposure to chimpanzees' blood from hunted or butchered infected meat. Indeed, recent research has shown that almost 60% of primate meat sold in West Africa is infected with SIVs ^[31]. Importantly, SIV infection is associated with a high viral load in its primate hosts, however, most of the time infected animals remain asymptomatic. In contrast, HIV-1 and HIV-2 infection of humans causes CD4⁺ T cell depletion and leads to AIDS. However, only HIV-1 is responsible for the global epidemic while HIV-2 is less virulent and is confined to West Africa, spreading more recently in India and Europe ^[32].

Statistical and evolutionary analyses have demonstrated that HIV-1 has resulted from at least three episodes of cross-species transmission from chimpanzees to humans generating the three groups: N (non-M, non-O) solely observed in a small population of Cameroon, O (Outlier) concentrated in West Africa and M (Major) spread globally ^[33, 34]. The latter is probably the oldest HIV-1 group with its origin extrapolated by phylogenetic analysis back to the 1930s and is also certainly the most transmitted group, representing 95% of the HIV-1 isolates found in infected individuals worldwide. In addition, group M is divided into 9 subtypes or clades: A, B, C, D, F, G, H, J and K ^[35]. Different subtypes dominate in different parts of the world so that clade B is mainly observed in Europe and the Americas while clade C is predominant in Southern Africa, and A and D in central and eastern Africa. Thus, subtypes A and C are responsible for the vast majority of the current world epidemic ^[36]. Moreover, several subtypes have recombined in the genome of infected individuals generating a total of 72 circulating recombinant forms (CRF01-CRF72) ^[37]. However, because of the rapid virus evolution, the nomenclature system is regularly updated ^[38].

1.1.4 HIV/AIDS epidemiology

The Nobel Prize in Physiology or Medicine was attributed in 2008 to Françoise Barré-Sinoussi and Luc Montagnier for the discovery of HIV-1, a quarter of a century earlier. This Nobel Prize not only acknowledged the innovative research performed by these brilliant scientists but also recognised the major global health impact of HIV/AIDS epidemic. As of today, AIDS (the final stage of HIV-1 infection) has become the fourth biggest killer in the world: it accounts for 40 million deaths since the early 1980's. Nearly 37 million people are currently living with HIV (PLHIV) in the world (Figure 1) and 0.8% of the adults are aged between 15-49 years^[39]. In 2014, an estimated 1.1 million people had died from AIDS, and 2.1 million people were newly infected. Despite these alarming figures, the overall trend is towards a reduction in the number of deaths which has dropped by 45% since 2005, and the number of new infections which has fallen by 35% since 2000. However, the HIV/AIDS epidemic continues to disproportionately affect women and young girls with AIDS being the leading cause of death in the world among women in their reproductive age (15-49 years)^[40].



NOTES: Data are estimates. Prevalence includes adults ages 15-49.

SOURCES: Kaiser Family Foundation, based on UNAIDS, AIDSinfo, Accessed June 2016



Figure 1. Adult HIV Prevalence, 2015. Overview of the HIV/AIDS epidemic compiled with information from WHO, UNAIDS and the Kaiser Family foundation's Global Health Policy division^[40]. Taken from The Kaiser Family Foundation's website, 2015.

Although HIV/AIDS is a global health issue, the epidemic is unequally distributed, affecting all the strata of the population in sub-Saharan Africa with a prevalence exceeding 10%; in many countries, such as Swaziland it is up to 27.7% ^[41]. Consequently, sub-Saharan Africa presents the highest burden of the disease, accounting for over two thirds of the PLHIV and including 59% of women ^[42]. In the rest of the world, HIV/AIDS is concentrated among key populations: MSM, Black, Asian and ethnic minorities (BAME), injecting drug users (IDUs), prisoners and sex workers. In Western and Central Europe, just under a million people (0.2% prevalence) are infected with HIV-1 but the rates of transmission have been increasing, particularly among MSM, and the number of people unaware of their status is alarmingly high ^[43]. For example, in the UK about 110,000 people are living with HIV and almost a quarter of them are unaware of their positive status ^[43]. Other parts of the world have a prevalence ranging from 0.1% in the Middle East and North Africa to 1% in the Caribbean ^[39].

1.1.5 HIV-1 genome organisation and protein products

HIV-1 is a lentivirus from the *Retroviridae* family which infects cells of the immune system, particularly CD4+ T cells and macrophages; without antiretroviral drugs, the progressive depletion of infected T cells irreversibly leads to AIDS ^[44]. The HIV genome consists of a pair of positive single-stranded RNA molecules of about 9.2kb. Common to all retroviruses, HIV-1 encodes for three main structural genes: *gag*, *pol* and *env* (Figure 2). The capsid proteins are encoded by *gag* which is translated into a 55kDa precursor (p55) and then processed by viral proteases into polyproteins: matrix (MA, p17), capsid (CA, p24), nucleoplasmid (NC, sp7), p6 proteins and the spacer peptides SP1 (p1) and SP2 (p2) ^[45]. *Pol* encodes for the viral enzymes; reverse transcriptase, proteases and integrase. The Gag-Pol fusion precursor protein is produced by ribosomes near the 3' end of *gag* and processed by viral proteases, including the reverse transcriptase (RT, p66 and p51), the protease (PR, p10) and the integrase (IN, p32) ^[45] (Table 1). The Env precursor (gp160) is cleaved by a cellular protease during trafficking to the cell surface into viral glycoproteins consisting of a surface subunit (SU, gp120) and a transmembrane subunit (TM, gp41).

Additionally, the HIV-1 genome encodes for regulatory proteins Tat and Rev, essential for gene expression and accessory proteins Vif, Vpr, Vpu, and Nef. The coding sequences of the HIV-1 genome are flanked by two long terminal repeats (LTR), comprising the regions R and U5 at the 5' end, and R and U3 at the 3' end (Figure 2) ^[45].

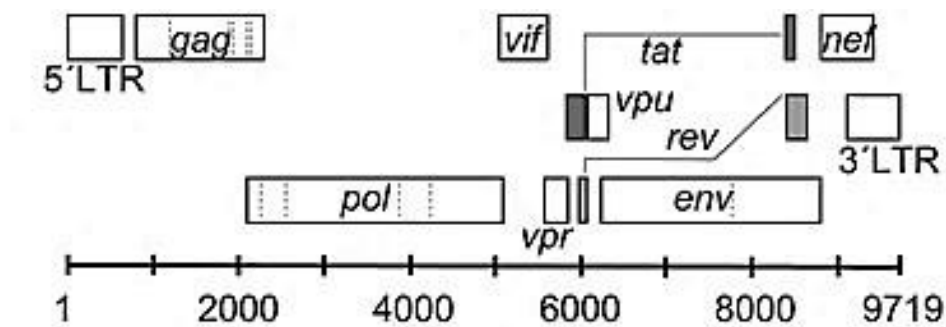


Figure 2. Organisation of an HIV-1 RNA genome. Regions coding for HIV-1 proteins are shown in rectangles and polyprotein-domain junctions are presented as solid vertical lines^[46]. Taken from Ode et al. 2015.

Table 1. Classification and function of the HIV-1 proteins

Protein type	Precursor	Viral protein	Full names	Size (kDa)	Localisation	Main functions and involvements
Structural	Gag Gag-Pol	MA	Matrix	17	Virion	- Virus replication - Env incorporation into virion - Particle assembly
		CA	Core capsid	24	Virion	- Structure the virion core - Coats the viral RNA - Viral replication
		NC	Nucleocapsid	7	Virion	Encapsulates viral RNA
		p6	-	6	Virion	Incorporates Vpr into virion
	Gag-Pol	PR	Protease	15	Virion	Cleaves protein precursors
		RT	Reverse Transcriptase	66	Virion	Catalyse the reverse transcription of vRNA
		RNAse H	-	51	Virion	Degrades RNA template
		IN	Integrase	31	Virion	Integration of viral DNA into host DNA
	gp160	gp120	Envelope surface subunit	120	Virion Plasma membrane	- Interacts with CD4 and co-receptors - Mediates viral entry
		gp41	Envelope Trans-membrane subunit	41	Virion Plasma membrane	Mediates virus and cell membrane fusion
Regulatory	No precursor	Tat	Trans-Activator of Transcription	16/14	Nucleus	- Binds to the trans-activating response element (TAR) - Enhances HIV-1 transcription
		Rev	Reverse	19	Nucleus Cytoplasm	- Interacts with Rev response element in <i>env</i> - Shuttles mRNA from nucleus to cytoplasm
Accessory		Vif	Viral protein F	23	Cytoplasm Virion	- Regulates virion maturation and infectivity - Inhibits the cellular restriction factors APOBEC 3G
		Vpr	Viral protein R	10-15	Nuclear membrane Virion	- Nuclear translocation - Inhibits cell division - Promotes cell differentiation
		Vpu	Viral protein U	16	Plasma membrane	- Inhibits host cell Tetherin - Promotes virion release, CD4 degradation, Env maturation, cell apoptosis
		Nef	Negative factor	25	Plasma membrane	- Enhances virion production - Down regulates CD4 and MHC class I molecules

1.1.6 Structure of an HIV-1 virion

The HIV-1 genome and regulatory proteins are contained in a cone-shaped core composed of about 2,000 copies of the viral protein p24 which is called a capsid. The capsid is surrounded by a matrix constituted of viral protein p17 which is essential for the integrity of the virion particle ^[47, 48]. The matrix shell is immediately beneath a bilayer membrane derived from the host cell which incorporates cellular proteins and lipids but also HIV envelope proteins. The resulting HIV-1 virion exhibits a spherical shape of a diameter of 100-150 nm, ^[47, 48]. Each viral particle contains 7 to 14 Env glycoprotein spikes ^[49, 50] (Figure 3). The functional envelope spike is crucial for viral entry into the target cell ^[51]. Nonetheless, the nascent virion has to undertake a further round of maturation to become infectious ^[51]. In the immature virion, Gag is anchored in the membrane by a myristoylated group at its N-terminal and forms a thick protein layer that renders the viral particle relatively rigid compared to the mature virion ^[52]. During the maturation process, about 5,000 molecules of Gag polyproteins are cleaved by the viral protease (PR) into NC, CA, MA and p6 peptide. After liberation of the different Gag components, the CA immediately assembles and forms hexagonal lattices of a fullerene cone, containing regulatory and accessory proteins, and the viral genome bound to NC as a ribonucleoprotein complex. The matrix proteins remain underneath the viral membrane and form the conical capsid core. Thus, the mature virion contains spikes at its surface and a capsid core that maintains the viral genome and reverse transcriptase in a closed environment, protecting them from host recognition ^[52].

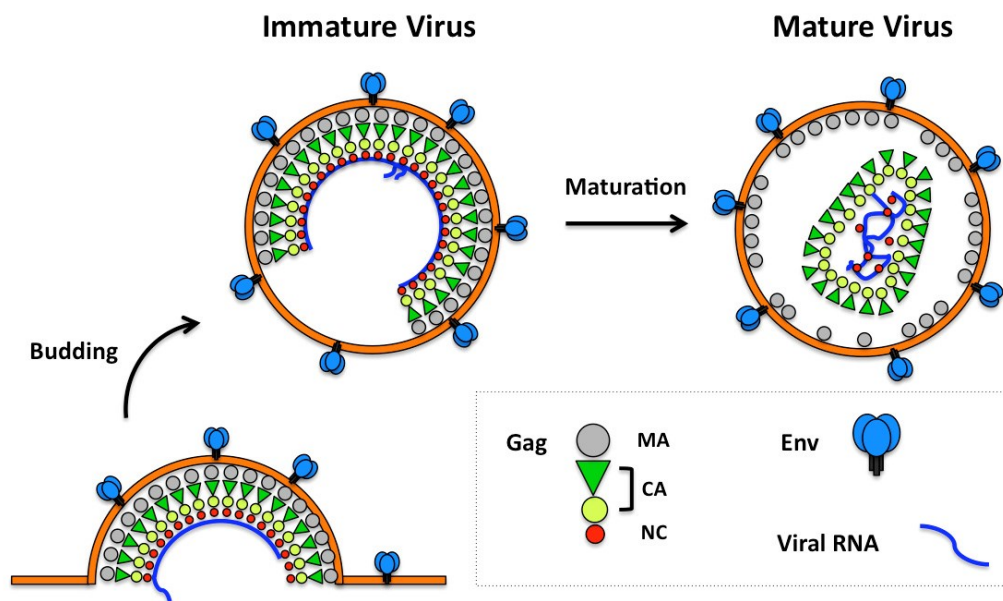


Figure 3. Immature and mature HIV-1 virion. Simultaneously to viral budding, the viral protease cleaves Gag and Gag-Pol into several polypeptides which lead to major changes in virion morphology and viral maturation. The diagram on the left panel represents immature HIV-1 virion and the one on the right panel, mature HIV-1 virion comprising a conical HIV-1 capsid core with two copies of single-stranded RNA molecules and viral proteins required for infection such as the reverse transcriptase and integrase. Envelope (Env); matrix (MA) and nucleocapsid (NC) ^[53]. Taken from Zhang et al. 2015.

1.1.7 Replication cycle

The replication life cycle of HIV-1 can be divided into an early and a late stage. The early phase of the HIV-1 life cycle is initiated by the binding of the HIV-1 virion to the CD4 receptor expressed on specific immune cells including T-helper cells, dendritic cells and macrophages ^[54, 55]. This is followed by a fusion of the viral and cellular membranes and the release of the capsid core into the cell. The released HIV-1 RNA is reverse transcribed into double stranded DNA that is then translocated into the nucleus and incorporated in the host chromatin as a provirus ^[56]. Viral DNA integration marks the beginning of the late phase of the HIV-1 life cycle. From this step, the host cell machinery is hijacked to produce *de novo* virions which assemble in the cytoplasm and bud from the cell. The maturation of the viral particles by viral proteases into fully infectious HIV-1 virions completes the replication cycle.

1.1.7.1 HIV-1 envelope structure and factors responsible for viral entry

HIV-1 displays spike proteins on its surface called HIV-1 envelope proteins (Env) that belong to class I viral fusion proteins ^[57]. Env is similar in structure to hemagglutinin (HA) protein of influenza and like all proteins of this class, it is trimeric ^[58, 59]. The Env precursor gp160 is produced in the host endoplasmic reticulum as a 160 kilodalton (kDa) protein comprising about 850 amino acid residues ^[60]. Thereby, it undergoes the first round of N-glycosylation and oligomerises into trimers of glycoproteins. Subsequently, gp160 transits to the trans-Golgi where it is further glycosylated and cleaved by furin-like proteases into a trimeric heterodimer of gp120 surface unit (SU) and a gp41 transmembrane protein (TM) non-covalently linked ^[61] (Figure 4). The surface unit (gp120) contains approximately 20-25 N-linked glycans and gp41 which contains about 3-4 N-linked glycans; the number and position vary according to the viral strain. The resulting Env complex is heavily glycosylated with carbohydrates representing nearly half of its molecular weight conferring a protective shield against the immune system. The glycoprotein gp120 binds to the target cell receptors and induces conformational changes that are required for the fusion machinery. This is orchestrated by gp41 and results in viral and cell membrane fusion ^[54].

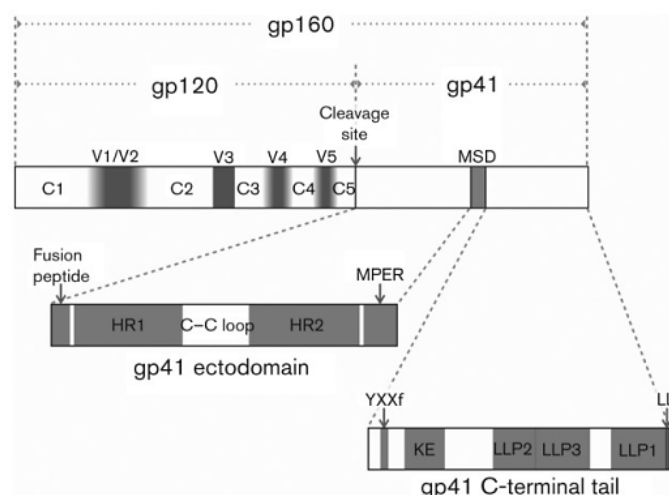


Figure 4. Structure of HIV Env. The HIV Env structure is presented with the immature polyprotein gp160 and the mature cleaved protein gp120 and gp41. The Env gp120 comprises five constant regions (C1-C5) interspersed with variable domains (V1V2-V5). The shading in the variable domains shows the range of variable region length, with V1/V2 ranging from 50 to 90 amino acid residues, V4 from 19 to 44 and V5 from 14 to 36 amino acid residues, while V3 does not vary substantially in size. The Env gp41 ectodomain is more conserved, and comprises a fusion peptide F, heptad repeat 1 and 2 domains (HR1 and HR2, respectively), C-C loop and the membrane proximal external region (MPER)). The gp41 C-terminal tail (CTT) includes the Kennedy epitope (KE) and the lentivirus lytic peptide sequences called LLP1, LLP2 and LLP3. Functional endocytic motifs are named YXXΦ (near the N terminus) and LL (at the C terminus) ^[62]. Taken from Steckbeck JD et al. 2012.

gp120

Env trimer is essential for viral entry into cells and mediates the interaction with CD4 and chemokine co-receptors expressed on the cell surface ^[63]. For many years, researchers have tried to elucidate the structure of Env as this protein plays a pivotal role in HIV-1 infection and therefore represents a major vaccine target. The investigation has been hindered by the presence of a dense glycan shield masking protein structure, the high variability of Env amino acid sequences, and the presence of non-functional Env forms at the viral surface ^[50]. Nonetheless, cryo-electron microscopy has enabled the characterisation of the global architecture of soluble Env glycoprotein complexes and Env spikes on the HIV-1 membrane (Figure 5) ^[64, 65]. In addition, crystallography studies provided critical information about how Env interacts with co-receptors and neutralising antibodies ^[58, 66].

Functional Env trimers are comprised of three gp41 molecules responsible for membrane fusion and three gp120 molecules that contain the CD4 and the co-receptors binding sites ^[64]. These latter are chemokine receptors that belong to the family of G protein-coupled receptors such as GPR1, GPR15, CCR2, CCR3, CCR5, and CXCR4 ^[67]. Sequence analyses revealed that gp120 consists of five conserved regions (C1-C5) intercepted by five highly variable sequences (V1 –V5) encoding for the variable loops ^[68] (Figure 4). X-ray crystallography approaches, initiated by Kwong et al., in the presence of CD4 and a monoclonal antibody 17b provided information about the atomic architecture of gp120 ^[58]. The monoclonal antibody (mAb) 17b is able to recognise discontinuous conserved epitopes preferentially exposed following CD4 ligand binding to gp120 and has been pivotal in HIV-1 Env structural analyses ^[69].

Researchers demonstrated that gp120 comprises of an inner domain linked by a highly-conserved bridging sheet to a more variable outer domain ^[58]. The inner domain presents helical characteristics and comprises of the C1 and C5 domains that interact with gp41. The bridging sheet forms a β -sheet constituted of four antiparallel β strands deriving from the C4 domain and the V1/V2 stem. The latter is adjacent to the outer domain which contains the stem of the V3, V4 and V5 loops and forms a barrel exposed on gp120 surface. The conserved regions C1, C2 and C5 interact with each other and form a central discontinuous protein backbone while the variable domains present disulphide bonds at their base and form loops protruding from the protein backbone. All the V loops of Env present high variability in amino acid components and protect to some extent against recognition by neutralising antibodies. However, the V3 loop which protrudes from the outer domain is of particular interest as it interacts with the CD4 co-receptor on the target's cell surface ^[58].

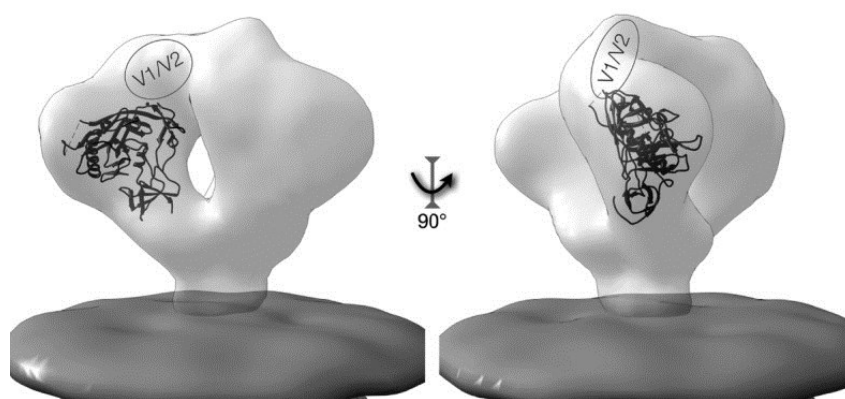


Figure 5. Schematic representation of HIV-1 gp120 core structure. The structure of HIV-1 Env is depicted with the relative emplacement of a gp120 subunit within the virion-associated trimeric structure. The HIV-1 Env is presented in light grey and the membrane in darker grey at the bottom of the figure^[62]. Taken from Steckbeck JD et al. 2012.

Crystal structure analyses of Env interaction with soluble CD4 molecules have provided information about the CD4 binding site (CD4bs). The latter is located in a hydrophobic cavity between the inner domain, the bridging sheet and the outer domain (Figure 6). The CD4bs is highly conserved and contains a V3 loop, a β -sheet and some surrounding amino acid residues^[66]. Analysis of the interaction between the CD4 receptor site and gp120 has suggested that the bridging sheet and the V3 loop bind to the N-terminal region of CD4 and that the extracellular region of CD4 interacts with the V3 tip. Unlike the other variable loops of gp120, the V3 loop is relatively well conserved normally containing 35 residues in length and protrudes at 30 Å from Env surface. In addition, the amino acid sequence of the V3 loop determines the virus tropism: if the 11th and 25th residues of V3 are negatively charged HIV-1 belongs to the R5 tropic and otherwise to the X4 tropic^[70]. Thus, HIV viruses are divided into two groups: R5 viruses that bind to the CCR5 beta-chemokine receptor for viral entry into macrophages and CD4+ T cells and X4 viruses that bind to the CXCR4 alpha-chemokine receptor to replicate in primary CD4+ T cells and not macrophages^[71-73].

gp41

gp120 is the most exposed part of HIV-1 Env and is non-covalently linked to gp41 via its N- and C- termini. The transmembrane protein gp41 is essential for viral fusion with the host cell membrane. It contains a long cytoplasmic tail of about 150 residues that serves as an anchor for the Env spike on the viral surface. In the gp160 precursor, the C5 domain of gp120 is immediately followed by the fusion peptide sequence at the N-terminus of gp41 which comprises of a domain rich in hydrophobic residues similar to those observed in influenza ^[59]. This fusion peptide sequence is adjacent to an ectodomain comprising of an N-terminal and a C-terminal heptad repeat region (NHR and CHR, respectively), which is also involved in the fusion machinery. The C-terminal region of the gp41 cytoplasmic tail contains five putative N-glycosylation sites which have been shown to be required for efficient gp160 cleavage. A conserved disulphide bond present between the NHR and CHR has been shown to be important for interaction with gp120 ^[74].

Native envelope spike

Despite critical advances in defining the anatomic architecture of HIV-1 gp120 monomers bound to antibodies in the presence or absence of CD4, significant gaps remain in the understanding of the structure and dynamics of the native HIV-1 spike displayed on viral surfaces. Biochemical and mutagenesis studies have contributed to the development of theoretical models of Env trimer structure, but recent improvements in cryoelectron tomographic research has provided an experimental model of the native spike ^[50]. These models have brought insights to the quaternary structure of Env trimers exposed on viral surfaces in three different states: unbound, CD4-bound and antibody-bound states at high resolution (20 Å) ^[75]. Particularly, Liu et al. found that in the unliganded state, the V1/V2 and V3 regions on each gp120 monomer are in proximity of the Env apex and that the three gp41 proteins form a mushroom-shaped structure at the base of the gp120 trimer ^[48].

This study also suggested that the variable loops (V1-V4) are probably glycosylated as they are, in general, situated in solvent accessible regions. Additionally, they demonstrated that Env trimers undergo conformational changes upon CD4 and 17b ligand binding from a “closed” unliganded state to an “open” state. Ligand binding induces rotations of each gp120 monomer of about 45° around an axis parallel to the central threefold axis, which is associated with an out-of-plane rotation of approximately 15°.

Interestingly, in some viral strains such as SIV_{CP-MAC}, Env was already present in this open state without CD4 ligand ^[75]. This finding provided an explanation as to why some viral strains were capable of infecting a target cell in a CD4-independent manner ^[75].

Similar techniques used to describe native Env structure were used to determine the distribution and number of spikes present on the virion surface. Zhu et al. discovered that a small number of Env complexes 14 ± 7 are embedded in the viral membrane while HIV-1 virions bud from the cell ^[50]. These findings were in concordance with protein measurements and previous estimations ^[49]. They found that the average inter-spike spacing was about 15nm and that Env spikes were sometimes organised in clusters which suggests that they might have constraints to diffuse in the viral membrane ^[50]. Besides, it has been demonstrated that increased spike numbers correlated with enhanced infectivity by improving fusion efficiency with the host membrane ^[76]. However, clustering might facilitate antibody-mediated neutralisation by promoting bivalent cross interactions ^[77].

Researchers then investigated the causes of the unusually low spike density compared to other viruses such as influenza. With this in mind, a number of studies performed a substitution of the natural gp120 signal sequence in site-directed mutagenesis experiments and demonstrated that a low level of Env was mainly due to the positively charged amino acids present in the signal sequence ^[78, 79]. Moreover, recent evidence suggested that a dileucine motif present in the cytoplasmic tail of gp41 might mediate endocytosis of Env via a clathrin-mediated endocytosis pathway reducing the number of Env spikes exposed at the viral surface ^[79].

HIV cellular receptors

Soon after the HIV/AIDS outbreak in 1984, researchers identified a receptor expressed on specific human cell type surfaces involved in HIV-1 infection ^[44]. This receptor was known as CD4, a 55KDa molecule member of the immunoglobulin superfamily which comprises a cytoplasmic tail, a transmembrane domain and four Ig-like extracellular domains (D1-D4)^[80].

Experimental studies demonstrated that only the D1 domain of CD4 was responsible for the interaction with HIV-1 gp120. The CD4 receptor is mainly expressed on a subset of T cells called CD4⁺ T helper cells and its main function is to bind MHC class II molecules. HIV-1 has the ability to replicate in CD4 expressing cell populations thereby making CD4⁺ T cells the principal viral targets for infection. However, CD4 receptors are also exposed on other cell types such as dendritic cells (DC), monocytes, macrophages, microglia cells, thymocytes and other T cells subsets ^[81]. In addition to the CD4 receptor, it was demonstrated that viral entry required binding to specific co-receptors: CXCR4 alpha-chemokine receptor to replicate in primary CD4⁺ T cells (X4 viruses), and CCR5 beta-chemokine receptor for entry into macrophages and CD4⁺ T cells (R5 viruses) ^[71-73]. Furthermore, some viruses are capable of recognising both CXCR4 and CCR5 (X4R5 viruses) and many other chemokine co-receptors including CCR2 and CCR8 ^[82]. However, the extent to which these co-receptors are utilised *in vivo* remains unclear.

1.1.7.2 Early phase of HIV-1 life cycle

A considerable amount of research has been done over the past few decades to elucidate the HIV-1 life cycle and identify potential antiretroviral drug targets. HIV-1 entry is initiated by gp120 binding to CD4 expressing cell populations. Env interacts with the host co-receptors, predominantly CCR5 (R5 virus), although later in disease the virus can evolve to use CXCR4 (X4 virus and/or R5X4 viruses) ^[83]. Virus infection can often be facilitated by additional non-covalent interactions with cell attachment factors such as glycosphingolipid galactosylceramide (GalC), heparan sulphate and mannose-binding lectins (e.g. DC-SIGN). Although not required for viral infection, binding to these attachment cell factors can significantly increase the probability of virion-cell encounters and enhance infection. The type of attachment factors used by HIV-1 is not clearly understood and varies according to the virus strain and target cell type ^[84-86]. CD4 binding to Env induces gp120 conformational changes and exposure of the V3 loop. CCR5/CXCR4 then interacts via its N-terminus with the base of V3 and the bridging sheet of gp120. This triggers the exposure of the N-terminal helical regions of gp41 which form a pre-fusion coil-coil structure that inserts in the cell membrane (Figure 6) ^[87]. Prior to membrane fusion, a highly stable six-helix bundle is formed between the fusion peptide and the gp41 transmembrane region and results in pore

formation. This pH-independent fusion of the viral and cellular membranes mediated by the activated gp41 is then followed by the release of the viral core into host cytoplasm ^[56].

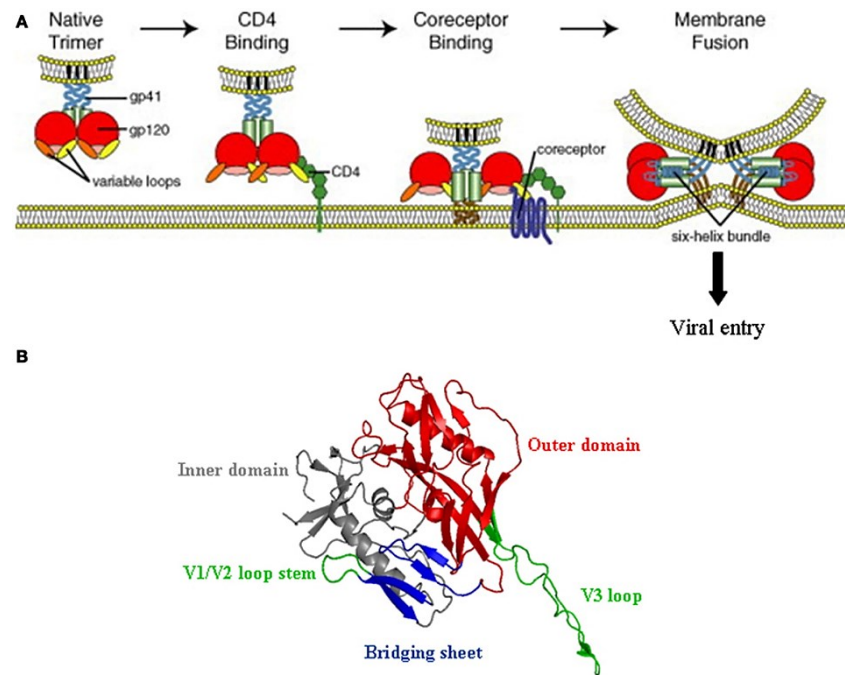


Figure 6. Diagram representing HIV-1 entry mechanism. (A) HIV entry into the target cell is divided into four steps: (1) the heterotrimer of gp120 and gp41 which form Env bind to the CD4 receptor on the target cell. (2) This induces conformational changes in the gp120 structure and allows co-receptor binding via the V3 loop of Env. (3) This triggers gp41 activation and initiates membrane fusion mediated by gp41 insertion into the target cell membrane. (4) Finally, a six-helix bundle is formed and complete membrane fusion allows the viral core to be released into the host cytoplasm. (B) Representation of the three-dimensional structure of gp120 in the CD4-bound conformation, including the inner and outer domains, the V1/V2 loop stem, and the four β strands (CD4 induced bridging sheet in blue) that together with the V3 loop (in green) contribute to co-receptor selectivity and interaction ^[87]. Taken from Connell et al. 2013.

Following capsid core entry into cells, the viral capsid is uncoated and the viral RNA is reverse transcribed into DNA. However, it is still unclear whether uncoating occurs in the cytoplasm or at the nuclear membrane. Experimental studies have suggested three potential mechanisms by which the viral capsid uncoating process might operate after entry into cells^[88]. The first model, based on early biochemical studies, supports the idea that the defragmentation of p24 molecules (CA) from the viral core occurs immediately, and almost completely, following entry to cell due to local pH changes and a simple dilution effect^[89]. In the second model, based on imaging approaches, researchers proposed that the capsid core disassembly takes place in the cytoplasm but is incomplete and some CA proteins remain associated with the Reverse Transcription Complex (RTC) which recruits critical host factors to mediate the reverse transcription process^[90]. The last model suggests that the viral core keeps its integrity until it reaches the nuclear pore complex (NPC) allowing the replicating viral RNA genome to be protected from cytosolic host DNA sensors^[91]. Only the last two models are in concordance with recent evidence that CA proteins play a role in late events of the HIV-1 replication cycle including nuclear import and viral genome integration^[92]. Additionally, these models are not exclusive from each other and may both occur depending on the cell type and the cycle stage^[88].

In all cases, the uncoating process is associated with the formation of RTC which comprises of the viral RNA genome, RT, MA, IN, NC, Viral protein R (Vpr) and various host proteins^[89]. The RTC mediates the retrotranscription of the viral RNA into proviral double-stranded DNA^[93] (Figure 7). Initiation of reverse transcription is triggered by the binding of a host transfer RNA (tRNA, lys3); the 3' end is base-paired and complementary to the primer binding site (PBS) at the 5' end of the viral RNA^[94]. The reverse transcription process is error-prone due to the lack of proofreading ability of the RT and can result in double-stranded (ds) viral DNA presenting high-diversity in nucleotide content. On average, HIV RT creates one error during each round of replication^[95]. Therefore, the number of mutations correlates with the amount of virus produced each day. In some individuals, this could result in over 1 billion mutated virions produced *de novo* per day^[95].

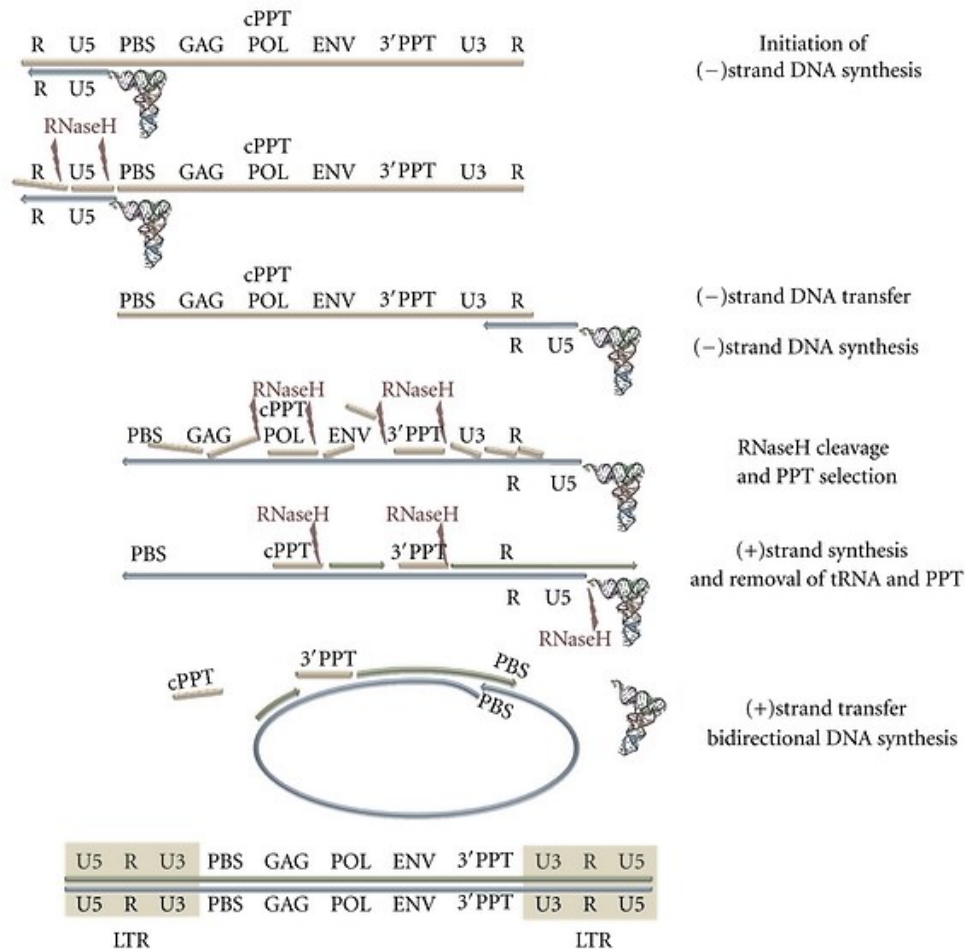


Figure 7. HIV-1 RNA reverse transcription. The reverse transcription of single-stranded viral RNA into a provirus double-stranded DNA can be divided into eight stages. First, the host cell tRNA (Lys3) binds to the primer binding site at the 5' end of the + strand of the viral RNA (orange), (stage 1). RT initiates reverse transcription and produces a (-) strand DNA using tRNA as primer. The RNase H digests the RNA template, (stage 2). Then, the (-) strand DNA transfer occurs between the R sequences at both ends of the single-stranded RNA genome, allowing further elongation of the (-) strand DNA with further degradation of the RNA template, (stage 3). DNA synthesis continues and two sequences resistant to RNase H degradation and rich in purine (cPPT and 3'PPT) are left (stage 4). Subsequently, these sequences are used as primers to synthesise (+) strand DNA (green), (stage 5). Synthesis of the (+) strand DNA continues until the first 18 nucleotides of the tRNA:DNA hybrid and the RNase H digest the primers PPT and release the primer binding site from the (+) strand, (stage 6). Thus, there is strand transfer between the primer binding site sequence of the (+) strand DNA and the primer binding site on the (-) strand DNA. Elongation of the (+) and (-) strands results in a linear double-stranded viral DNA with long terminal repeats (LTRs) newly formed, (stage 8) ^[93]. Taken from Esposito et al. 2012.

Once generated, the linear ds DNA is integrated into the host genome as a provirus by PIC-associated integrases and the host chromatin-binding protein Lens Epithelium-Derived Growth Factor (LEDGF) (Figure 8). The PIC comprises of the ds DNA associated with viral proteins including MA and Vpr, and with several host proteins such as NUP153, NUP358, CPSF6, and/or TNPO3 ^[96-98]. The first step of ds DNA integration is called “3’ processing”: the terminal dinucleotide from each 3’ end of the viral cDNA are removed by IN. The second step is named “strand transfer”: IN catalyses the ligation of the 3’ end to the 5’O-phosphate end of the host DNA (Figure 7) ^[93]. Molecular analyses have shown that viral integration occurs preferentially into transcriptionally active regions and might be promoted by increased chromatin accessibility allocated to these regions ^[99, 100]. However, Chun et al. demonstrated that only a very small proportion of the cDNA molecules are integrated into the host chromatin ^[101]. Quantitative estimation of integration levels suggests that only one or two molecules of viral DNA are integrated per cell ^[102]. The newly integrated viral DNA or provirus is irreversibly present in the host chromosome. Depending on the host cell type and the activation status, the provirus either undergoes replication process or remains latent, generating viral reservoirs disseminated all over the body ^[103]. Viral cDNA integration into the host chromatin announces the end of the HIV’s early life cycle phase.

1.1.7.3 Late phase of HIV-1 life cycle

Following viral integration in the host DNA, the HIV-1 genome can be replicated and transcribed by the cellular transcription machinery which uses the 5’ LTR as a eukaryotic transcriptional unit. The latter functions as a promoter and contains the initiator (Inr), TATA-box, three sp1 sites and two NF-κB binding sites capable of enhancing the reaction process ^[104]. The process of viral transcription is catalysed by cellular RNA polymerase II in association with HIV-1 Trans-Activator of Transcription protein (Tat) and host transcription factors including positive transcription elongation factor b (P-TEFb), and is completed by the 3’ LTR ^[105, 106]. After recruitment of the RNA polymerase II by NF-κB and other transcription factors, the HIV-1 promoter elements help to position the RNA polymerase II at the initiation site of transcription and the formation of the transcription complex allowing transcription to proceed ^[107]. Therefore, viral transcription efficiency depends on the

nuclear level of NF- κ B: latent T cells that exhibit a basal level of viral transcription present low level of NF- κ B; and activated T cells for which the transcription is upregulated express high nuclear concentration of NF- κ B ^[108]. In addition, the viral Tat protein identified in 1987 by Kao et al. has been shown to play a crucial role in viral transcription ^[109]. Tat upregulates the RNA polymerase II activity by binding to the transactivation response (TAR) located downstream of the 5' end of HIV-1 RNA. Cyclin T1 recruited by Tat interacts with the CDK9 kinase, also known as Tat-associated kinase (TAK) of the P-TEFb ^[110]. The TAK phosphorylates the C-terminal domain of the RNA Pol II, which dramatically upregulates viral transcription and allows the formation of full-length HIV-1 RNA molecules.

The resulting mRNA is produced as a single molecule and spliced by the cellular machinery. The first mRNA produced is spliced multiple times and leads to the production of viral proteins including Rev, Nef and Tat which, by a mechanism of retroaction, enhance viral transcription ^[105]. However, due to the limited number of splicing sites in the viral RNA, the cellular machinery generates RNA copies of various lengths: unspliced, totally-, partially- and multiply- spliced molecules ^[111]. Unspliced and partially spliced RNA transcripts are exported out of the nucleus by Rev which mediates viral mRNA transport by binding to the Rev Response Element (RRE) ^[112]. Partially spliced mRNA are transported to the cytoplasm and translated into Vpr, Vif, Vpu and Env. Two molecules of unspliced RNA transcripts are incorporated into newly formed virions to constitute the viral genome along with the mRNA for Gag and Pol synthesis ^[111].

Protein production and virion assembly occur in the cytoplasm mainly immediately underneath the cell membrane. Gag polyproteins are translated as monomers from unspliced viral mRNA by ribosomes and play a crucial role in virion formation. The early stage of viral assembly involves two principal functional assembly domains present in Gag molecule: a membrane domain (M) which allows Gag binding to lipids in the plasma membrane; and an interaction domain (I) responsible for Gag multimerisation resulting in about 1,500 molecules in the immature virion ^[113]. This I domain recruits Gag-Pol polyproteins, tRNA primer and the RNA molecules into the newly formed viral particle, and by interacting with MA and the cytoplasmic tail of gp41 allows Env complex incorporation ^[114]. In addition, Nef and Vpu induce host CD4 receptor downregulation and destruction to avoid interaction with Env ^[115, 116].

The late stage of viral particle formation brings into play the late assembly domain (L) comprising the PTAP motif located in the protein p6 of Gag ^[117]. To induce viral budding from the cell, the host multivesicular body pathway is hijacked following PTAP motif interaction with TSG101 which recruits the ESCRT-1 complex and causes membrane budding and scission processes in the cell ^[118]. Then, during viral budding from the cell, nascent virions undergo a maturation process initiated by PR cleavage of Gag, resulting in infectious viral particles that are ready to infect new cell targets ^[119].

1.1.7.4 Cellular restriction factors of HIV-1 replication

Several cellular restriction factors of HIV-1 replication that act upon viral entry have been identified while researchers were investigating the role of HIV-1 accessory proteins. Among them, the apolipoprotein B messenger RNA (mRNA)-editing enzyme catalytic polypeptide-like 3 (APOBEC3) family of proteins are the most known potent inhibitors of HIV-1, particularly APOBEC3G ^[120], tripartite-motif-containing 5 α (TRIM5 α) ^[121] and tetherin/bone marrow stromal cell antigen 2 (BST2)/CD317 (tetherin) ^[120, 122, 123]. APOBEC3G presents polynucleotide (RNA or DNA) cytidine deaminase activity which is responsible for its antiviral functions. Researchers have shown that APOBEC3G incorporates into assembling HIV-1 virions, associates with the RTC and deaminates cytidine residues in the nascent minus-strand cDNA, thereby inhibiting reverse transcription ^[120]. This results in guanosine-to-adenosine hypermutation of viral plus-strand DNA and causes a loss of genetic integrity. Additionally, APOBEC3G actions reduce the amount of viral DNA that accumulates in infected cells through a mechanism which remains unclear ^[124]. Nonetheless, the antiviral activity of APOBEC3G is strongly inhibited by the viral protein Vif which targets this restriction factor for ubiquitination and proteasome degradation, hence reducing the level of virion-incorporated APOBEC3G molecules ^[120].

TRIM5 α belongs to the family of TRIM-containing proteins that have been identified in different species of primates including owl monkeys and rhesus macaques, and exert their antiviral functions by interacting with CA proteins of retroviruses ^[121]. The mode of action of TRIM5 α against HIV-1 is not clear, but various studies have shown that TRIM5 α binding to CA upon viral entry can disturb the uncoating process and interfere with reverse transcription and viral replication ^[90].

Tetherin is a membrane protein that alters viral release from infected cells resulting in virion internalisation, accumulation in the endosome and eventual degradation ^[125]. Similar to other restriction factors, the mechanism underlying tetherin's antiviral action is poorly understood. However, HIV-1 Vpu protein has been identified as an antagonist of tetherin whereas SIV, which lacks Vpu, uses Nef to inhibit tetherin action ^[115, 123]. A representation of the HIV-1 replication cycle and restriction factor targets is presented in Figure 8.

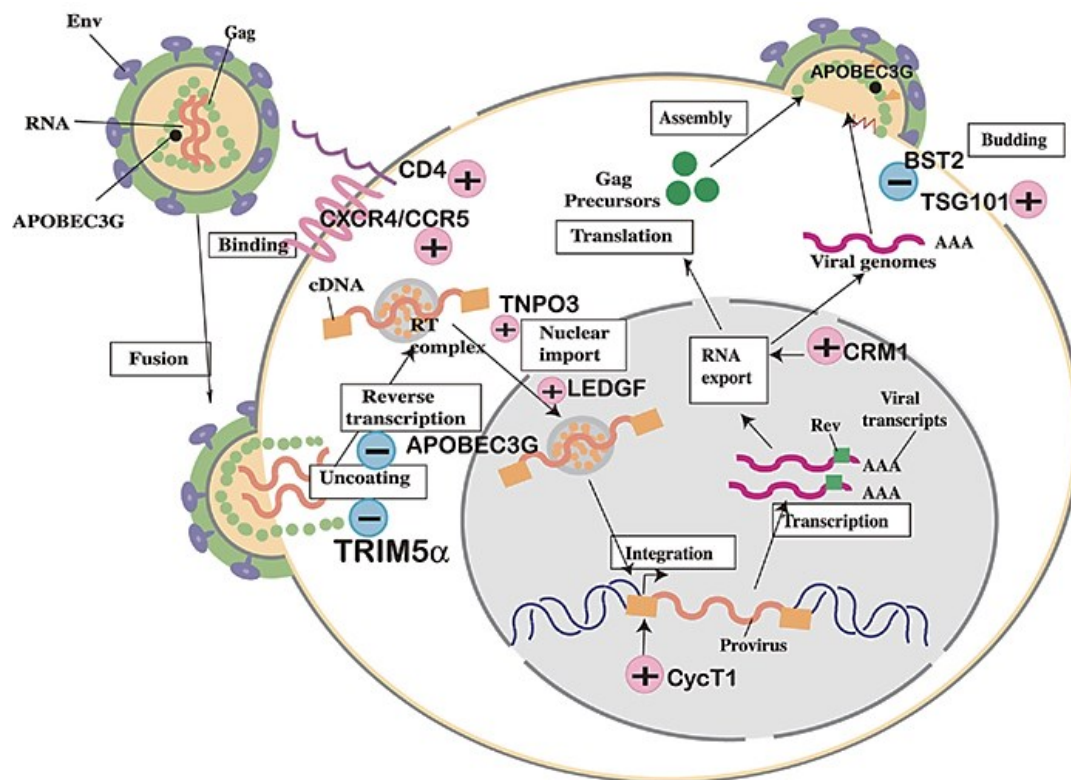


Figure 8. HIV-1 replication cycle and restriction factor targets. HIV-1 virion interacts with the target cell via Env binding to CD4⁺ T cell receptor and chemokine receptor (CCR5 or CXCR4) on the host cell surface. This induces Env spike conformational changes resulting in viral and cellular membrane fusion. The viral content is then introduced into the cell cytoplasm, and following uncoating, the RNA genome is released. After reverse transcription, the pre-integration complex (PIC) is formed and translocated into the cell nucleus. The viral DNA is integrated into the host chromosomal genome as a provirus by PIC-associated integrases and host chromatin-binding protein lens epithelium-derived growth factor (LEDGF) and Transportin 3 (TNPO3). Transcription of the provirus is operated by host RNA polymerase II and Cyclin T1 (CycT1) (a component of the CDK9/pTEFb transcription factor complex positive transcription elongation factor b (P-TEFb)). The viral mRNA is spliced resulting in the production of mRNA of different sizes which are exported to the cytoplasm, and for the largest of them by the host protein CRM1. Then viral mRNAs are translated into viral proteins which are incorporated into the viral genome and assembled as virions ready to bud from the host cell. Tetherin also known as BST2 is an interferon-inducible membrane protein that inhibits the budding of virus particles from infected cells. Tsg101 protein, plays a significant role in the cellular vacuolar protein sorting (Vps) pathway and is required for HIV-1 budding from the cell. APOBEC3 is a cellular restriction factor that incorporates into assembling HIV-1 virions, associates with the RTC and deaminates cytidine residues in the nascent minus-strand cDNA, thereby inhibiting reverse transcription. (+) Positive factors required for viral replication. (-) Negative factors that inhibit viral replication [126]. Taken from Nakayama et al. 2012.

1.2 Clinical phases and pathogenesis of HIV-1 infection

The natural course of HIV-1 infection is characterised by a long incubation period and a slow disease progression that results in depletion of CD4 expressing lymphocytes, a systemic immune activation and a dysregulation of the immune system. Numerous cohort studies across the world have demonstrated that in the absence of antiretroviral treatment (ART), AIDS onset occurs within ten years after infection ^[127, 128]. This suggests that HIV-1 pathogenesis follows a common mechanism which is independent of human and viral diversity ^[129]. Human studies and non-human primate models, such as rhesus macaques have provided reliable *in vitro* and *in vivo* models to study HIV-1 pathogenesis and the different stages of HIV-1 infection ^[130-132]. The latter can be divided into three main steps based on immune responses and viral load: acute infection, chronic infection and the development of AIDS (Figure 9).

1.2.1 Establishment of primary infection

HIV-1 is predominantly transmitted by sexual intercourse through genital or rectal mucosal surfaces ^[133]. Mathematical models of random viral evolution and sequences analyses indicate that HIV-1 transmission is due to a single founder virus in nearly 80% of individuals while the remaining individuals are infected with two to five distinct viruses ^[134]. Recent evidence has shown that HIV-1 preferentially uses the CCR5 co-receptor rather than CXCR4 ^[131]. The causes of this selection have been linked to the presence of high proportions of CCR5+CD4+ activated and memory T cells residing or migrating in/to the mucosa, in response to HIV-1 invasion, thus providing favourable conditions for R5 virus initial and preferential expansion ^[135, 136]. Additionally, recent research has demonstrated that HIV-1 transmission was biased towards the selection of viruses with amino acids that increase virus fitness ^[15]. It has been demonstrated that transmission efficiency varies according to viral load of the infected patient and can be up to 10-fold higher during acute infection compared to the chronic phase of infection ^[18, 137]. However, it is not clear whether HIV-1 is exclusively transmitted by cell-free virions or by infected cells; and if transmission occurs via transcytosis through an intact mucosal epithelium or via microabrasion, and/or ulceration in the mucosa ^[138].

Early studies suggest that Dendritic cells (DCs) and Langerhans cells (LCs) expressing CCR5 and CD4 receptor may be the first cells to be infected by HIV-1 in the mucosa ^[139, 140]. These cells express C-type lectins such as DC-SIGN which are capable of capturing HIV-1 particles and transferring them to CD4⁺ T cells through virological synapses ^[141, 142]. This process, called trans-infection might facilitate viral dissemination to susceptible CD4⁺ T lymphocytes and contribute to the formation of a small founder population of infected cells within a few hours after viral exposure ^[131, 143]. Other studies have proposed that macrophages present within the epithelial membranes might support initial viral replication immediately after exposure, although it is not clear how this localised infection might then further amplify ^[142, 144].

Conversely, recent data from an *ex vivo* study has demonstrated that transmitted/founder virus isolated in acute infection preferentially replicates in CD4⁺ T cells rather than macrophages or DCs indicating that CD4⁺ T cells are the primary targets of HIV-1 infection in the mucosa ^[145]. Indeed, small numbers of CD4⁺ T cells have been observed beneath intact epithelium, which can be attributed to previous or ongoing immune responses ^[144]. Moreover, microabrasions/ulcers in the cervicovaginal tissue caused by sexual intercourse could favour the presence of activated CD4⁺ T cells near mucosal surfaces rendering them susceptible to viral infection ^[144]. Therefore, the primary target cells of HIV-1 infection in the mucosa might depend on different factors including; the type of mucosal tissue, the integrity of the mucosa and the presence of co-infections or inflammation.

Following successful HIV-1 infection of primary target cells, the virus must form an initial infection focus that can result in either an abortion of infection or exponential viral expansion. Therefore, to be effectively transmitted the virus must spread to target cells with propagation rates greater than one ^[146]. This viral expansion is promoted by inflammatory and innate immune responses that recruit activated CD4⁺ T cells and other immune target cells at the initial site of infection. In addition, researchers have demonstrated that local immune activation caused by co-infection with other sexually transmitted infections (STI) such as bacterial vaginosis, enhances the risk of contracting HIV-1 due to a greater number of immune target cells present in the sexual mucosa favouring HIV-1 dissemination ^[14, 17]. Similarly, abrasion or wounds promote virus crossing by compromising the integrity of the mucosal epithelial barriers. This would amplify viral propagation by triggering inflammatory responses ^[147].

During the first few days following sexual transmission of HIV-1, the infection grows rapidly from the portal of viral entry but remains localised in the genital tissue. Then, the virus spreads to local draining lymph nodes and the whole system via the bloodstream. This period which lasts about ten days is called the eclipse phase, as the viral RNA is not yet detectable in the systemic compartment by conventional methods ^[134]. Viral expansion is initially limited by the relatively low numbers of CD4⁺ T cells in the primary infection foci. DCs have been identified as playing a major role in the establishment of HIV-1 infection and subsequent virus systemic dissemination ^[139]. A study performed in rhesus macaques has shown that specific plasmacytoid DCs are capable of enhancing SIV infection by secreting chemokines, such as MIP-3 α which is responsible for the recruitment of CD4⁺ T cells to the mucosa. In this experiment, the infection could be aborted using inhibitors of the production of chemokines by DCs, that prevented the attraction of CD4⁺ T cells to the initial site of infection ^[148].

The end of the eclipse phase is characterised by the systemic dissemination of HIV-1 to the lymphoid tissues (LT). The main function of the DCs and macrophages is to capture and present antigen to the LT. They are thought to contribute to viral entry into the lymphatic system by trafficking captured virus to the LT, particularly the spleen and the gut-associated lymphoid tissue (GALT), which presents a high level of CD4⁺CCR5⁺ T cells ^[149]. Additionally, recent evidence has indicated that founder viruses are resistant to the antiviral actions of interferon alpha which facilitate uncontrolled viral replication in the early stage of infection ^[150].

These findings suggest that a window of opportunity exists for a vaccine to successfully eliminate HIV-1 infection. This window of possible intervention occurs at the beginning of the acute infection phase before systemic dissemination.

1.2.2 Acute infection

The acute infection phase occurs approximately 2-4 weeks following HIV-1 transmission and is characterised by the appearance of flu-like symptoms in 50 to 90% of infected individuals (Figure 9). These symptoms predict a more rapid disease progression and are of non-specific nature such as lymphadenopathy, muscle ache, weight loss, fever,

fatigue and rashes, which makes HIV diagnosis difficult to establish ^[151]. At the cellular level, the beginning of the acute phase termed primary HIV infection (PHI) is manifested by systemic infection with an exponential increase in viral load (VL) that can be detected in plasma at levels as high as 10^6 RNA copies/mL and 10^{12} virions generated per day ^[152]. Compared to the viraemia peak, which takes place 3-6 weeks following HIV-1 transmission, the number of CD4⁺ T cells declines dramatically in the initial mucosal site of infection and partially in the periphery ^[153]. Consequently, this first reduction in CD4⁺ T cells is often unnoticed by conventional detection methods. However, the GALT which contains over 60% of all CD4⁺CCR5⁺ T cells present in the body is particularly affected by CD4⁺ T lymphocyte death. This important cell loss in the GALT is now recognised as one of the main indicators of PHI ^[149]. In some cases, this initial dramatic reduction of CD4⁺ T cells in the GALT severely compromises the immune system and is accompanied by opportunistic infections, such as pneumonia and oesophageal candidiasis.

It has been demonstrated that VL attains its maximum level when 30 to 60% of all memory CD4⁺ T lymphocytes become infected and die from direct infection, or apoptosis due to the lack of co-stimulatory signals ^[154, 155]. However, a small proportion of infected CD4⁺ T cells become quiescent forming a viral reservoir of latently infected cells that is disseminated all over the body and can persist for several years. These viral reservoirs are resistant to antiretroviral drug treatments and their persistence makes the total eradication of HIV in infected individuals highly challenging ^[108].

The VL spontaneously decreases 2-4 weeks following transmission by 100- to 1000-fold in RNA copies/mL, before stabilising weeks or months later during the asymptomatic chronic phase of infection ^[129]. This reduction in viraemia coincides with the emergence of HIV-1-specific immune responses and is followed by a rebound in CD4⁺ T cell count although to an inferior level than prior infection ^[156]. These observations support the idea that the initial depletion in CD4⁺ T cells is mainly due to the direct killing and cytolytic effects of HIV-1. This is further supported by *in vitro* experiments using activated peripheral blood mononuclear cells (PBMCs) which demonstrate that high levels of primary virus replication can cause CD4⁺ T cell death even in the absence of syncytial formation ^[129].

As mentioned above the viraemia peak is associated with the elicitation of a strong HIV-1-specific cellular immune response. This cell-mediated immunity against HIV-1 involves

natural killer (NK) cells, CD8⁺ T cells (also known as Cytotoxic T lymphocytes (CTLs)) and CD4⁺ T-helper cells ^[157]. Several studies have suggested a significant role for CTL responses due to the temporal association between the appearance of HIV-specific HLA-restricted CTL and the initial dramatic drop in VL in various cohorts ^[156]. Additionally, investigators have indicated that CTL responses drive HIV-1 viral escape. This was observed with the apparition of CTL epitope escape mutants following the reduction in VL ^[158]. Alternatively, it has been suggested that the strong depletion in CD4⁺ T cells during PHI may be responsible for the reduction in viraemia due to exhaustion of activated CD4⁺ T cell substrate. Furthermore, antibodies (Abs) against HIV-1 infection have been suggested as potentially responsible for the drop in VL as they start to be detectable in plasma at this stage ^[129]. These initial humoral immune responses involve non-neutralising antibodies which are first directed against p24 molecules, then against Env ^[159]. Although these antibodies are rarely capable of neutralising the autologous virus, those targeted against Env may mediate ADCC function contributing to CD4 T cell depletion. The extent to which ADCC may impact on the initial reduction of peak viraemia is unclear.

Thus, the acute phase of HIV-1 infection is characterised by a peak in VL which is associated with a transient decline in CD4⁺ T cells and is followed by a dramatic drop in viraemia, most likely due to the generation of HIV-1-specific CD8⁺ CTL responses. An alternative hypothesis is that the transient reduction in CD4⁺ T cells may be mediated by the direct killing of HIV-1 itself, which is then curtailed by the emergence of cellular immunity, resulting in a recovery in CD4⁺ T cells ^[160].

1.2.3 From a chronic infection to AIDS

1.2.3.1 Pathogenesis of CD4⁺ T cell decline

The chronic phase of HIV-1 infection persists for a median of ten years without ART ^[127]. This intermediary stage is characterised by an absence of clinical symptoms and is also called the asymptomatic phase. Following the rebound in CD4⁺ T cells count observed at the end of the acute phase infection, the chronic phase of HIV-1 infection is manifested by a slow and irreversible decline in the number of CD4⁺ T cells at a rate of up to 25-60 cells/ μ L

per year until the development of AIDS ^[153]. During this period, the VL measured by RT-PCR is highly variable amongst HIV-1 infected patients and ranges from <50 copies RNA/mL to >106 copies RNA/mL. Despite this, the high VL observed in most untreated individuals, the number of HIV-1 infected CD4⁺ T cells circulating in the peripheral blood remains relatively low ^[156, 161, 162].

Studies of SIV infection in non-human primate models (NHP) such as mangabey monkeys and rhesus macaques have provided reliable models to further the understanding of HIV-1 pathogenesis ^[154]. SIV infection is manifested by a high VL and significant CD4⁺ T cells losses, particularly in the GALT, like in HIV-1 infection ^[154]. However, SIV-infected NHPs do not progress to AIDS and do not manifest signs of high chronic immune activation as observed in HIV-1 infected individuals ^[163]. These findings suggest that the decline in CD4⁺ T-cells that occurs during the chronic phase of HIV-1 infection is unlikely to be solely mediated by the direct killing of HIV-1. Researchers have suggested that the systemic chronic immune activation caused by HIV-1 infection is the leading cause of the irreversible slow decline of CD4⁺ T cells and the onset of AIDS ^[164]. Models of HIV-1 pathogenesis also involve the increase in lymphocyte death by apoptosis or necrosis, the sequestration of lymphocytes into lymphoid organs, and the decreased production of lymphocytes.

Systemic chronic immune activation

Although, the direct relationship between CD4⁺ T cell depletion and chronic immune activation has not been fully established, chronic immune activation is considered as one of the most critical drivers of CD4⁺ T cells losses and the development of AIDS ^[164]. This state of chronic immune activation is manifested by: an increased secretion of inflammatory cytokines, such as TNF-alpha and IL-6, heightened expression of phenotypic markers on circulating B- and T-lymphocytes (e.g., CD38 or HLA class II molecules), and greater levels of activated lymphocytes ^[165]. Previous studies have reported that the level of activated CD8⁺ T cells was a stronger predictor of shorter survival in HIV-1 infected patients than VL or CD4⁺ T cell count ^[166]. These findings support the observations that systemic immune activation is a major correlate of reduced lymphocyte proliferation and is associated with increased morbidity and mortality amongst ART-treated patients ^[164]. Furthermore, they observed that signs of chronic immune activation tend to be reduced with ART which implies a role of

HIV-1 replication in the induction of high level of immune activation. Finally, researchers found that the concomitant use of the immune suppressant cyclosporine A and antiretroviral drugs, was associated with a more sustained CD4⁺ T cell restoration than with ART alone ^[167].

Intensified T lymphocytes death

HIV-1 is capable of inducing the death of both infected T cells and uninfected bystander T cells through mechanisms which are mediated by HIV-1 proteins particularly heightened immune activation ^[165]. Uninfected activated T cells die from abnormal apoptosis induced by several types of stimuli. These involve the direct cytotoxicity of HIV proteins (e.g., Env, Nef or Tat) and HIV-1 induced inflammatory signals ^[153]. These stimuli may also induce the death of HIV-1-infected T cells; however the latter are more likely to die from the cytolytic effects of HIV-1. Furthermore, numerous studies have demonstrated that in the absence of ART, CD4⁺ T cell depletion results principally from the death of uninfected, rather than HIV-1 infected, T cells ^[153].

Interestingly, some studies have indicated that HIV-induced T cell apoptosis leads to heightened levels of HIV-1 replication by the Bcl10/MALT1/CARMA complex, a process which is dependent on active caspase 8 ^[160]. This observation indicates that HIV-1 has evolved a mechanism to overcome the potential limiting replication factor caused by the massive death of uninfected bystander CD4⁺ T cells.

Sequestration in lymphoid tissue

The lymphoid tissue is particularly affected during HIV infection even at the earliest stage of infection, as observed with generalised lymphadenopathy in some individuals during the acute phase of infection ^[168]. Without ART, lymph nodes show signs of inflammation, characterised by an increased expression of inflammatory cytokines which is a direct reflection of the high-level of HIV-1 replication in these tissues. The inflammation of the lymphoid organs is also manifested by enhanced expression of adhesion molecules which alter the architecture of the lymphoid tissues and progressively causes sequestration of lymphocytes in these organs. The sequestration of lymphocytes in lymphoid tissues

contributes to the dramatic reduction in CD4⁺ T cells observed in the peripheral blood of HIV-1 infected patients.

Diminished production

There is some evidence of HIV-1 impaired lymphocyte production as indicated by the loss of primitive hematopoietic progenitors in the bone marrow of HIV-1 infected patients^[169]. Moreover, researchers have indicated that the poor CD4⁺ T cells restoration during ART in some HIV-1 patients may be due to reduced thymic function. These observations imply that dysfunctional thymic function in these HIV-1 infected patients may reduce the production and replenishment of CD4⁺ T cells^[170].

Thus, without ART, HIV-1 causes indirect and direct deleterious effects on the immune system which contribute to the irreversible decline of CD4⁺ T cells and the onset of AIDS. Nonetheless, numerous studies have demonstrated individual variability and significant differences in rates of disease progression amongst HIV-1 infected individuals^[171].

1.2.3.2 Genetic factors contributing to slow disease progression

Researchers have categorised HIV-1 infected patients as rapid, moderate, slow or long-term non-progressors (LNTPs), or elite controllers, according to the duration of their asymptomatic phase. LNTPs represent less than 1-5% of the total HIV population in the world; they maintain high CD4⁺ and CTL counts and are capable of controlling the infection for more than 8 years before progressing to AIDS. “Elite” or “natural” controllers accounts for less than 1% of all PLHIVT cell; they suppress viral replication below 50 copies/mL of HIV RNA in plasma, maintain high CD4⁺ and CD8⁺ T cell counts and control the infection for more than 15 to 20 years^[172]. Multiple factors play a role in delaying the progression to AIDS in LNTPs and elite controllers including individual genetic factors and viral parameters.

The level of VL at the set-point, the rate of CD4⁺ T cells decline, the quality and magnitude of CTL responses and the emergence of escape mutants are all important indicators of progression rates to AIDS. Disease progression is also influenced by genetic predispositions that influence the efficiency of the humoral and cellular immune responses

^[173-175]. 1% of the Caucasian population present a homozygous mutation in the gene CCR5 ($\Delta 32$) resulting in the absence of the CCR5 receptor at cell surfaces ^[176]. This genetic mutation confers a relative resistance to HIV infection and slow progression towards AIDS. Other genes have been associated with a slower progression to AIDS, such as *CCR2 I64*, *KIR3DS1* and certain HLA haplotypes: *HLA-B*57*, *HLA-B*27* ^[176-179]. In contrast, some HLA haplotypes have been linked to rapid disease progression and include HLA-B35, HLA-B21 and HLA-B8 ^[180, 181]. Researchers estimate that 90% of the genetic and non-genetic factors influencing AIDS progression in HIV-infected individuals remain to be elucidated ^[176].

However, even among LNTs, elite controllers or other HIV-1 infected people with a predisposition that reduces disease progression, in the absence of ART, HIV infection ultimately leads to AIDS.

1.2.4 The final stage of the infection: AIDS

The hallmark of AIDS is the drop of the CD4⁺ T cells count below a threshold of 200 cells/ μ L in plasma ^[156]. Once this threshold is passed the immune system is severely compromised, and opportunistic infections or cancers may occur.

The beginning of AIDS is associated with a decline in CTLs, a deterioration of the architecture of lymphoid organs and a loss of thymic function ^[162]. This eventually leads to a severe immunosuppression, and the infected individuals become susceptible to a wide range of malignancies and opportunistic infections including Kaposi's sarcoma, cervical cancer, herpes Simplex, candidiasis, toxoplasmosis and tuberculosis ^[2]. The latter is the highest cause of death among HIV-1 infected patients and one of the biggest killers in the world. HIV-1 can also infect the central nervous system through CD4-independent HIV-1 infection of astrocytes causing HIV-associated neurocognitive disorders such as HIV-associated dementia (HAD) ^[182]. Overall, in the absence of ART, HIV pathogenesis causes a generalised immunodeficiency which aggravates irreversibly over time and ultimately causes death ^[129, 183, 184].

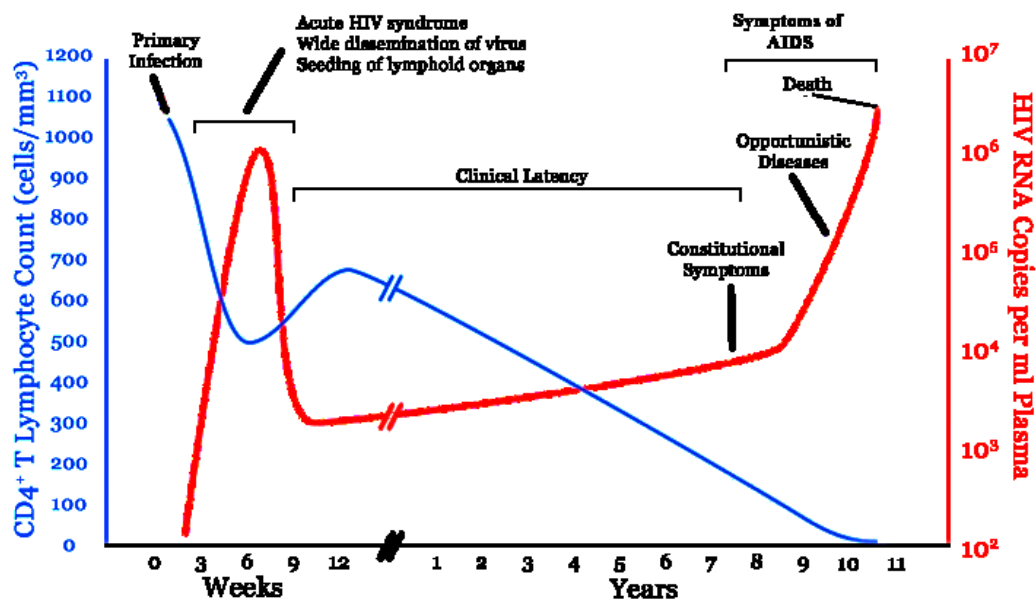


Figure 9. Representation of the different phases of HIV-1 infection. HIV-1 infection is established within approximately ten days following viral exposure. The acute phase begins after systemic dissemination and is characterised by a rapid increase in viral load (red line) which peaks at about 2-3 weeks following transmission and coincides with a decline in CD4⁺ T cells count (dark green line). At this stage, HIV-1 infected individuals present flu-like symptoms and the moment when the antibodies against HIV-1 become detectable in the plasma is called seroconversion. The appearance of adaptive immune responses is characterised by the increase of Cytotoxic T lymphocytes (CTLs) and coincides with a dramatic reduction of the viral load to a set point which is a predictor of disease evolution. This drop in viraemia is followed by an increase in CD4⁺ T cell counts and marks the beginning of the asymptomatic phase or clinical latency. The set point is usually indicated by the level of viral load when the neutralising antibodies (Nabs) start to emerge. Despite the sustained efforts of the humoral and cellular antiviral responses the viraemia continues to increase and causes an irreversible CD4⁺ T cell depletion to a level below 200 cells/ μ l. From this point, the infected individuals enter in the late stage of the infection called AIDS which occurs, on average, ten years following transmission in the absence of antiretroviral treatment. This exhaustion of the immune system is associated with the appearance of opportunistic infections and cancers ultimately causing death ^[185]. Taken from Pantaleo et al. 1993 ^[186].

1.3 The adaptive immune responses

HIV-1 is a human infection that infects specific immune cells; the same cells whose the primary function is to develop immunity against viral infections. In addition to these features, the virus employs various mechanisms to escape immune responses by evolving rapidly and by producing proteins that hijack cellular constituents to promote infection. The cellular and antibody responses to HIV-1 are described below with an emphasis on the mechanisms of immune evasion that cause a generalised failure of the immune system and lead to AIDS.

1.3.1 Cellular adaptive immune responses and pathogenesis of HIV

The end of the acute phase of HIV-1 infection is indicated by the appearance of CD4⁺ and CD8⁺ T cell responses and the establishment of a chronic phase of infection. HIV-1-specific cellular immune responses have been associated with a durable control of infection [157, 162]. Nevertheless, without antiretroviral treatment, these immune responses ultimately lose the battle against HIV-1. Understanding the mechanisms underlying the success and failure of the cellular immune responses is crucial for the development of vaccines against HIV-1 infection [183].

1.3.1.1 CD4⁺ T cell responses

HIV-1 tropism for CD4⁺ T cells is probably the most obvious mechanism that the virus uses to escape immune responses. The extent of CD4⁺ T helper cells depletion is predictive of the evolution of the disease progression to AIDS [153]. This correlation is due to the critical role of CD4⁺ T cells in orchestrating immune responses. CD4⁺ T cells are activated following the interaction of their T cell receptor (TCR) recognising their cognate peptide presented in the context of antigen-MHC II. Following activation CD4⁺ T cells differentiate into specific subsets depending on the type of cytokines present in their environment. The primary role of CD4⁺ T lymphocytes is to release cytokines that modulate immune responses, in particular those that induce antibody isotype class switching and activate the maturation of CD8⁺ T cells [187]. The activation of CD4⁺ T cells upon antigen recognition, trigger T cell differentiation into T helper (Th) cell subsets which recruit and stimulate other effector

immune cells (macrophages, B cells and CTLs) ^[153]. Initially, activated CD4⁺ T helper cell subsets were divided into two groups Th1 and Th2 based on their cytokine secretion patterns ^[188]. Th1 cells secrete various cytokines including IFN γ , IL-2 and IL-3, which activate cytotoxic effector cells and promote B cell production of IgG₁; while Th2 cells secrete diverse interleukins which stimulate antibody responses. However, this dichotomous separation of Th cells has recently been re-assessed, particularly, since the discovery of two new types of activated CD4⁺ T helper cells: Th17 and regulatory T cells (Treg) which are involved in autoimmunity, cancer and inflammation. These CD4⁺ T helper cell subsets are not spared during HIV-1 infection and also suffer important losses ^[189]. Despite their susceptibility to HIV-1 infection, there is some evidence that CD4⁺ T cells may be involved in the control of the disease. Studies of LTNPs show that low viral loads were associated with strong CD4⁺ T cell responses ^[190, 191]. In addition, it has been reported that LTNPs present similar levels of CD4⁺ T cell populations in the gut mucosa to that of HIV-uninfected controls while this tissue is the most affected by Th cell depletion in chronically infected patients ^[190].

A recent study has shed light on a novel subset of CD4⁺ T cells that cooperate with CTLs in the cytolytic control of HIV-1 replication ^[192]. These cells present significant transcriptional and phenotypic differences compared to HIV-1-specific Th1 cells but share similar characteristics with CD8⁺ T cells. The emergence of these cytotoxic CD4⁺ T cells coincided with the early viral set points suggesting they are implicated in early control of viral replication. Nonetheless, CD4⁺ T cells irreversibly decline during infection which leads to AIDS ^[153].

The first phase of CD4 T cell decline is a preferential and severe depletion of CD4⁺ T cells within the gastrointestinal tract during acute infection, particularly of CD4⁺/CCR5⁺ memory T cells. In contrast, the central memory (TCM) and CD4⁺ naive (TN) T cell in the blood and secondary lymphoid tissues are relatively spared from destruction ^[153]. The second phase leads to a redistribution of CD4⁺ memory T cells due to a migration of proliferating CD4⁺ TCM and early transitional effector memory (TTrM) T cells to the sites severely affected by this destruction. In the last phase, TTrM and TCM cells progressively fail to maintain CD4⁺ memory T cell populations which ultimately leads to CD4⁺ T effector cell insufficiency and the onset of AIDS ^[165].

Thus, CD4⁺ T cells are the major target of HIV-1 and their irreversible decline during chronic infection may suggest a more important role of CTL responses.

1.3.1.2 CTL responses

The emergence of CTLs in the body that are capable of directly killing HIV-1 infected cells coincides with the early decline in VL observed in most infected patients ^[157]. A range of evidence suggests that HIV-1-specific CTL responses, largely CD8⁺ T cell responses, play a significant role in this initial reduction of viraemia, and also in the control of the VL during the chronic phase of infection ^[162, 183, 193]. Activated CD8⁺ T cells differentiate into cytotoxic T cell lymphocytes (CTLs) capable of recognising and eliminating HIV-1 infected cells ^[194]. The interaction between the CD8 T cell receptor (TCR) and antigen-MHC class I molecule complexes triggers CTL release of perforin and granzymes onto target cells ^[194]. These molecules perforate the target cell membrane and trigger cell death by apoptosis. CD8 cells can also trigger apoptosis through the Fas-Fas-L pathways. In addition, CTLs secrete various anti-viral cytokines including TNF α and IFN γ which limit the spread of infection, and RANTES, MIP1- α , and MIP-1 β which are ligands of CCR5 that prevent or reduce viral entry.

An over-representation of the HLA class I alleles B*27 and B*57 have been reported in the vast majority of LTNPs cohorts, highlighting the importance of the associations between particular MHC alleles and CTL control of HIV-1 infection ^[176, 180, 181]. The role of CD8⁺ T cells in restricting HIV-1 replication has been further demonstrated by several non-human primate studies ^[157, 195]. Schmitz et al. showed that CD8⁺ T cell depletion (by administration of anti-CD8 bNAbs) resulted in a 10 to 100 fold increase in VL and a more rapid disease progression in SIV_{mac}-infected macaques ^[196]. The loss of anti-CD8 bNAbs over time resulted in CD8⁺ T cell population recovery and restoration of immunological control ^[197].

It has been suggested that the conservation of CD4⁺ T cell populations in LTNPs helps to maintain higher levels of HIV-1-specific CTLs compared to progressors ^[193]. In addition, it has been proposed that the CTLs of LTNPs are better at recognising HIV-1 infected CD4⁺ T cells that contain endogenous virus that is less efficient at MHC downregulation ^[193]. There is strong evidence that CTL responses exert selective pressure that drives HIV-1 immune escape ^[158, 198]. In this context, higher levels of viral replication in chronic infection drive higher viral mutation rates increasing the selection of variants that escape immune recognition by CTLs ^[158].

However, other studies have demonstrated that the CD8⁺ T cells of progressors and LTNPs present similar frequencies, breadth, avidity and abilities to recognise the autologous virus ^[199]. These findings suggest that additional functional qualities of CD8⁺ T cells differ between progressors and LTNPs. Indeed, Migueles et al. demonstrated that the qualitative features of the HIV-1-specific CD8⁺ T cell response in antiretroviral-treated patients (VL <50 copies/mL) and untreated patients (LTNPs) were different ^[200]. They found that the per-cell cytotoxicity, proliferation and polyfunctionality capacities of HIV-1-specific CD8⁺ T cells were greater in LTNPs compared to progressors although HIV-1-specific CD4⁺ T cell proliferations were similar. In addition, *in vitro* experiments showed that the CD8⁺ T cells of LTNPs exhibited a greater ability to suppress HIV-1 replication than those of progressors ^[201]. These findings were corroborated by the observation that the HIV-1-specific CD8⁺ T cells isolated from LTNPs up-regulated perforin and granzyme B expressions which conferred greater cytotoxic capacity ^[201, 202]. In spite of strong CTL responses during HIV-1 infection, ultimately CD8⁺ T cells fail to clear the infection in both chronically infected patients and LTNPs ^[203]. This is compounded by the ongoing decline in CD4 T cell help, in part as a consequence of CTL-mediated killing of infected CD4 cells ^[194]. Lack of CD4 T cell help and continuous activation of CD8⁺ T cells leads to CD8 T cell dysregulation and exhaustion, as evidenced by reduced cytolytic capacity, poor proliferation, and reduced expression of important signalling molecules that mediate TCR activation.

In summary, while CD8⁺ T cells might be pivotal in the control of HIV-1 infection, they also accelerate progression towards AIDS by favouring CD4⁺ T cell depletion over time. Moreover, vaccine strategies that have attempted to elicit strong virus-specific CD8⁺ T cells in humans have thus far been unsuccessful ^[204], although this may reflect the paucity of epitopes recognised and the magnitude of induced responses. It was suggested that focusing on CD8⁺ T cells (which act after the cell has been infected) for potential prophylactic vaccines might be limiting compared to antibodies ^[205].

1.3.2 Humoral immune responses

1.3.2.1 Generation of antibody diversity

The generation and maintenance of antibody-secreting cells (ASCs) as well as the production of specific antibodies are the basis of the humoral immune response and are crucial for the vast majority of successful vaccination strategies ^[206]. Antibodies (Ab) are immunoglobulins (Ig) that can be found either as soluble proteins exclusively produced by a population of terminally differentiated B cells called plasma cells; or as membrane-bound proteins called B cell receptors (BCR) which are solely exposed on B cell surfaces ^[207]. B cells present various functions; they can recognise antigen via their BCR, present antigen to helper T cells, and produce antibodies of different isotypes and diversity directed against the detected antigen (Ag) ^[208]. The remarkable diversity of the B cell antibody repertoire results from three distinct genetic alterations: variable (V), diversity (D) and joining (J) (V(D)J) recombination, class switch recombination (CSR) and somatic hypermutation (SHM).

B cells are generated throughout life and their development progresses through different stages and checkpoints. Early B cell development occurs in primary lymphoid organs (e.g. human foetal liver and bone marrow) and the following maturation steps in secondary lymphoid organs (e.g. mucosa-associated lymphoid tissue (MALT) and the lymph nodes). In primary lymphoid tissues, B cell development includes the pro-B cell, pre-B cell and immature B cell stages. Pro-B cells derive from pluripotent hematopoietic stem cells which can differentiate into all lineages of blood cells, including B cells. Early B cell development is characterised by the recombination of V, D and J gene segments in the H chain locus and the V and J in the L chain loci (Figure 10). These gene segment rearrangements occur primarily in the foetal liver and adult bone marrow and result in a diverse repertoire of VDJ_H and VJ_L encoding for BCRs expressed on the pro-B cell surfaces ^[209]. At a first check point, surrogate L chain (SLC) pair with the Ig H chain to form the pre-BCR. During a second checkpoint, autoreactive pre-BCR Ig H chains are eliminated and Ig L chains are expressed and paired with Ig H chains forming a BCR exposed on immature B cells. At this stage, each immature B cell expressing only one BCR (IgM) undergoes a selection process which involves receptor editing or deletion to prevent the development of B cells that recognise self-antigen. Selected B cells leave the primary lymphoid tissues as translocational B cells and migrate to the spleen where they undergo a fourth checkpoint,

while continuing their development to become mature follicular B cells (or marginal-zone B cells) ^[208].

Only mature B cells that circulate in the periphery, or reside in lymphoid organs, can be probed for their ability to bind to a foreign antigen via their BCRs. Antigens are either free or presented by APCs such as DCs and macrophages. A BCR consists of a membrane-bound antibody binding to Ag and a heterodimer Ig α /Ig β (CD79) that transduces an activation signal. Upon Ag interaction with BCR complex, activation signals are transduced through immuno-receptor tyrosine-based activation motifs (ITAM) of CD79. This leads to B cell differentiation and proliferation into antibody-secreting plasma cells and memory B cells, and hypermutation of genes coding for the BCR to improve antigen binding ^[210].

Some antigens such as viral proteins induce B cell activation with the help of T cells and are called T cell-dependent (TD) Ag ^[211]. These antigens are endocytosed, degraded and displayed by MHC class II molecules on the B cell surface where they can be recognised by follicular T helper cells (T_{FH}) cells. The recognition of these peptides by T_{FH} cells induces CD28 and CD40L expression on the T cell surface. CD40L then interacts with CD40 expressed on the B cell surface causing further B cell activation and production of cytokines that induces cell proliferation. This eventually leads to B cell expansion, class-switch recombination (CSR) and affinity maturation by somatic hypermutation (SHM) and is accompanied by the generation of high-affinity B cells, memory B cells and long-lived plasma cells ^[208].

CSR is the process that exchanges the initially expressed constant domain (C μ) exons for an alternative set of downstream constant region genes, C γ , C α or C ϵ , and results in the expression of antibodies that have the same antigen specificity but with different functions (Figure 10). SHM introduces point mutations at high rates in the variable region of an antibody which enables the selection of B cells that produce an antibody with higher affinity ^[212]. A sustained and up-regulated activation provided by T helper cells within germinal centres (GCs) is required for B cell expansion and subsequent CSR and SHM events. Without T cell help, B cell activation is incomplete and results in cell apoptosis “anergy” due to a lack of survival signals. Thus, the constant loss of CD4⁺ T helper cells during HIV infection results in B cell dysregulation and delayed antibody responses ^[153].

Alternatively, some B cells presented outside GCs can be activated by a T cell-independent mechanism that involves the production of antibodies following a

multimerisation of BCR complexes ^[213]. However, the antibodies resulting from this mechanism present a lower affinity and are less diverse compared to those generated by T cell-dependent activation. Interestingly, in the case of HIV/SIV, scientists demonstrated that despite the inability of antigen protein to induce a T cell-independent humoral response, membrane-bound trimeric Env was able to activate B cells by a T cell-independent mechanism. Thus, in HIV/SIV infection, it was observed that Abs directed against Gag protein decline during infection whereas antibodies targeting Env remain consistently high. Such mechanism is likely to participate in viral immune evasion through promoting a high Ab response against Env while preventing T cell-dependant Ab affinity maturation ^[214].

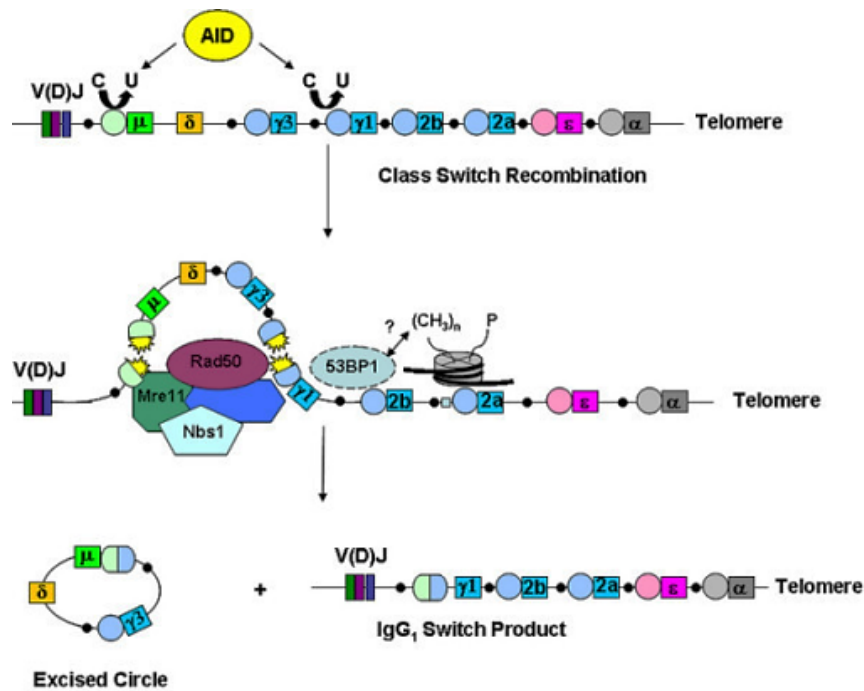


Figure 10. V(D)J and class switch recombination. In the upper panel, the variable (V_H), diversity (D_H), and joining (J_H) gene segments assembled to form the antibody variable region. This process called V(D)J recombination consists of cleavage of the recombination signal sequences localised between the gene segments by the recombination-activating gene 1 (RAG1)–RAG2 complex. The cleaved exons are joined by the action of several proteins called non-homologous end-joining (NHEJ) proteins. A promoter upstream of the locus controls the expression of the rearranged VDJ segment and the μ heavy chain (not shown). The latter assembles with a light chain hence forming an IgM antibody. Then, the constant regions of the heavy chain (C_H) and light chain (L_H) can be exchanged for an alternative set of downstream C_H -region exons such as C_γ , C_α or C_ϵ through a process called class switch recombination (CSR), here IgM to IgG. This process orchestrated by the enzyme activation-induced cytidine deaminase (AID), leads to lesions at the switch regions (circles) upstream of a given IgH gene, which is converted to DNA double-strand breaks (DSBs). These DSBs recruit various factors, such as Mre11, Nbs1 and Rad50, which are involved in the resolution of the DNA break. During this process, 53BP1 is thought to play a role of anchor at the IgH locus involving interactions with nucleosomes near the break through various histone modifications. This might modulate chromatin in this area to facilitate DNA repair. CSR results in the replacement of the μ exon with another constant region gene (IgG1 shown) and the excision of the intervening sequences as an episomal circle. CSR allows the expression of antibodies that have the same antigen specificity but are of a secondary C_H isotype (IgG, IgA or IgE) and thereby present different effector functions. The recombined variable region (VDJ) of both the heavy and light chains also undergo a high rate of point mutations via a process called somatic hypermutation (SHM) catalysed by the AID (not shown) ^[215]. Taken from Adams et al. 2006.

1.3.2.2 Antibody structure and function

Human antibodies share a common structure that consists of multimeric complexes of two proteins: the light (L) and heavy (H) chains. There are five types of H chains (μ , γ , α , ϵ and δ) generating IgM, IgG, IgA, IgE and IgD respectively (Figure 11). Additionally, antibodies contain either a κ or a λ L chain, adding more variability to the Ig repertoire ^[216]. Moreover, Abs can be divided into: (1) an antibody binding fraction (Fab) containing the variable (V) domains responsible for Ag recognition; and (2) a constant fraction (Fc) consisting of the constant (C) domains which defines Ab classes (or isotypes) and confers effector functions ^[216]. Despite the high degree of homology of their Fc portion, Ab effector functions can vary greatly depending on the isotype (Table 2). Ab effector functions are mediated by the interaction of the Fc domain with components of the complement pathway, or Fc receptor complexes (FcRs), which connect the innate and humoral immune systems ^[217].

During class-switch recombination (CSR) the Fc portion is changed while the Fab portion is conserved. Therefore, a given ASC can generate Abs presenting different effector functions but sharing the same antigen specificities ^[218]. The type of antibody generated by an ASC is determined by a balance of cytokines and the cell environment. The spleen and secondary lymphoid organs provide conditions that favouring the production of Abs of the IgG class whereas, in the MALT, the IgA isotype is preferentially produced ^[218].

IgM

The initial B cell expansion following HIV infection occurs rapidly and produces antibodies of weak affinities, mainly IgM directed against the most abundant HIV protein: Gag p24 molecules ^[219]. Then, isotype class switching might occur to generate a more sophisticated antibody response that involves Abs of other classes presenting various anti-viral properties and higher binding affinities ^[218]. IgM antibodies are expressed as monomers on the B cell surface and play the role of receptor; or are secreted as multimers circulating in serum and secretions. Multimeric IgM antibodies are formed from five or six monomers linked by a joining (J)-chain and can be exported at mucosal surfaces upon J-chain binding to a secretory component expressed by epithelial cells. The generation of IgM antibodies does not require isotype class switching or affinity maturation, although there is evidence that once generated they can be further matured ^[220]. Therefore, IgM antibodies bind antigens

with a weak affinity compensated by a strong avidity and mediated by their ten binding sites ^[221]. Besides, IgM antibodies are particularly effective at activating the classical pathway of the complement system. This innate mechanism “complements” the action of Abs and phagocytic cells to eliminate foreign pathogens and up-regulate immune responses. The classical pathway can be initiated by binding of the Fc domain of an IgM (or two IgG antibodies) to the C1 component. This activation triggers a signalling cascade involving over 30 proteins and leading to the lysis of viral membranes, infected cells or bacteria ^[222].

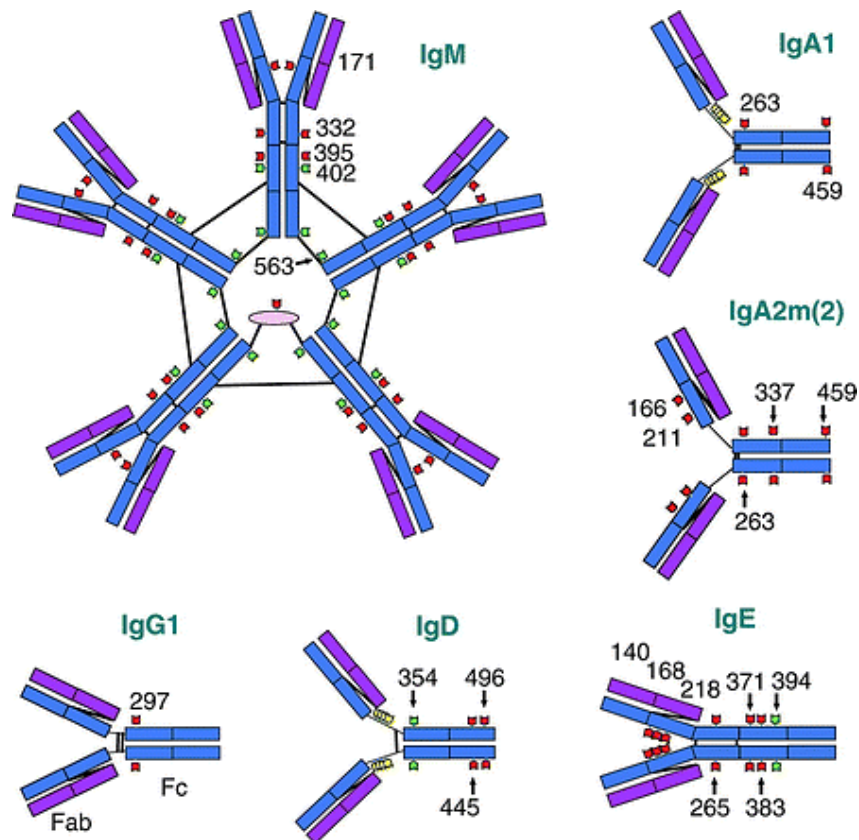


Figure 11. Structure and glycosylation sites of the five human antibody classes. The diagram illustrates the structure and glycosylation sites of human IgM, IgG, IgD, IgE and IgA₁ and IgA. Light chains are shown in purple, heavy chains in blue, IgM J chain in pink, and interchain disulphide bridges are shown in black. Sites of N-glycosylation are presented with complex (red) or oligomannose (green) glycans according to the glycan analysis. Clusters of O-glycans are presented in yellow ^[223]. Taken from Arnold et al. 2007.

IgD

Similar to IgM, the IgD isotype is produced early in the infection before class-switch recombination (CSR) occurs. IgD antibodies are solely expressed as monomers and are found as either receptors on mature B cells or as soluble Ab in serum where they represent a small proportion (0.25%) of all Abs. Mature B cells co-express IgD and IgM while naive B cells exclusively display the IgM isotype on their surface. The functional differences between IgD and IgM on mature B cells are not well understood as knockout mice deficient in IgD gene expression do not carry any abnormalities. However, IgD antibodies are thought to play a role in immune surveillance and allergic reactions ^[224]. Nonetheless, the action of IgM alone and/or in combination with IgD is not sufficient to clear pathogen invasion, and some activated B cells undergo CSR which contributes to enhanced humoral immune responses. This process is initiated by the activation-induced cytidine deaminase and results in the substitution of the constant region μ ($C\mu$) and $C\delta$ encoding for IgM and IgD isotypes with $C\alpha$, $C\gamma$ or $C\epsilon$ to produce IgG-, IgA- or IgE-antibodies, respectively ^[225]. IgM producing cells are not replaced following CSR and this antibody response declines over time, a few weeks after the beginning of the infection. Despite its potential, effectiveness as a neutralising agent in the early stage of infection, IgM is not as versatile as IgG or IgA, which undergo further rounds of affinity maturation ^[221].

IgG

IgG is the major antibody produced in blood and extracellular fluids (75% of the total antibody in serum) and plays a significant role in the control of infection. The interaction of IgG with any foreign antigen leads to pathogen immobilisation, recognition and degradation by phagocytic immune cells such as macrophages and DCs ^[216]. There are four types of $C\gamma$ domains which generate IgG₁, IgG₂, IgG₃ and IgG₄ subtypes, named in order of their proportions in serum: 66%, 23%, 7% and 4%, respectively ^[226] (Figure 12). All IgG subtypes except IgG₄ are capable of activating the classical pathway of the complement system ^[222]. These subtypes differ not only by their proportion in serum but also by their half-lives, affinity to Fc receptors expressed on phagocytic cells and efficiency at activating the complement system.

IgG subtypes share over 90% homology in their Fc portion but vary greatly in the structure of their hinge domain that separates the Ab Fab and Fc portions which plays a

crucial role in Ab flexibility ^[226]. The Fc portion of IgG antibodies can bind to specific types of FcRs called FcγRs. There are six types of FcγRs which differ by their structures and binding affinities to each IgG subtypes ^[227]. Specifically, IgG₄ can only bind with a low affinity to FcγRI and II; IgG₂ binds exclusively to FcγRII while both IgG₁ and IgG₃ can interact with all FcγRs. The FcγRI presents the highest affinity to IgG Fc domains while FcγRII and III have a lower affinity and interact preferentially with Ag-IgG complexes. The interaction of these complexes with FcγRs can trigger antibody-dependent cell-mediated cytotoxicity (ADCC) and/or antibody-mediated phagocytosis. The type of activity elicited depends on the glycosylation pattern of the IgG Fc domain interacting with the FcγRs and the cell types expressing the FcγRs (e.g. macrophages, DCs and NK cells) ^[223]. Previous studies have reported that fucosylation of IgG antibodies can inhibit ADCC functions ^[228]. In the case of HIV infection, investigators have demonstrated that a non-fucosylated anti-Env mAb enhanced ADCC activity, compared to the wild-type mAb; however this did not correlate with improved *in vivo* protection ^[229].

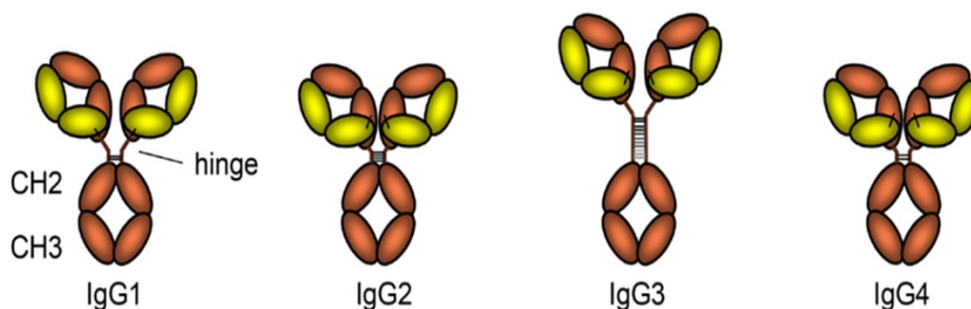


Figure 12. The four human IgG subclasses. Representation of the four human IgG subclasses: IgG₁, IgG₂, IgG₃ and IgG₄. The linkage between the heavy (in orange) and light (in yellow) chains, the length of the hinge and the number of disulphide bridges connecting the two heavy chains are shown ^[226]. Taken from Vidarsson et al. 2014.

IgE

All IgG subtypes are produced in response to infection although they can present contradicting properties which could impede immune responses. Recent developments have allowed us to understand how IgG subtypes could effectively work together to confer a protective humoral immune response ^[230]. Collins and Jackson proposed in their “Temporal Model of human IgE and IgG function” that IgE serves to alert the immune system of an

infection and that IgG subtypes are produced in a pre-determined sequential order in response to infection ^[231]. Indeed, they suggested that the early response to pathogen invasion could induce the production of IgE antibodies. The latter are primarily involved in allergic and inflammatory responses, and their effector functions are mediated through the interaction with two types of FcεR: FcεRI expressed on DCs, basophils and mast cells and FcεRII present on B cells, eosinophils and LCs. The binding of IgE antibodies to these receptors can induce phagocytosis and degranulation, and modulate B cells to induce either a T_H1 or a T_H2 response. In the temporal model, IgE antibodies induce the dispersion of mast cells to function as sentinels alarming the immune system to the onset of an infection ^[232]. In parallel, activated B cells switch from IgM to IgG₃, and, despite their different effector function, IgG₃- and IgM-antibodies join forces to prevent pathogen propagation. Then, later in the infection, IgG₁ followed by IgG₂ subtypes, with higher affinities, are generated. If the pathogens persist IgG₄ antibodies are eventually produced to dampen the inflammatory responses. This temporal model of human IgE and IgG function was recently supported by sequencing analysis ^[233].

IgA

IgA is the most abundant immunoglobulin in humans, particularly in external secretions such as breast milk, saliva, tears and mucus of the bronchial, digestive and genitor-urinary tracts ^[205]. As HIV-1 infection is principally transmitted through rectal and genital mucosal tissues; determining the potential role of IgA against HIV-1 acquisition might provide a critical insight into the development of an effective HIV vaccine ^[205]. There are two subtypes of IgA in humans: IgA₁ and IgA₂, which differ by the structure of their heavy chain and their proportions in serum: 85% and 15%, respectively ^[234] (Figure 13). The relatively low concentration of IgA₂ is principally due to its lower synthetic rate and higher catabolic rate compared to IgA₁, which shortens its half-life in serum. Despite this, IgA₂ is the most abundant subtype in mucosal secretions. This might be explained by the high sensitivity of IgA₁ to degradation by bacterial proteases present in large amounts at mucosal surfaces and secreted by the commensal microflora. Also, IgA₁ antibody presents a longer hinge region compared to IgA₂ which serves as a binding site for proteases (Figure 13). Both IgA subtypes are present as monomers in serum and as dimers in secretions ^[235].

Dimeric IgA (dIgA) and other polymeric forms are IgA monomers are linked by a joining chain (J-chain) produced by mucosally activated B cells in the lamina propria. To be exported, dIgA molecules bind to the polymeric immunoglobulin receptor (pIgR) via the J-chain. This allows transcytosis through the epithelial cells and their secretion into the luminal space of the gastrointestinal tract, and other mucosal surfaces. The antibody-binding domain of the pIgR is then cleaved off from the membrane domain resulting in a secretory component covalently linked to the dIgA antibody (a secretory dIgA (SIgA)) ^[236] (Figure 13).

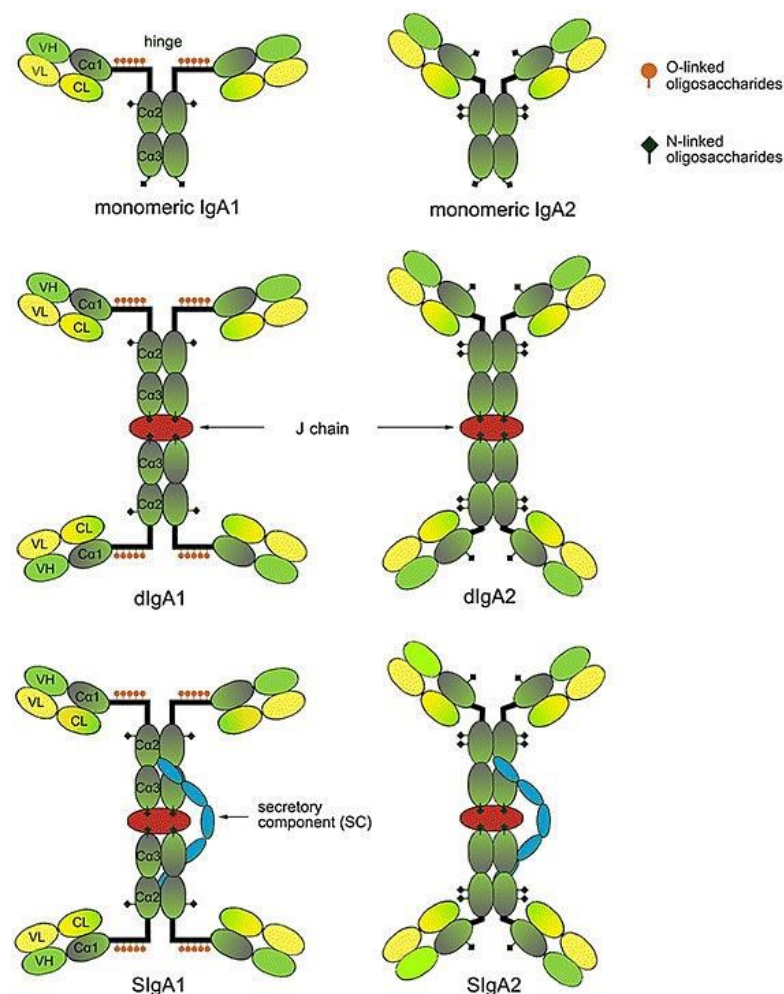


Figure 13. Structure of human IgA antibodies. The diagrams represent the different forms of human IgA₁ and IgA₂ antibodies: monomers (mIgA₁ and dIgA₂), dimers (dIgA₁ and dIgA₂), respectively and secretory forms (SIgA₁ and SIgA₂). Heavy chain (green), light chain (yellow), Joining (J) chain (red), secretory component (SC), (blue) and in the IgA₁ hinge region are shown O-linked oligosaccharides (orange). N-linked oligosaccharides are depicted in both IgA₁ and IgA₂ molecules ^[237]. Taken from Zhou et al. 2014.

IgA antibodies are thought to be a crucial component of mucosal immune responses to numerous pathogens. However, their role in the prevention of HIV-1 infection remains unclear^[235, 237]. In the recent RV144 Thai vaccine trial, Env-specific mIgA antibodies were shown to correlate with an increased risk of HIV-1 acquisition^[238]. Researchers suggested that mIgA antibodies impeded IgG mediated ADCC functions^[238-240]. The effector functions of IgA antibodies are mediated through the interaction with various host receptors including FcαRI, pIgR, Fcα/μR^[236, 241]. It has been suggested that the controversial role of IgA in antibody responses to infection might involve the FcαRI (CD89) receptor^[242]. IgA binding to FcαRI (or CD89) expressed on eosinophils, monocytes, macrophages and DCs triggers mechanisms of immune defence such as ADCC, phagocytosis and release of activated oxygen species and cytokines^[243]. Ag-IgA complex interaction with CD89 induces receptor cross-linking and elicits diverse effector functions that are usually protective but, in some cases, can also be detrimental to the cells. In myeloid cells, CD89 can interact with the immunoreceptor tyrosine-based activation motif (ITAM)-containing FcγRs which transduces an activation signal into the cell^[244]. However, it has been proposed that some cells express CD89 in a form which does not associate with ITAM-containing FcγRs. Therefore, IgA binding to these receptors does not trigger activation signals. This could explain the inhibitory and anti-inflammatory effects that have been attributed to IgA^[242, 244].

IgA and IgM are both capable of binding to Fcα/μR. The function of this receptor is not clear but is thought to participate in the coordination of the immune response in some mucosal tissues^[245]. The N- and O-linked glycans on IgA have been shown to bind to sugars receptors on microbes preventing their interaction with mucosal surfaces^[246]. Although the capacity of IgA to activate the classical pathway of the complement system has been debated, it has also been demonstrated that IgA antibodies lack the residues identified in the Fc domains of IgG and IgM which are responsible for complement pathway activation^[246]. Moreover, as suggested in some papers, IgA activation by the alternative complement pathway using recombinant or denatured IgA appears to be typically artificial. In contrast, native IgA-antigen complexes have been shown to inhibit complement activation triggered by IgM and IgG^[247, 248]. Besides, it has been suggested that IgA antibodies interact with other receptors such as secretory component specific eosinophil receptors, the M-cell receptor, and the transferrin receptor CD71. However, the effector functions elicited by these interactions have not been elucidated^[236].

Table 2. Properties of the human antibody isotypes.

Antibody	IgG				IgA		IgM	IgD	IgE
Heavy chain	γ				A		μ	δ	ε
H chain	γ ₁	γ ₂	γ ₃	γ ₄	α ₁	α ₂	None	None	None
MW (KDa)	150	150	170	150	160/ 385	160/385	970	180	190
Form	M	M	M	M	M,D, S	M,D, S	M,P, S	M	M
Complement fixation	weak	weak	strong	no	no	no	strong	no	no
Fc receptor binding	strong	weak	strong	strong	yes	yes	yes	no	yes
Serum concentration (mg/mL)	11.4	6.9	1.7	1.4	3.0	0.6	2.5	-	0.0005

M (monomeric), D (dimeric), S (secretory) and P (pentameric).

1.4 Structural features of Env that confer antibody resistance

1.4.1 Non-functional Env forms

The antibodies generated in response to HIV are diverse regarding structures and anti-viral properties. Nevertheless, only certain types of antibodies can prevent virions from infecting the host target cells. These antibodies block the viral and cellular membrane fusion process that is mediated by Env and the co-receptors; thereby neutralising viral infectivity. Such Abs, directed against the two viral envelope proteins gp120 and gp41, are so-called neutralising antibodies (NAbs) ^[161]. NAbs play a critical role in controlling viral infection and participate in the protective effect of a number of efficient vaccines ^[206]. Despite the overwhelming amount of evidence that NAbs can prevent HIV-1 acquisition in animal models, this has not yet been proven in humans; and their potential in limiting established infection remains unclear ^[249]. This is mainly due to the incredible antigenic diversity of HIV-1 Env that jeopardises NAbs efficiency. Env genes from viruses belonging to the same clade can differ by up to 20%, and these discrepancies are even more pronounced amongst viruses of distinct clades (up to 35%) ^[250]. During infection, mutations in Env genes accumulate, particularly, within the variable loops. NAbs that bind to these sites apply a selection pressure that drives the evolution of Env sequences towards immune system escape ^[251, 252]. So far, all HIV vaccine approaches that attempted to elicit broad and potent NAbs have failed, and have instead induced a great amount of non-neutralising antibodies

(nNAbs) that are directed against non-functional forms of Env including gp120/gp41 monomers and gp41 “gp41 stumps” (Figure 14) ^[253]. These features of Env confer resistance to antibody responses during natural infection and hamper the development of an efficient vaccine against HIV-1 infection ^[254].

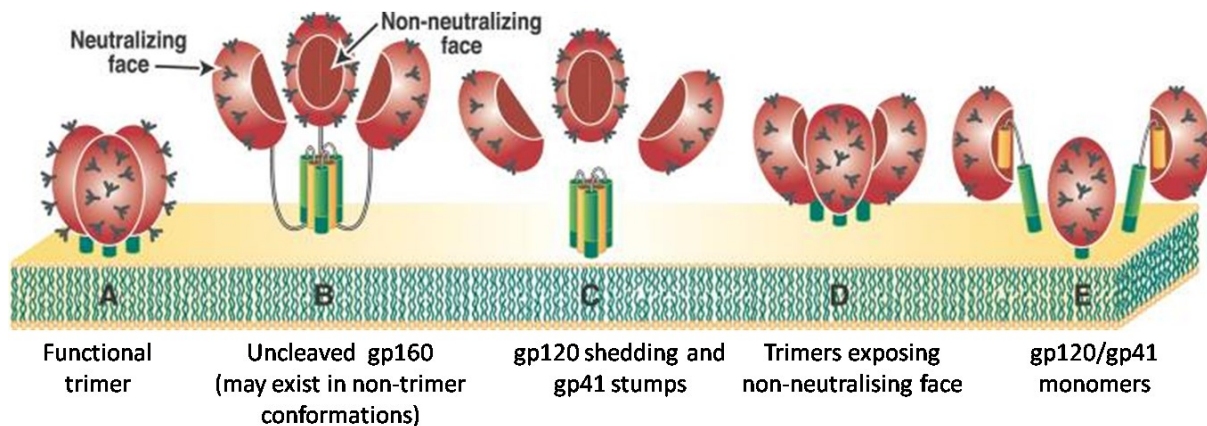


Figure 14. Representation of the potential forms of Env on the viral surface. HIV-1 gp120 protein is represented in red and carbohydrate moieties are shown in green as “tree”-like structure. HIV-1 Env gp41 transmembrane protein is depicted in yellow (N-terminal domain) and in green (C-terminal domain) separated by a disulphide-constrained loop. The membrane-proximal external region (MPER) of gp41 is shown in dark green ^[253]. Taken from Moore et al. 2006.

1.4.2 The silent, non-neutralising and neutralising faces of HIV-1 Env

HIV-1 Env surface protein, gp120, presents antigenic features that induce in infected individuals, the production of both non-neutralising and neutralising antibodies ^[255]. Researchers have identified three antigenic regions of gp120 that can elicit these two types of antibodies: the non-neutralising and “silent” neutralising faces ^[256]. The silent face is a large heavily glycosylated region situated in the outer domain of gp120, where potential epitopes are masked. This results in low immunogenicity hence its designation as immuno “silent”. In contrast, the non-neutralising face of gp120 is strongly immunogenic and elicits high Ab responses. However, as it is involved in the interaction with gp41, this surface is inaccessible to antibodies in the native HIV spike and is rather exposed on non-functional

Env. Therefore, Abs recognising epitopes on this face are thought to be incapable of neutralising functional virions and are so-called nNAbs. It has been suggested that the accessibility and not the quantity of non-functional Env such as gp120/gp41 or shed gp120 monomers is responsible for the high nNAbs titres observed in infected individuals ^[253].

The neutralising face of gp120 contains the conserved CD4bs, portions of the inner and outer domains and the bridging sheet. This face comprises essential structural sites for viral entry that are well-conserved amongst HIV strains and the newly generated virus quasiespecies in infected individuals. Nonetheless, these neutralising sites are not very accessible for antibodies. Notably, the CD4bs that mediates the interaction between gp120 and CD4 on target T cells is embedded within the core of gp120 and is difficult for bulky antibodies to access ^[255]. Besides, gp41 presents a domain close to the membrane called the membrane proximal external region (MPER), which can elicit neutralising antibodies. However, this site is almost inaccessible in the native spike and is predominantly exposed during the viral entry process ^[257]. Additionally, gp41 comprises three immunodominant regions called cluster I, II and III that are accessible on non-functional Env or in the post-fusion Env spike and are therefore non-neutralising epitopes ^[258, 259].

1.4.3 Mechanisms of resistance to antibody-mediated neutralisation

HIV-1 Env presents structural characteristics that prevent antibody recognition and participate in immune evasion:

- N-glycans added to gp120 during post-translational reactions cover the vast majority of the exposed “silent” face; and as these carbohydrates are produced by the host cell, they are not recognised as antigen but as “self” by the immune system and are therefore immunosilent ^[255, 260]. The exceptions are the 2G12 monoclonal antibody, and the recently discovered “PGT” antibodies that include glycans in their epitopes ^[261-263]. In addition, animal studies using SIV deficient in glycans suggest that Env carbohydrates might inhibit the development of Ab responses ^[264, 265].
- NAb access to the CD4bs is limited because of sterical hindrance: the CD4bs is recessed within the gp120 core and occluded by the V1/V2 loops and N-linked glycans. Similarly, the co-receptor binding site is hidden by the V3 loop and by carbohydrates ^[266].

- Conserved regions of Env that could act as potent epitopes, such as gp41 fusion peptide and the co-receptor binding site are only transiently exposed following CD4 interaction with gp120^[256]. Moreover, the importance of these conformational epitopes has been confirmed by the observations that CD4-independent viruses can fuse to the target cell membrane using the co-receptor binding site alone^[267-269]. Thus, these types of viruses were shown to be more vulnerable to neutralisation due to increased exposure of their co-receptor binding sites^[270].
- Antibody binding to the membrane protein gp41 is limited by a glycan shield and gp120 located above^[255]. The MPER which has been identified as an NAb epitope is difficult to access and may suffer from sharing epitope similarities with intestinal microbiota, thereby subverting antibody recognition^[257].
- The variable loops which are structurally more accessible are important targets for NAb; however, their sequences are highly variable and their structure greatly flexible which render them prone to rapid immune evasion by selection pressures^[258, 271].
- Antibody recognition of receptor binding sites induces conformational changes that mask the co-receptor binding site. This “conformational masking” facilitates neutralisation escape^[256]. However, the affinity of some antibodies is enhanced upon gp120-CD4 interaction; this rare type of Ab is known as a CD4-inducible antibody^[69, 272].

1.4.4 Conformational masking of receptor binding sites

Analysis of the thermodynamics of the interaction between Env and the co-receptors, CD4 and CCR5 versus between Env and specific antibodies has provided a better understanding of the mechanism of neutralisation resistance^[273]. Thermodynamic studies can determine whether a biochemical reaction such as a protein-protein interaction is energetically favourable or not by measuring the Gibbs free energies (ΔG) using an isothermal titration calorimeter. The ΔG present two components: the enthalpy (ΔH) which is the total internal energy of the system and the entropy ($-T\Delta S$) which is the disorder that acts to disrupt the system^[273].

Env conserved neutralisation epitopes are recessed within the native spike while the rest of the structure is firmly packed in a “closed” conformation that is energetically favourable ^[256, 273]. CD4 and CCR5 interaction with Env induce extensive conformational changes in gp120 architecture that have a high unfavourable entropy cost of about 50 kcal mol⁻¹. This high entropy cost might be a mechanism of defence against antibody attack. Monoclonal NABs binding to the CD4bs or co-receptors incur an entropy cost of about 25-30 kcal mol⁻¹ ^[256]. To bind to these receptor binding sites, neutralising antibodies must stabilise them by inducing energetically unfavourable conformational changes similar to those caused by gp120 binding to CD4. In contrast, non-neutralising antibodies that bind to monomeric gp120/gp41 do not incur negative energy and are therefore favoured during affinity selection and are abundantly produced during natural infection ^[273]. It might be expected that the high entropy cost necessary for viral interaction with the CD4 receptor would reduce the capacity of HIV-1 virions to enter into target cells. However, Kwong et al. suggest that the negative energetic cost to adopt CD4-bound conformation is compensated by a strong avidity between HIV-1 virion and membrane-bound CD4 ^[256]. The membrane confers a restriction in movement within a two-dimension space that allows membrane-bound CD4 to attain the avidity required to overcome the energetically unfavourable conformational changes in gp120. Free-floating antibodies and soluble CD4 diffuse readily and have a limited ability to bind Env with the strong avidity necessary to stabilise the highly flexible structure. Consequently, the thermodynamics of HIV-1 Env renders the native spike largely inaccessible to antibody-mediated neutralisation ^[256].

1.5 (Broadly) neutralising antibodies

Despite numerous sophisticated mechanisms of resistance to Ab neutralisation that Env has evolved, most HIV-1 infected people are capable of mounting neutralising Ab responses ^[161]. *In vitro* measurements of the activity of NABs isolated from patients have enabled the identification of potent antibodies that can neutralise viruses from diverse clades and at low concentrations. These antibodies termed broadly neutralising antibodies (bNABs) are produced by about 20-25% of HIV-1 infected individuals ^[274, 275]. In addition, studies have demonstrated that these bNABs can appear years after infection and often provide little or no benefits to patients ^[276, 277].

Therefore, there has been an increased interest in these bNAbs as to whether they can be elicited by a vaccine and protect against HIV-1 transmission ^[254, 278]. This increased interest has been fuelled by several non-human primate studies where passively administered bNAbs protected against SIV and SHIV-1 challenges ^[279, 280]. However, more research is required to determine the quantities and specificities of bNAbs necessary to confer sterilising protection in humans ^[281, 282]. BNAbs that are monoclonal antibodies (i.e. recognise a single epitope), have been of particular use to characterise HIV-1 and to provide insights for vaccine development. These recently discovered bNAbs present a range of specificities and unique features that bypass the structural resistance mechanisms of HIV-1 Env. They usually have undergone multiple rounds of affinity maturation. Structural analyses suggest that they bind to only a small number of amino acid residues (10-20) or N-linked glycans (1-2). In addition, they often present a particular long heavy chain complementarity determining region 3 (HCDR3) loop that might increase their flexibility ^[283]. Some bNAbs are also autoreactive, but it is not clear whether it is a side effect resulting from chronic immune activation or a potential useful attribute ^[284].

BNAbs epitopes on gp120

Most bNAbs can be categorised into four groups based on the location of their epitope on the HIV-1 spike (Figure 15):

- CD4bs-directed bNAbs
- V1/V2 loop bNAbs that recognise glycopeptide epitopes
- V3 loop N-linked glycans directed bNAbs
- bNAbs that target epitopes on gp41 located in the MPER ^[258]

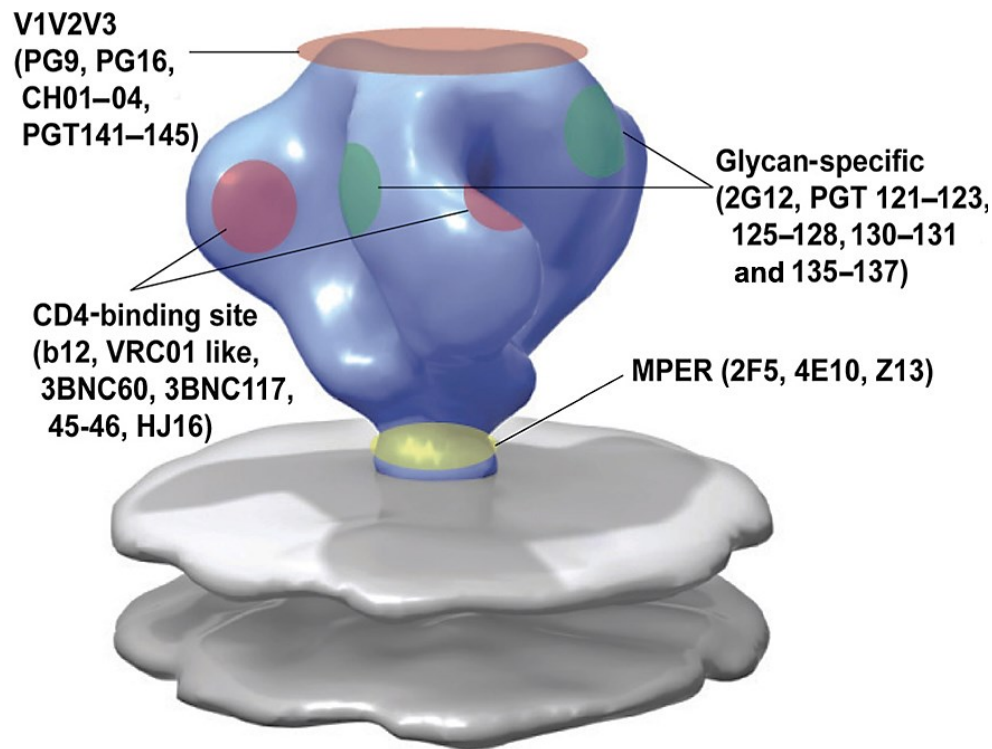


Figure 15. Broadly neutralising epitopes on HIV-1 Env protein. The targets of bNAbs can be separated into several groups: (1) IgG anti-CD4-binding site, (2) IgG anti-V1V2 site and anti-N-linked glycan V3 site, and (4) IgG anti-gp41 membrane proximal external region (MPER) ^[285]. Taken from Euler et al. 2012.

The CD4bs presents different features which render it one of the most attractive targets for antibody-mediated neutralisation ^[286, 287]. Crucial for attachment to the target cell, the CD4bs is highly conserved across the HIV-1 clades; particularly at position 43 of its amino acid sequence where a phenylalanine dominates the interaction with the CD4 molecule ^[288]. The typical example of NAb directed against the CD4bs was isolated from a phage library in 1994 and was called IgG₁b12 (b12) ^[289]. Crystallisation analysis of b12 in complex with gp120 molecules provided a better understanding of its neutralisation mechanism. It was primarily thought that the long HCDR3 of b12, that results from unusual V(D)J recombination events, was extended into the recessed CD4 binding site where it contacted the Phe43 and conferred broad neutralising activity. However, co-crystal structures revealed that only the heavy chain of b12 interacts with gp120 and that the HCDR3 is not central to the neutralisation mechanism, making only substantial contact with the periphery of the CD4bs ^[290]. Nonetheless, b12 is not a strong bNAb. It is highly active

against B clade HIV strains but can neutralise only 35% of circulating viruses ^[291]. Since this time, many other CD4bs bNAbs with similar modes of action to b12 have been identified, such as CH103 ^[292].

In addition to these bNAbs, more potent CD4bs bNAbs have been recently discovered. They use another neutralisation mechanism that structurally imitates the CD4 receptor and include VRC01-like antibodies ^[258, 293]. These particular bNAbs interact with gp120 via extensive contact between the epitope and the second heavy chain CDR (H2CDR) of the antibody. This H2CDR is encoded by the immunoglobulin heavy-chain variable (V_H) and characterised by an exceptional level of somatic mutations ^[294]. The V_H genes which are associated with the generation of VRC01 antibody class are found in most human groups; therefore it is theoretically possible to induce these potent CD4-mimicking antibodies in these populations. Interestingly, most of the CD4bs antibodies isolated in patients did not convey neutralising activity such as b6 and b13 and were not capable of preventing infection.

Thus, some researchers suggest that antibodies binding to both monomeric gp120/gp41 and the native spike are non-neutralising, and that only those that can exclusively recognise Env trimers, are neutralising ^[77]. Therefore, these types of neutralising antibodies are quaternary antibodies where they bind to conformational structures only present in the Env trimer or which have been induced upon interaction with the CD4 receptor.

Several antibodies binding to CD4-induced epitopes or originally present in CD4-independent HIV strains have been identified ^[69]. The best known among them is the 17b mAb that has played a significant role in the elucidation of the HIV-1 Env structure ^[58]. 17b is a CD4-induced (CD4i) Ab where the epitope overlaps the CD4 and co-receptor binding site. Additionally, 17b presents a broad neutralising activity which implies that its epitope is well-conserved across HIV-1 clades. CD4i bNAbs present long HCDR3 loops, rich in sulphated tyrosines which mimic the N-terminal peptide of gp120. The CCR5 co-receptor binding site interacts with the N-terminal peptide of gp120 via negatively-charged sulphated tyrosines. The Fab domains of 17b demonstrate higher neutralisation activity *in vitro* than the whole IgG molecule, suggesting that the Fc portion might impose steric constraints that restrict access to the epitope ^[295]. Thus, CD4i antibodies can neutralise the virus, only in the

presence of CD4 molecules which limits its potential for intervention. However, it was suggested that CD4i bNAbs might play a crucial role during natural infection by neutralising newly-generated CD4-independent virions and by applying a selection pressure that maintain the CD4 tropism ^[296].

Several neutralising antibodies from different studies that bind amino acid residues and glycans have recently been discovered. These NABs directed against the V1/V2 loop harbour an unusually long H3CDR of over 25 amino acid residues. The latter penetrates the glycan barrier of gp120 to bind to amino acid residues located on the C strand of the V1/V2 loops and to nearby glycans. PG9, PG16 and the “PGT” 25-128, 130 and 131 antibodies belong to this category of bNAbs which is characterised by an outstanding neutralisation breadth and a superior activity compared to previously described bNAbs ^[262, 263]. Particularly, PG9 and PG16 are known to be quaternary antibodies that interact preferentially with Env trimers as they recognise; conserved regions of the V1/V2 and V3 loops, an epitope overlapping the CD4bs, and potentially the CCR5 binding site ^[297, 298].

Antibodies directed against the V3 loop present a moderately long HCDR3 and have undergone extensive somatic hypermutations ^[283]. This results in the generation of a high-affinity binding antibody capable of overcoming the high sequence variability of the V3 loop ^[299]. Notably, 447-52D one of the most effective anti-V3 Abs presents the unusual characteristic of the neutralising HIV strains, X4 and R5 which contain the GPGR at the motif apex of the V3 loop. Primary isolates of Clade B and the tissue culture lab adapted strain (TCLA), which largely present the GPGR sequence, are easily neutralised by 447-52D. Therefore, viruses from other subtypes that do not carry this motif are relatively insensitive to 447-52D which seriously restrains its neutralisation breadth. However, this might be compensated by the fact that 447-52D interaction with the GPGR motif is predominantly mediated by side-chain residues of the V3 loop and the base of HCDR3 which enables this antibody to bind various V3 sequences ^[300].

The glycan shield of gp120 protects against antibody recognition by providing a surface that is recognised as “self” by the immune system and therefore is immunologically silent. Nonetheless, a unique antibody named 2G12 capable of binding a cluster of conserved carbohydrate moieties present on the silent face of gp120 has been identified ^[261]. 2G12 presents an unusual structure where the two heavy chains (V_H) of each Fab have

interchanged positions and are interlocked resulting in a novel paratope at four domains that can interact with oligomannose residues over an extended surface ^[261]. Attempts to elicit similar highly potent NAb have failed so far. This may be because of the differences in glycosylation processing between recombinant monomeric and trimeric Env with native spike ^[301, 302].

The transmembrane protein gp41 is poorly immunogenic mainly due to steric constraints imposed by the adjacent gp120 molecules and glycans that limit antibody access to this target. Consequently, anti-gp41 antibodies are mainly non-neutralising except those directed against the MPER including 4E10, 2F5 and Z13e1 ^[280]. These bNAbs bind to two adjacent conserved sequences close to the viral membrane that are only transiently exposed during the fusion process upon receptor interaction. Crystal structure analyses of 2F5 and 4E10 revealed that they bear long HCDR3 with hydrophobic surfaces that can bind anionic phospholipids of the viral membrane ^[303]. Thus, the presence of the viral membrane is likely to be required for the neutralisation activity of these antibodies; and vaccination strategies that attempted to elicit 2F5/4E10-like Abs based on their amino-acid sequences alone have failed so far ^[304].

Overall, the various attempts to develop a vaccine that elicit bNAbs have been unsuccessful and such goals seem unlikely to be achievable soon. Therefore, there has been an increased interest in improving the understanding of antibody-mediated virus neutralisation.

1.6 Defining antibody-mediated virus neutralisation

Since the 1930s, researchers have drawn up theories about how antibodies neutralise virus infectivity, and have tested the mechanism, kinetics, stoichiometry and efficacy of virus neutralisation ^[305, 306] to answer this question. Largely, neutralisation is known as a reduction of infectivity resulting from the direct interaction between antibodies and viral particles, which cause an interruption of the viral replication cycle prior vRNA synthesis ^[307, 308]. Initially, this definition was limited to antibodies and their by-products (e.g. Fab, fragments, and F(ab')₂), this definition has now been extended to similar activities exerted by any molecules (lectins, receptors, defensins etc.) where binding to the virion leads to a loss of infectivity ^[309-311]. However, as this thesis focuses on antibody interaction

with HIV-1 we will only address antibody-mediated virus neutralisation. Although the concept above of virus neutralisation is widely accepted, this definition has been further expanded by some authors to other modes of action which have raised many controversies. Here some of them that have been reviewed by Klasse ^[305]:

- Antibodies that prevent virion attachment to the cell by binding to viral cellular receptors and/or co-receptors. Klasse argued that these antibodies should not be termed as neutralising antibodies as they do not bind to the virus but instead they should be termed infection-blocking antibodies ^[305].
- Antibodies that blocks virion budding from infected cells thereby acting at the late stage of the viral cycle. This mode of action has been described for antibodies targeting the neuraminidase expressed on the viral surface of Influenza. Antibodies binding to this enzyme block the digestion of neuraminic acid moiety of the receptor for the virus and therefore the release of influenza virions from the cell ^[312]. Nonetheless, some researchers proclaim that, according to the strict definition of neutralisation, these antibodies do not act prior to viral replication, and therefore should not be categorised as neutralising antibodies ^[305].
- Antibody-virus interaction that leads to Antibody-dependent cell-mediated viral inhibition (ADCVI) and Antibody-dependent cellular cytotoxicity (ADCC) highlighted the importance of a strict definition of neutralisation ^[313]. These mechanisms are observed only in the presence of effector cells and cannot be termed as antibody-mediated neutralisation.
- Antibodies that inhibit cell-to-cell transfer of viral particles through virological synapses ^[314]. This inhibition can be observed with NAbs but also with Abs that bind to cellular structure involved in these synapses, and therefore cannot be defined as a neutralisation mechanism ^[315].
- Antibody-mediated viral aggregation (AMVA) has been originally defined as a distinct mechanism from neutralisation although depending on the ratio of virion/antibody it can cause a reduction in infectivity ^[307]. Basically, at low concentration of Abs, the

aggregation of viral particles predominates whereas at higher concentrations epitopes on virions become saturated and cross-linking of viral particles can no longer occur ^[316].

Thus, according to the strict definition of neutralisation, antibodies neutralise by either blocking viral attachment to the cell, or by interfering with other compulsory events take place upon attachment. These events could be conformational changes, interaction with co-receptor, fusion and endocytosis, but are always prior to viral replication ^[305].

Another fundamental aspect that impacts on neutralisation is the theory of occupancy ^[308, 317]. Burton et al. suggested that antibodies prevent virions binding to the cell through steric hindrance and proposed that the ability of an antibody to neutralise may be determined by the level of occupancy of sites at viral surface independently of epitope specificity ^[318]. However, some HIV-1 Env antibodies are not capable of neutralising regardless of level of occupancy. Additionally, it was observed that nNABs that interact with epitopes overlapping b12 binding site do not interfere with its neutralisation activity although they presented higher affinities ^[319]. Researchers proposed that these nNABs bind to epitopes which are not exposed on functional Env trimers such as monomeric gp120/gp41 or uncleaved gp160 trimers ^[253]. The detection of non-functional forms of Env at the surface of primary isolates and infected cells confirmed this hypothesis; and researchers suggested that these epitopes might be responsible for the abundant production of nNABs in chronically infected patients ^[320]. This has been corroborated by the failure of vaccines based on monomeric gp120 to induce NABs and to confer protection ^[204]. Besides, Yang et al. demonstrated that occupancy was a crucial factor for neutralisation ^[321]. Measurements of antibody neutralisation of viruses presenting mixtures of neutralisation-sensitive and neutralisation-resistant envelope glycoproteins showed that HIV-1 Env trimer is inactivated by the binding of a single antibody molecule.

Therefore, a virion is neutralised when all the functional Env trimers are bound to a NAB. This model was applicable to NABs with different potency and to various HIV strains ^[321]. In addition to epitope characteristics and occupancy level, neutralisation is predominantly affected by the antibody affinity. *In vitro* experiments showed that weak interactions between antibody and virions lead to viral escape from neutralisation ^[322]. It

was suggested that HIV-1 has evolved non-neutralising dominant epitopes to enable nNAbs to block adjacent neutralising epitopes and to bind with stronger affinities than NAb^s ^[323].

1.7 Non-neutralising antibodies

Non-neutralising antibodies are abundantly and universally produced during natural HIV-1 infection and act by signalling viral particles for destruction by the innate immune system. Recently, Robinson suggested that the selection of HIV-1 variants among circulating strains, with limited ability to elicit bNAbs, select for viruses entering the target cells less quickly ^[324]. Indeed, the sequestration of the conserved neutralisation epitopes might increase the number of Env conformational changes required for viral entry. Therefore, this potentially opens a wider window of opportunity for non-neutralising antibodies to tag viral particles for destruction through other antibody-mediated effector functions ^[324].

These functions are mediated by the binding of the Fc portion of IgG antibodies to FcγR expressed on specific immune cells including macrophages, neutrophils, NK, Langerhans, mast and myeloid cells. Researchers found that some non-neutralising bNAbs were capable of inhibiting the replication of HIV-1 primary isolate (PI) in immature Dendritic Cells (iDC) and macrophages ^[325]. The mechanism used by nNAbs could not be measured by the “classical” neutralisation assay involving the use of phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMC) as target cells. They used instead flow cytometry to measure the inhibitory activities of both nNAbs and bNAbs with macrophages and iDC as target cells. *In vivo*, these two cell types might play a role in spreading the infection: macrophages as a reservoir of HIV virions, and iDCs as the first target cells following mucosal transmission ^[326, 327]. Interestingly, they found that the neutralising activity of the selected bNAbs were 16- to 12,000-fold higher when iDCs or macrophages were used as targets instead of PBMC. Moreover, nNAbs that presented no neutralising activity against HIV-1_{BaL} *in vitro* were capable of inhibiting viral replication in macrophages and iDCs ^[325].

To understand the neutralisation mechanism underlying this enhanced activity; they compared the inhibitory effects of Fab fragments to whole IgG molecules with or without inhibitors of FcγRs. Thereby, they demonstrated that the observed increased inhibitory

activity was mediated by FcγRs expressed on iDCs and macrophages. It is likely that, IgG-FcγR interaction triggers endocytosis and degradation of opsonised virions leading to a reduction of the infectivity^[325].

Thus, they defined a novel type of nNAbs that were termed non-neutralising inhibitory Abs (nNiAbs), different from Nabs that are capable of both mechanism of inhibition: Fab- and FcγR- mediated. However, not all the nNAbs present this function. So far only those targeting the principal immunodominant domain (PID) of gp41 and the linear region of the V3 loop have been identified as nNiAbs. Besides, earlier studies showed that nNAbs isolated from infected patients were capable of ADCC activity. In addition, there is some evidence that non-neutralising antibodies were capable of complement-mediated inactivation (CMI)^[328].

In consequence, the development of vaccines that elicit nNAbs responses has become more attractive due to the inhibitory potential of this type of response and the feasibility of this approach compared to vaccine inducing bNAbs. Nonetheless, no studies have shown so far that nNAbs could confer *in vivo* protection against HIV infection. In addition, as mentioned above, only a subset of nNAbs has a protective effect *in vivo*; and developing a vaccine that induces nNiAbs might be as challenging as developing a vaccine that elicits bNAbs.

It has been indicated that nNAbs participate in virus immune evasion and even enhanced natural infection by exhausting immune resources with the production of ineffective antibodies and by facilitating virus entry and/or virus replication in target cells^[329, 330]. The latter mechanism called antibody-dependent enhancement (ADE) of infection has been reported for several viruses including the dengue virus (DENV) and HIV-1 and can potentially make the difference between an efficient and a harmful vaccine candidate^[331, 332].

In HIV-1 infection, ADE occurs via nNAbs-virion complex interactions with the complement system and/or Fc receptor and leads to increased infection and accelerated disease progression^[329]. In a recent study, Willey et al. investigated the effects of complement on antibodies and viruses from patients with acute HIV-1 infection using T cell lines that express a receptor for complement components (CR) named Complement

Receptor type 2 (CR2) ^[332]. They showed that autologous nNabs directed against HIV-1 Env that present during acute infection could increase viral infectivity by up to 350-fold by stabilising virion-target cell interactions ^[332].

Additionally, they reported that viruses which had evaded neutralising responses presented a greater capacity for complement-mediated ADE (CADE) and that those autologous antibodies were capable of CADE with heterologous viral isolates ^[332]. CR1, CR3 and CR4 have also been reported as capable of CADE in other studies ^[330, 333]. However, although biologically conceivable, the role of ADE in natural HIV infection remains unclear.

Therefore, further research is required to elucidate the ADE mechanisms occurring *in vivo*. There is also a need to develop assays that can predict the probability that vaccine-induced or passively-administrated antibody will lead to protection or enhanced infection ^[329].

1.8 Systemic antibody responses during HIV-1 infection

The humoral immune response to HIV-1 infection consists of diverse antibody isotypes with various antiviral functions. However, this adaptive immune response is delayed and appears long after the establishment of infection ^[277]. Recent findings regarding early events following HIV-1 acquisition reported that early B cell dysregulation caused by the CD4⁺ T cell depletion, might be responsible for the delayed and inefficient antibody responses ^[219, 277]. During acute HIV-1 infection, antibodies are generated against the viral proteins encoded by *env*, *gag* and *pol* genes ^[219]. B cell responses emerge within a week after viral load detection and consist initially of antibody–antigen complexes. This is rapidly followed by the appearance of the first free Env-specific antibodies: anti-gp41 IgM (approximately five days after the onset of the symptoms), then isotype class recombination occurs and anti-gp41 IgM antibodies are switched to IgG and IgA.

Two weeks later, the first gp120-specific antibodies appear and are directed against different Env epitopes in a sequential order. The first wave of IgG antibodies is specific for the immunodominant region of gp41 and the V3 loop of gp120 ^[219]. IgG antibodies against *gag* proteins emerge between two weeks (p24) and a month (p55) after viral RNA detection in plasma while Abs against the integrase can take almost two months to appear. Interestingly, Tomaras et al. found that antibody isotype kinetics varied depending on the

subjects and were detected either in the classical sequential order IgM<IgG<IgA or simultaneously^[219].

Thus, they suggested that the simultaneous emergence of IgM, IgG and IgA might be due to a T cell-independent mechanism of B cell activation occurring in response to the initial severe CD4⁺T cell depletion^[219]. Furthermore, they proposed that early antibody class switch recombination (CSR) may be triggered by gp120 carbohydrates binding to C-type mannose receptors on B cells which activate the activation-induced cytidine deaminase (AID). Besides, analyses of the antiviral functions of these first antibodies demonstrated that not only were they able to bind virions but also to activate the complement system. Nonetheless, mathematical modelling analyses of the early IgM/IgG antibody responses revealed that these first antibodies did not present neutralising activity and tended to have no effect on the VL. Additionally, *in vitro* experiments showed that antibodies generated during the first five weeks after VL detection were unable to inhibit viral replication and did not mediate viral neutralisation^[334].

It takes between one to several months (depending on the virus clade), to detect the first NAb against the infecting (autologous) viral strain in HIV infected patients; about 3-8 weeks for clade C and two months for clade B^[335-337]. A recent study investigating the different neutralising Env-specific IgG subtypes (IgG₁₋₄) revealed that IgG₁ was predominantly elicited in chronically infected patients with clade B and C HIV-1 viruses. This confirmed the idea that during chronic infection, the T helper response was skewed towards Th2^[338]. Moreover, they reported that the IgG₁ antibody subclass presented the greatest responses against the viral proteins encoded by *env*, *gag* and *pol* and mediated ADCC and were also important activators of the complement system^[338].

Another study performed on TZM-bl cells demonstrated that the anti-MPER IgG₁ antibody exhibited a stronger neutralising activity than IgG₃ in the presence of the four FcγRs^[339]. However, recent evidence suggested that IgG₃ Abs may be responsible for the IgG-mediated protection observed in the RV144 Thai trial. Indeed, Tomaras et al. analyses showed that the V1/V2-specific IgG₃ responses in protected vaccinated individuals were higher than in controls. This might be linked to the greater ADCC ability of IgG₃ compared to all the other isotypes^[340]. Together, this indicates that the type of IgG subclasses responses may influence antiviral outcomes against HIV-1 infection.

The first neutralising IgG antibodies generated in response to HIV-1 infection target the V3 loop of Env and in rare cases the V2 loop^[219]. As mentioned previously the variable loops are prone to high-levels of mutations which often occur through a single mutation and/or rearrangements of the glycan shield preventing NAb access to their epitopes^[256]. In addition, there is evidence that NAb exert a selection pressure which drives neutralisation evasion, causing the generation of viral quasi-species, in the way that newly generated viral particles are resistant to earlier NAb^[341, 342].

Studies of isolated NAb and autologous viral strains from different time-points demonstrated that NAb responses are always a-step behind the rapidly evolving viruses^[161, 343]. However, the potency of NAb tends to increase throughout the course of infection and NAb capable of recognising heterologous viral strains have been reported in numerous cohorts^[262, 276, 338, 344]. Only about a quarter of PLHIV develop bNAb; duration of infection and host or viral genetic characteristics have been proposed as factors affecting the emergence of this type of antibody response^[77, 276, 304]. Crystallography analyses of bNAb in interaction with HIV-1 Env have enabled the identification of epitopes that could be targeted by novel vaccines. These epitopes include the CD4bs, MPER, V1/V2 and V3 loops, and CD4i sites^[290, 345].

Nevertheless, studies have demonstrated that bNAb can take years after infection to appear and have a limited effect on the infection^[292, 335, 346]. Data from SIV challenges in rhesus macaques suggested that antibody responses should occur within 10 days following transmission to be able to control or eliminate the infection^[277]. Therefore, during the acute phase, the role of NAb in effectively controlling HIV-1 replication remains unclear. In fact, the initial drop in viraemia has been attributed to the action of CD8⁺ T cell, innate immune factors and even to some activities of non-neutralising antibodies^[157]. Early studies, reported higher NAb titres in LTNP compared to rapid progressors and suggested that NAb might contribute to disease control^[347, 348]. However, these studies failed to clinically demonstrate whether it was NAb and/or other factors that limited infection spread^[349, 350]. Moreover, a recent study by Li et al. described lower ADCC activity in chronically infected individuals compared to elite controllers which suggests that non-neutralising antibodies might play a crucial role in the control of infection^[351].

In addition, it was reported that broad neutralising activity was significantly more common amongst chronically infected patients than in ECs and that NAb responses did not

correlate with slower disease progression ^[249, 352]. This has been supported by the recent observation that the modest protection (31.2%) observed in the Thai RV144 efficacy trial did not correlate with neutralisation ^[340]. Nonetheless, there is a growing body of evidence in non-human primate studies that NAb passively administered can prevent SIV and SHIV-1 infection in the primate models ^[353, 354]. Additionally, studies of highly-exposed seronegative individuals (HESN) showed that the resistance to HIV-1 acquisition was associated with the presence of neutralising SIgA antibodies ^[355-357].

Thus, contrary to most pathogens, the protective antibody responses to HIV-1 is relatively inefficient and arises too late to be able to limit virus spread. Neutralising antibodies appear months to years after transmission and fail to control viraemia in the face of the rapidly evolving virus. Nonetheless, most researchers agree that if (broadly) NAb were present before or induced immediately upon, HIV-1 transmission they may prevent the infection. Therefore, one of the most important challenges in HIV-1 vaccine development is to elicit antibodies before viral exposure that can block HIV-1 transmission, particularly at the mucosal surface which is the main portal of viral entry ^[205, 254].

1.9 Mucosal antibody responses and correlates of protection

The majority of HIV-1 acquisition occurs through sexual intercourse, and HIV-1 enters the body predominantly via the genital or rectal mucosal surfaces ^[16, 21]. IgA are thought to play a crucial role in mucosal immune responses. Nonetheless, during acute HIV-1 infection, IgA appear greater than ten days after VL detection and disappear rapidly within weeks ^[358]. However, an effective HIV-1 vaccine must elicit mucosal Ab responses that are present prior or immediately following viral transmission ^[359]. Despite IgA importance in mucosal immunity, its role in the prevention of HIV-1 acquisition remains unclear. Data from different sources have highlighted correlations between IgA responses and prevention/control of HIV-1 infection ^[360]. A recent study revealed that HIV-1-specific IgA responses were predominant in chronically infected patients producing bNAbs ^[338]. Another study detected V1/V2-specific bNAbs of IgA class in the saliva and genital secretions of HIV-1-infected patients ^[361].

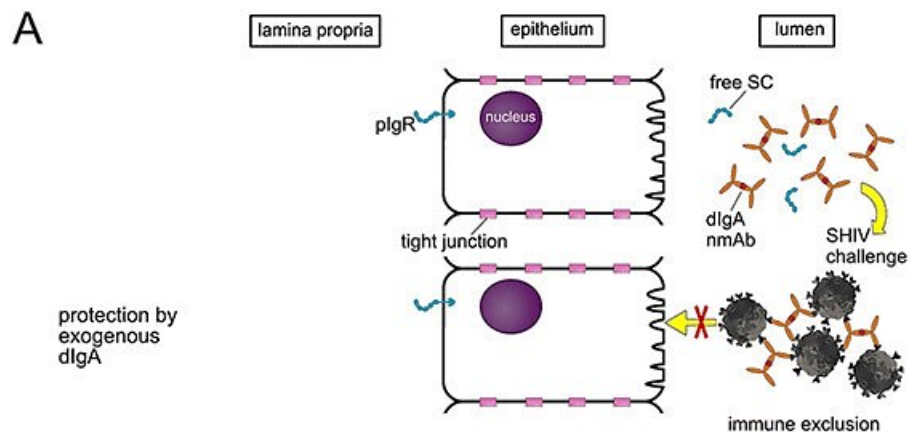
Additionally, the presence of mucosal dIgA has been associated with resistance to infection in highly exposed protected seronegative individuals (HEPS) ^[362-364]. In particular, a research study of sex workers in Kenya found a positive correlation between the presence of neutralising IgA in genital secretions and the protection from HIV-1 acquisition ^[365]. However, these early studies remain controversial as later investigations on sex workers failed to demonstrate that mucosal IgA antibodies were neutralising ^[363]. Instead, the presence of HIV-1-specific IgA antibodies was correlated with repeated HIV-1 exposures and *in vitro* analyses suggested that these antibodies were unable to confer resistance to HIV-1 infection ^[363]. Nonetheless, these studies were based only on the ability of IgA to neutralise HIV-1 virions while there are various other mechanisms through which IgA might provide protection against HIV-1 acquisition. Indeed, IgA possesses various anti-viral properties that could be exploited in a vaccine context. IgA might prevent virus interaction with the epithelium, mediate viral aggregation and inhibit virion movements across the mucosal barriers leading to "immune exclusion"^[366] (Figure 16). Several recent studies showed that Env-specific IgA can inhibit HIV-1 transcytosis through epithelial cells ^[367-369]. In addition, it has been demonstrated that SIgA can neutralise the infectivity of transcytosed virions through epithelial cells ^[369]. Moreover, HIV-1 specific IgA antibodies capable of ADCC and ADCVI have been found in cervicovaginal fluids ^[370, 371] (Figure 16).

Viral aggregation of HIV-1 virions in mucus, or at the mucosal surfaces has been proposed as an alternative mechanism to classical neutralisation to block HIV-1 transmission at the initial portal of viral entry ^[372] (Figure 16). There are strong suggestions that viral aggregation restricts virion mobility in mucus limiting their diffusion towards the underlying epithelium ^[361, 372, 373]. Likewise, the interaction of mucin proteins with the secretory component of dIgA bound to virions may trap virus-antibody complexes in mucus and prevent their interaction with epithelial cells ^[374]. Moreover, viral aggregation might reduce the total number of virions through complex formation and render viral particles bigger and "more visible" by the immune system. This might cause an increase in uptake by dendritic and macrophage cells and lead to viral phagocytosis and destruction. Similarly, viral aggregate diffusion through intracellular spaces or via transcytosis across epithelial barriers might also be limited ^[375]. Recent findings have shown that dIgA might be able to aggregate HIV-1 virions, however, these findings are preliminary and need to be fully explored ^[375]

(Figure 16). Similarly, an experimental study demonstrated that non-specific pentameric IgM antibodies could aggregate influenza virions in the presence of complement and cause a significant reduction of the viral load ^[376].

In contrast, high levels of mIgA antibodies correlated with increased risk of infection in the RV144 trial. It was proposed that the mIgA antibodies may have perturbed IgG effector functions (ADCC) in the vaccinated individuals. Nevertheless, mucosal IgA responses were not measured during the clinical trial, and thus it was not possible to determine whether systemic specificity, function and concentration were reflective of mucosal responses ^[238]. Therefore, these findings underlined the lack of understanding of IgA role in HIV-1 infection for which the effector functions might vary depending on the structure (monomeric vs multimeric) and the biological compartment (systemic vs mucosal). These investigations are integral components of this thesis and aim to provide information for the potential use of dIgA for pre-exposure prophylaxis and the rational design of vaccines.

Passive immunization with monoclonal dIgA



Vaccine-induced IgA: protective mechanisms

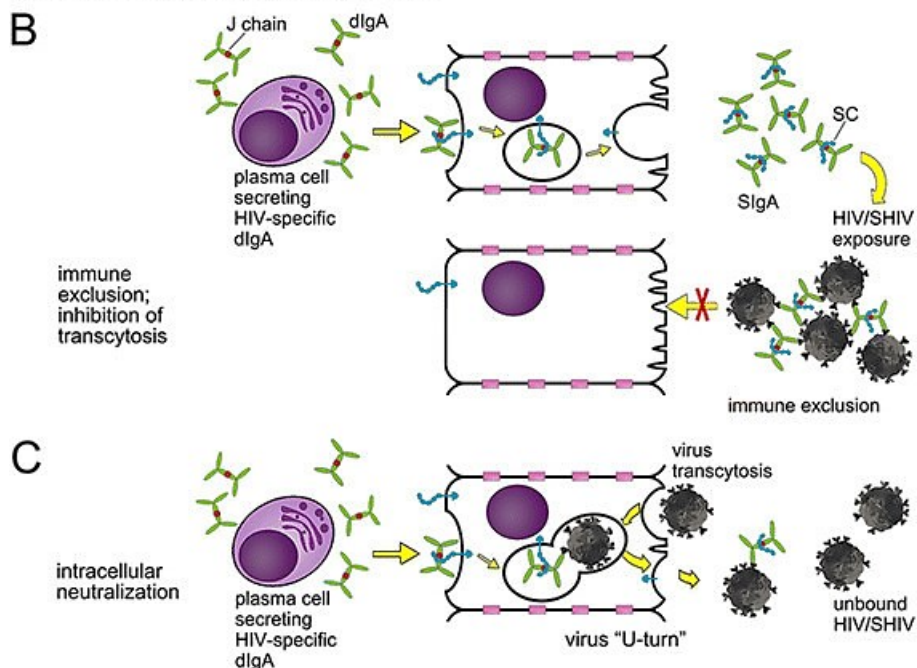


Figure 16. Protective role of IgA against HIV/SHIV infection at mucosal surfaces. A. Passive immunisation with a neutralising monoclonal dIgA. Neutralising monoclonal dIgA antibodies administered in the mucosal lumen of macaques can bind to SHIV and form large Ab-virions aggregates, preventing SHIV particles to cross the epithelial barrier. B and C. Protection against HIV/SHIV infection by locally produced IgA in HEPS or vaccine-induced IgA. B. HIV/SHIV specific dIgA produced by plasma cells in the lamina propria, binds to plgR (in blue) on the basolateral surface of epithelial cells. The plgR mediate the export of dIgA across the epithelial cells to the mucosal lumen. The secretory component (SC) of SIgA derived from the plgR. SIgA prevents HIV/SHIV by immune exclusion and inhibition of transcytosis mechanisms. C. Intracellular neutralisation. dIgA-plgR complex interacts with HIV/SHIV inside the epithelial cell and then the virion-dIgA complex is excreted into the mucosal lumen^[237]. Taken from Zhou et al. 2014.

1.10 Anti-HIV treatments

HIV-1 infection is probably the most studied virus in the world, nonetheless the development of a vaccine capable of blocking HIV-1 transmission seems difficult to achieve soon. Alternative prevention strategies (i.e. circumcision, microbicides, and pre- and post-exposure oral prophylaxis) may provide ways to contain the epidemic, yet ultimately a prophylactic vaccine to eradicate HIV/AIDS is needed ^[377].

1.10.1 Highly Active Antiretroviral Therapy (HAART)

Since the discovery of HIV-1 as the causative agent of AIDS, tremendous efforts have been made for the development of effective antiretroviral therapy (ART) which can suppress HIV-1 replication to undetectable levels. Increased understanding of the HIV-1 life cycle has provided knowledge and tools to extend the arsenal of ART drugs approved by the FDA from the first anti-HIV drug Zidovudine in 1987, to over 35 today ^[378]. These pharmacological agents have been classified into six groups based on their mode of action and resistance profiles: Fusion Inhibitors, chemokine co-receptor antagonists, nucleoside/nucleotide reverse transcriptase inhibitors (NRTI), Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs), HIV integrase strand transfer inhibitors, and Protease Inhibitors (PIs) ^[379].

The viral entry into the cell is the first event in HIV-1 life cycle and is mediated by the interaction of HIV-1 Env with the CD4 receptor and CCR5/CCX4 co-receptors expressed on target cells ^[80, 129]. Several ART drug classes target this initial step: entry and fusion inhibitors and chemokine co-receptor antagonists ^[380-382]. While the viral entry into the cells takes only an hour, the following reverse transcription step extends to over 10h which increases the opportunities for drug interventions ^[63, 89]. The reverse transcriptase (RT) which requires deoxyribonucleoside triphosphates (dNTPs) to catalyse viral DNA synthesis is the main target of anti-HIV drugs. Two distinct classes of antiretroviral agents inhibit RT activity: analogues of native nucleoside substrates (NRTIs) and small molecules that bind to the noncatalytic allosteric pocket on the enzyme (NNRTIs) ^[93]. The last step of the reverse transcription is catalysed by the pre-integration complex (PIC) and targeted by the newest approved ARV medicines: integrase inhibitors (INIs). These inhibitors block the integration of viral DNA into the host chromosome ^[383]. The last group of ARV drugs intervene during the

final stage of the viral cycle and prevent the proteolysis of viral polyproteins. They retain Gag molecules in their uncleaved states which interrupt the maturation and the production of progeny virions ^[51, 384]. These inhibitors are the most potent ARV drugs ever developed ^[385].

Initially, HIV-1 infected patients were given one of the ARV drugs at a time, but in the middle of the 1990s combination of three antiretroviral agents of different classes also known as “highly active antiretroviral therapy” or HAART was demonstrated to be more efficient at suppressing viral replication by several studies ^[386-388]. In 2013, the WHO guidelines recommended giving HAART to patients with CD4 counts less than 500 cells/mm³ in plasma mainly due to the high drug toxicities and the emergence of drug resistances ^[389]. Nonetheless, the recent -Short Pulse AntiRetroviral Therapy At seroConversion (SPARTAC) trial demonstrated that giving drugs to newly HIV-infected individuals for 48 weeks of treatment in the early stages of infection presented numerous advantages compared to no early treatment. This trial was conducted between 2004 and 2010 and enrolled 366 patients from Australia, Brazil, Italy, South Africa, Spain, Uganda, UK and Ireland. The results showed that early treatment increased CD4+ T cell count and reduced the VL in patients, and that the earlier treatment was started the better health outcome. Although these findings support the idea that early treatment is more beneficial to HIV-1 infected subjects, more research needs to be done to understand the mechanism underlying these advantages (most likely limiting latent reservoir size and early depletion of CD4 T cells) ^[175, 390, 391].

1.10.2 Pre-Exposure Prophylaxis

The administrations of ARV drugs to people who are at a high risk of contracting HIV (pre-exposure prophylaxis (PrEP)) have been shown to significantly lower the risk of HIV transmission by a few randomised placebo-controlled trials ^[392, 393]. Currently, the most widely used PrEP regimen corresponds to a daily administration of a combination of tenofovir disoproxil fumarate (TDF) and emtricitabine (FTC) also known as Truvada. Particularly, the PROUD study which enrolled 544 HIV-negative MSM across England, demonstrated recently that daily Truvada reduced the risk of HIV infection by 86% for MSM ^[392]. A similar study called IPERGAY, performed in France showed equivalent reductions in the risk of contracting HIV-1 amongst MSM with an average of 15 pills of Truvada taken per

month ^[393]. Following these exciting findings, the French Ministry of Health authorised, for the first time in Europe, access to PrEP for the most at-risk populations. Although, the use of PrEP for key populations has provided promising results, it has been associated with increased rates of side effects, lack of adherence, and has raised concerns regarding the development of drug resistance and increased risk behaviours ^[394, 395].

1.10.3 Post-Exposure Prophylaxis

After possible exposure to HIV, high dose of ARV drugs known as Post-Exposure Prophylaxis (PEP) may cause an abortion of the infection and reduce the risk of becoming HIV positive. PEP consists of a combination of 2-3 antiretroviral agents that must be taken within 72h of potential exposure and be continued for a month. Nonetheless, the effectiveness of PEP has never been evaluated in a human control trial due to ethical reasons; yet several scientists support the idea that PrEP should be given to high-risk individuals who present for PEP as this might further decrease their risk of contracting HIV ^[396].

1.11 Circumcision and Microbicides

Although the efficiency of ARV drugs in reducing the risk of HIV-1 transmission has been well established, the ability and feasibility of providing such treatments to over 35 million of people living with HIV (PLHIV) around the world has been the subject of much debate. Despite research advances and government supports, PrEP and PEP remain relatively expensive for most developing countries which are the most severely affected by HIV/AIDS ^[394]. Therefore, cheap and reliable alternative or complementary strategies are still under developments, particularly for women often unable to successfully negotiate male consent for condom use or mutual monogamy ^[397].

Although the use of condoms remains the most effective barriers against HIV and other sexual transmitted infections, various studies have shown that circumcision can reduce the risk of acquisition by up to 66% among men and that is one of the most affordable interventions for large populations ^[398]. Nonetheless, in resource poor settings, women are the most affected by HIV, and the risk of heterosexual transmission is higher from men to women than *vice versa* ^[42]. For women, one of the biggest potential advances

in HIV prevention would be the development of topical formulations called microbicides that can block HIV infection at sexual mucosal surfaces. Notably, the Centre for the AIDS Program of Research in South Africa (CAPRISA) 004 trial showed that the use of 1% vaginal gel formulation of the nucleotide reverse transcriptase inhibitor, tenofovir, reduced the risk of HIV-1 acquisition among women by 39%. This reduction reached 54% in women with high gel adherence ^[397]. However, the recent results of FACTS 001 Phase III Trial of Pericoital Tenofovir 1% Gel failed to show efficacy in preventing HIV infection in women (Rees et al. CROI 2015). Up to lately there was no effective microbicides that had been licensed, and efforts have focused on the evaluation of cheap microbicide compounds with proven efficacy and limited side effects ^[399]. However, recently the FDA has approved a reverse transcriptase inhibitor (dapivirine) as a microbicide ^[400]. Dapivirine is a NNRTI ARV drug that has been developed as a vaginal ring that only needs to be replaced once a month to provide a discreet and easy-to-use new method of protection for women. The efficacy of this ARV as a microbicide is currently being assessed in two Phase III trials called The Ring Study and ASPIRE ^[401]. These trials involved more than 4,500 women volunteers from over 20 sites across Southern and Eastern Africa. The recent findings of The Ring Study indicate that a monthly vaginal ring containing dapivirine reduced the risk of HIV-1 acquisition by up to 37% among African women, with greater efficacy correlating with higher adherence rates ^[402]. This novel microbicide gives hope to millions of women around the world to protect themselves from sexual acquisition of HIV-1 infection ^[400].

Thus, although the development of various alternative preventive strategies have provided ways to limit the spread of HIV infection, reduce the mortality rate and improve the quality of life for PLHIV; they are unlikely to eradicate HIV/AIDS worldwide. Therefore, a prophylactic vaccine is urgently needed to achieve such a goal.

1.12 Antibody-based vaccines and passive immunisation

HIV-1 infection is mainly transmitted through sexual intercourse; nonetheless increased knowledge and awareness of the risk of contracting HIV through unprotected sex has not been sufficient enough to reduce the spread of HIV/AIDS ^[282]. Although the number of new HIV infection has decreased over the last decade, the figures remain high with 2 million people newly infected in 2014 ^[39]. ART associated with other alternative strategies may not be able to eradicate HIV alone while an effective and safe vaccine has the potential to end the epidemic. Most investigators in the field agree that an effective HIV-1 vaccine should induce both cellular and neutralising antibody responses, at the mucosal sites of transmission, before systemic dissemination. Although a strong cellular response to HIV-1 infection will be required to control systemic infection, these types of responses might not be essential to prevent viral entry from mucosal surfaces ^[403, 404]. Alternatively, studies in NHPs models using SHIV strains have suggested that the passive administration of potent bNAbs could be an effective way to prevent or control HIV-1 infection ^[279, 353].

1.12.1 Vaccine strategies to elicit protective cross-neutralising antibodies

The efficiency of the vast majority of licensed vaccines has been strongly correlated with the induction of neutralising antibody responses. Over the past 30 years, the field of HIV vaccine research has attempted to develop a vaccine that can elicit bNAbs and confer sterilising immunity, particularly at mucosal surfaces ^[254, 278]. Therefore one of the main challenges of HIV-1 vaccine research is the design of immunogens that can induce bNAbs ^[323]. Advances in this field have been limited by the relatively rare occurrence of these antibodies (20-30% in PLHIV) and their delayed appearance which can take months to years to evolve during natural infection ^[276]. These aspects have been related to immune impairment caused by the HIV-1 infection itself and/or to the poor immunogenicity of Env as an antigen ^[278].

To date none of the various Env immunogens that have been developed and tested in clinical trials with healthy subjects were able to induce bNAbs ^[204]. These observations imply that the dysregulation of the immune system during HIV-1 infection might not be the primary reason for the lack of bNAbs responses in most patients. This idea was supported by a recent study which revealed that a bNAb, called CH103 co-evolved with the autologous

viral stain. Indeed, it was observed that the increase in viral diversity occurred just before the development of enhanced antibody breadth. Consequently, researchers suggested that viral immune evasion might drive the development of bNAbs among HIV-1 infected individuals ^[292]. Thus, these findings raise questions about the feasibility of developing HIV-1 Env immunogens that can induce bNAbs responses in healthy individuals in the absence of viral evolution.

The immunogen used in the first Phase I HIV vaccine trial, in 1987, was a recombinant gp160 protein produced by an insect cell-line expression system transfected with a baculovirus vector ^[405]. Since then, over 30 candidate vaccines have been assessed in more than 200 Phase I and II clinical trials ^[403]. Nonetheless to date, only three vaccine strategies have reached the phase III in humans: soluble gp120 Env as an immunogen to elicit NAbs, adenovirus vector to induce strong CTL responses and a combination of both strategy using gp120 Env and a viral vector to elicit humoral and cellular immune responses ^[204].

The first phase III HIV studies, VAX003 and VAX004 were conducted in large groups of IDUs in Thailand and predominantly in MSM in Canada, respectively ^[406]. These two placebo-controlled efficacy trials used two recombinant (r)gp120 proteins from different viral clades in an attempt to induce protective immunity. In the Thai trial VAX003, IDUs were given seven intramuscular injections of AIDSVAX B/E every six months which contained one rgp120 from a CXCR4 TCLA strain (MN) and a CCR5 PI (A244) with alum as adjuvant ^[406, 407]. The vaccine regimen was very similar in VAX004, except that participants received AIDSVAX B/B (VaxGen) which comprises a MN and a GNE8 rgp120 from two different subtype B strains ^[407]. Analyses of the trial outcomes revealed that the vaccine did not induce protective effects. These vaccines failed to elicit NAb responses which have been previously associated with prevention of HIV infection in numerous non-human primate studies ^[279, 353, 354]. Nonetheless, various approaches to elicit bNAbs are still under investigations and involved immunogens that mimics the viral spike, epitope immunogens and immunogens that target B cell ontogeny ^[258].

1.12.2 Vaccine strategies to induce CTL responses

Following the failure of these antibody-inducing vaccines to provide protection, scientists shifted the focus of research towards development of an HIV vaccine that could induce strong CTL responses to control the infection and delay the progression to AIDS ^[204]. Several studies have suggested that an HIV vaccine that could induce CTL responses might be sufficient to reduce HIV incidence although it might not confer sterile mucosal protection ^[408, 409]. DNA plasmid and viral vectors are known to be particularly efficient at eliciting T cell responses which have been shown to correlate with a lower viral set point and prolonged survival of rhesus monkeys infected with SIV ^[410]. The most famous HIV vaccine trial which tested this approach in humans is the HIV Vaccine Trials Network (HVTN) 502 also known as the STEP study ^[411]. Here, they used an adenovirus type 5 (Ad5) vector which contained *gag*, *pol* and *nef* genes. Early results of a phase 2b efficacy trial showed that CD8⁺ T cell responses were induced among participants but revealed also that HIV incidence was increased among some vaccine recipients. These observations, along with the lack of protective effect, prompted the interruption of the trial. Study participants who had a previous immunity against Ad5 vector or were uncircumcised presented increased risk of contracting HIV, which underscored the importance of choosing the right viral vector ^[411]. Additionally, this vaccine induced responses to 2-3 epitopes on the virus while to confer protection against HIV-1 infection a vaccine would likely need to target many more ^[111].

However, a recent study by Hansen et al. that used SIV protein-expressing Rhesus Cytomegalovirus (RhCMV) vectors might have rekindled the CTL vaccine based approach ^[412]. In this study, 13 out of 24 rhesus macaques receiving either RhCMV vectors alone or RhCMV vectors followed by adenovirus 5 (Ad5) vectors (versus 0 of 9 DNA/Ad5-vaccinated rhesus macaques) demonstrated stringent and durable virological control of SIV infection (undetectable VL and ≥ 1 year protection) ^[412]. Subsequent analyses revealed that this vaccine regimen was able to induce and maintain SIV-specific effector memory T cell responses that can interrupt SIV infection prior to the viral expansion required for efficient immune evasion ^[413]. Moreover, this vaccine elicited SIV-specific CD8⁺ T cells that recognise unusual, broad and highly promiscuous epitopes called supertopes, including dominant responses to epitopes restricted by class II MHC molecules that are hardly ever observed in CD8⁺ T cell responses to any other pathogens ^[414].

Recently, the vaccine field attempted to combine both approaches to induce humoral and cellular responses which resulted in the most successful HIV vaccine trial so far: the RV144 Thai trial ^[415]. This study enrolled over 16,000 participants and provided the proof-of-concept that developing an effective HIV vaccine was an achievable goal. The vaccine regimen was based on a classical prime-boost strategy with four injections of a canarypox vector (ALVAC-HIV, Sanofi Pasteur) to prime the immune system followed by two administrations of the previously used rgp120 vaccine (AIDSVAX B/E) to boost immune responses. Interestingly, this regimen did not induce either NAbs or strong CTL responses and instead elicited predominantly nNAbs and CD4⁺ T cell responses ^[416]. Nonetheless, a modest efficacy of 31.2% was observed and subsequent tremendous efforts have been made to identify the immune correlates of risk in those that received the vaccine ^[340]. Analyses of serum samples suggested that V1/V2-directed IgG may have contributed to protection against HIV while high systemic levels of Env-specific IgA might have interfered with the vaccine induced protective antibody responses. Besides, some researchers argued that this vaccine regimen conferred a moderate protection partly because the study population had originally a very low incidence of HIV infection ^[417]. Therefore, this raises questions about the efficacy of this approach with vaccine recipients with more frequent viral exposure and high-risk behaviours such as MSM, IDUs and sex workers ^[204].

However, the RV144 trial provided critical insights about which immune responses might be valuable in an HIV vaccine. Consequently, global vaccine efforts now aim at inducing both bNAbs and CTL responses systemically and at the mucosal sites of infection. Besides, these findings highlighted the lack of understanding of the structural and functional differences between IgG and IgA isotypes, both neutralising and non-neutralising in HIV infection which are the primary focus of this thesis.

1.12.3 Passive immunisation of HIV-1-specific antibodies

The passive transfer of human bNAbs that present the ability to neutralise a broad range of HIV strains may prevent or control HIV-1 infection. This idea has been supported by several studies which demonstrate that passive immunisation of bNAbs in NHP models of simian/HIV-1 (SHIV) infection can protect against intravenous and mucosal challenge^[279, 353]. These bNAbs include the gp120-specific b12^[418, 419], 2G12^[279], PG16^[420] and VRC01^[421] and gp41-specific 4E10 and 2F5^[280] which have been used in this thesis. Additionally, a study that used the recently discovered V3 glycan-dependent bNAb PGT121 has provided further hope by demonstrating that sterilising protection against vaginal mucosal SHIV challenge in macaques was an achievable goal^[422]. These encouraging results observed in NHP, have been followed by clinical studies in HIV-1 infected patients using first and second generations of bNAbs. Notably, Bar et al. found that the passive administration of VRC01 slightly delayed plasma viral rebound after interruption of ART compared to controls^[423]. Additionally, the bNAb called 3BNC117 specific for the CD4bs was shown to significantly reduce viraemia for up to 28 days post treatment, interruption^[424] and in another study this bNAb significantly improved neutralising responses to heterologous tier 2 viruses amongst HIV-1 patients.

Therefore, both NHP and human studies have shown that passive immunisation of bNAbs can help to control HIV-1 infection and/or suppress viral load in HIV-1 infected patients. However, some of these studies have also reported, the emergence of resistant viral strains and pre-existent resistant viruses to the administered bNAbs^[423, 425, 426]. These findings provide evidence that bNAbs administered passively can drive viral escape which highlights both the potential and the limits of the use of bNAbs as immunotherapy to control HIV-1. Nonetheless, passive transfer of bNAbs may provide an alternative approach to ART for the treatment of HIV-1 infected patients by reducing the burden of daily medication, side effects, toxicity and resistance to antiretroviral drugs^[379]. Additionally, the passive transfer of bNAbs could be an alternative approach to PrEP and help prevent HIV-1 acquisition^[427]. However, further research is needed to improve the understanding of the mechanisms and specificities that mediate efficient antibody antiviral function against HIV-1. This research may provide insights into the selection of bNAbs presenting the best

abilities to prevent and/or control HIV-1 infection, which is an integral component of this thesis.

1.13 Thesis outline

A large body of evidence suggests that broadly neutralising and non-neutralising HIV-1-specific antibodies could prevent HIV-1 transmission. In the context of HIV-1 infection, Env trimers exposed on viral surfaces have been identified as the most desirable target for an Ab-based vaccine against HIV-1 ^[254, 345]. Additionally, researchers in the field have suggested that an efficient vaccine against HIV-1 infection should act at the mucosal surfaces, where HIV-1 enters into the body in the vast majority of the cases, following sexual intercourse ^[205, 282, 366]. IgA is known to be the first line of defence against pathogens invasion at the mucosal surface ^[235, 236]. Despite the pivotal place of IgA in mucosal immunity, its potential role in the prevention and/or control of HIV-1 infection remains unclear. Furthermore, very little is known about how IgA may interact with other antibodies isotypes (e.g. IgG) found at the mucosal surface, and which of these antibody isotypes is the most effective to block HIV-1 infection. Besides, there are four types of IgG (IgG₁₋₄) and two forms of IgA (IgA₁₋₂) and while IgG only exists as monomer, IgA can be found as monomer or dimer, with the dimeric form being predominant in the mucosal compartment. The impact of antibody isotype and these isoform differences on antibody antiviral function is not well understood. Conversely, the impact of epitope specificity and accessibility on antibody antiviral function against HIV-1 infection has been reported by several studies ^[262, 428-430]. However, it is still not clear to what extent epitope properties influence antibody ability to capture and neutralise virions.

In addition to these structural characteristics, the ability of antibodies to act rapidly against HIV-1 invasion might be of fundamental importance to prevent sexual HIV-1 acquisition. In spite of several decades of research, very little is known about the time required for antibody-virus interaction to last to inhibit HIV-1 infection. Based on the above observations, it was hypothesised that antibody epitope specificity, accessibility, isotype (IgG vs IgA) and structure (monomeric vs dimeric) might influence HIV-1 Env-specific antibody antiviral function. Moreover, it was thought that the kinetic parameters of antibody-virus interactions may impact on antibody antiviral functions and that IgG and IgA (mIgA or dIgA) bNAbs recognising the same epitope may differ in their ability to bind,

capture and neutralise HIV-1 virions. Furthermore, it was postulated that the combination of IgG and IgA bNAbs of identical specificity might weaken antiviral efficiency due to competition for the same Env sites. Conversely, it was posited that the mixture of bNAbs targeting discrete epitopes from the same isotype might improve antiviral activities. Finally, it was proposed that an extended antibody-virus incubation time prior to the measurement of virion capture and neutralisation efficiency, may improve antibody antiviral outcomes by increasing the chance of antibody-virus encounter. The hypotheses of this research have been divided into eight interlinked aims, and the means employed to tackle them are described here:

Aim 1: To assess the impact of epitope specificity and accessibility on antibody ability to capture virions and to neutralise infectious virions (iVirions). The ability of a panel of IgG and a panel of IgA (mIgA or dIgA) antibodies recognising a range of HIV-1 Env epitopes were compared for their ability to capture purified HIV-1 particles using a novel liquid-phase virus capture assay (VCA) and to neutralise iVirions by standardised TZM-bl assay.

Aim 2: To evaluate the impact of isotype and structure on antibody ability to capture virions and to neutralise iVirions. The ability of a panel of HIV-1 Env-specific IgG, mIgA and dIgA bNAbs recognising the same epitopes were compared for their capacity to capture virions and to neutralise iVirions.

Aim 3: To investigate whether the combination of IgG and IgA bNAbs of identical specificity may weaken antiviral efficiency due to competition for the same Env sites. Competitive enzyme-linked immunosorbent assay (ELISA) and competitive VCA were developed to determine whether IgG and IgA bNAbs recognising the same epitope compete for binding to HIV-1 Env and capturing HIV-1 virions. To evaluate the potential impact of this competition on antiviral outcomes, the proportions of virions captured and the neutralisation activities of mixtures of IgG and IgA (mIgA or dIgA) bNAbs, targeting the same epitope, were also compared to the results obtained with each mAb taken separately

Aim 4: To investigate whether mixtures of bNAbs targeting discrete epitopes, from the same isotype, improve antiviral activities. The amounts of virions captured and the neutralisation activities of combinations of IgG, mIgA and dIgA bNAbs targeting distinct epitopes, from the same isotype and structure were compared to the results obtained with each mAb alone.

Aim 6: To examine the impact of antibody-virus incubation time on virion capture and neutralisation. VCA and TZM-bl neutralisation assay were performed using the panels of HIV-1 Env-specific IgG, mIgA and dIgA antibodies without pre-incubation with virus and after 1h and 24h incubation.

Aim 7: To measure and compare the kinetic parameters of the interaction of the panels of HIV-1 Env-specific IgG, mIgA and dIgA antibodies. Bio-Layer Interferometry (BLI) instrument was used to determine the kinetic constants of the panel of antibodies to soluble HIV-1_{BaL} Env gp140 trimer. The parameters measured were the binding affinity constant (K_D), association constant rate (K_{on}) and dissociation constant rate (K_{off}). Comparison analyses of the kinetic constants of the different panels of antibodies were also performed to assess the influence of epitope specificity, accessibility, isotype and structure on antibody-HIV-1 Env interaction.

Aim 8: To study the relationship between the kinetic parameters of antibody-virus interactions and antiviral function and how this may be impacted by antibody-virus incubation time. Spearman r correlation analyses were performed between each kinetic parameter and the quantities of virions captured and the neutralisation activity by the different panels of antibodies and for the three antibody-virus incubation times tested.

In the first part of this thesis, the impact of epitope and antibody structural features on antibody ability to capture virions and neutralise infectious virions is investigated. The second part of this thesis studies the influence of these antibody characteristics on the kinetic constants of antibody-HIV-1 Env interaction. The last part of the thesis examines the relationship between antibody-virus interaction and antibody-mediated antiviral function.

The ultimate goal of this investigation was not only to improve the understanding of the potential role of IgG and IgA antibodies in the prevention and control of HIV-1 infection but also to anticipate the impact of Env-specific IgG and IgA antibodies in passive immunisation studies and as vaccine-induced antibodies. This research may provide proof-of-concepts for the developments of an effective antibody-based vaccine and an immunotherapy against HIV-1 infection.

Chapter 2

Materials and Methods

2.1 Mammalian cells and tissue culture maintenance

Three types of cells were used: PM-1 and TZM-bl cells which were provided by the National Institute for Health AIDS Research and Reference Reagent Program (NIH AIDS Reagent Program) and Chinese hamster ovary/dihydrofolate reductase (CHO/DHFR) cells which were obtained from American Type Culture Collection (ATCC), CRL-9096.

PM-1 cell line

PM-1 cell line is derived from a CD4⁺ clone called Hut78 T cell. It is susceptible to a wide range of HIV-1 isolates, including biologically pure macrophage (M phi)-tropic isolates, such as HIV-1_{BaL} ^[431]. PM-1 cell line is generally used for viral production and is cultured in Roswell Park Memorial Institute Medium (RPMI) supplemented with 4.5 g/L glucose, L-glutamine, 10 % (v/v) foetal-calf serum (FCS) (Sigma) (heat inactivated at 56°C for 1h), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen Life Technologies) which forms a complete medium.

TZM-bl cell line

TZM-bl cell line also known as JC53-bl is a commonly used HIV-1 indicator cell line. It is developed from a CD4⁺ HeLa sub-clone that endogenously expresses CXCR4, high levels of CD4/CCR5 and contains β-galactosidase and luciferase reporter cassettes controlled by an HIV-1 LTR promoter. These two reporter genes are activated by HIV TaT expression and enable the quantification of viral replication in infected cells. TZM-bl cells are subcultured every four days by trypsinisation and maintained in complete Dulbecco's Modified Eagle's Medium (DMEM) at 37°C with 5% CO₂ in a humidified incubator ^[432].

Chinese Hamster Ovary (CHO) cells

Chinese Hamster Ovary (CHO) cells are a cell line derived from the ovarian cells of Chinese hamsters that have been isolated in 1957 by T. Puck ^[433]. This cell line has been engineered to enable the selection of integrated plasmid DNA into the host chromosome and generate a stable transfected cell line. This mammalian cell expression system called DHFR-CHO cell line lacks the enzyme dihydrofolate reductase (DHFR) which is required for purine synthesis. DHFR-CHO cells are maintained in complete DMEM (Invitrogen) and

subcultured twice to three times per week. This cell line was used for antibody production. A plasmid expressing the gene of interest and a plasmid encoding for DHFR were co-transfected into DHFR-deficient CHO cells. Transformed cells, with the appropriate vectors, were then selected by their ability to grow in purine-free medium, CD OptiCHO (Invitrogen).

2.2 Virus culture, quantification and purification

HIV-1_{BaL} was donated by Dr Robert Gallo, Dr Mikulas Popovic and Dr Suzanne Gartner. ΔEnvHIV-1 pseudovirus was donated by Dr Deborah King. PM-1 cells were used to produce a viral stock of HIV-1_{BaL} instead of PBMC to prevent the influence of donor variability potentially observed with viruses grown in PBMC. Briefly, 5×10^6 PM-1 cells were resuspended in 1mL of high-titre virus stock and incubated overnight at 37°C with 5% CO₂. 10mL of fresh complete RPMI medium was then added to the cells and cultured for three days before replacing with 20mL of fresh medium containing 5×10^6 cells. After a total of 7 days of culture, an additional 10×10^6 cells were introduced in 40mL of fresh medium and cultured for 3-4 more days while the supernatant fluid was harvested and conserved at -80°C. An aliquot of virus was centrifuged at 2,500rpm for 10 minutes to pellet the cells and debris. A proportion of the virus was used to test for p24 content by p24 ELISA and infectivity by TZM-bl luciferase assay (Promega). Viral stocks were then stored in 1mL aliquots in sterile cryotubes either at -80°C or in vapour phase liquid nitrogen.

Alternatively, to perform assays outside Category III containment level with HIV-1_{BaL}, infectious viral stocks were inactivated with 2mM aldrithiol (AT-2) (Sigma) for 2h at 37°C in a water bath and stored at -80°C. An aliquot was tested for infectivity by TZM-bl luciferase assay. Prior to performing the luciferase assay, plates were seeded with 100μL/well of TZM-bl cells (5×10^5 cells/mL) and the assay was performed following manufacturer's instructions (Promega). The viral stock was judged as successfully inactivated when the luciferase production was less than three times the background. Inactivated virus stocks were concentrated using 100KDa molecular cut-off columns (Millipore) up to 1mL. Concentrated aliquots of the same stock were pooled together and resuspended in Phosphate Buffered saline (PBS). Each 1mL of AT-2 inactivated HIV-1_{BaL} was purified over 100μl of 20% sucrose cushion at 20,000 g for 60 min in a table top microcentrifuge. Supernatant fluids were

removed, and the sucrose fractions containing HIV-1_{BaL} virions were resuspended in PBS. 10µL of anti-CD45 beads (Miltenyi Biotec, Germany) were added to every 500 µL of virus to remove cell microvesicles and incubated at 4°C with gentle mixing overnight. CD45⁺ microvesicles depletion was performed with LD magnetic columns according to instructions. Purified virions were concentrated over 20% sucrose cushions in a table top microcentrifuge and resuspended in PBS. Viral stocks were tested for p24 concentration, aliquoted and frozen at -80°C for binding and capture assays.

2.3 Antibodies and recombinant proteins

The polyclonal HIVIG (IgG derived from the pooled plasma of HIV-infected individuals) and the panel of monoclonal antibodies (bNAbs) 2G12, 2F5, PG9, PG16, 7B2, CH31, PGT136, 447-52D, VRC01 and VRC03 were obtained from the Centre for HIV-1 and AIDS Vaccine Immunology (CHAVI) (Table 3). b12 bNAbs were provided by Dr Dennis Burton, Scripps Research Institute (La Jolla, United States) and 4B3 was a kind gift from Dr Raphaele El Habib and Dr Christiane Moog, Institut Pasteur (Paris, France). IgG and IgA controls were purchased from Polymun Scientific (Austria). CH31 dIgA₁ was produced by me at the Duke Human Vaccine Institute (DHVI) within the team of Dr Larry Liao (USA). Briefly, CHO/DHFR cells that express J Chain were transiently cotransfected with the plasmid pCH31A1H encoding for CH31 dIgA₁, and pvf5k encoding for DHFR using polyethylenimine (PEI) (Polysciences Inc.). Supernatant fluid from transfected cells was harvested after 4 to 5 days of culture and CH31 dIgA₁ was purified by Peptide M affinity chromatography. Purified CH31 bNAbs were quantified with a Nanodrop instrument. CH31 mAb purity and dimeric conformation were confirmed by Western blot from native and SDS-PAGE gels. HIV-1_{BaL} Env gp140 protein (from Clade B) was kindly provided by Dr Larry Liao.

Table 3. Antibodies used in the different assays

Antibodies	Brief description of epitopes	Isotypes used	References
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b12	CD4bs on gp120	IgG ₁ , mIgA ₂ , dIgA ₂	[290, 434]
CH31	CD4bs on gp120, VRC01-like mAb	IgG ₁ , mIgA ₂ , dIgA ₂ , dIgA ₁	[435]
2F5	human cluster II mAb to gp41 MPER, binds ELDKWA	IgG ₁ , mIgA ₂ , dIgA ₂	[280]
7B2	human cluster I mAb to immunodominant region of gp41	IgG ₁ , mIgA ₂ , dIgA ₂	[436]
4B3	human cluster I mAb, gp41 epitope (579-613) SGKLIC	IgG ₁	[437]
2G12	gp120 carbohydrates at glycosylation residues in C2, C3, C4, and V4, gp120 V3	IgG ₁	[261, 438]
PG16	gp120 V1-V2, quaternary structure	IgG ₁	[439, 440]
PG9	gp120 V2-V3, quaternary structure	IgG ₁	[439, 440]
PGT136	V3 and V4 glycans, gp120 V3	IgG ₁	[297]
VRC01	CD4 mimicry by VH1–2-derived heavy chain	IgG ₁	[293, 435]
4E10	human cluster II mAb to gp41 MPER, binds NWFDIT	IgG ₁	[280]
VRC03	CD4 mimicry by VH1–2-derived heavy chain	IgG ₁	[293, 435]
HIVIG	Derived from pooled plasma of HIV-infected individuals	IgG ₁	[441]
447-52D	gp120 V3 loop, binds KSIHIGPGRAF	IgG ₁	[300, 442]
Control IgG	Polyclonal antibody from pooled normal human serum	IgG	
Control IgA	Polyclonal antibody from pooled normal human serum	IgA	

2.4 HIV-1 p24 ELISA

The HIV-1 p24 antigen ELISA was performed using prepared quantification kits from Aalto Bio reagents to measure the quantity of HIV-1_{BaL} virions or ΔEnvHIV-1 pseudovirus. This assay is a Twin-site sandwich ELISA that has been taken from a previous method developed in the early 1990's^[443]. Prior to performing the assay, the coating antibody called D7320 (a sheep anti-HIV-1-p24 gag), was resuspended in 1mL of distilled water to a concentration of 2mg/mL. Then, D7320 was diluted in 400mL of 0.1M NaHCO₃ to a concentration of 5μg/mL. 100μL/well of D7320 were added in white, opaque high-binding 96-well plates (Costar). The plates were covered, wrapped with saran wrap and incubated overnight at room temperature (RT). A blocking buffer was prepared with 2% milk in Tris-Buffered Saline (TBS) (Sigma) and 100μl/well of this solution was added to the plates. After 1h incubation at RT, the plates were frozen at -20°C; coated plates could be stored for several weeks.

To perform the p24 ELISA D7320 coated plates were thawed at RT. The buffer in the wells was discarded and the plates were washed once with TBS. Then, p24 standard was diluted in 0.1% Empigen (Sigma) and TBS (Sigma). Prior to assay measurement, HIV-1 samples were treated with Empigen to lyse the virus, release and linearise the proteins including p24 proteins. Samples, standards and controls were then added to the plates (100µL/well) for 3h at RT. Secondary antibody (BC 1071-AP) was diluted (1:8000) in TBS containing 20% sheep serum (Sigma) and 0.05% Tween (Sigma) resulting in a the conjugate solution. After a washing step with TBS, 100µl of conjugate was added per well and incubated for 1h at RT. After four washes with 0.1% Tween in PBS (Sigma), and two washes with ELISA light assay buffer (T1023, chemiluminescent substrate for alkaline) (Aalto), 50µL/well of CSPD with Sapphire Substrate (Aalto) was added and incubated for 30min at RT. Plates were read on a luminometer.

2.5 Biolayer interferometry binding assays

To determine the parameters that influence antibody-HIV-1 interaction, kinetic constants of the interaction between a panel of antibodies (Table 3) and HIV-1_{BaL} Env gp140 or whole HIV-1_{BaL} virions were performed using the Bio-Layer Interferometry (BLI) instrument (Fortebio) (Figure 17). The BLI uses a label-free technology for real time measurement of the association and dissociation constant rates (k_{on} and k_{off}); and the binding affinity constant (K_D) of antibody-antigen (Ab-Ag) interaction ^[428] (Figure 17). Compared to ELISA or SPR, the BLI was shown to be faster, easier to implement and to enable the measurement of biomolecular interactions even in crude mixtures. Kinetic parameters of Ab-Ag interactions were measured in an eight-step assay, in triplicate. Prior to running the experiment on the BLI, Streptavidin (SA) biosensors (Fortebio) were rehydrated in assay buffer (1 x PBS, 0.01% BSA, 0.02 % v/v Tween 20) for at least 10min. The initial baseline was established by immersion of the SA biosensors for 30secs in 400µL of assay buffer. 4µL of *Galanthus nivalis* lectin conjugated with Biotin (Biotin-GNA) (Sigma) were immobilised onto SA biosensors for 150secs (loading step). After a washing phase of 45secs, biosensors were immersed into a tube containing HIV-1_{BaL} Env gp140 (50µg/mL) or HIV-1_{BaL} virions (15ng/mL of p24) for 360secs (association phase) and then in a tube of buffer only for 360s (dissociation phase). The duration of the different phases was optimised

and all measurements were performed at RT. Results were analysed with the software, BLItz Pro 1.1.0 28 (ForteBio) using a curve fitting 1:1 binding model. Binding kinetic curves were represented in nm as a function of time. Negative binding was reported when the binding signals were below 0.1 nm.

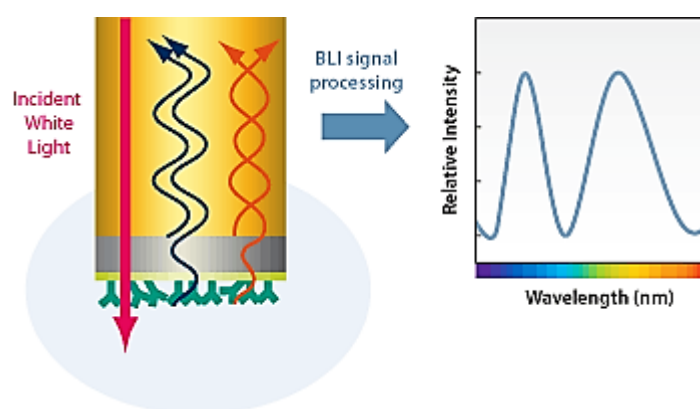


Figure 17. Biolayer interferometry basic principles. Light is reflected from two surfaces: a layer of immobilised macromolecules and an internal interference layer. Any change in the number of molecules interacting with the biosensor tip causes a shift in the interference pattern that can be measured in real time. The wavelength shift is a direct measure of the change in thickness of the protein layer^[444]. Taken from Wesley Straub, 2016^[445].

2.6 Competitive ELISA

A competitive ELISA was developed to determine whether IgG, mIgA₂ and dIgA₂ bNAbs that present the same epitope specificity, compete for interaction with HIV-1_{BaL} Env gp140 (BaL gp140). Medium-binding ELISA plates (Greiner Bio-One) were coated with 50µL per well of BaL gp140 (1µg/mL) diluted in Dulbecco's PBS (DPBS) (Gibco). The plates were sealed and incubated for 1h at 37°C. After four washes with PBST (0.05% Tween 20 in PBS), the plates were blocked by adding 200µL per well of assay buffer (1% BSA, 1x PBST), sealed and incubated for 1h at 37°C. Fresh antibody samples, standards and controls were prepared in assay buffer by a 5-fold dilution series of seven dilution steps, in triplicates. A volume of 50µL of samples, standards or controls were then applied to the plate and incubated for 1h at 37°C. Final antibody concentrations ranged from 0.0003 to 5µg/mL. Precisely, HIV-1-specific IgG antibodies were incubated for 1h at 37°C with BaL gp140 either alone or with their mIgA₂ or dIgA₂ counterparts recognising the same epitope. A total of five different ratios of IgG/mIgA₂ and IgG/dIgA₂ were tested: 10:1, 2:1, 1:1, 1:2 and 1:10 while the concentration of BaL gp140 was the same in all assays (15ng/mL).

To measure the ability of mIgA₂ and dIgA₂ bNAbs to inhibit the binding of their IgG counterpart to BaL gp140, antibody binding detection was performed with goat α-Human IgG HRP (Sigma) diluted (1:15000) in assay buffer. To determine the capacity of IgG to block mIgA₂ or dIgA₂ bNAbs interaction with BaL gp140, antibody detection was measured with goat α-Human IgA HRP (Sigma) diluted (1:10000) in assay buffer. Following incubation, the plates were washed four times as previously and the colorimetric signal was produced using the TMB substrate (KPL), and absorbance read at 450nm using a microplate reader (BMG Labtech).

The percentage of inhibition of IgG mAb binding to BaL gp140 due to the presence of unlabelled mIgA₂ or dIgA₂ bNAbs, was calculated for each ratio. The amplitude of IgG mAb binding signal in the presence of mIgA₂ or dIgA₂ mAb was compared to the amplitude of the binding signal with IgG alone. In addition, the percentage of inhibition of the binding to BaL gp140 of mIgA₂ or dIgA₂ bNAbs due to the presence of IgG, was assessed by comparing the binding signals with or without IgG.

2.7 Immuno-bead Viral Capture Assays

Purified HIV-1 virions at a concentration of 500ng/mL of p24 were incubated for 0h/less than 5 min, 1h or 24h at 37°C with 10µg/mL of antibody, in a final volume of 100µL of MACS buffer containing PBS, 1mM EDTA and 0.1% BSA (Miltenyi Biotec). Protein G Sepharose High Performance columns (Protein G HP SpinTrap) (GE Healthcare Life Sciences) present a strong affinity for the Fc region of IgG and were used to capture IgG antibodies. IgA capture columns were prepared by adding 250µL of IgA Capture Select Antibody Affinity Resin (Life Technologies) into MicroSpin G-50 Columns (GE Healthcare Life Sciences). Alternatively, for capturing both IgG and IgA antibodies in the competitive viral capture assay, 250µL of IgA Capture Resin were applied to Protein G HP SpinTrap columns and gently mixed by inversion. Binding, neutralisation and elution buffers were obtained by GE Healthcare Life Sciences and diluted to a final concentration of 1x from the stock solutions according to manufacturers' instructions.

All centrifugations were done at 100g for 30s using a table top microcentrifuge with a loosened lid and bottom cap removed. IgG and IgA columns were placed in 2mL collection tubes and centrifuged to remove preserving buffer. After priming, fresh columns with 500µL of binding buffer, antibody-virus mixtures were applied, and the columns were gently mixed for 4-5 min at room temperature. After centrifugation, columns were washed twice with 300µL of binding buffer to remove unbound virions. The flow-through from these two washes were pooled together and constituted the negative fraction. Collection tubes for the positive fraction were prepared by adding 30µL of neutralisation buffer to each tube. Then, 400µL of elution buffer was incorporated in each column and the columns were gently mixed by inversion and centrifuged. The elution step was performed twice, and the two elutions were pooled together to form the positive fraction. Both negative and positive fractions (NF and PF, respectively) were then treated with Triton-X100 (1%) to lyse virions. Each separation was performed, in triplicate and at least three experiments were performed for each condition tested. The proportion of virions in each fraction was determined by a p24 ELISA, and the amount of p24 present in the positive fraction was expressed proportional to the total of the positive and negative fractions using the formula: virion capture (%) = $(PF / (NF+PF)) \times 100$. HIV-1 p24 antigen ELISA was performed as described above using prepared quantification kits from Aalto Bio reagents and carried out according to the manufacturer's instructions.

2.8 Antibody-mediated neutralisation assays

Neutralisation of HIV-1_{BaL} was measured with the panel of antibodies using a modified version of the standardised TZM-bl assay protocol, developed and optimised by Montefiori et al. at Duke University^[446, 447]. Briefly, this assay measures the neutralisation in TZM-bl cells as a function of a reduction in Tat-induced luciferase gene expression after a single round of virus infection. Antibodies, at a final concentration ranging from 0.39µg/mL to 50µg/mL, were pre-incubated with HIV-1_{BaL} for 0h/less than 5 min, 1h or 24h at 37°C. During the incubation, TZM-bl cells were trypsinised, counted and resuspended in complete DMEM medium at a final concentration of 10⁵cells/LI. Then, 100µL of TZM-bl cells were added to each well containing 100µL of antibody-virus mixtures, in triplicate. In the negative control wells, antibody-virus mixtures were replaced by 100µL of complete medium. Positive control wells contained 50µL of complete medium and 50µL of virus. 96-well plates were incubated for 72 hours at 37°C with 5% CO₂. After incubation, cells were washed with 200µL/well of PBS. 100µL of Promega lysis buffer were added to every well and plates were frozen for at least 2h at -80°C to ensure full cells lysis. Luciferase activity was measured as relative light units (RLU) on a luminometer following manufacturer's instructions.

2.9 Statistical analyses

Statistical analyses were performed with GraphPad Prism v7.0. All comparisons were performed by two-way ANOVA-Tukey's test with 95% Confidence Intervals (CI). All correlation studies were determined by two-tailed Spearman analysis. 1-way ANOVA was used to determine the variance in TCID₅₀ in viral neutralisation assays.

Chapter 3

Capture and neutralisation of HIV-1 virions by envelope-specific antibodies

3.1 Introduction

The primary target for an antibody-based vaccine against HIV-1 infection is the envelope protein (Env) exposed on the virion surface. This is required for viral entry into susceptible cells and therefore infection. Vaccine-elicited broadly neutralising antibodies (bNAbs) directed against Env, that can block HIV-1 acquisition at mucosal surfaces are an important goal in HIV prevention ^[254, 258]. However, despite three decades of research, the development of such vaccines is still to achieve. During the natural course of HIV-1 infection, most HIV-1 infected individuals are capable of mounting neutralising antibody responses but only about a quarter of them develop bNAbs ^[276]. Although bNAbs might help in controlling the infection, they arise months to years after the infection which is too late to confer protection. Therefore, there has been a growing interest in vaccine-induced bNAbs for protection against HIV-1 transmission ^[254, 258]. This increased interest has been fuelled by several non-human primate studies where passively administered bNAbs protected against SIV and SHIV-1 challenges ^[279, 320, 353, 381]. Consequently, the investigation of bNAbs interaction with HIV-1 Env may provide critical information for the rational design of effective vaccines against HIV-1 infection.

Up to now, all HIV vaccine approaches that have tried to induce antibodies with high potency and cross-clades neutralisation activity have been unsuccessful, and have elicited high levels of non-neutralising antibodies (nNAbs) instead ^[204, 254, 258]. These nNAbs are universally and abundantly produced among HIV-1-infected individuals and target predominantly non-functional forms of Env including gp120/gp41 monomers as well as gp41 stumps ^[253, 320]. Several studies have shown that the vast majority of viral particles in cell-free supernatants are not infectious because there are defective and/or do not enter into contact with a susceptible cell ^[448, 449]. Indeed, it has been demonstrated that HIV-1 viral stocks prepared *in vitro* contain diverse populations of HIV-1 viruses, that form quasi-species composed of infectious virions (iVirions) and defective non-infectious virions (dVirions) ^[428]. An early study that used a reverse transcriptase assay and a virus-induced cytotoxicity assay has estimated that the ratio of infectious to defective virions in HIV-1 viral stocks ranged from 1:1 to 1:100, depending on the viral strain and host cells, although it remained constant with a given virus-cell system ^[448]. A more recent study has suggested that the ratio of infectious to defective virions have been overestimated, and is rather in the order of one to eight. This is due to the more frequent occurrence of cell-to-cell infections

that are thought to be more efficient ^[449]. Therefore, the large amount of nNABs among infected subjects has been related to the presence of these dVirions that express non-functional forms of Env, but also to the high variability and flexibility of the functional Env forms, that blurs humoral immune responses ^[204, 254, 258]. Indeed, although HIV-1 infection has been shown to be established by one or two virions in most infected people ^[134], every single viral isolate expresses a highly variable Env molecule at its surface ^[250, 253]. Several mechanisms have been identified as factors contributing to Env diversity at the viral surface and include; the variability of Env oligomerisation state (monomer, dimer, trimer of gp120 and gp41), conformation (e.g. open/close), post-translational modifications (the level and specificity of Env glycosylation ^[250, 253]), and the number and distribution of Env on the viral surface ^[50].

HIV-1 Env is generated in the host endoplasmic reticulum as a precursor protein called gp160 ^[60]. Here, it assembles as a trimer before transiting to the trans-Golgi where it is cleaved by furin-like proteases in a trimeric heterodimer containing three gp120 surface units (SU) non-covalently linked to three gp41 transmembrane proteins (TM) ^[61]. However, the degree of cleavage catalysed by furin-like proteases is variable and may result in uncleaved or partially cleaved gp160 molecules that are assembled into budding virions and exposed on the viral surface. In addition, the non-covalent link between gp41 and gp120 is relatively unstable and may lead to gp120 shedding leaving gp41 stumps exposed on the viral surface. Consequently, Env can be expressed on the viral surface as a monomer, dimer or trimer of gp120/gp41 or gp41 “stumps” that have lost gp120, which all contribute to the variability of Env oligomerisation state and functionality ^[250, 253].

Functional (trimer of gp120/gp41) and non-functional (monomer or dimer of gp120 and/or gp41) Env forms are incorporated at random on budding virions and the number of functional Env present, as well as their disposition on the viral surface, impact on infectivity ^[253, 321]. Some virions may acquire fewer functional Env forms than required to mediate receptor binding and membrane fusion. The functional copies may also not be distributed in a cluster which would facilitate infectivity. This would suggest that the viral protein might not be in adequate proximity to enable the formation of the viral entry complex. Additionally, some of the functional incorporated Env forms might decay or disassemble making them defective and some virions may also be bald particles lacking Env proteins

^[253]. Moreover, while Env gp160 precursor transits into the host endoplasmic reticulum and Trans-Golgi, it undergoes intensive N- and O- glycosylation which may vary in number, localisation and specificity, depending on the host cell ^[78]. This provides an array of possible glycosylation patterns of Env and results in a highly diverse glycan shield that masks potential epitopes ^[342]. As Env glycans are generated by the host cell and cover the vast majority of gp120 surface, they are not recognised as antigen but as “self” by the immune system which makes them poorly immunogenic ^[342]. Nonetheless, some exceptional antibodies such as 2G12 and the recently discovered “PGT” antibodies including PGT136 can recognise glycans present on the gp120 Env surface ^[438, 450].

In addition to the glycan shield that hides critical epitopes, the structure of the functional Env molecule limits antibody access to major epitopes, such as the CD4bs which is embedded within the core of gp120 ^[290]. Furthermore, certain neutralising epitopes that are targeted by Nabs, including the co-receptor CCR5 or CXCR4 binding site and the fusion peptide of gp41 are only transiently exposed during the viral cell attachment and fusion processes ^[256]. Therefore, the high flexibility and the conformational changes undergone by Env upon binding to receptors, limit bNAbs access to key epitopes. Nevertheless, a small number of bNAbs that target crucial epitopes have been isolated from patients, such as those targeting the gp120 CD4bs: b12, VRC01, VRC03 and CH31; the gp41 Membrane Proximal External Region (MPER): 2F5 and 4E10, and the gp120 V1/V2 loops directed bNAbs: PG9 and PG16 ^[251, 293, 451].

Furthermore, the IgG and IgA isotypes of some of these bNAbs are commercially available, which enables the study of the impact of antibody isotype and structure on antiviral function possible. This investigation might be of fundamental importance as both IgG and IgA are present at the mucosal portal of HIV-1 entry, and IgA is known to be the first line of defence against pathogens invasions through mucosal surfaces ^[226, 236]. In spite of the central position of IgA in the mucosal immunity, very little is known about the role of IgA in protection against HIV-1 infection ^[237]. Moreover, it is still not well understood how IgA may interact with other antibodies isotypes (e.g. IgG) present at mucosal surfaces and which of these isotypes is the most effective to block HIV-1 infection ^[236, 238]. Furthermore, there are different forms of IgG and IgA: four types of IgG: IgG₁₋₄ and two forms of IgA₁₋₂ which differ particularly by the length of their hinge region. IgG is the main isotype found in serum and

only exists as a monomer, whilst IgA is the most abundant isotype in the mucosal compartment and exist as a monomer and a dimer; the dimeric IgA (dIgA) molecule being the predominant structure in the mucosal compartment and monomeric IgA (mIgA) in serum ^[236, 238].

The impact of isotype, structure and isoform of these recently isolated bNAbs on antiviral functions against HIV-1 remain to be defined. Accordingly, the study of the interaction between HIV-1 virions and the isolated HIV-1-specific bNAbs (as well as nNAbs of different isotypes, structure and isoforms) may provide critical insights into the design of immunogens that can elicit bNAbs rather than nNAbs. Such research may also provide key information for the selection of bNAbs that prevent HIV-1 infection, or treat HIV-1 infected individuals via passive immunisation.

Based on these observations, the objective of this chapter was to study the factors that mediate efficient antiviral function against HIV-1 by Env-specific antibodies. For this purpose, different panels of IgG, mIgA and dIgA antibodies comprising bNAbs and nNAbs, targeting a range of Env epitopes were used. These panels comprised 14 IgG₁ (IgG) antibodies targeting six different types of HIV-1 Env epitopes: (1) gp120 CD4bs, (b12, CH31, VRC01 and VRC03), (2) gp120 glycans and V3 (2G12, PT136 and 447-52D), (3) gp120 V1/V2 (PG9 and PG16), (4) gp41 Membrane Proximal External Region (MPER), (2F5 and 4E10), (5) gp41 cluster I (7B2 and 4B3) and (6) polyclonal (HIVIG). HIVIG is a polyclonal NAb, 7B2 and 4B3 are nNAbs and the 11 other antibodies are bNAbs. The panel of antibodies tested also included 9 IgA antibodies: the mIgA₂ and dIgA₂ isoforms of b12, CH31, 2F5 and 7B2 and the dIgA₁ isoform of CH31 (Figure 18).

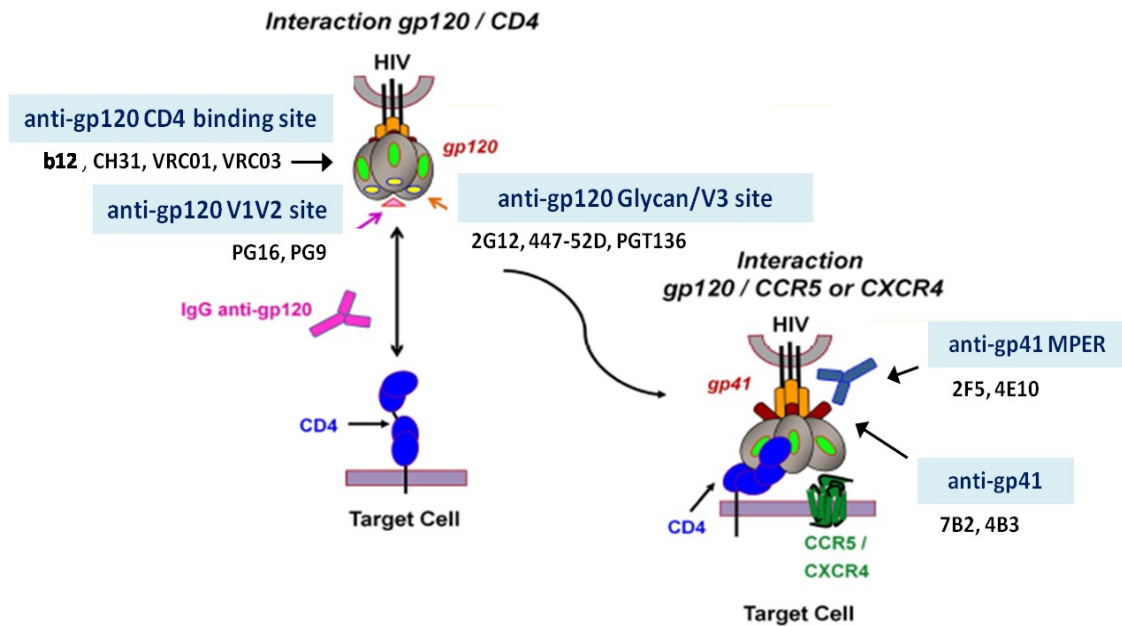


Figure 18. HIV-1 Env structure and epitopes targeted by the panel of antibodies. The panel of antibodies tested in the different assays bind to various epitopes on HIV-1 envelope (Env) gp120 and gp41 which can be categorised into five groups: (1) gp120 CD4-bs, (2) gp120 V1/V2 site, (3) gp120 N-linked glycan and V3 site, (4) gp41 Membrane Proximal External Region (MPER) and (5) gp41 cluster I. The CD4 binding site is presented in green, the variables (V) V1 V2 and V3 sites in yellow and gp41 sites in red, orange, and black (transmembrane domain). On the right panel HIV-1 Env is presented in its unbound state (close conformation) and on the left panel in interaction with the CD4 receptor expressed on the target cell surface and with co-receptor CCR5 or CXCR4 ^[452]. Adapted from Su et al. 2014.

The antibody panels were compared using an antibody viral capture assay (VCA) and the TZM-bl neutralisation assay. VCA has been used for over a decade to characterise immune responses to virions and infected cells. All VCAs involve the capture of a virus on a solid support by antibodies, ligands or beads. However, VCAs differ from each other by the method employed to quantify the virions captured. In this study, virions were lysed with a detergent to release their p24 contents which were then measured by p24 ELISA. Another method to quantify the amount of virions captured is measuring the amount of viral RNA by qRT-PCR. VCAs have played a significant role in the discovery of the presence of both functional and non-functional Env forms on HIV-1 viral particle surface. Previous studies have indicated that although only HIV-1 Env-specific NAbS can neutralise infectious virions

(iVirions), both NAb and nNAb can capture virions^[428, 453, 454]. This indicated that antibody virion capture function “occupies a unique immunological space”^[453] and may provide critical information into understanding the relationship between antibody-virus interaction and antiviral function. Moreover, this research highlights the lack of understanding of the relationship between antibody ability to capture viral particles and to neutralise iVirions^[453].

Based on the observations mentioned above, the first aim of this chapter was to evaluate the influence of epitope specificity and accessibility on IgG and IgA antibodies abilities to capture and neutralise HIV-1 virions (**Aim 1**). The second aim was to study the relationship between antibody ability to capture virion and to neutralise iVirions (**Aim 2**).

It was hypothesised that the epitope features required to mediate efficient virion capture might differ from those necessary to mediate potent neutralisation. Particularly, it was anticipated that epitope accessibility would be the major factor influencing virion capture while epitope specificity would be the predominant factor impacting on neutralisation potency. It was postulated that the ability of antibodies to capture virions would correlate with neutralisation.

Moreover, it was thought that the ability of NAb to efficiently neutralise iVirions captured, and/or to selectively capture iVirions over dVirions, is critical to their capacity to prevent infection of susceptible cells. To assess this potential, an antibody-mediated virion capture neutralisation index (AVCNI) was devised to quantify the ability of an antibody to selectively capture and neutralise iVirions and dVirions. This indicator was calculated using the ratio virion: neutralisation (%) / capture (%). It was proposed that this novel index would provide an effective way to classify bNAbs; bNAbs with the highest AVCNI values would be most effective against HIV-1 infection. A recent study that has investigated the immune correlates for risk of infection for the RV144 HIV-1 vaccine efficacy trial demonstrated that while plasma IgG against the HIV-1 Env V1/V2 were protective, HIV-1 Env-specific plasma IgA responses directly correlated with the risk of infection^[238, 340]. In addition, the results of this research showed that antibody-dependent cellular cytotoxicity (ADCC) also inversely correlated with risk of HIV acquisition, but only in the presence of low plasma Env-specific IgA. Furthermore, post hoc analyses revealed that Env-specific plasma IgA/IgG ratios were higher in HIV-infected than in uninfected RV144 vaccine recipients^[340]. Correlation analysis

showed that vaccine-induced Env-specific mIgA may have attenuated IgG protective responses through competition for the same HIV-1 Env binding sites which might have diminished IgG mediated ADCC effector function^[238].

As a result, the influence of antibody isotype on antibody capturing and neutralising HIV-1 virions was investigated by first comparing IgG and IgA bNAbs presenting the same epitope specificity; secondly, by assessing the impact on antibody function of the combination of IgG and IgA (mIgA or dIgA) bNAbs recognising the same epitope at equal concentration; and thirdly by determining how this impact might be due to IgG and IgA bNAbs competition for the same Env sites (**Aim 3**). It was hypothesised that dIgA molecules with twice more binding sites than mIgA and IgG might have an increased ability to capture and neutralise HIV-1 particles. Moreover, it was postulated that the combination of IgG and IgA (mIgA or dIgA) bNAbs, sharing the same epitope specificity, might mitigate antiviral outcomes. This might be due to differences in antiviral efficiencies between IgG and IgA bNAbs, and competition between IgG and IgA bNAbs presenting identical specificity for the same Env binding sites.

While the studies mentioned above suggest that the combination of bNAbs binding to the same Env epitopes and of different isotypes (IgG and IgA) may diminish antiviral function, mounting evidence indicates that the combination of bNAbs targeting distinct epitopes may enhance antiviral functions against HIV-1 infection^[427, 428, 455]. Several researchers have suggested that due to the extraordinary diversity of HIV, combinations of bNAbs binding to discrete HIV-1 Env epitopes would likely be required to confer protection against HIV-1 infection^[427, 428, 455]. Moreover, despite two decades of research since the discovery of the first bNAbs, very little is known about how nNAbs and bNAbs may interact to control or prevent HIV-1 infection, and how the combination of nNAb with a bNAb may impact on antiviral activities against HIV-1. Indeed, both bNAbs and nNAbs may be elicited during the natural course of HIV-1 infection^[275, 346]. However, while nNAbs are universally and abundantly produced among HIV-1 infected individuals, only about 20-25% of them develop bNAbs^[276, 338].

Based on these observations the fourth aim of this chapter was to explore different combinations of IgG and IgA bNAbs that may increase antiviral function. For this purpose, the combinations of IgG, mIgA₂ and dIgA₂ bNAbs with another bNAb targeting a discrete

epitope and of the same isotype were tested, as well as a mixture of bNAb with a nNAb (**Aim 4**). It was proposed that the combinations of IgG, mIgA and dIgA bNAbs targeting distinct epitopes (and of the same isotype) may improve antiviral function against HIV-1. It was also hypothesised that the combination of a bNAb and a nNAb, at the ratio 1:1, might reduce antiviral function, if they compete for the same Env site. However, it was postulated that the impact of these combinations on antiviral function would be influenced by the antibodies' isotype, structure and specificity.

In addition, to antibody epitope specificity, accessibility, isotype and structure, the ability of antibody to act quickly against HIV-1 virions might be of critical importance to prevent sexual HIV-1 acquisition. NHP studies have suggested that SIV/HIV-1 may cross mucosal barriers within an hour to several hours after exposure ^[418, 456]. However, the time required for HIV to cross sexual mucosal barriers in human remains to be defined. In addition, very little is known about the necessary time-frame within which antibodies interact with viruses to prevent HIV-1 infection, and how time affects antibody antiviral function.

As a result, the final aim of this study was to determine the effects of antibody-virus incubation time on antibody ability to capture and neutralise virions, and to assess how these effects might be influenced by other antibody factors (**Aim 5**). It was proposed that extended antibody -virion incubation time (T=24h) might correlate with enhanced antiviral function by increasing the chance of antibody-virus encounter, and/or by causing spontaneous or antibody-mediated, gp120 shedding from viral surface which may irreversibly inactivate iVirions. It was also proposed that a short incubation time (T=0h/less than 5 minutes) would highlight the potential importance of the kinetics of antibody binding relative to the standard 1h antibody-virus incubation time and extended incubation.

The ultimate goal of these investigations was to improve the understanding of the parameters of antibody-virus interaction that control antibody antiviral function, to inform the rational design of antibody-based vaccine strategies, and to provide insights into the selection of bNAbs for preventative and therapeutic passive immunisation. In summary, this chapter comprises of five interlinked aims:

Aim 1: To assess the impact of epitope specificity and accessibility on IgG ability to capture and neutralise virions.

Aim 2: To study the relationship between antibody ability to capture virion and to neutralise infectious HIV-1 virions.

Aim 3: To determine the influence of antibody isotype and structure on antibody capacity for capturing and neutralising HIV-1 virions.

Aim 4: To explore different combinations of IgG and IgA bNAbs that may increase antiviral function.

Aim 5: To evaluate the effects of antibody-virus incubation time on antibody ability to capture and neutralise virions.

The first part of this chapter examines the influence of epitope specificity and accessibility on antibody ability to capture virions and to neutralise iVirions (Aim 1), and studies the relationship between virion capture and neutralisation (Aim 2). The second part evaluates the potential impact of isotype and structure on antiviral function and explores different combinations of IgG and IgA (mIgA or dIgA) antibodies that could enhance antiviral outcomes (Aim 3 and 4). The third part appraises the influence of antibody -virion interaction time on antiviral function and assesses how this might be affected by antibody epitope specificity, isotype and structure (Aim 5).

3.2 Results

3.2.1 Influence of epitope specificity and accessibility on virion capture and neutralisation functions

Virion capture and neutralisation are thought to be two distinct antiviral functions. Based on this, it was hypothesised that the epitope characteristics necessary to mediate efficient virion capture might differ from those needed to mediate potent neutralisation. The first aim of this chapter was to investigate the relative influence of epitope specificity and accessibility on antibody ability to capture virions and to neutralise iVirions. For this purpose, the proportions of HIV-1_{BaL} virions captured (measured by VCA), and the neutralisation activities against HIV-1_{BaL} determined by TZM-bl assay for a panel of IgG antibodies and a panel of IgA bNAbs were compared (Figure 18). Two distinct panels of IgG and IgA antibodies were used as it was thought that antibody isotype might influence antibody function. In the first part of this chapter, the focus was on the impact of epitope specificity and accessibility on antiviral function.

Comparison of the proportion of virions captured by the panel of HIV-1 Env-specific IgG antibodies indicates that IgG targeting the same category of epitope presented a comparable capacity for virion capture (Figure 19). Here, was no significant difference in the amount of virions captured between the following IgG antibodies: b12, VRC01 and CH31 (gp120 CD4bs-specific), PG9 and PG16 (gp120 V1/V2-specific), 2G12, PGT136 and 447-52D (gp120 V3/glycans-specific), 2F5 and 4E10 (gp41 MPER-specific) (Figure 19). However, the gp120 CD4bs directed VRC03 captured a significantly lower amount of virions ($33.9 \pm 2.3\%$) than its counterparts b12 and VRC01 ($46.6 \pm 2.8\%$ and $46.9 \pm 2.9\%$, respectively, both $p < 0.005$) (Figure 19). The gp41 cluster I-specific 4B3 captured fewer virions than its 7B2 counterpart ($41.5 \pm 3.1\%$ vs $49.7 \pm 2.0\%$, $p < 0.05$).

The panel of IgG antibodies were ranked according to the proportions of virions that they captured. This ranking indicated that IgG antibodies binding to gp120 V1/V2 and V3/glycans appeared to present greater capacity to capture virions than those directed against the gp120 CD4bs and gp41 MPER-specific IgG antibodies; and those binding to the gp120 CD4bs than those targeting gp41 MPER (Table 4). Additionally, the nAb gp41 cluster I-specific 7B2 captured a higher quantity of virions ($49.7 \pm 2\%$) than the gp41 MPER-specific bNAbs 2F5 and 4E10 ($36.5 \pm 2\%$ and $28.8 \pm 5.1\%$, all $p < 0.005$) (Table 4). These results suggest

that although both antibody epitope specificity and accessibility might influence the ability of antibody to capture HIV-1 virions, antibody accessibility might be the predominant factor impacting on virion capture. Overall, IgG antibodies directed against the same group of epitopes presented similar capacities for virion capture, and antibody targeting readily accessible epitopes (e.g. gp120 V1/V2 and gp120 V3/glycans and gp41 cluster I), showed greater virion capture than those recognising occluded epitopes (e.g. gp120 CD4bs and gp41 MPER) [58, 457].

Study of the proportion of virions captured by the IgA panel indicates that antibody structure and specificity influenced the amount of virions captured. It was observed that the CD4bs-specific b12 dIgA₂, CH31 dIgA₂ and dIgA₁ captured comparable levels of virions (Figure 20). Likewise, the CD4bs-specific b12 mIgA₂ and CH31 mIgA₂ captured a similar amount of virions. However, b12 dIgA₂ captured a significantly greater proportion of virions than b12 mIgA₂ ($38.1 \pm 1\%$ vs $27.8 \pm 1.3\%$, $p < 0.005$); CH31 dIgA₂ and dIgA₁ captured more virions than CH31 mIgA₂ ($34.5 \pm 0.8\%$ and $34.1 \pm 0.6\%$ vs $29.1 \pm 1.9\%$, $p < 0.05$) and 7B2 dIgA₂ captured more virions than 7B2 mIgA₂ ($29.1 \pm 1.9\%$ vs $14.9 \pm 3.4\%$, $p < 0.0001$). This indicates that antibody structure (monomeric vs dimeric) impacted on the quantity of virions captured. Additionally, the ranking of IgA bNAbs according to their ability to capture virions suggest that dIgA bNAbs capture more virions than mIgA bNAbs (Table 5). Nevertheless, no significant differences were observed between the proportions of virions captured by 2F5 mIgA₂ and 2F5 dIgA₂, which suggests that the impact of antibody structure on antibody virion capture function was also influenced by antibody epitope specificity. Moreover, the ranking of IgA bNAbs suggests that gp120 CD4bs-specific IgA (b12 and CH31) bNAbs captured more virions than gp41 MPER-specific IgA (2F5) bNAbs, which further indicates that epitope accessibility might be an important factor influencing antibody virion capture.

Together, these findings suggest that epitope accessibility is the dominant factor influencing IgG virion capture with antibody binding to readily accessible epitope (e.g. gp120 V3/glycans and gp120 V1/V2) presenting enhanced virion capture over those targeting occluded epitopes (e.g. gp41 MPER). However, for IgA both antibody structure and accessibility appear to modulate the ability to capture virions, with dIgA antibodies presenting greater ability to capture virions than their mIgA₂ counterparts of identical specificity.

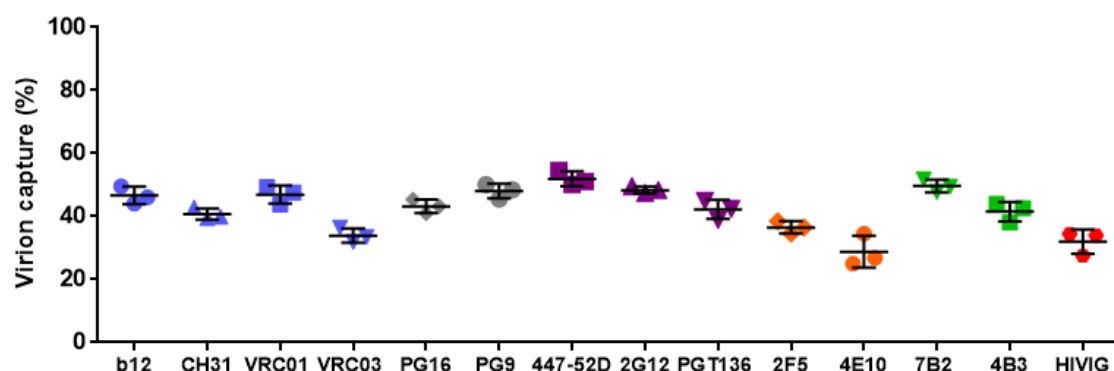


Figure 19. Virion capture by a panel of HIV-1 Env-specific IgG antibodies. The proportion of HIV-1_{BaL} virions captured by a panel of IgG antibodies was measured using the antibody virion capture assay and quantified by p24 ELISA. The panel of 14 IgG antibodies were grouped into six categories according to their Env epitope specificities: gp120 CD4bs (blue), gp120 V1/V2 (grey), gp120 V3 /glycans (purple), gp41 MPER (orange), gp41 cluster I (green) and several epitopes (red) for the HIVIG polyclonal antibody. Percentages of virion captured are shown as means $\pm$ SEM (n=3) of three independent experiments. Comparison analyses were performed with two-way ANOVA-Tukey's Multiple Comparison Test (MCT) with 95%CI.

Table 4. Ranking of the virus capture capacity of a panel of HIV-1 Env-specific IgG antibodies

IgG	Epitope specificity	Virion capture (%)
447-52D	gp120 V3 /glycans	51.9 $\pm$ 2.4
PGT136	gp120 V3 /glycans	50.9 $\pm$ 3.6
7B2	gp41 cluster I	49.7 $\pm$ 2
2G12	gp120 V3 /glycans	48.3 $\pm$ 1.1
PG9	gp120 V1/V2	48.1 $\pm$ 2.3
VRC01	gp120 CD4bs	46.9 $\pm$ 2.9
b12	gp120 CD4bs	46.6 $\pm$ 2.8
PG16	gp120 V1/V2	43.2 $\pm$ 2.2
4B3	gp41 cluster I	41.5 $\pm$ 3.1
CH31	gp120 CD4bs	40.8 $\pm$ 1.8
2F5	gp41-MPER	36.5 $\pm$ 2
VRC03	gp120 CD4bs	33.9 $\pm$ 2.3
HIV-IG	Polyclonal	32.1 $\pm$ 3.8
4E10	gp41-MPER	28.8 $\pm$ 5.1

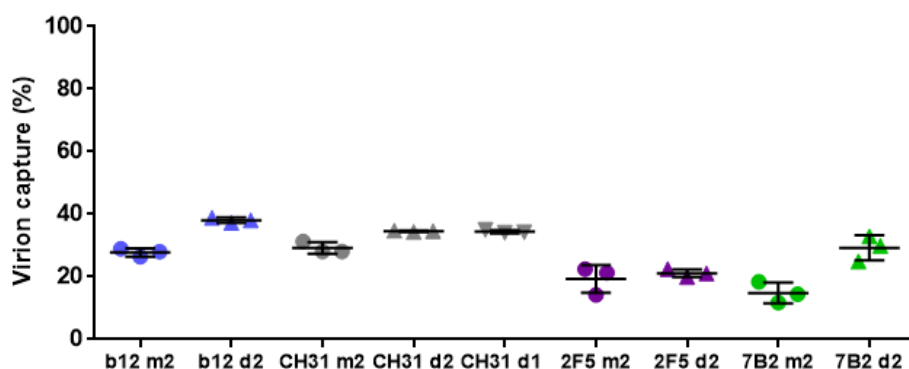


Figure 20. Virion capture by a panel of HIV-1 Env-specific IgA antibodies. The proportion of HIV-1_{BaL} virions captured by a panel of IgA antibodies was measured using the antibody virion capture assay and quantified by p24 ELISA. The panel of 9 IgA bNAbs comprised of: the mIgA₂ (m2) and dIgA₂ (d2) isoforms of b12, CH31, 2F5 and 7B2 and the dIgA₁ (d1) isoform of CH31 which were grouped into three categories according to their Env epitope specificities: gp120 CD4bs (blue and grey), gp41 MPER (purple), gp41 cluster I (green). Percentage of virions captured are shown as means $\pm$ SEM (n=3) of three independent experiments. Comparison analyses were performed with two-way ANOVA-Tukey's MCT with 95%CI.

Table 5. Ranking of the virus capture capacity of a panel of HIV-1 Env-specific IgA antibodies

IgA	Epitope specificity	Virion capture (%)
b12 dIgA₂	gp120 CD4bs	38.1 $\pm$ 1
CH31 dIgA₂	gp120 CD4bs	34.5 $\pm$ 0.8
CH31 dIgA₁	gp120 CD4bs	34.1 $\pm$ 0.6
7B2 dIgA₂	gp41 Cluster I	29.4 $\pm$ 4
CH31 mIgA₂	gp120 CD4bs	29.1 $\pm$ 1.9
b12 mIgA₂	gp120 CD4bs	27.8 $\pm$ 1.3
2F5 dIgA₂	gp41-MPER	21.2 $\pm$ 1.3
2F5 mIgA₂	gp41-MPER	19.2 $\pm$ 4.4
7B2 mIgA₂	gp41 Cluster I	14.9 $\pm$ 3.4

Analysis of the TZM-bl neutralisation assay revealed that each IgG of the panel presented a unique neutralisation profile (Figure 21). Notably, it was observed that b12 (gp120 CD4bs-specific) and 447-52D (gp120 V3-specific) neutralised virtually all the iVirions in the entire antibody concentration range tested. Conversely, nNAbs targeting the occluded gp41 cluster I (4B3 and 7B2) did not neutralise HIV-1_{BaL}.

To better compare the differential abilities of the panel of IgG antibodies to neutralise HIV-1_{BaL} virions and to assess how this antiviral function might be influenced by epitope specificity, the percentages of neutralisation of the panel of 14 HIV-1 Env-specific IgG antibodies, were calculated by non-linear regression for the same antibody concentration used in the virion capture experiment (10µg/mL).

The results indicate that IgG antibodies that bound to the same category of epitope, presented significantly different neutralising activities (Figure 22). The four gp120 CD4bs b12, VRC01, CH31 and VRC3 bNAbs presented distinct neutralisation activities at the antibody concentration of 10µg/mL ($100\pm0.02\%$, $93.9\pm0.9\%$, $52.5\pm3.4\%$ and $85.7\pm1.4\%$, respectively, all $p<0.05$). Among the gp120 CD4bs antibodies, CH31 presented the lowest neutralisation activity compared to b12, VRC01 and VRC03 (all $p<0.0001$) and b12, the greatest neutralisation activity (all $p<0.05$). Likewise, the gp120 V1/V2-specific IgG antibodies, PG16 and PG9 neutralised significantly different amounts of infectious virions ($46.6\pm0.3\%$ vs $88.4\pm1.6\%$, $p<0.0001$). Additionally, the V3 specific antibody 447-52D ($99.2\pm0.03\%$) presented a considerable greater neutralisation activity than its counterparts of the same epitope group, 2G12 and PGT136 which had similar neutralisation activities ($49\pm3.0\%$ and $50.5\pm3\%$, both $p<0.0001$). In contrast, IgG antibodies targeting the gp41-MPER (2F5 and 4E10) presented comparable neutralising potencies, and those recognising the gp41 cluster I (4B3 and 7B2) did not neutralise. It was observed that the polyclonal HIV-IG presented the lowest neutralising activity among all the IgG antibodies tested except 4E10, 4B3 and 7B2.

Therefore, these data indicate that IgG antibodies directed against the same type of epitope presented distinct neutralising activities as observed with IgG antibodies recognising the gp120 CD4bs, gp120 V1/V2, gp120 V3/glycans and gp41 cluster I classes of epitopes. This suggests that epitope specificity was the major factor influencing neutralisation. However, the ranking of the panel of IgG antibodies according to their neutralisation activity indicate that IgG bNAbs targeting gp120 sites (CD4bs, V1/V2 and

V3/glycans) presented greater neutralisation activity than those targeting gp41 (MPER and cluster I) and the polyclonal HIV-IG (Table 6). These data suggest that epitope accessibility influences neutralisation potency as MPER and cluster 1 epitopes are occluded in the pre-fusion conformation of Env^[457-459].

Comparison of the neutralisation curves for the panel of HIV-1 Env-specific IgA antibodies indicate that mIgA₂ and dIgA₂ bNAbs with the same epitope specificity, presented comparable neutralisation curves across the range of antibody concentration tested, except for b12 dIgA₂ which appeared to neutralise a higher proportion of iVirions than its mIgA₂ equivalent (Figure 23). Analysis also revealed that while the CD4bs-specific b12 and CH31 mIgA and dIgA antibodies presented distinct neutralisation potencies, 2F5 mIgA₂ and 2F5 dIgA₂ showed comparable neutralisation activities as well as 7B2 mIgA₂ and 7B2 dIgA₂ which did not neutralise (Figure 24).

Moreover, ranking of the panel of IgA bNAbs according to their neutralisation activities (b12 IgA>>CH31 IgA>2F5 IgA>7B2 IgA) indicates that epitope specificity is a dominant determinant of IgA-mediated neutralisation (Table 7). Furthermore, it was observed that b12 dIgA₂ neutralised significantly more virions than its mIgA₂ counterpart ($98.4\pm0.57\%$ vs $71.5\pm0.21\%$, $p<0.0001$). Similarly, CH31 dIgA₂ and dIgA₁ presented greater neutralisation potencies than their mIgA₂ counterparts ($33.6\pm2.5\%$ and $31.7\pm0.7\%$, respectively, vs $26.1\pm2.5\%$, $p<0.01$). This suggests that the IgA molecule structure (monomeric vs dimeric) influenced the neutralisation activity, and that the dimeric conformation of dIgA molecule may confer advantages for neutralising virions. Nonetheless, greater differences were observed between IgA antibodies targeting different epitopes than between IgA antibodies binding to the same epitope and of different structure. Together, these results indicate that although IgA structure, as well as epitope accessibility, influenced neutralisation potency, epitope specificity appeared to be the predominant factor impacting on neutralisation activity among the panel of IgA antibodies.

Thus, these results were relatively different from those of the VCA. While epitope accessibility was the main factor influencing IgG and IgA antibodies virion capture, epitope specificity was the predominant parameter influencing neutralisation. These data suggest that antibody structure (monomeric vs dimeric) has a significant impact on IgA capacity to capture and neutralise iVirions, with greater antibody function observed for dIgA antibodies compared to their mIgA antibodies equivalents.

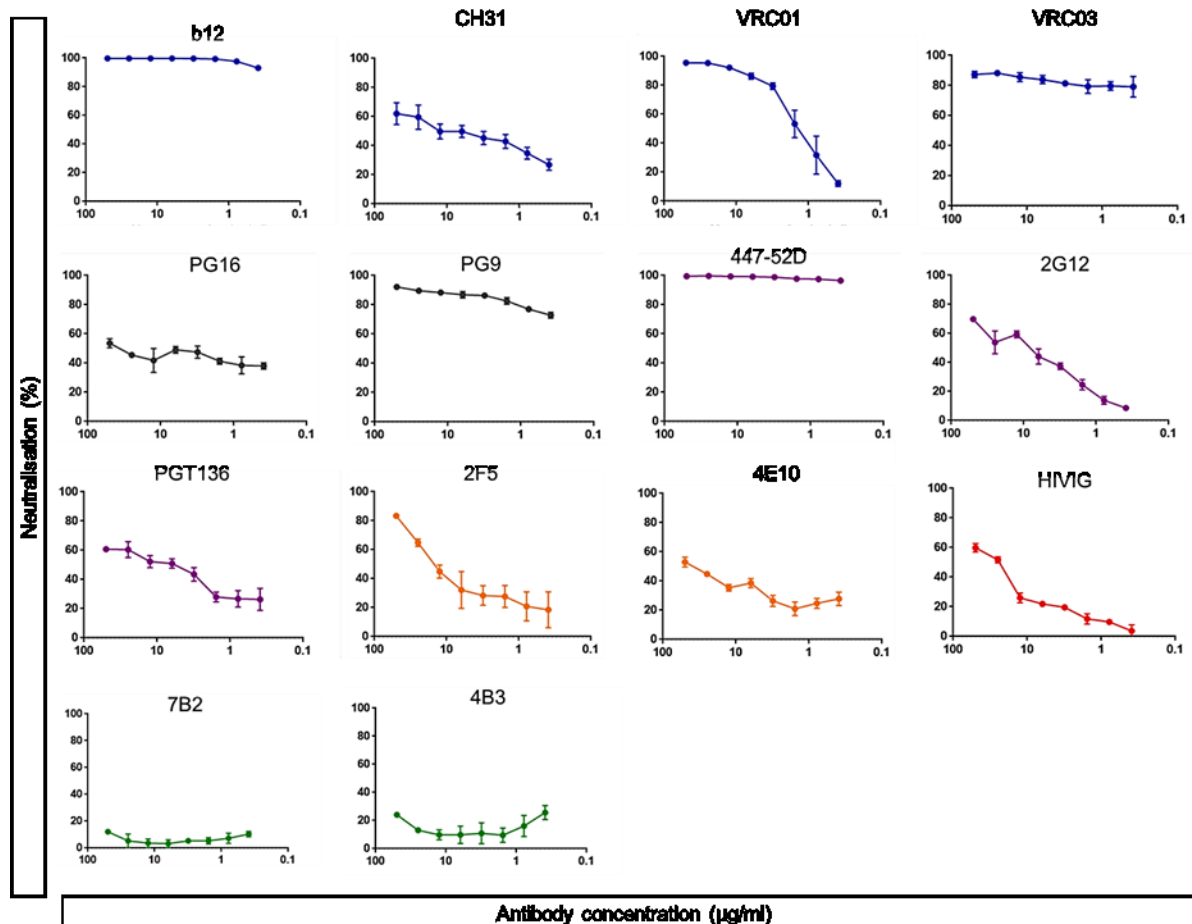


Figure 21. Neutralisation curves of a panel of HIV-1 Env-specific IgG antibodies. Neutralisation of HIV-1_{BaL} infection of TZM-bl cells by a panel of HIV-1 Env-specific IgG antibodies measured by TZM-bl assay. The data presented are obtained from three independent experiments and each sample was tested in triplicate. The panel of 14 IgG antibodies were divided into six categories according to their Env epitope specificities: gp120 CD4 binding sites (blue), V1/V2 (grey), gp120 V3/glycan (purple), gp41 Membrane Proximal External Region (MPER), (orange), gp41 cluster I (green) and several epitopes (red) for the HIVIG polyclonal antibody.

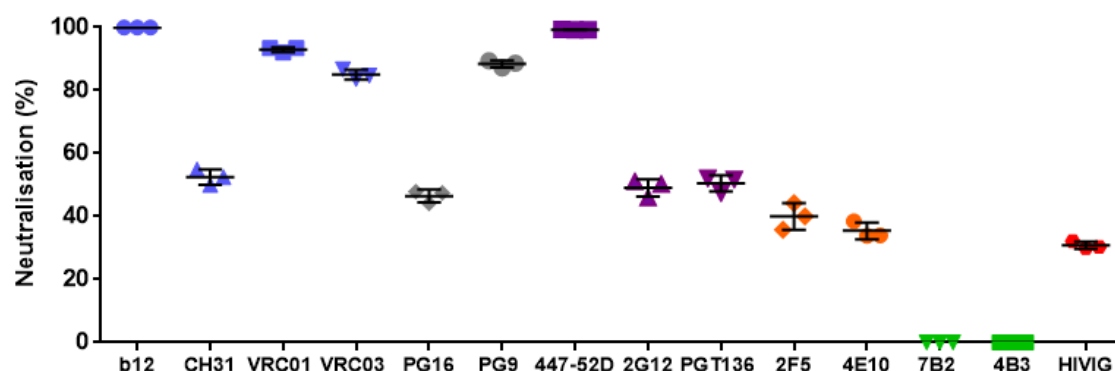


Figure 22. Neutralisation activities of a panel of HIV-1 Env-specific IgG antibodies. The percentages of neutralisation against HIV-1_{BaL} infection (10µg/mL antibody) were calculated by non-linear regression of the neutralisation curves. The 14 IgG antibodies of the panel were divided into six categories according to their epitope specificity: gp120 CD4bs (blue), V1/V2 (grey), gp120 V3/glycans (purple), gp41 MPER (orange), gp41 cluster I (green) and several epitopes (red) for the HIVIG polyclonal antibody. The percentage of neutralisation is shown as means $\pm$ SEM (n=3) of three independent experiments. Comparison analyses were performed with two-way ANOVA-Tukey's MCT with 95%CI.

Table 6. Ranking of the neutralisation activity of a panel of HIV-1 Env-specific IgG antibodies.

IgG	Epitope specificity	Neutralisation (%)
b12	gp120 CD4bs	100 $\pm$ 0.02
447-52D	gp120 V3/glycans	99.2 $\pm$ 0.03
VRC01	gp120 CD4bs	93.9 $\pm$ 2.4
PG9	gp120 V1/V2	88.4 $\pm$ 1.6
VRC03	gp120 CD4bs	85.7 $\pm$ 1.4
CH31	gp120 CD4bs	52.5 $\pm$ 3.4
PGT136	gp120 V3/glycans	50.5 $\pm$ 3
2G12	gp120 V3/glycans	49 $\pm$ 3
PG16	gp120 V1/V2	46.6 $\pm$ 0.3
2F5	gp41-MPER	40 $\pm$ 2.8
4E10	gp41-MPER	34 $\pm$ 3.3
HIV-IG	Polyclonal	30.9 $\pm$ 1.3
7B2	gp41 linear epitope	0
4B3	gp41 linear epitope	0

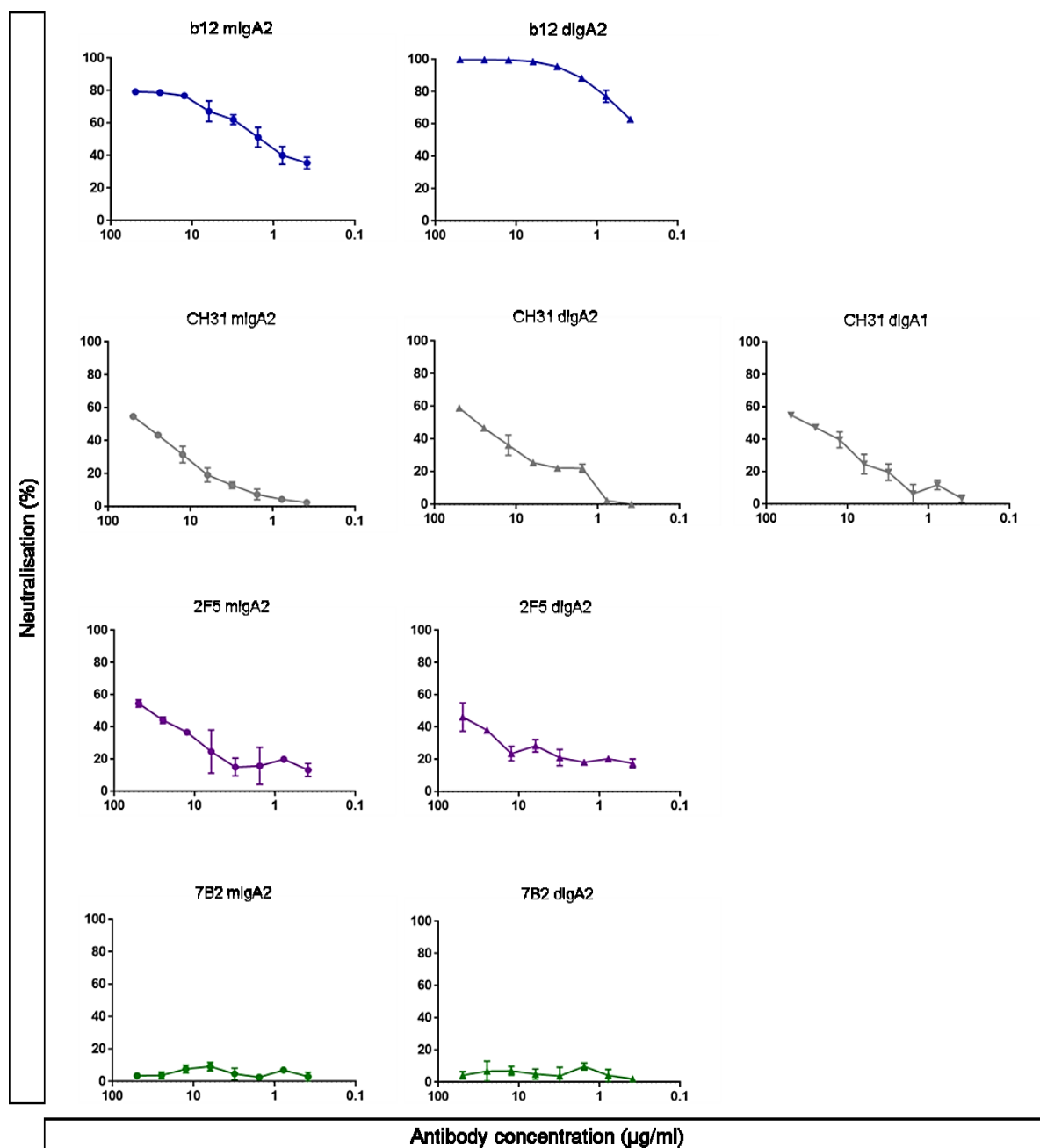


Figure 23. Neutralisation curves of the panel of HIV-1 Env-specific IgA antibodies.
Neutralisation of HIV-1_{BaL} infection of TZM-bl cells by a panel of HIV-1 Env-specific IgA antibodies measured by TZM-bl assay. The data presented are obtained from three independent experiments with each sample tested in triplicate. The panel of 9 IgA antibodies comprised of the mIgA₂ and dIgA₂ isoforms of b12, CH31, 2F5 and 7B2 and the dIgA₁ isoform of CH31 which were divided into three categories according to their Env epitope specificity: gp120 CD4bs (blue and grey), gp41 MPER (purple), gp41 cluster I (green).



Figure 24. Neutralisation activities of a panel of HIV-1 Env-specific IgA antibodies. The percentages of neutralisation against HIV-1_{BaL} infection were calculated by non-linear regression of the neutralisation curves for each IgA antibody in the panel for a concentration of 10µg/mL. The panel of 9 IgA bNAbs comprises the mIgA₂ and dIgA₂ isoforms of b12, CH31, 2F5 and 7B2 and the dIgA₁ isoform of CH31, which were grouped into three categories according to their Env epitope specificity: gp120 CD4bs (blue and grey), gp41 MPER (purple), gp41 cluster I (green).

Table 7. Ranking of the neutralisation activity of a panel of HIV-1 Env-specific IgA antibodies

IgA	Epitope specificity	Neutralisation (%)
b12 dIgA₂	gp120 CD4bs	98.4±0.6
b12 mIgA₂	gp120 CD4bs	71.5±0.2
CH31 dIgA₂	gp120 CD4bs	33.6±2.5
CH31 dIgA₁	gp120 CD4bs	31.7±0.7
CH31 mIgA₂	gp120 CD4bs	26.1±2.5
2F5 dIgA₂	gp41 MPER	23±1.9
2F5 mIgA₂	gp41 MPER	19.3±1.2
7B2 dIgA₂	gp41 PID	0
7B2 mIgA₂	gp41 PID	0

3.2.2 Relationship between antibody virion capture and neutralisation

The second aim of this chapter was to study the relationship between antibody ability to capture virions and to neutralise iVirions. For this purpose, Spearman r correlation analyses were performed between the proportions of virions captured, and the percentage of neutralisation by the panel of 12 IgG bNAbs and 7 IgA bNAbs, for a concentration of 10 μ g/mL of antibody. The analyses show that virion capture and neutralisation significantly correlated with a greater amount of virions captured, resulting in higher neutralisation activity for both IgG and IgA antibody panels (all, $p < 0.05$) (Figure 25).

Interestingly, two distinct groups of IgG antibodies could be observed along the non-linear regression line, (Figure 25A). The first group on the left side comprises seven IgG antibodies: HIV-IG (polyclonal), 4E10 and 2F5 (gp41 MPER-specific), PG16 (gp120 V1/V2-specific), 2G12 and PGT136 (gp120 V3/glycans-specific), and CH31 (gp120 CD4bs-specific). The second group of IgG on the right side includes five IgG bNAbs: the gp120 V1/V2-specific PG9; gp120 V3-specific 447-52D; and the gp120 CD4bs-specific VRC03, VRC01 and b12 (Figure 25A). Remarkably, while the first group of IgG antibodies appears to neutralise similar proportion of captured virions, the second group of IgG bNAbs appears to neutralise about twice more iVirions than they captured. Similarly, two groups of IgA antibodies could be observed along the non-linear regression line (Figure 25B). On the left side, the first group comprises CH31 (gp120 CD4bs-specific) and 2F5 (gp41 MPER-specific) mIgA and dIgA antibodies which neutralised comparable amounts of virions to those captured. On the right side, the second includes b12 mIgA₂ and b12 dIgA₂ (gp120 CD4bs-specific) which appears to neutralise over twice more virions than they captured.

Taken together, these results indicate that the second group of IgG and IgA antibodies might present the ability to selectively capture iVirions over dVirions, and/or to neutralise efficiently the iVirions that they captured. In contrast, the first group of IgG and IgA antibodies might not be selective, and capture indistinguishably iVirions and dVirions, and/or do not efficiently neutralise iVirions captured. Therefore, these results suggest that the ratio neutralisation (%) / Virion capture (%) might enable to quantify the selective ability of an antibody to capture iVirions and to neutralise them, compared to capture of dVirions, and/or to capture iVirions without efficiently neutralising them.

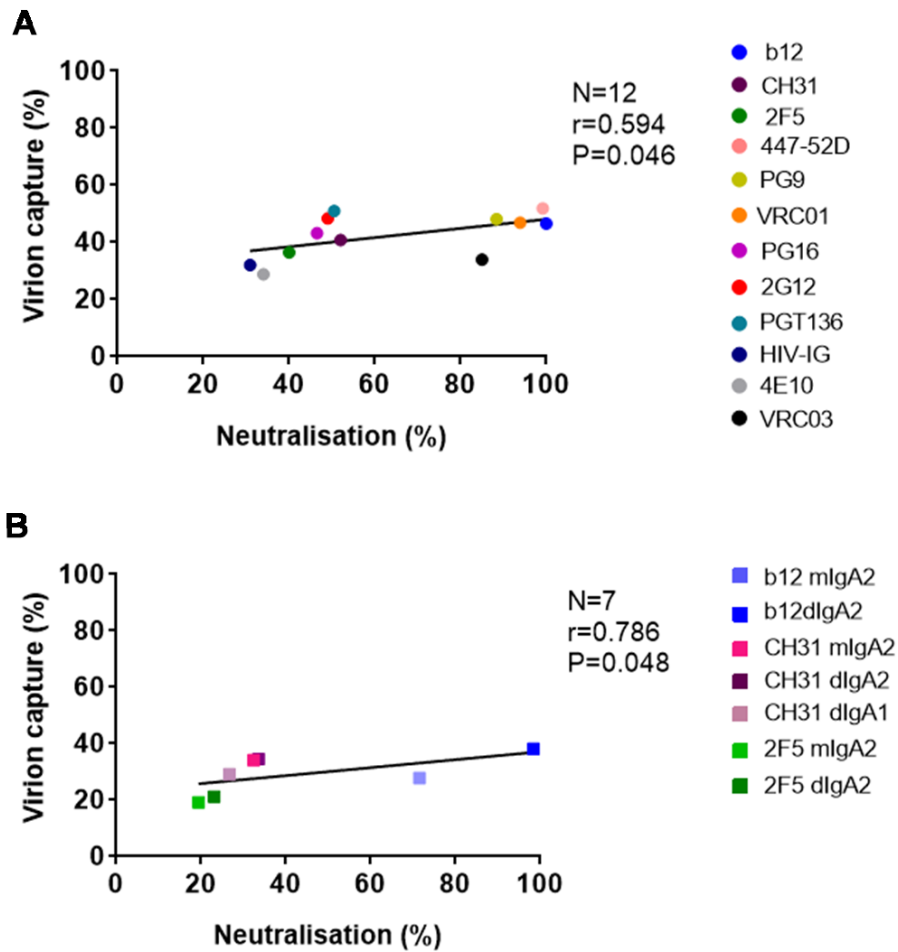


Figure 25. Correlation analysis between virion capture and neutralisation. Spearman r correlation analysis was performed between the proportion of virion captured and of infectious virions neutralised by the panel of HIV-1 Env-specific IgG bNAbs (A) and by the panel of IgA bNAbs (B). The first panel comprises 12 IgG bNAbs recognising distinct HIV-1 Env epitopes: gp120 CD4bs (b12, CH31, VRC01 and VRC03), gp120 V1/V2 (PG9 and PG16), gp120 V3/glycans (2G12, PGT136 and 447-52D), gp41 MPER (2F5 and 4E10) and several epitopes (the polyclonal HIV-IG). The second panel includes 9 IgA bNAbs: the mIgA₂ and dIgA₂ isoforms of b12, CH31, 2F5 and 7B2 and the dIgA₁ isoform of CH31. N=number of antibodies, r = coefficient of correlation and P= exact p-value.

Based on the above observations, an antibody virion capture neutralisation index (AVCNI) was devised to assess the ability of a bNAb to efficiently neutralise iVirions captured, and/or to selectively capture iVirions over dVirions. It was postulated that an AVCNI >1 indicates a high capacity to neutralise iVirions captured, and/or a preference for capturing and neutralising iVirions over dVirions; an AVCNI=1 shows a lack of efficient neutralisation, and/or no selective preference between iVirions or dVirions; an AVCNI <1 designates a poor neutralisation capacity, and/or a preference for dVirions; and an AVCNI=0 signifies no neutralisation activity and/or no iVirions capture. It was thought that this index would be an effective tool for the selection of bNAbs presenting the best abilities to prevent HIV-1 infection. Using this index, the best bNAb would be the one presenting the highest AVCNI value, and which is able to neutralise 100% of iVirions. This would indicate that this bNAb, by capturing the lowest amount of virions, achieves the highest neutralisation activity. This would imply that this bNAb presents a high selectivity for iVirions over dVirions and efficiently neutralises all the iVirions captured and present in solution.

The results of this analysis show that PG16 (gp120 V1/V2-specific), 2F5 (gp41 MPER-specific), HIV-IG (polyclonal), and the two gp120-specific glycans IgG antibodies, 2G12 and PGT136, presented an AVCNI of about 1 (Figure 26A). This indicates that they were not able to efficiently neutralise the iVirions that they captured and/or had no preferential capture of iVirions compared to dVirions (Figure 26A). Conversely, the gp120 CD4bs-specific VRC03, b12 and VRC01, as well as the gp120 V1/V2-specific PG9, and the gp120 V3-specific 447-52D presented significantly greater AVCNI values than the aforementioned IgG antibodies (AVCNI>1). This implies that they efficiently neutralised the iVirions that they captured and/or strongly selected iVirions compared to dVirions (all $p < 0.05$). Additionally, it was observed that CH31 and 4E10 had an AVCNI slightly above 1 (1.28 ± 0.01 and 1.26 ± 0.4 , respectively,) which suggests a weaker neutralisation efficiency compared to the IgG antibodies mentioned above and/or a modest preference for iVirions over dVirions. Moreover, the gp41 cluster I-specific IgG antibodies, 7B2 and 4B3, present AVCNI values of 0 which is in agreement with the fact they are nNAbs (Table 8).

The ranking of the panel of IgG antibodies, as a function of their AVCNI, indicates that VRC03 and b12 presented the highest AVCNI (2.52 ± 0.28 and 2.14 ± 0.18 , respectively) (Table 8). However, while b12 (gp120 CD4bs-specific) was able to neutralise virtually all the

iVirions present ($100\pm0.02\%$), VRC03 (gp120 CD4bs-specific) neutralised just fewer than 90% of the iVirions ($85.7\pm1.4\%$) for an antibody concentration of $10\mu\text{g/mL}$ ($p<0.0001$) (Table 7). This suggests that of our panel of IgG antibodies, b12 was the most potent bNAb and presented the best ability to prevent HIV-1_{BaL} infection of TZM-bl cells. Moreover, it appears from this ranking that overall IgG antibodies targeting the CD4bs presented the highest AVCNI. This highlights the importance of epitope specificity in prevention of viral infection, and is in accord with the key role of the CD4bs on infectivity^[63, 287].

Comparisons of the AVCNI of the panel of IgA antibodies revealed that whereas b12 mIgA₂ and b12 dIgA₂ (gp120 CD4bs-specific) presented an AVCNI well above 1, CH31 (gp120 CD4bs-specific) and 2F5 (gp41 MPER-specific) mIgA and dIgA presented an AVCNI of around 1 (Figure 26B). This indicates that b12 IgA antibodies were able to efficiently neutralise iVirions captured and/or presented a high selectivity for iVirions compared to dVirions. Conversely, CH31 and 2F5 IgA antibodies had a low neutralisation capacity and/or no selectivity for iVirions over dVirions (Figure 26B). Furthermore, it was observed that whereas b12 dIgA₂ presented a significantly greater ability to capture virions and neutralise iVirions than b12 mIgA₂; b12 dIgA₂ and b12 mIgA₂ had a comparable AVCNI, which indicates that they presented identical capacity to neutralise iVirions captured and/or selectivity for iVirions. Similarly, whereas CH31 dIgA₂ and CH31 dIgA₁ captured a greater proportion of virions than CH31 mIgA₂, they all showed comparable AVCNI. Additionally, 2F5 mIgA₂ and 2F5 dIgA₂ which presented similar abilities to capture and neutralise iVirions, also presented comparable AVCNI values. Overall, this indicates that IgA structure (monomeric vs dimeric) does not influence IgA ability to select iVirions over dVirions, and/or to efficiently neutralise iVirions captured.

The ranking of the panel of IgA antibodies according to their AVCNI shows that b12 mIgA₂ and b12 dIgA₂ presented the highest AVCNI (Table 9). Nonetheless, previous analysis showed that for a same antibody concentration, b12 dIgA₂ presented a greater neutralisation activity than its mIgA₂ counterpart ($98.4\pm0.6\%$ vs $71.5\pm0.2\%$, $p<0.0001$). Consequently, this indicates that b12 dIgA₂ was the most potent IgA antibodies from the panel of IgA bNAbs tested. Additionally, although b12 and CH31 bNAbs bind to the CD4bs, they presented significantly different AVCNI values. This supported the idea that epitope

specificity is the predominant factor influencing antibody ability to efficiently neutralise iVirions captured, and/or to select iVirions over dVirions.

Therefore, the comparisons of the AVCNI of the panel of IgG and IgA antibodies indicate that b12 IgG and b12 dIgA₂ were the most potent bNAbs of our antibody panels. These results suggest that epitope specificity was the predominant factor impacting on the AVCNI. Additionally, this indicates that the devised AVCNI is an effective tool to quantify antibody ability to neutralised iVirions captured and/or selectively capture iVirions compared to dVirions; and therefore to select bNAbs that present the best abilities to prevent HIV-1 infection. Finally, the findings of this research imply that antibody isotype may impact on antibody function as IgG and dIgA antibodies recognising the same epitope appeared to capture and neutralise different proportions of virions. In light of these results, subsequent analyses aimed to compare the antiviral abilities of IgG and IgA bNAbs presenting the same epitope specificity.

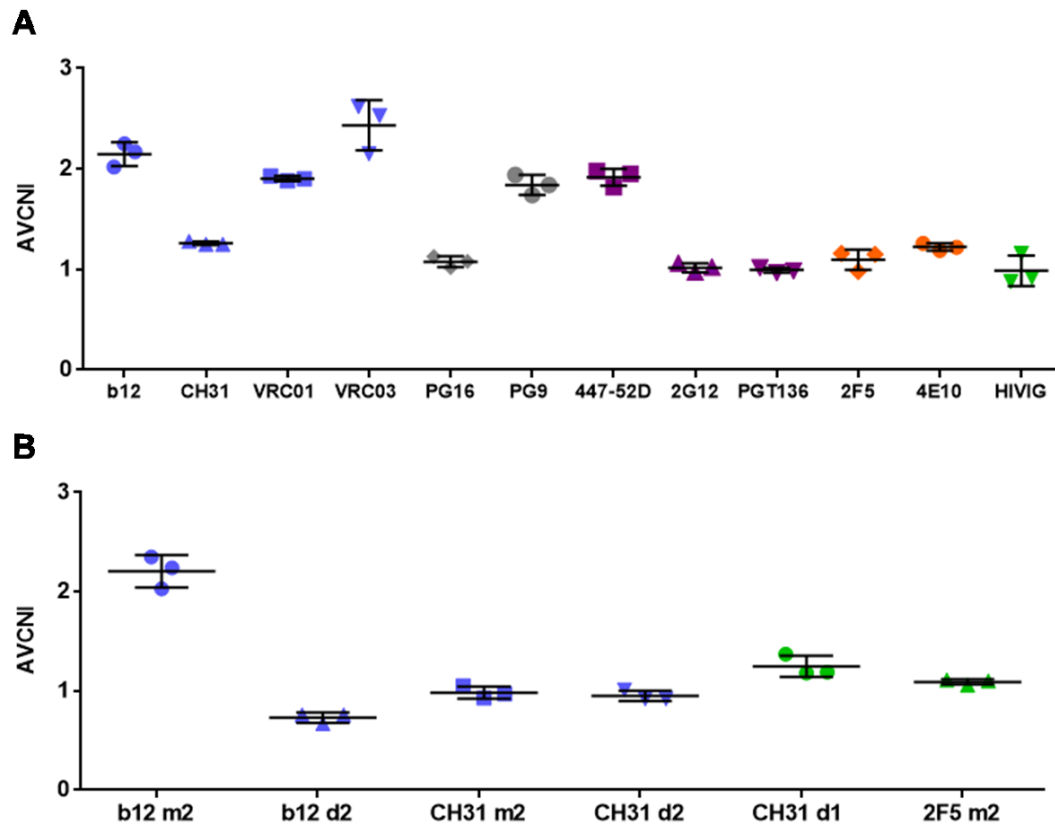


Figure 26. Comparisons of the AVCNI of a panel of HIV-1 Env-specific IgG and IgA antibodies. The Antibody Virion Capture Neutralisation Index (AVCNI) of a panel of 12 IgG (A) and 9 IgA (B) antibodies was calculated using the ratio neutralisation (%)/virion capture (%), of HIV-1_{BaL} virions, for an antibody concentration of 10µg/mL. The first panel comprises 12 IgG bNAbs recognising distinct HIV-1 Env epitopes: gp120 CD4bs (b12, CH31, VRC01 and VRC03), gp120 V1/V2 (PG9 and PG16), gp120 V3/glycans (2G12, PGT136 and 447-52D), gp41 MPER (2F5 and 4E10) and several epitopes (the polyclonal HIV-IG). The second panel includes 9 IgA bNAbs: the mIgA₂ and dIgA₂ isoforms of b12, CH31, 2F5 and 7B2 and the dIgA₁ isoform of CH31. An AVCNI=1 indicates a lack of efficient neutralisation of iVirions captured and/or no selective preference between iVirions or dVirions, and an AVCNI <1 indicates a poor neutralisation capacity of iVirions captured and/or a preference for dVirions.

Table 8. Ranking of the panel of HIV-1 Env-specific IgG antibodies according to their AVCNI

IgG	Epitope specificity	AVCNI
VRC03	gp120 CD4bs	2.52±0.28
b12	gp120 CD4bs	2.14±0.18
VRC01	gp120 CD4bs	1.91±0.03
447-52D	gp120 V3 /glycans	1.89±0.09
PG9	gp120 V1/V2	1.85±0.16
CH31	gp120 CD4bs	1.28±0.01
4E10	gp41-MPER	1.26±0.4
PG16	gp120 V1/V2	1.1±0.1
2F5	gp41-MPER	1.09±0.08
HIV-IG	Polyclonal	1.0±0.19
2G12	gp120 V3/glycans	1.0±0.05
PGT136	gp120 V3/glycans	1.0±0.02
7B2	gp41 cluster I	0
4B3	gp41 cluster I	0

Table 9. Ranking of the panel of HIV-1 Env-specific IgA antibodies according to their AVCNI

IgA	Epitope specificity	AVCNI
b12 dIgA₂	gp120 CD4bs	2.61±0.09
b12 mIgA₂	gp120 CD4bs	2.59±0.16
2F5 mIgA₂	gp41-MPER	1.13±0.05
2F5 dIgA₂	gp41-MPER	1.08±0.04
CH31 dIgA₂	gp120 CD4bs	0.99±0.08
CH31 dIgA₁	gp120 CD4bs	0.92±0.03
CH31 mIgA₂	gp120 CD4bs	0.88±0.02
7B2 dIgA₂	gp41 cluster I	0
7B2 mIgA₂	gp41 PID	0

3.2.3 Impact of isotype on antibody ability of to capture and neutralise HIV-1 virions

The third aim of this chapter was to examine the influence of antibody isotype on antiviral function. It was hypothesised that antibody isotype might influence antibody function, and that IgG, mIgA₂ and dIgA₁₋₂ antibodies recognising the same epitope may differ in their ability to capture virions and to neutralise iVirions. Furthermore, it was posited that the combination, at the ratio 1:1, of IgG and IgA (mIgA or dIgA) bNAbs, sharing the same epitope specificity, might alleviate antiviral outcomes due to competition for the same Env-binding sites. Thus, the impact of isotype on antiviral function was investigated, firstly, by comparing the abilities of IgG and IgA bNAbs, presenting the same epitope specificities, to capture and neutralise virions; secondly, by measuring the impact on antibody function of the combination of IgG and IgA bNAbs recognising the same epitope; and thirdly, by determining in what extent this might be related to competition between IgG and IgA antibodies binding to the same sites using competitive VCA.

Comparisons of the amounts of virions captured by b12, CH31, 2F5 and 7B2 IgG, mIgA₂ and dIgA₁₋₂ bNAbs, recognising the same epitopes, show that the gp120 CD4bs-specific b12 and CH31 IgG bNAbs, captured higher proportions of virions ($46.6 \pm 2.8\%$ and $40.8 \pm 1.8\%$, respectively) than their mIgA₂ ($27.8 \pm 1.3\%$, $p < 0.005$ and $29.1 \pm 1.9\%$, $p < 0.01$, respectively) and dIgA₂ counterparts ($38.1 \pm 1\%$, $p < 0.01$ and $34.5 \pm 0.8\%$, $p < 0.05$, respectively) (Figure 27). Similarly, the 2F5-IgG isotype (gp41 MPER-specific) captured nearly twice more virions ($36.5 \pm 2\%$) than its mIgA₂ ($19.2 \pm 4.4\%$) and dIgA₂ counterparts ($21.2 \pm 1.3\%$), (both, $p < 0.01$). Moreover, whereas the gp41 cluster I-specific, 7B2 IgG captured $49.7 \pm 2.1\%$ of virions, 7B2 mIgA₂ and 7B2 dIgA₂ captured only $14.9 \pm 3.4\%$ and $29.4 \pm 4\%$ of viral particles, respectively.

Thus, b12, CH31, 2F5 and 7B2 IgG bNAbs captured significantly more virions than their mIgA₂ and dIgA₂ counterparts. Taken together, this suggests that in addition to antibody structure, the isotype influences antibody virion capture capacity, with IgG antibodies presenting greater virion capture ability than their mIgA₂ and dIgA₁₋₂ equivalents.

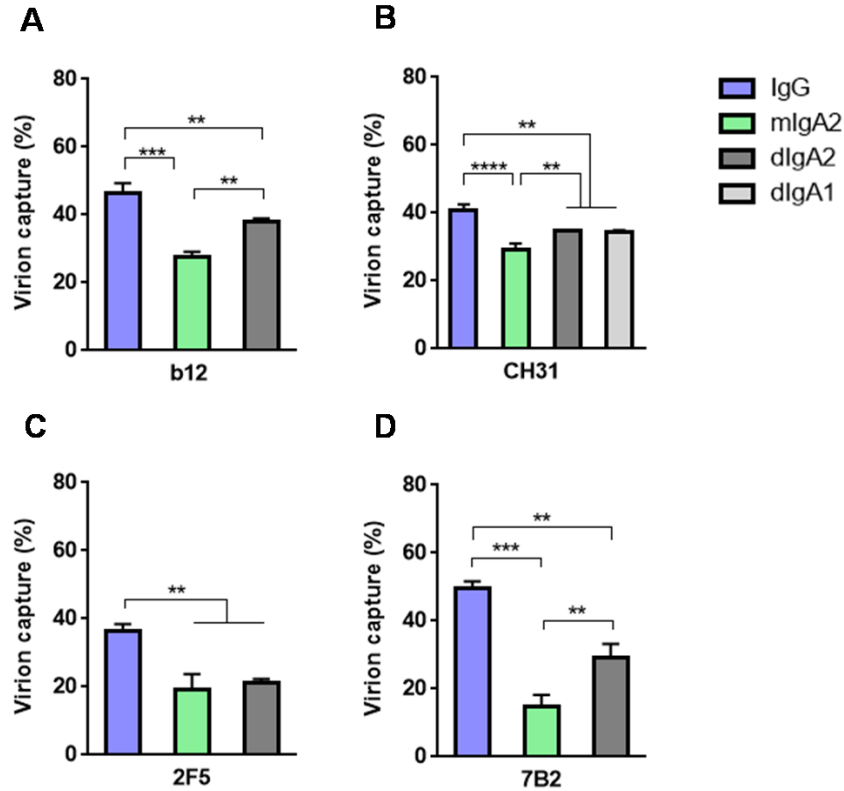


Figure 27. Comparisons of the virion capture ability of HIV-1 Env-specific IgG and IgA antibodies. IgG (blue), mIgA₂ (green) and dIgA₁₋₂ (grey) forms of b12 (A), CH31 (B), 2F5 (C) and 7B2 (D) antibodies, at a concentration of 10µg/mL, were incubated for 1h at 37°C, with purified HIV-1_{BaL} virions (500ng/mL). Antibodies were then immobilised on a Protein G HP SpinTrap or IgA Capture Resin columns and the amount of virions captured by each individual antibody was assessed by p24 ELISA. P-value results are presented as *p<0.05, **p<0.01, ***p<0.005, ****p<0.001.

To investigate the influence of isotype on antibody ability to neutralise iVirions the IgG and IgA isotypes of b12, CH31, 2F5 and 7B2 antibodies were compared for their capacity to inhibit HIV-1_{BaL} infection of TZM-bl indicator cells. The neutralisation curves of the gp120 CD4bs-specific b12 and CH31 bNAbs suggest that b12 and CH31 IgG antibodies presented greater ability to inhibit HIV-1_{BaL} infection of TZM-bl cells than their mIgA₂ and dIgA₁₋₂ counterparts (Figure 28). Similarly, the results indicate that the gp41 MPER-specific, 2F5 IgG presented a greater capacity to reduce HIV-1_{BaL} infection of TZM-bl cells than its mIgA₂ and dIgA₁₋₂ equivalents. In contrast, the data show that the gp41 cluster I-specific 7B2 IgG as well

as its mIgA₂ and dIgA₂ counterparts were not able to neutralise HIV-1_{BaL} virions which is in concordance with the fact that these bNAbs had been reported in the literature as being nNAbs^[436, 437] (Figure 28).

To better compare the effectiveness of the IgG, mIgA₂ and dIgA₁₋₂ isotypes of b12, CH31, 2F5 and 7B2 antibodies to neutralise HIV-1_{BaL} infection of TZM-bl cells, the neutralisation percentages reached by each mAb for a concentration of 10µg/mL were calculated and compared (Table 10). As an antibody concentration of 10µg/mL was also used in the antibody VCA (Table 11), this enabled the study of the relationship between total virion capture and neutralisation for a same antibody concentration.

The gp120 CD4bs-specific b12 IgG presented a significantly greater neutralisation activity than its mIgA₂ counterpart, with 30% more neutralisation potency observed with b12 IgG (100±0.02%) compared to b12 mIgA₂ (71.5±0.2, $p<0.0001$) and b12 dIgA₂ (98.4±0.6%, $p<0.05$) (Table 10). Additionally, CH31 IgG (gp120 CD4bs-specific) neutralisation activity was twice higher (52.5±3.4%) than the neutralisation activities of its mIgA₂ (26.1±2.5%, $p<0.01$) and just under twice greater than those observed with its dIgA₂ (33.6±2.5%) and dIgA₁ (31.7±0.7%) equivalents (both $p<0.05$). Likewise, the gp41 MPER-specific 2F5 IgG neutralised twice more virions than its mIgA₂ and dIgA₂ equivalents (both $p<0.05$) (Table 10).

Therefore, b12, CH31 and 2F5 IgG antibodies presented considerably greater abilities to neutralise HIV-1_{BaL} than their mIgA₂ and dIgA₁₋₂ counterparts (Table 10). Taken together, these findings indicate that antibody structure and isotype influence antibody ability to neutralise iVirions, with IgG antibodies presenting a greater ability to inhibit HIV-1_{BaL} infection of TZM-bl cells than their mIgA₂ and dIgA₁₋₂ equivalents. Moreover, this further suggests that the dimeric structure might confer an advantage to neutralise iVirions compared to the monomeric structure, depending on the epitope targeted. This might compensate for the lower ability of the IgA isotype to neutralise HIV-1_{BaL} virions compared to the IgG isotype.

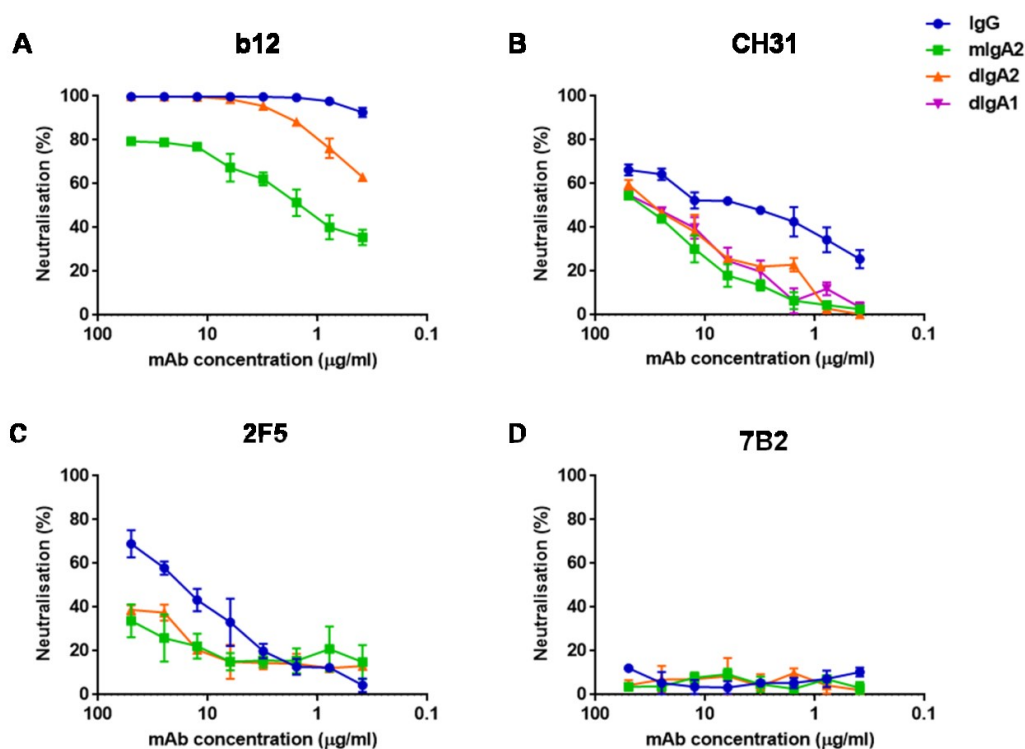


Figure 28. Neutralisation of HIV-1_{BaL} by IgG and IgA antibodies targeting the same epitope.

The IgG (blue), mIgA (green) and dIgA (orange) forms of b12 (A), CH31 (B), 2F5 (C) and 7B2 (D) antibodies were diluted with a two-fold serial dilution at a concentration starting from 50μg/mL and incubated for 1h at 37°C with purified HIV-1_{BaL} (500ng/mL). Antibody-virus mixtures were tested in a TZM-bl neutralisation assay. The percentages of neutralisation were calculated as a reduction in infectivity compared to the virus control. The data shown are representative results obtained from three independent experiments and samples were tested in triplicates.

Table 10. Neutralisation of HIV-1_{BaL} by a panel of HIV-1 Env-specific IgG and IgA antibodies

Antibody	Neutralisation (%) with 10μg/mL of antibody		
	IgG	mIgA ₂	dIgA ₂
b12	100±0.02	71.5±0.2	98.4±0.6
CH31	52.5±3.4	26.1±2.5	33.6±2.5 and 31.7±0.7*
2F5	40±2.8	19.3±1.2	23±1.9
7B2	ND	ND	ND

*Neutralisation (%) with 10μg/mL of antibody calculated for CH31 dIgA₁
 ND, Not Determined (as 7B2 is a non-neutralising antibody).

Table 11. Proportion of HIV-1_{BaL} virion captured by a panel of IgG and IgA antibodies

Antibody	Virion capture (%) with 10µg/mL of antibody		
	IgG	mIgA ₂	dIgA ₂
b12	46.6±2.8	27.8±1.3	38.1±1
CH31	40.8±1.8	29.1±1.9	34.5±0.8 and 34.1±0.6*
2F5	36.5±2	19.2±4.4	21.2±1.3
7B2	49.7±2.1	14.9±3.4	29.4±4

*Virion capture (%) with 10µg/mL of antibody calculated for CH31 dIgA₁

Based on the observation that isotype and structure influence antibody ability to capture and neutralise virions, the AVCNI of the panel of IgG and IgA antibodies was calculated, to assess the influence of isotype and structure on the ability to neutralise iVirions captured, and/or to selectively capture iVirions over dVirions. Interestingly, comparisons of the AVCNI for the panel of IgG and IgA bNAbs show that while the gp120 CD4bs-specific b12 IgG presented greater ability to capture and inhibit HIV-1 infection than its mIgA₂ and dIgA₂ counterparts, b12 mIgA₂ and dIgA₂ had a significantly greater capacity to neutralise iVirions captured and/or to selectively capture iVirions over dVirions than b12 IgG (both $p < 0.05$) (Table 12). In contrast, CH31 IgG (gp120 CD4bs-specific) which captured more virions and neutralised more iVirions than its IgA counterparts, also presented a significantly greater AVCNI compared to its IgA equivalents (all $p < 0.05$). Moreover, while 2F5 IgG (gp41 MPER-specific) presented greater abilities to capture virions and neutralise iVirions than its mIgA₂ and dIgA₂ equivalents; it had comparable AVCNI values than its IgA counterparts, (Table 12).

Overall, these data suggest that antibody isotype influence antibody ability to neutralise captured iVirions and/or to selectively capture iVirions, with IgG and IgA antibodies, recognising the same epitope, presenting distinct AVCNI. However, the results further indicate that the influence of isotype on the AVCNI might be also dependent on the epitope specificity. It was observed that antibody of distinct isotype and recognising the same epitope may present comparable AVCNI. Additionally, the predominant influence of the epitope specificity on the AVCNI was further supported, by the observation that the two gp120 CD4bs-specific bNAbs (b12 and CH31) presented considerably distinct AVCNI. Moreover, it was previously shown that the conformation of an antibody (monomeric vs

dimeric) might not impact on the AVCNI with mIgA and dIgA antibodies of identical specificity presenting similar AVCNI. In total, these results suggest that epitope specificity in a close relationship with antibody isotype might impact on the ability of antibody to neutralise captured iVirions and to selectively capture iVirions. Thus, the AVCNI might provide important insights into the selection of bNAbs which present the best potential to prevent HIV-1 infection.

Table 12. Antibody virion capture neutralisation index of the panel of IgG and IgA antibodies

Antibody	Antibody virion capture neutralisation index (AVCNI)		
	IgG	mIgA ₂	dIgA ₂
b12	2.14±0.18	2.59±0.16	2.61±0.09
CH31	1.28±0.01	0.88±0.02	0.99±0.08 and 0.92±0.03*
2F5	1.09±0.08	1.13±0.05	1.08±0.04
7B2	ND	ND	ND

*AVCNI calculated for CH31 dIgA₁

ND: Not determined as 7B2 is a non-neutralising antibody.

3.2.4 Combination of IgG and IgA antibodies sharing the same specificity

Based on the above observations, it was hypothesised that the presence of IgG and IgA (mIgA or dIgA) antibodies sharing the same epitope specificity, at the ratio 1:1, as can be found at the mucosal portal of viral entry might attenuate antiviral function against HIV-1 infection. Indeed, it was postulated that as IgG antibodies presented a greater ability to capture and neutralise HIV-1 virions than their IgA equivalents, the combination of IgG antibodies with IgA antibodies might mitigate IgG effector functions, due to competition between IgG and IgA antibodies for the same Env-binding sites.

To assess the impact of the mixture of IgG and IgA antibodies, sharing the same epitope specificity, on virion capture function, HIV-1_{BaL} virions were incubated with b12, CH31, 2F5 and 7B2 IgG, mIgA₂ and dIgA₁₋₂ antibodies either alone or in combinations of IgG and IgA (mIgA or dIgA) antibodies targeting the same epitope. It was observed that the

combination of IgG and mIgA₂ antibodies, binding to the same epitope, captured fewer virions than IgG antibodies alone, but more than mIgA₂ antibodies taken separately (Figure 29). Particularly, the gp120 CD4bs-specific b12 IgG captured significantly fewer virions combined with its mIgA₂ equivalent than alone ($38.2 \pm 1.8\%$ vs $46.6 \pm 2.8\%$, $p < 0.01$) and significantly higher proportions of virions than b12 mIgA₂ taken separately ($27.8 \pm 1.3\%$, $p < 0.005$), (Figure 29A). Interestingly, the proportions of virions captured by CH31, 2F5 and 7B2 IgG antibodies alone or combined with their dIgA₁₋₂ equivalents were not significantly different except for b12 IgG. While b12 IgG captured fewer virions when combined with its dIgA₂ equivalent ($41 \pm 1.4\%$ vs $46.6 \pm 2.8\%$, $p < 0.05$), b12 dIgA₂ captured similar quantity of virions alone or with b12 IgG.

Consequently, these data indicate that the combination of IgG and IgA (mIgA or dIgA) antibodies, sharing the same epitope specificity, might mitigate the overall amount of virion captured compared to IgG antibodies alone. Moreover, as overall, mIgA antibodies presented a lower ability to capture virions than their dIgA counterparts, the combination of IgG and mIgA antibodies, had a greater negative impact on the virion capture by IgG antibodies, compared to IgG and dIgA mixtures.

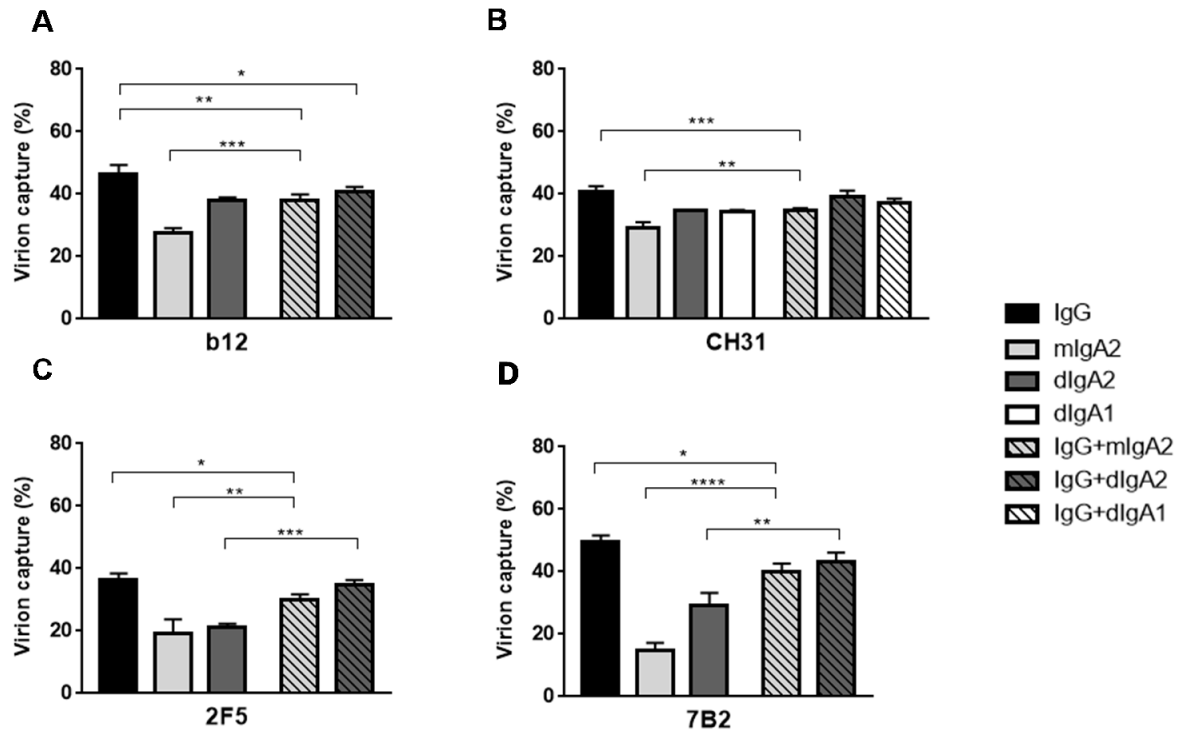


Figure 29. Virion capture by mixtures of IgG and IgA antibodies targeting the same epitope. b12 (A), CH31 (B), 2F5 (C) and 7B2 (D) IgG (black), mIgA₂ (light grey) and dIgA₂ (dark grey), (and dIgA₁ in white for CH31 only) antibodies were incubated either alone or in combinations with IgA (for IgG) or IgG (for IgA) antibodies, recognising the same epitope at equal concentration (10µg/mL) and with purified HIV-1_{BaL} virions (500ng/mL) for 1h at 37°C. Antibodies were then immobilised on a column containing both Protein G and IgA Capture Resin and the amount of virions captured by each individual antibody or combination of antibodies was assessed by p24 ELISA. Tukey's MCT using 95% CI was used, and p-value results were presented as *p<0.05, **p<0.01, ***p<0.005, ****p<0.001.

To determine whether the combination of IgG and IgA (mIgA or dIgA) antibodies sharing the same epitope specificity, reduces the neutralisation activity compared to IgG antibodies alone, TZM-bl neutralisation assay was performed with HIV-1_{BaL} and the panel of IgG, mIgA₂ and dIgA₁₋₂ isotypes of b12, CH31 and 2F5. Prior addition of the TZM-bl cells, HIV-1_{BaL} was incubated for an hour with the IgG, mIgA₂ and dIgA₁₋₂ either alone or in combinations of IgG and IgA (mIgA or dIgA) antibodies of identical specificity, at the ratio 1:1. Then, the level of inhibition of HIV-1_{BaL} infection by each antibody or antibody mixtures was measured as a reduction of RLU compared to the positive control with virus only and expressed as percentage of neutralisation (Figure 30). To compare virion capture to neutralisation for a same antibody concentration, the percentage of neutralisation obtained for each antibody for a concentration of 10µg/mL was also calculated (Table 13).

Interestingly, while the mixture of the gp120 CD4bs-specific b12 IgG with its mIgA₂ counterpart presented a significantly lower neutralisation activity than b12 IgG alone (98.8±0.03% vs 100±0.02%, $p<0.05$), the combination of b12 IgG with its dIgA₂ showed similar level of neutralisation than b12 IgG alone (Table 13). In contrast, there were no significant differences in the neutralisation activities of CH31 IgG (gp120 CD4bs-specific) and 2F5 IgG (gp41 MPER-specific) taken separately, or mixed with their mIgA or dIgA equivalents. The mixtures of CH31 and 2F5 IgG and IgA (mIgA or dIgA) antibodies presented a greater neutralisation activity than each IgA (mIgA or dIgA) antibody alone (all $p<0.05$).

Therefore, it was observed that only the combination of the gp120 CD4bs directed b12 IgG and b12 mIgA₂ attenuated neutralisation outcome with a significantly lower neutralisation activity observed with the mixture than with b12 IgG alone.

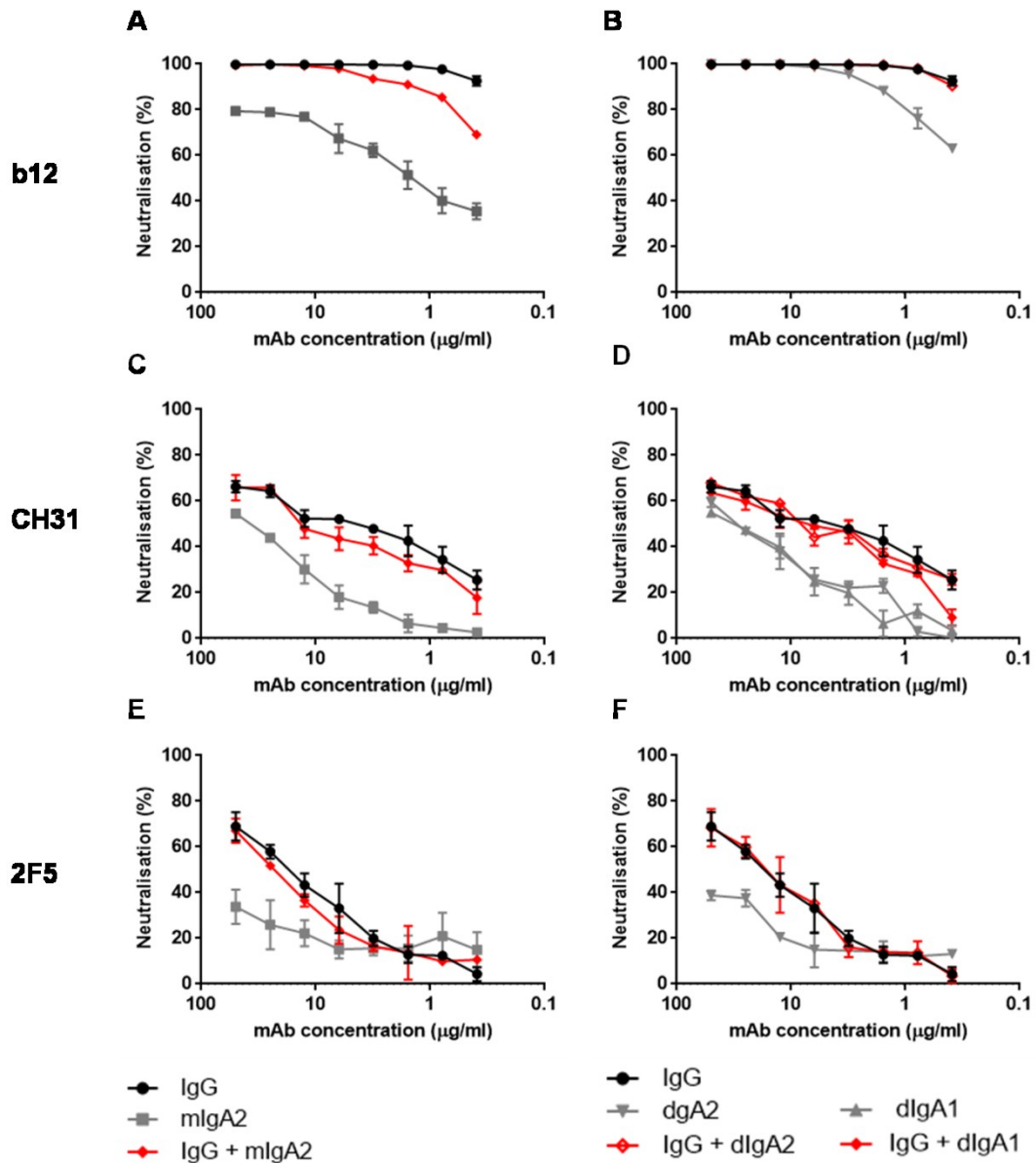


Figure 30. Neutralisation by mixtures of IgG and IgA antibodies targeting the same epitope. The curves show on the left panel, the percentage of neutralisation of HIV-1_{BaL} infection in TZM-bl cells, mediated by b12, CH31 and 2F5 IgG (black) and mIgA₂ (grey) alone or in combination at the ratio 1:1 (red); and on the right panel the same results with dIgA (in grey). The data are representative results obtained from three independent experiments and samples were tested in triplicates. Results obtained with 7B2 antibodies are not shown as they are nNABs.

Table 13. Effect of combination of IgG and IgA recognising the same epitope on neutralisation

Neutralisation for 10 µg/mL of antibody (%)					
Antibody	IgG	mIgA ₂	dIgA ₂	IgG+mIgA ₂	IgG+dIgA ₂
b12	100±0.02	71.5±0.2	98.4±0.6	98.8±0.3	100±0.05
CH31	52.5±3.4	26.1±2.5	33.6±2.5 and 31.7±0.66*	50.7±0.07	54.1±0.5 and 52±1.2*
2F5	40±2.8	19.3±1.2	23±1.9	34.3±4	40.4±6.4
7B2	ND	ND	ND	ND	ND

*Neutralisation (%) with 10µg/mL of antibody calculated for CH31 dIgA₁

ND, Not Determined (as 7B2 is a non-neutralising antibody).

Table 14. Effect of combination of IgG and IgA recognising the same epitope on virion capture

Virion capture (%) with 10µg/mL of antibody					
Antibody	IgG	mIgA ₂	dIgA ₂	IgG+mIgA ₂	IgG+dIgA ₂
b12	46.6±2.8	27.8±1.3	38.1±1	38.2±1.8	41±1.4
CH31	40.8±1.8	29.1±1.9	34.5±0.8 and 34.1±0.6*	34.7±0.9	39.3± and 37.4±1.2*
2F5	36.5±2	19.2±4.4	21.2±1.3	29.9±2	35±1.4
7B2	49.7±2.1	14.9±3.4	29.4±4	40.2±2.4	43.3±2.9

*Virion capture (%) with 10µg/mL of antibody calculated for CH31 dIgA₁

To investigate how the combination of IgG and mIgA₂ antibodies, at the ratio 1:1, impacted on the ability to neutralise iVirions captured and/or to selectively capture iVirions; the AVCNI of each IgG and IgA antibodies combination tested was calculated and compared to the AVCNI of each antibody taken separately (Table 15).

While b12 mIgA₂ and b12 dIgA₂ (gp120 CD4bs-specific) presented a significantly greater AVCNI than b12 IgG (both $p < 0.05$), the AVCNI of b12 IgG and b12 IgA (mIgA₂ or dIgA₂) mixtures were comparable to those observed with b12 mIgA₂ and b12 dIgA₂ taken separately, and greater than the one observed with b12 IgG alone ($p < 0.05$) (Table 15). Similarly, although CH31 IgA antibodies (gp120 CD4bs-specific) presented a significantly lower AVCNI than CH31 IgG (all $p < 0.05$), the AVCNI of the mixture of CH31 IgG and CH31 IgA (mIgA₂ or dIgA₁₋₂) were comparable to the AVCNI of CH31 IgG alone and significantly greater than observed with CH31 IgA (mIgA₂ or dIgA₂) taken separately (all $p < 0.05$). Moreover, the AVCNI of the 2F5 IgG and 2F5 IgA (mIgA₂ or dIgA₂) combinations were equivalents to the AVCNI of each 2F5 bNAb alone.

Therefore, the results show that the AVCNI of IgG and IgA antibodies mixtures were equal to the AVCNI of the antibody isotype presenting the highest AVCNI value. This suggests that when combining IgG and IgA antibodies, that recognise the same epitope, the AVCNI values were not additives. Furthermore, this suggests that there might be competition between IgG and IgA antibodies for capturing and neutralising iVirions, and that it was the antibody that presented the highest AVCNI which might win the competition. Taken together, this implies that the AVCNI can quantitatively represent the ability of bNAbs or mixtures of bNAbs to neutralise captured iVirions and/or to selectively capture iVirions and that thus might reflect the affinity of an antibody for iVirions.

Overall, the results indicate that IgG antibodies presented a greater ability to capture virions and to neutralise virions than their mIgA₂ and dIgA₂ equivalents. Moreover, it was observed that dIgA antibodies presented enhanced antiviral capture and neutralisation than their mIgA counterparts, depending on the epitope specificity. This suggests that both antibody isotype and structure (monomeric vs dimeric) influence antiviral function. Moreover, the results indicate that IgG and IgA antibodies recognising the same epitope compete for binding and capturing virions. In addition, the data show that the combination of IgG and IgA antibodies, at the ratio 1:1, might mitigate IgG effector functions depending on the antibody epitope specificity and structure, and the antiviral function measured. This suggests that the mixture of IgG and IgA antibodies of identical specificity does not enhance antiviral function, and that, on the contrary, it has either no effect or attenuates antiviral abilities. Furthermore, the results indicate that the devised AVCNI enables to efficiently quantify the neutralisation of captured iVirions, and/or preferential capture of iVirions for individual antibody and combination of antibodies. Additionally, the study of the AVCNI further implies that epitope specificity, in a close relationship with antibody isotype, influences the ability to neutralise iVirions captured and/or to select iVirions.

Table 15. Impact of combination of IgG and IgA antibodies binding to the same epitope on the AVCNI

Antibody virion capture neutralisation index (AVCNI)					
Antibody	IgG	mIgA ₂	dlgA ₂	IgG+mIgA ₂	IgG+dlgA ₂
b12	2.14±0.18	2.59±0.16	2.61±0.09	2.58±0.17	2.52±0.06
CH31	1.28±0.01	0.88±0.02	0.99±0.08 and 0.92±0.03*	1.36±0.05	1.38±0.08 and 1.39±0.1
2F5	1.09±0.08	1.13±0.05	1.08±0.04	1.16±0.24	1.14±0.12
7B2	ND	ND	ND	ND	ND

*AVCNI calculated for CH31 dIgA₁.

ND, Not Determined (as 7B2 is a non-neutralising antibody).

3.2.5 Competition between IgG and IgA antibodies of identical specificity for capturing and neutralising HIV-1 virions

Based on the observations that mixture of IgG and IgA antibodies, recognising the same epitope, may alleviate antiviral function, the objective of the present study was to determine to what extent this was due to competition between IgG and IgA antibodies for the same epitopes. For this purpose, a competitive VCA was developed to investigate whether the panel of b12, CH31, 2F5 and 7B2 IgG antibodies competed with their mIgA₂ and dIgA₁₋₂ equivalents to capture HIV-1_{BaL} virions.

The results show that b12, CH31, 2F5 and 7B2 mIgA₂ antibodies were able to inhibit virion capture by their IgG equivalents (Figure 31). Particularly, it was observed that the gp120 CD4bs-specific b12 IgG captured significantly less virions in the presence of its mIgA₂ equivalent than alone (35.9±1.6% vs 46.6±2.8%, p<0.005), (Figure 31A, left panel); and that 7B2 mIgA₂ (gp41 cluster I-specific) inhibited virion capture by its IgG counterpart by 13±3.8% (p<0.01), (Figure 31D, left panel). However, while the data show that b12 dIgA₂ and 7B2 dIgA₂ significantly reduced the proportion of virions captured by their IgG equivalents (39.5±1.2%, p<0.01) and (40.2±3.1%, p<0.05) respectively, CH31 and 2F5 IgG antibodies captured comparable levels of virions alone or in presence of their dIgA₂ counterparts, (Figure 31 C and D).

Furthermore, it was found that the panel of IgG antibodies tested were able to reduce the amount of virion captured by their mIgA₂ equivalents. For instance, the CD4bs-specific CH31 IgG reduced the proportion of virion captured by its mIgA₂ equivalent by

10±4%, $p<0.005$, (Figure 31B, second panel). Similarly, the gp41 MPER-specific 2F5 mIgA₂ captured fewer virions in the presence of its IgG counterpart than alone (14.9±3.4% vs 12.2±1.3%, $p<0.05$). In contrast, the data indicate that while b12 and CH31 IgG antibodies were not able to block virion capture by their dIgA₁₋₂ equivalents, 2F5 and 7B2 IgG antibodies significantly reduced the amount of virion captured by their dIgA₂ counterparts (both, $p<0.05$).

Therefore, whereas b12, CH31, 2F5 and 7B2 mIgA₂ antibodies presented the ability to reduce virion capture by their IgG equivalents only b12 and 7B2 dIgA₂ antibodies demonstrated this capacity. Conversely, while all the IgG antibodies of the panel were able to reduce the proportion of virion captured by their mIgA₂ counterparts, only 2F5 and 7B2 IgG antibodies were able to inhibit virion capture by their dIgA₂ equivalents. Taken together, these results indicate that mIgA₂ antibodies were stronger inhibitors of IgG antibodies mediated-virion capture than dIgA₁₋₂; and that IgG antibodies presented a greater ability to block the capture of virions by their mIgA₂ equivalents than their dIgA₁₋₂ counterparts. Consequently, the competition was more pronounced, between IgG and mIgA₂ antibodies than between IgG and dIgA₂ antibodies recognising the same epitope.

In all, these findings imply that the ability of a given antibody to inhibit virion capture by another antibody isotype, of identical-specificity, might be dependent of the antibody isotype and structure (monomeric vs polymeric) as well as the motif targeted.

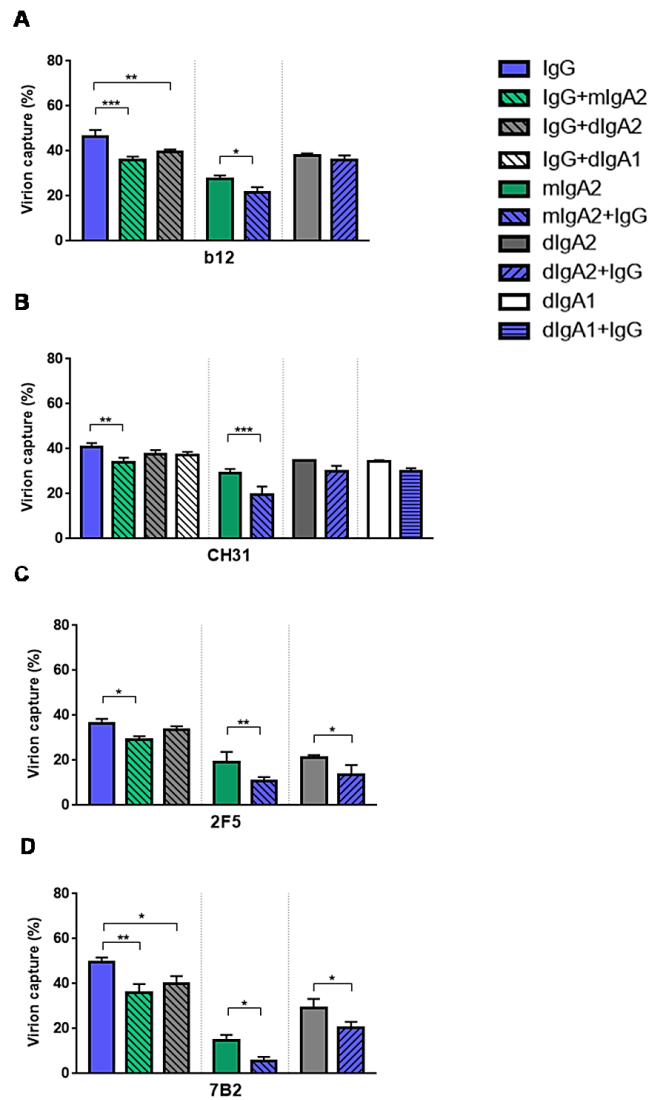


Figure 31. Competitive antibody viral capture assay between IgG and IgA antibodies recognising the same epitope. The histograms show, on the left panel, mIgA₂, dlgA₂ and dlgA₁ inhibition of IgG mediated-virions capture; on the middle panel, IgG inhibition of mIgA₂ virion capture, and on the right panel IgG inhibition of dlgA₂ and dlgA₁ virion capture. On the left panel, b12 (A), CH31 (C), 2F5 (E) and 7B2 (G) IgG (blue) antibodies were incubated either alone or in combinations with mIgA₂ or dlgA₂ (or dlgA₁ for CH31 only), recognising the same epitope (10μg/mL) and with purified HIV-1_{BaL} virions (500ng/mL) for 1h at 37°C. IgG alone or combined with their IgA equivalents were then immobilised on IgG-specific column and only IgG-bound virions were eluted and quantified by p24 ELISA. On the middle and right panel, mIgA₂ (green), dlgA₂ (grey) or dlgA₁ (white) antibodies were incubated either alone or with IgG presenting the same epitope specificity (10μg/mL each) and purified virions HIV-1_{BaL} virions. IgA alone or mixed with their IgG counterparts were immobilised on IgA-specific column and IgA-bound were eluted and quantified by p24 ELISA. Comparisons of the amount of virions captured were performed by Tukey's MCT using 95% CI and p-value results were presented as *p<0.05, **p<0.01, ***p<0.005, ****p<0.001.

3.2.6 Study of antibody combinations that may improve antiviral function

3.2.6.1 Combination of bNAbs recognising distinct epitopes

The results of the preceding experiments show that IgG and IgA antibodies, recognising the same epitopes, competed for capturing HIV-1 virions, and that the combination of IgG and IgA (mIgA or dIgA) bNAbs, of identical specificity, could either mitigate IgG antiviral function or have no impact, and equal the antiviral capacity of IgG taken separately. Based on these data, it was proposed that, on the contrary, the combination of antibody targeting discrete epitopes, from the same isotype (IgG or IgA), at the ratio 1:1, might result in an additive effect with an increase of the quantity of virions captured, compared to each IgG or IgA alone, due to lack of competition for the same Env sites. To test this hypothesis, the proportion of virions captured by the gp120 CD4bs-specific bNAbs b12 and CH31 were compared to the amount of virions that they captured when combined with the gp41 MPER-specific bNAb 2F5. These comparisons were done for each isotype of b12, CH31 and 2F5 antibodies.

The mixtures of b12 and 2F5 as well as of CH31 and 2F5 bNAbs captured significantly more virions than each of these IgG, mIgA₂ and dIgA₂ taken separately (Figure 32). b12 and 2F5 IgG antibodies combined captured 73.7±4.2% of virions while they captured separately 46.6±2.8% and 36.5±2% of virions, respectively (both $p<0.0001$) (Table 17). Likewise, whereas CH31 IgG and 2F5 IgG captured 40.8±1.7% and 36.5±2% of virions respectively, when mixed they captured 46.8±1.8% of virions (both $p<0.05$). Similarly, increases in the proportion of virions captured were observed with the combinations of the mIgA₂ and dIgA₂ isotypes of b12 and 2F5 as well as of CH31 and 2F5 compared to each of these IgA antibodies taken separately (all $p<0.05$). Furthermore, analyses show that the mixture of b12 and 2F5 IgG antibodies captured significantly more virions than the combinations of their mIgA₂ or dIgA₂ equivalents (both $p<0.005$). In contrast, while CH31 IgG and 2F5 IgG captured more virions than their respective mIgA₂ and dIgA₂ counterparts, the mixtures of CH31 and 2F5 mIgA₂ and dIgA₂ antibodies captured comparable amount of virions. This indicates that the additive effect on virion captured by CH31 and 2F5 bNAbs was greater than with the IgA-isotype than with the IgG-isotype.

Taken together, these results suggest that the combinations of bNAbs directed against distinct epitopes and of the same isotype increased the overall amount of virions captured, with additive effects more or less important depending on the epitopes targeted and antibody isotype.

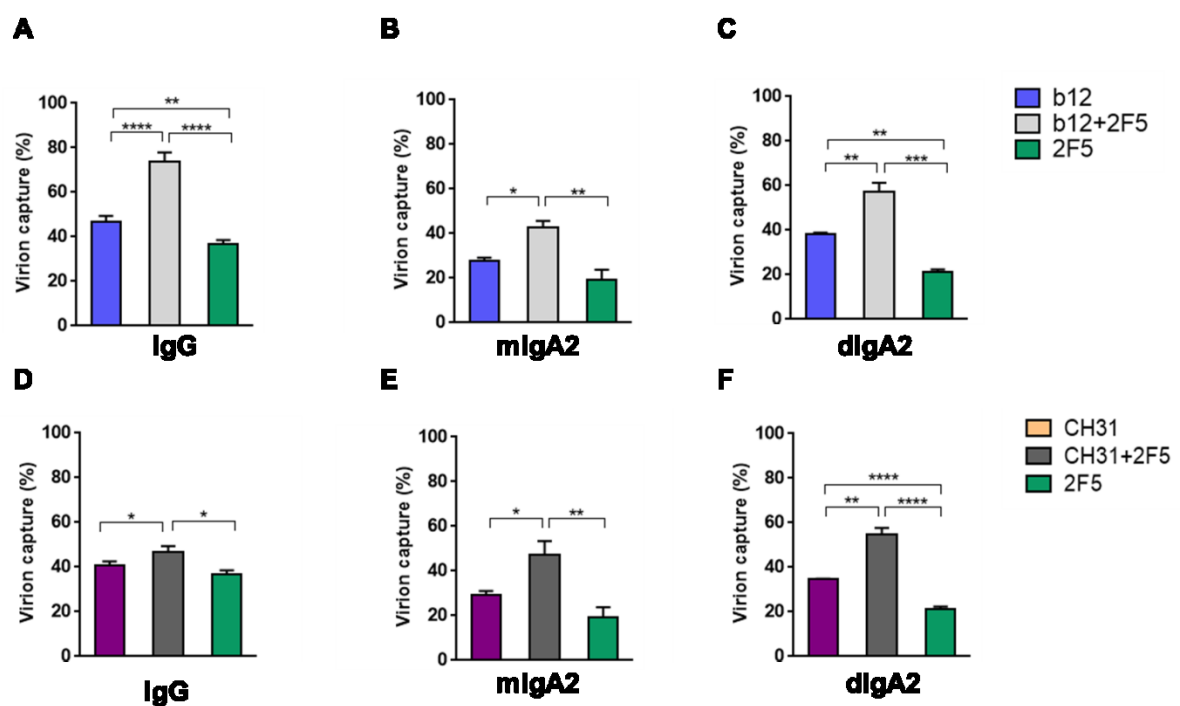


Figure 32. Virion capture by mixtures of antibodies with distinct epitope specificities and same isotype. A panel of IgG (A), mIgA₂ (B) and dIgA₂ (C) isotypes forms of b12 (blue), CH31 (orange) and 2F5 (green) antibodies were incubated either alone or in combinations as follow, b12 and 2F5 or CH31 and 2F5 with HIV-1_{BaL} virions (500ng/mL) for 1h at 37°C. The proportions of virion captured by each antibody or antibody mixtures are represented in the histograms. The results are representative of three independent experiments of triplicates samples. Comparisons of the amount of virions captured with antibody alone or antibody mixtures were performed by Tukey's MCT using 95% CI and p-value results were presented as *p<0.05, **p<0.01, ***p<0.005, ****p<0.001.

Subsequently, TZM-bl neutralisation was performed to compare the neutralisation against HIV-1_{BaL} of mixtures of b12 (gp120 CD4bs-specific) and 2F5 (gp41 MPER-specific) and of CH31 and 2F5 bNAbs, to the neutralisation obtained with each bNAb alone. While b12 IgG, mIgA₂ and dIgA₂ bNAbs presented similar neutralisation curves alone or when mixed with 2F5 bNAbs of the same isotype, a notable increase in the neutralisation activity of CH31 IgG, mIgA₂ and dIgA₁₋₂ in combination with their 2F5 equivalents was observed (Figure 33). This increase appeared to be more important with CH31 and 2F5 mIgA₂ and dIgA₂ bNAbs than with their IgG counterparts. Interestingly, the percentages of neutralisation for the mixture of CH31 mIgA₂ and 2F5 mIgA₂ ($49.8 \pm 1.1\%$) was roughly equal to the sum of the neutralisation of each bNAb alone ($26.1 \pm 2.5\%$ and $19.3 \pm 1.2\%$, respectively) (Table 16). Similarly, the combination of CH31 dIgA₂ and 2F5 dIgA₂ neutralised ($59.5 \pm 2.1\%$) as much as the sum of the neutralisation of each of these bNAbs individually ($26.1 \pm 2.5\%$ and $19.3 \pm 1.2\%$, respectively). However, the mixtures of CH31 and 2F5 IgG antibodies neutralised only about 7% more than CH31 IgG alone ($52.5 \pm 3.5\%$ vs $59.5 \pm 2\%$, $p < 0.05$). Furthermore, it was observed that the neutralisation of the mixture of CH31 and 2F5 dIgA₂ antibodies ($59.5 \pm 2.1\%$) was comparable to that observed with the combination of their IgG equivalents ($61.2 \pm 1\%$), and significantly higher than the neutralisation of CH31 and 2F5 mIgA₂ antibodies ($49.8 \pm 1.1\%$, $p < 0.05$).

Thus, these results show that while the combinations of b12 (gp120 CD4bs-specific) and 2F5 (gp41 MPER-specific) bNAbs, at the ratio 1:1, did not enhance neutralisation, the mixture of CH31 and 2F5 bNAbs resulted in a significant increase of iVirions neutralised, with greater additive effects observed for CH31 and 2F5 IgA antibodies than with their IgG counterparts. Hence, these results indicate that the ability to increase the neutralisation by combining bNAbs targeting distinct epitopes, and of the same isotype, was not only dependent on the bNAbs epitopes specificity and isotype, but also on their differential neutralisation efficiencies.

Table 16. Neutralisation of mixture of antibodies with distinct epitope specificities and same isotype.

Antibody	Neutralisation (%) with 10µg/mL of antibody				
	b12	CH31	2F5	b12+2F5	CH31+2F5
IgG	100±0.02	52.5±3.4	40±2.8	100±0.01	61.2±1
mIgA ₂	71.5±0.2	26.1±2.5	19.3±1.2	74.5±0.7	49.8±1.1
dIgA ₂	98.4±0.6	33.6±2.5	23±1.9	99.5±0.07	59.5±2.1

Table 17. Virion capture by mixtures of antibodies with distinct epitope specificities and same isotype.

Antibody	Virion capture (%) with 10µg/mL of antibody				
	b12	CH31	2F5	b12+2F5	CH31+2F5
IgG	46.6±2.8	40.8±1.8	36.5±2	73.7±4.2	46.8±1.8
mIgA ₂	27.8±1.3	29.1±1.9	19.2±4.4	42.6±3	47.3±6.1
dIgA ₂	38.1±1	34.5±0.8	21.2±1.3	57±4.3	54.9±3.9

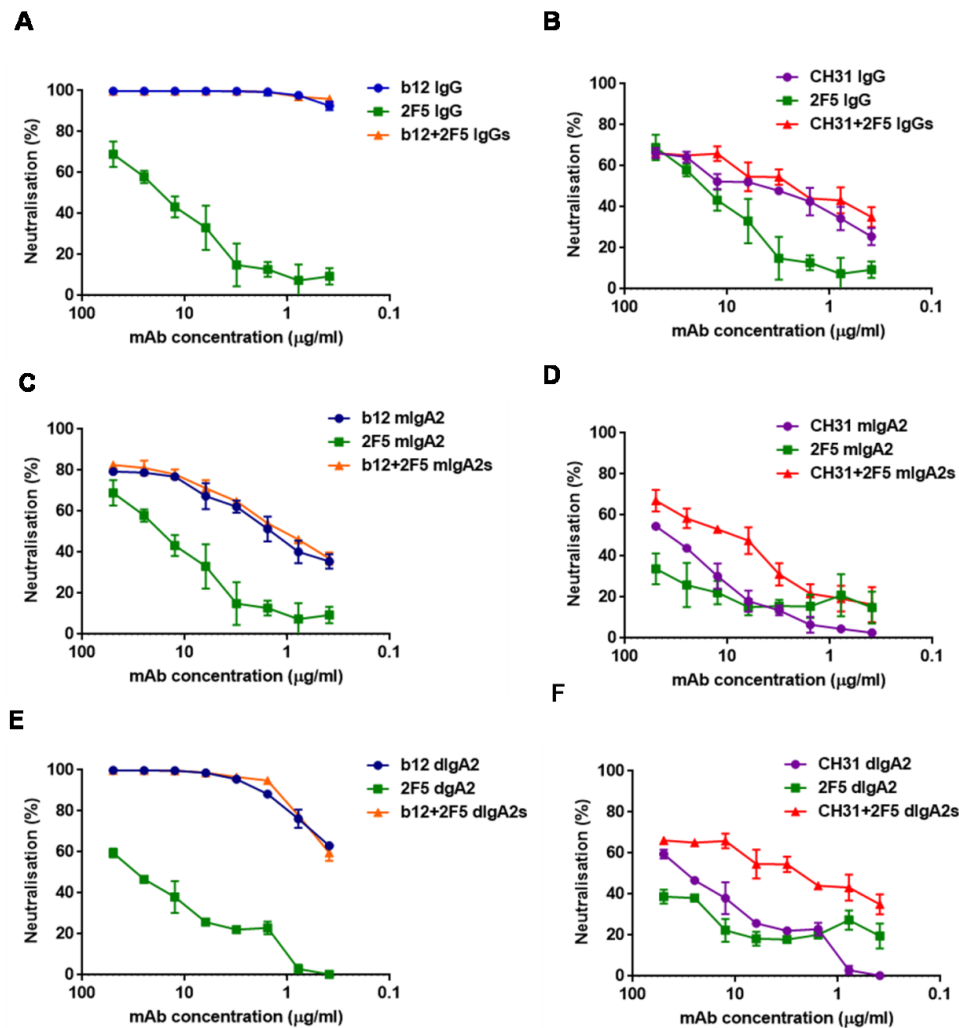


Figure 33. Neutralisation curves of combination of antibodies with distinct epitope specificities and same isotype. b12 (blue), CH31 (purple) and 2F5 (green) antibodies were incubated with HIV-1_{BaL} alone or in combination, and the percentages of inhibition TZM-bl cells infection mediated by each antibody or mixtures were calculated as a reduction in infectivity, compared to the virus control. These data are representative results obtained from three independent experiments and samples were tested in triplicates.

To investigate the impact of combining bNAbs on the ability to neutralise captured iVirions and/or to selectively capture iVirions; the AVCNI of each bNAb combination was calculated and compared to the AVCNI of each bNAb alone (Table 18).

The findings of this study show that the combination of b12 and 2F5 IgG, mIgA₂ and dIgA₂ antibodies, respectively, lead to significant reductions of the AVCNI compared to b12 bNAbs alone (all, $p < 0.05$) (Table 18). However, the mixture of b12 and 2F5 bNAbs all presented an AVCNI > 1 , which indicate that b12 and 2F5 bNAbs mixtures neutralised more efficiently iVirions captured and/or capture more iVirions compared to 2F5 bNAbs alone. Moreover, the results show that b12 and 2F5 IgA antibodies mixtures presented significantly higher AVCNI than the mixture of their IgG counterparts (both $p < 0.05$), which emphasises the influence of isotype on AVCNI. Conversely, no differences were observed between the AVCNI of CH31 and 2F5 IgG, mIgA₂ and dIgA₂ antibodies combined or alone. These data indicate that the combination of b12 and 2F5 bNAbs increased the overall proportion of virions captured without impacting on neutralisation, leading to a reduction of the AVCNI. Moreover, these findings show that CH31 and 2F5 bNAbs taken separately presented comparable AVCNI than CH31 and 2F5 bNAbs mixtures. This suggests a proportional increase in virions captured and of iVirions neutralised.

Together these data infer that combining bNAbs directed against different HIV-1 Env epitopes can affect the selective ability of the bNAbs mixtures to capture iVirions over dVirions, and/or the resulting neutralisation efficiency, compared to each bNAb taken separately. The impact of bNAbs mixtures on the AVCNI is influenced by the epitope targeted and antibody isotype. These findings also indicate that the combination of bNAbs recognising distinct epitopes results in additive effects with respect to their AVCNI values. Thus, these results demonstrate that the AVCNI could be used as a tool to assess the impact of the combinations of bNAbs on antiviral function.

Table 18. AVCNI of combination of antibodies with distinct epitope specificities and the same isotype.

Antibody	Antibody virion capture neutralisation index (AVCNI)				
	b12	CH31	2F5	b12+2F5	CH31+2F5
IgG	2.14±0.18	1.28±0.01	1.09±0.08	1.35±0.11	1.29±0.08
mIgA ₂	2.59±0.16	0.88±0.02	1.13±0.05	1.98±0.08	1.05±0.16
dIgA ₂	2.61±0.09	0.99±0.08	1.08±0.04	1.73±0.18	1.12±0.14

3.2.6.2 Combination of non-neutralising antibodies with broadly neutralising antibodies

The results of the preceding study indicate that the mixture of bNAbs binding to discrete epitopes and of the same isotype may increase virion capture and neutralisation. Based on these findings, the aim of the following work was to determine how the combination of a nNAb with a bNAb, at the ratio 1:1, impacts on antiviral activities against HIV-1. For this purpose, the panel of IgG and IgA isotypes of b12, CH31 and 2F5 bNAbs were incubated with HIV-1_{BaL} virions either alone or mixed with their 7B2 nNAbs equivalents. Then, VCA and TZM-bl neutralisation assay were performed to assess the impact of nNAbs on bNAbs antiviral functions.

The combination of b12 IgG, mIgA₂ and dIgA₂ bNAbs (gp120 CD4bs-specific) with their respective 7B2 (gp41 cluster I-specific) nNAbs equivalent, captured considerably higher proportions of virions compared to b12 bNAbs alone and 7B2 nNAbs taken separately (all $p < 0.05$) (Figure 34). Similarly, the mixtures of CH31 (gp120 CD4bs-specific) bNAbs with their 7B2 equivalents captured significantly more virions than CH31 bNAbs or 7B2 nNAbs alone (all $p < 0.05$) (Table 20). Moreover, the combination of 2F5 IgG (gp41 MPER-specific) with 7B2 IgG captured higher amount of virions ($53 \pm 2.8\%$) than 2F5 IgG alone ($36.5 \pm 2\%$ $p < 0.01$) and similar to 7B2 IgG alone ($49.7 \pm 2.1\%$). However, 2F5 and 7B2 IgA antibodies taken separately or mixed together captured similar proportions of virions.

Therefore, these data indicate that the combination of b12 and CH31 bNAbs with 7B2 nNAbs resulted in an increase of virions captured, and this for the three isotype tested (IgG, mIgA₂ and dIgA₂). Conversely, the mixture of 2F5 bNAbs with 7B2 nNAbs lead to similar percentages of virions captured to 7B2 nNAbs taken separately. Consequently, these results suggest that the combination of a nNAb with a bNAb, at equal concentration, may enhance virion capture outcome in the absence of competition for the same HIV-1 Env binding sites.

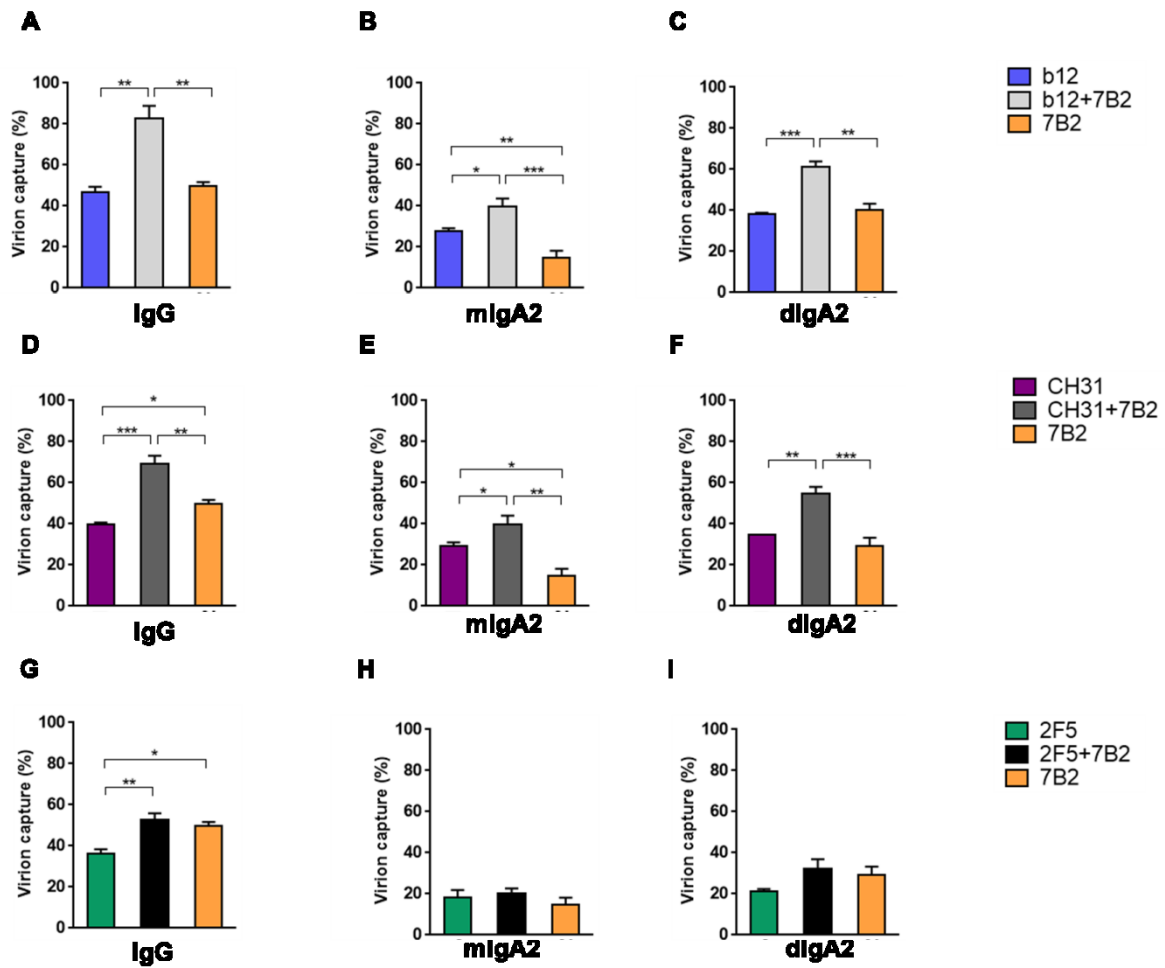


Figure 34. Virion capture by mixtures of antibodies with distinct epitope specificities and the same isotype. A panel of IgG (A), mlgA₂ (B) and dIgA₂ (C) isotypes forms of b12 (blue), CH31 (purple) and 2F5 (green) bNAbs were incubated either alone or in combination with their 7B2 nNAbs equivalent and HIV-1_{BaL} virions (500ng/mL) for 1h at 37°C. The proportion of viriond captured by each antibody or antibody mixtures are represented in the histograms. The results are representative of three independent experiments of triplicates samples. Comparisons of the amount of virions captured with antibody alone or antibody mixtures were performed by Tukey's MCT using 95% CI and p-value results were presented as *p<0.05, **p<0.01, ***p<0.005, ****p<0.001.

To determine whether the combination of nNAbs and bNAbs mitigate neutralisation, a TZM-bl neutralisation assay was performed with the panel of IgG, mIgA₂ and dIgA₂ isotypes of b12, CH31 and 2F5 bNAbs, incubated with HIV-1_{BaL} either alone or mixed with their 7B2 nNAb isotype equivalent. There were no differences between the neutralisation observed with b12 IgG, mIgA₂ and dIgA₂ bNAb (gp120 CD4bs-specific) alone or combined with their 7B2 nNAb (gp41 cluster I-specific) equivalent at equal concentrations (Figure 35). In contrast, while CH31 (gp120 CD4bs-specific) and 7B2 IgG antibodies combination neutralised similar percentages of iVirions to CH31 IgG taken separately, CH31 mIgA₂ and CH31 dIgA₂ neutralised a greater amount of virions when combined with their respective 7B2 counterparts than alone (both $p < 0.05$) (Table 19). Remarkably, it was observed that combinations of 2F5 (gp41 MPER-specific) and 7B2 IgG, mIgA₂ and dIgA₂ antibodies, all resulted in a loss of the neutralisation activity compared to 2F5 bNAb alone.

These results indicate that 7B2 did not interfere with b12 bNAb-mediated neutralisation; and therefore the combination of 7B2 and b12 bNAb had no effects on the neutralisation of b12 bNAb. Similarly, these results suggest that 7B2 IgG did not blocked CH31 IgG-mediated neutralisation. Moreover, these results imply that 7B2 mIgA₂ and 7B2 dIgA₂ might have facilitated CH31 mIgA₂ and CH31 dIgA₂ neutralisation. Furthermore, the analyses revealed that 7B2 nNAb inhibited 2F5 bNAb-mediated neutralisation, and this for all the isotypes tested. Together, this suggests that the two gp41-specific 2F5 and 7B2 bNAb might compete for interaction with HIV-1 virions and particularly for capturing iVirions.

Thus, the increase in virions captured observed when mixing bNAb and nNAb did not necessarily correlate with an increase in neutralisation. This might be due to the capture of dVirions. Overall, these results indicate that the mixture of bNAb and nNAb may increase antiviral function, depending on the epitope targeted, antibody isotype and antibody function measured.

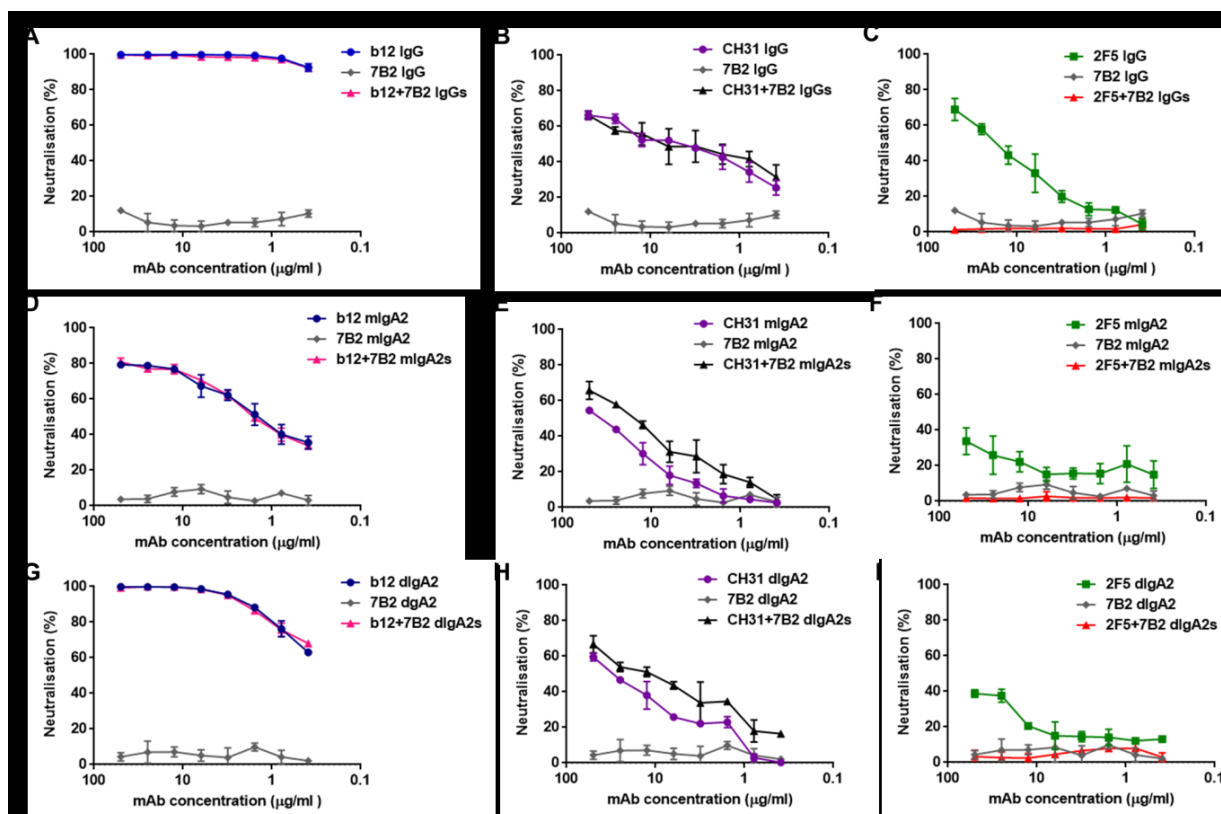


Figure 35. Impact of 7B2 on b12-, CH31- and 2F5-mediated neutralisation. The percentage of neutralisation of the different isotypes of b12, CH31 and 2F5 bNAbs alone, or in combination with their 7B2 nNAbs equivalent are shown on the left, middle and right panel, respectively. These data shown are representative of three independent experiments and samples were tested in triplicates.

Table 19. Effect on neutralisation of combining a nNAb with a bNAb

Neutralisation (%) with 10μg/mL of antibody						
Antibody	b12	b12+7B2	CH31	CH31+7B2	2F5	2F5+7B2
IgG	100±0.02	99.7±0.14	52.5±3.4	53.3±0.5	40±2.8	ND
mlgA ₂	71.5±0.2	86.8±1.1	26.1±2.5	42.0±0.1	19.3±1.2	ND
dlgA ₂	98.4±0.6	97.7±0.02	33.6±2.5	49.3±2.3	23±1.9	ND

ND, Not Determined (as 7B2 abrogated 2F5-mediated neutralisation).

Table 20. Impact on virion capture of combining a nNAb with a bNAb

Virion capture (%) with 10μg/mL of antibody							
Antibody	7B2	b12	b12+7B2	CH31	CH31+7B2	2F5	2F5+7B2
IgG	49.7±2.1	46.6±2.8	82.9±6	40.8±1.8	69.2±4	36.5±2	53±2.8
mlgA ₂	14.9±3.4	27.8±1.3	39.5±4.1	29.1±1.9	39.8±4.2	19.2±4.4	20.4±2.3
dlgA ₂	29.4±4	38.1±1	61±2.8	34.5±0.8	54.7±3.4	21.2±1.3	32.4±4.4

Then, the impact on the AVCNI of mixing bNAbs and nNAbs was assessed. b12 IgG, mIgA₂ and dIgA₂ antibodies presented AVCNI >2, indicating a high selectivity for iVirions capture and neutralisation. In contrast, the AVCNI of 7B2 nNAbs were null as there are nNAbs. It was observed that the mixtures of b12 and 7B2 IgG, mIgA₂ and dIgA₂ antibodies, respectively, presented substantially lower AVCNI values compared to b12 bNAbs taken separately (all $p < 0.05$). The previous data indicate that this was due to an increase of virions captured along with no increase in neutralisation. This suggests that the rise in virions captured observed with b12 and 7B2 antibodies mixtures might be due to an increase in dVirions captured, and/or a lack of neutralisation of iVirions captured, resulting in a reduction of the AVCNI for b12 and 7B2 antibodies mixtures compared to b12 alone. Nonetheless, the AVCNI of the combination of b12 and 7B2 bNAbs remained >1, which indicates that the mixtures still selectively captures iVirions over dVirions and/or neutralised most of the iVirions captured.

Similarly, the analyses show that the combination of CH31 IgG with 7B2 IgG significantly reduced the AVCNI compared to CH31 IgG alone (0.75 ± 0.03 vs 1.28 ± 0.01 $p < 0.01$). This diminution of the neutralisation/capture ratio was due to a higher proportion of virions captured by CH31 and 7B2 mixtures for an equivalent neutralisation activity than CH31 IgG alone. Furthermore, the AVCNI of CH31 and 7B2 IgG antibodies was <1 which indicates a preferential capture of dVirions and/or a lack of efficient neutralisation of iVirions. However, no differences were reported between the AVCNI of CH31 mIgA₂ and dIgA₂ antibodies alone or combined with their 7B2 equivalents. Additionally, the AVCNI of CH31 mIgA₂ and CH31 dIgA₂ were close to 1 which implied that they have no preferential selection of iVirions or dVirions. Yet, it was previously observed that the CH31 and 7B2 IgA antibodies (mIgA₂ or dIgA₂) mixtures captured and neutralised significantly more virions than each mAb alone. This suggests that the combination of CH31 IgA and 7B2 IgA led to a proportionate increase of the amount of virions captured and of virions neutralised, and therefore the AVCNI remained constant. This suggests that the rise in neutralisation activities observed with CH31 IgA and 7B2 IgA mixtures were due to an increase in the proportion of virions captured. These results also indicate that the AVCNI of the mixtures of

2F5 and 7B2 IgG, mIgA₂ and dIgA₂ were null as 7B2 nAbs abrogated 2F5 bAbs-mediated neutralisation, likely by blocking 2F5 access to gp41 sites.

Thus, these results show that when the combination of bAbs with nAbs, at the ratio 1:1, diminished the selective ability to capture and neutralise iVirions (AVCNI) compared to bAbs alone, this correlated with an increase of the proportion of virions captured with no changes in the amount of virions neutralised. Moreover, these data suggest that when the mixture of bAbs with nAbs, at the ratio 1:1, nullified the AVCNI, this was due to nAbs inhibition of bAbs mediated neutralisation. The results imply that when the combination of bAbs with nAbs at the ratio 1:1 did not impact on the AVCNI; this was an indicator of the beneficial impact of the nAbs virion capture, on bAbs-mediated neutralisation. Thus, this study suggests that the AVCNI could be used as an indicator of the antiviral outcomes for mixtures of bAbs with nAbs.

Table 21. Effect on the AVCNI of combining a nAb with a bAb

Antibody virion capture neutralisation index (AVCNI)						
Antibody	b12	b12+7B2	CH31	CH31+7B2	2F5	2F5+7B2
IgG	2.14±0.18	1.24±0.09	1.28±0.01	0.75±0.03	1.09±0.08	ND
mIgA ₂	2.59±0.16	1.78±0.05	0.88±0.02	0.99±0.04	1.13±0.05	ND
dIgA ₂	2.61±0.09	1.57±0.07	0.99±0.08	0.91±0.12	1.08±0.04	ND

ND, Not Determined (as 7B2 abrogated 2F5-mediated neutralisation).

3.2.7 Effects of antibody-virus incubation time on antiviral function

The findings of the first and second part of this chapter show that antibody epitope specificity, accessibility, isotype and structure play a critical role in antibody-mediated antiviral function. However, in addition to these parameters the ability of antibody to act quickly against HIV-1 virions might be of fundamental importance to prevent sexual HIV-1 acquisition ^[366]. Indeed, despite several decades of research, very little is known about how long it takes for HIV-1 particles to cross mucosal barriers and most importantly the time required to antibodies against HIV-1 to exert their antiviral function to prevent infection. One of the reasons for this lack of knowledge might be due to the fact that most antibody-

virus assay protocols such as ELISA, VCA and neutralisation assay are standardised to antibody-viral interaction over an hour period.

Here, it was proposed that by increasing the chance of antibody-virus encounter, longer incubation time may improve antibody ability to capture and neutralise HIV-1 virions while in contrast a shorter incubation period might reduce antibody antiviral function. Moreover, it was hypothesised that studying antibody antiviral function in a broader time frame might provide a better understanding of antibody-HIV-1 interaction and insights for the selection of antibody that present the best abilities to prevent HIV-1 infection. Thus, the aim of the third part of this chapter was to determine the impact of antibody-virus incubation time on antibody ability to capture virions, to neutralise infectious viral particles, and to assess how this might be influenced by antibody epitope specificity, accessibility, isotype and structure.

To examine the impact of antibody-virus incubation time on antiviral function and to evaluate how this might be affected by epitope specificity and/or accessibility, the panel of IgG antibodies and HIV-1_{BaL} virions were incubated for 0h (less than 5 min), 1h or 24h prior to performing the antibody VCA and TZM-bl neutralisation assays. A significant reduction in the amount of virions captured, the longer the incubation time, was observed for the gp120 V1/ V2-specific PG9 and gp120 V3/glycans-specific 2G12, PGT136 (Figure 36B), and with the polyclonal HIVIG (Figure 36D). Particularly, PG9 captured a significantly greater proportion of virions without pre-incubation with virions ($55.6 \pm 3.1\%$) than after 1h ($48.1 \pm 2.3\%$, $p < 0.05$) or 24h incubation ($37.1 \pm 2\%$, $p < 0.0001$). Similarly, more virions were captured by 2G12 without pre-incubation than after 24h incubation ($53.5 \pm 1.4\%$ vs $43.8 \pm 2\%$, $p < 0.001$). Moreover, PGT136 captured fewer virions after 24h incubation ($42.2 \pm 3\%$) compared to 1h incubation ($50.9 \pm 3.6\%$, $p < 0.05$) and compared to no incubation ($51.8 \pm 4.2\%$, $p < 0.01$). HIV-IgG captured a significantly lower proportion of virions after 1h ($32 \pm 3.8\%$, $p < 0.05$) and after 24h ($27.1 \pm 4.6\%$, $p < 0.01$) of incubation time. In contrast, no differences between the proportion of virions captured after 0, 1 and 24h antibody-virus incubation time were observed for the rest of the IgG antibodies panel.

Thus, the results of this analysis indicate that the increase in antibody-virus incubation time did not correlate with enhance antibody virion capture. In contrast, the

proportion of virions captured by the panel of IgG remained stable overtime except for PG9 (gp120 V1/V2), 2G12 and PGT136 (gp120 glycans-specific) and the polyclonal HIVIG for which there was a significant decrease with the time. Therefore, these results suggest that antibody epitope specificity influences the proportion of virions captured overtime. Moreover, these results indicate that after only few minutes of incubation most IgG antibodies of the panel had achieved their maximal antibody capture capacity.

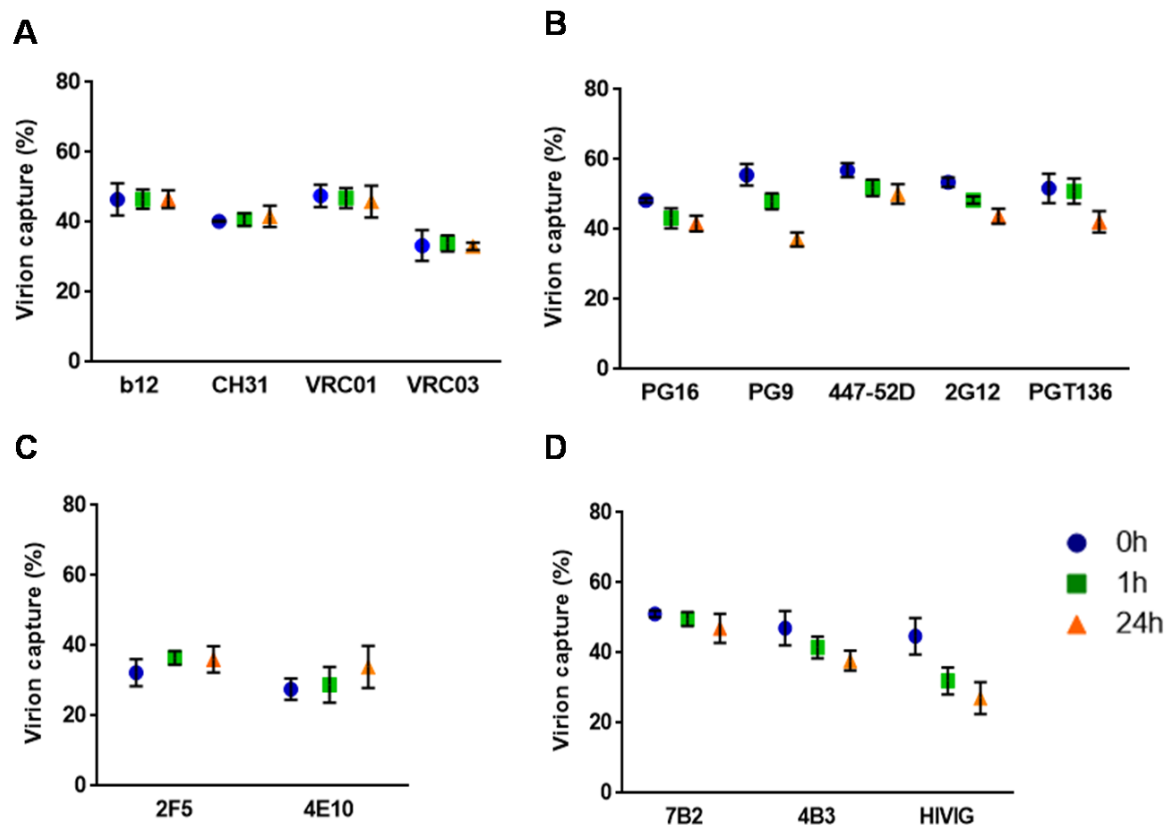


Figure 36. Impact of incubation time on IgG-mediated virus capture. The proportion of virions captured by HIV-1 Env-specific IgG antibodies was measured after 0h, 1h and 24h of incubation. HIV-1 Env-specific IgG antibodies were grouped into three groups according to the epitopes that they target: gp41 (A), V1/V2 V3 loops and glycans (B) and CD4 binding sites (C).

Comparisons of the neutralisation curves after 0, 1 and 24h of antibody-virus incubation show that neutralisation increased the longer the incubation time; except for b12 (anti-gp120 CD4bs) and 447-52D (anti-gp120 V3) which neutralised virtually 100% of iVirions across the range of concentrations and for all time points tested (Figure 37). Notably, these results indicate that each IgG antibody of the panel presented a unique neutralisation profile overtime.

The comparisons of the percentage of neutralisation for a concentration of 10µg/mL of antibody show that b12 and VRC03 (anti-gp120 CD4bs) neutralised similar proportions of virus overtime. Conversely, CH31 (anti-gp120 CD4bs) neutralised more iVirions the longer the incubation time with significantly greater levels of inhibition observed after 1h ($52.5 \pm 3.4\%$, $p < 0.01$) and 24h ($69.8 \pm 4.1\%$, $p < 0.0001$) compared to no incubation ($40.7 \pm 4.2\%$), and after 24h compared to after 1h ($p < 0.0001$), (Figure 38A). Similarly, VRC01 (anti-gp120 CD4bs) presented more potent neutralisation after 1h ($93.8 \pm 1.7\%$) and 24h ($99.1 \pm 0.03\%$) of pre-incubation than without pre-incubation ($59.5 \pm 6.6\%$, both $p < 0.0001$).

In addition, 4E10 (gp41 MPER-specific), PG16 (gp120 V1/V2-specific), 2G12 and PGT136 (anti-gp120 V3/glycans) presented the highest increases in neutralisation activity among the panel of IgG antibodies tested (Figure 38C). Particularly, PG16 inhibited substantially more iVirions after 24h pre-incubation ($96.4 \pm 0.6\%$) than after 1h ($46.5 \pm 1.7\%$) or without pre-incubation ($32.5 \pm 2.6\%$), (both $p < 0.0001$). In contrast, neutralisation by the gp120 V1/V2-specific PG9 was comparable for all incubation times. However, despite the increase in antibody-virus incubation 7B2 and 4B3 still did not neutralise.

These results further indicate that antibody-virus incubation time impacts differently according to the epitope specificity. Interestingly after 24h incubation, 11 IgG antibodies out of 12 neutralised over 70% of iVirions (except HIV-IG) while only five reached this level after 1h pre-incubation with virus (b12, VRC01, VRC03, PG9 and 447-52D). This shows a global increase in neutralisation potency overtime.

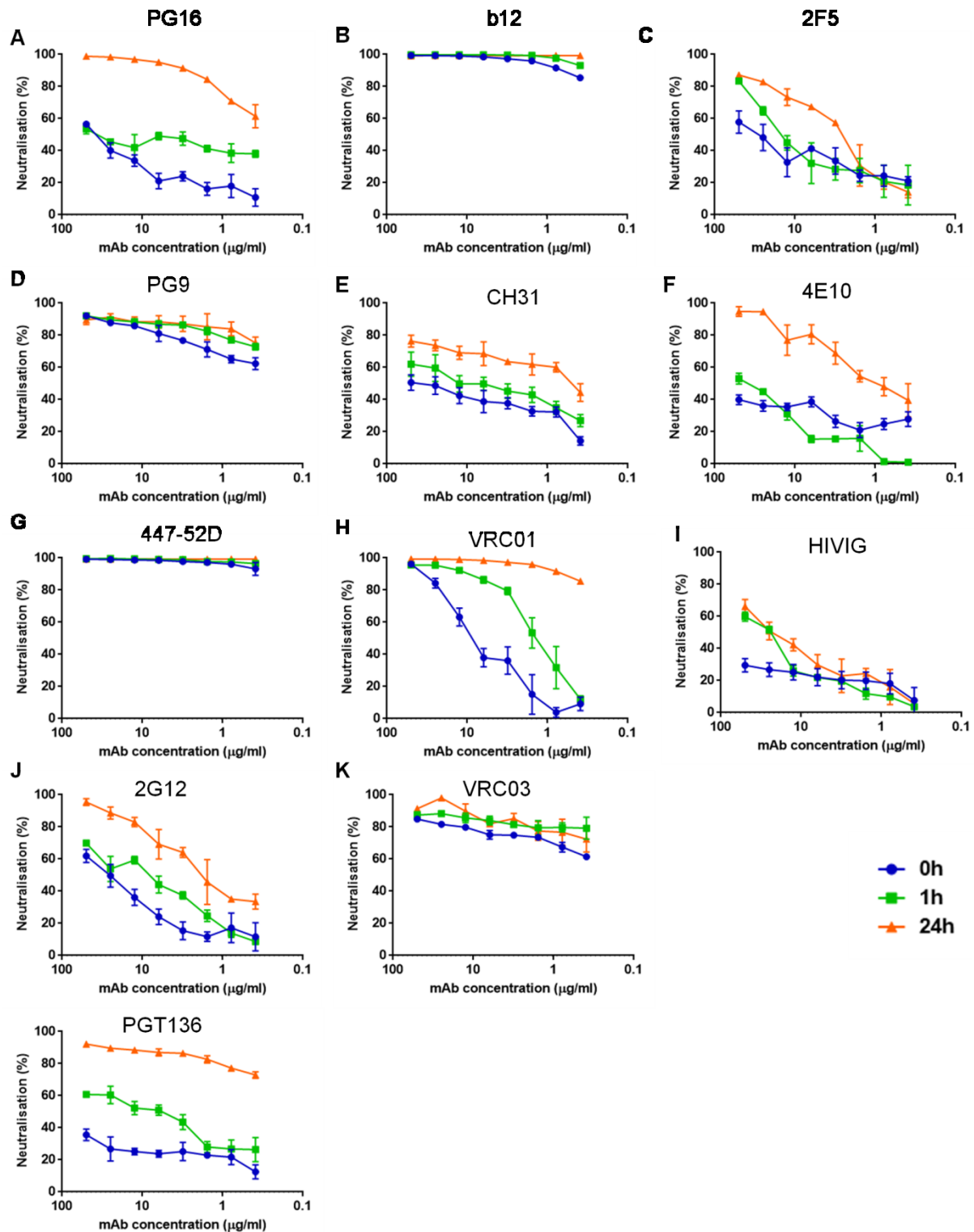


Figure 37. Impact of incubation time on IgG antibodies neutralisation curves. The neutralisation curves of b12, CH31 and 2F5 IgG, mIgA₂, dIgA₂ and dIgA₁ following 5 min (0h) (blue), 1h (green) or 24h (orange) incubation with HIV-1_{BaL}, prior the addition of TZM-bl cells. Neutralisation activity was measured by TZM-bl assay. The data presented are obtained from three independent experiments and each sample was tested in triplicates.

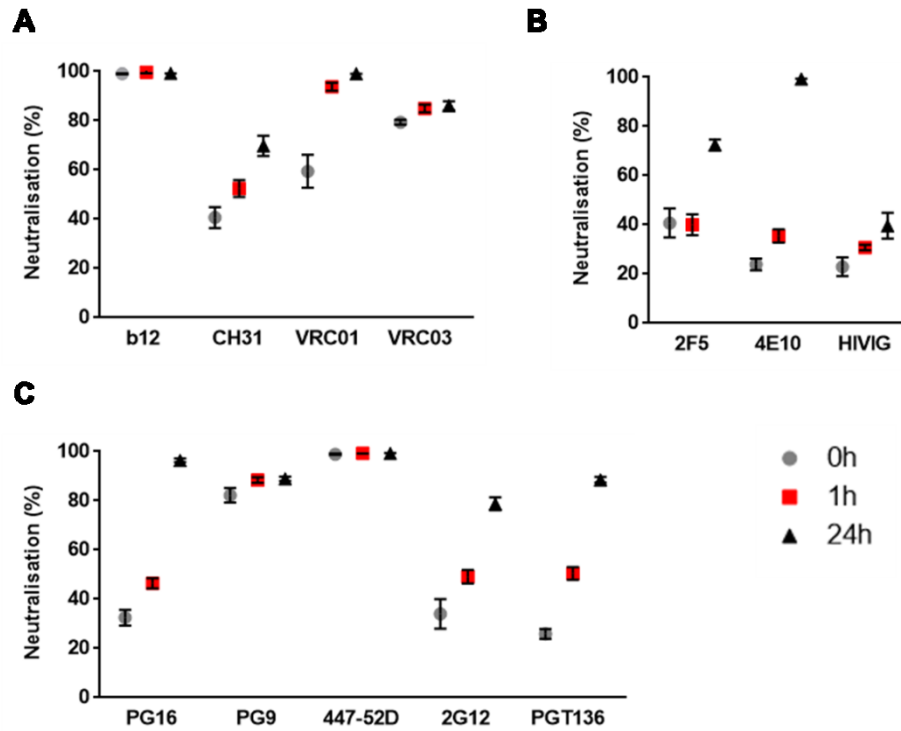


Figure 38. Impact of incubation time on IgG antibodies neutralisation. Graphs show the percentage of neutralisation for a panel of IgG antibodies (10µg/mL) after 0, 1 and 24h incubation with HIV-1_{BaL}, prior to addition of the TZM-bl cells. The panel of 12 IgG bNAbs were grouped according to their epitope specificity: (A) gp120 CD4bs directed, b12, CH31, VRC01 and VRC03, (B) gp41 MPER-specific 2F5 and 4E10, and the polyclonal HIVIG and (C) gp120 V1/ V2-specific PG16 and PG9, gp120 V3/glycans directed 447-52D, 2G12 and PGT136. TZM-bl neutralisation assay was repeated three times for each IgG of the panel and in triplicate samples.

Analysis shows that without pre-incubation with virus (T=0h) the amount of virions captured did not correlate with the percentage of neutralisation, it significantly correlated after 1h pre-incubation ($p=0.046$), and considerably more after 24h pre-incubation ($p=0.0096$) with virus (Figure 39).

Overall, these results demonstrate that the longer was the antibody-virus incubation time prior to performing antibody virion capture and TZM-bl neutralisation assays, the stronger the correlation between antibody virion capture and neutralisation.

Subsequent analysis of the AVCNI shows that the AVCNI of the panel of IgG antibodies tested increased the longer the antibody-virus incubation time, except for b12 and VRC03 (anti-gp120 CD4bs) and 447-52D (anti-gp120 V3) (Figure 40). This suggests that the neutralisation efficiency of iVirions captured increased with time and/or that more iVirions were captured overtime, for most IgG antibodies of the panel. However, this remained the same overtime for b12, VRC03 and 447-52D, as the later already captured virtually all the iVirions present. Together, this implies that the AVCNI measures the ability to neutralise iVirions captured and/or the amount of iVirions captured rather than the selectivity for iVirions over dVirions.

Thus, the most striking findings to emerge from this study were that the capture of virions by IgG antibodies remained stable overtime while their neutralisation activity and their AVCNI increased with the time.

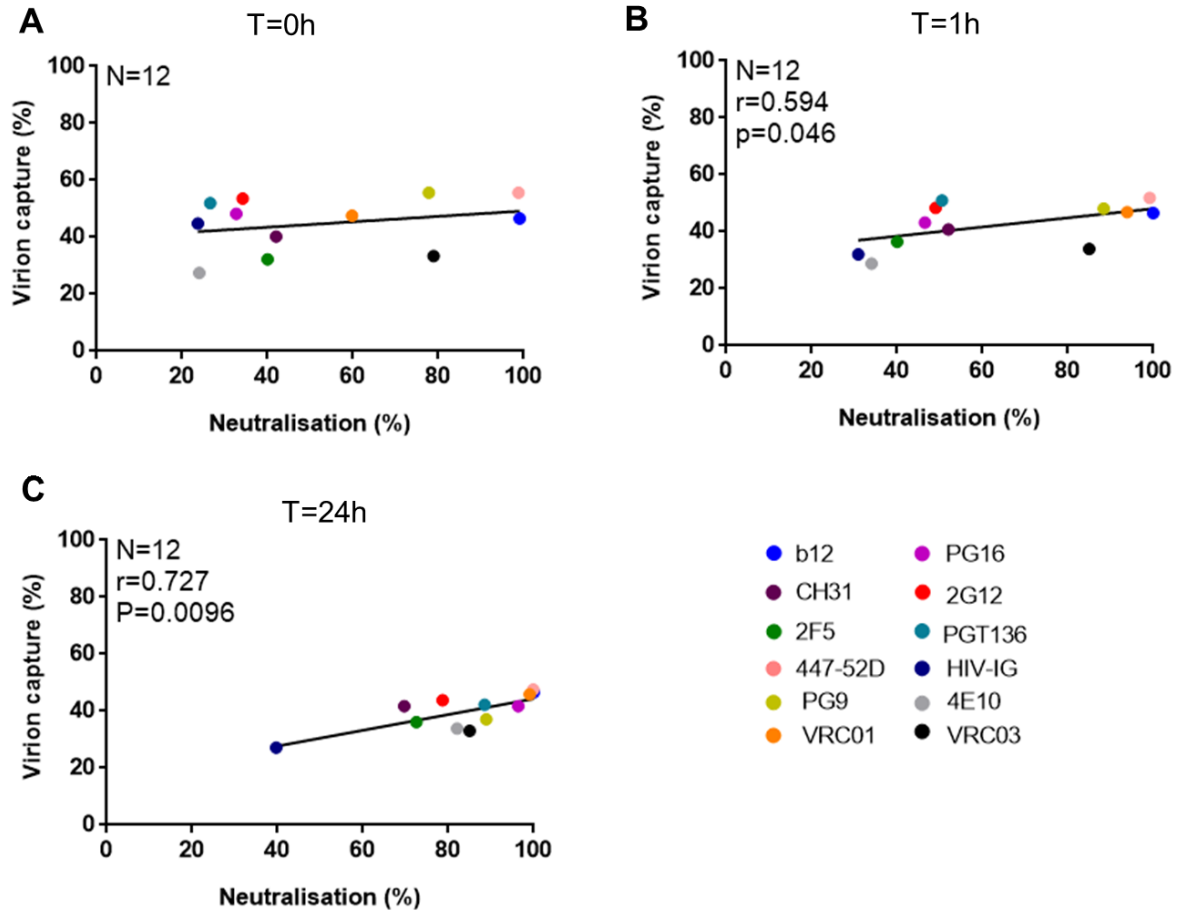


Figure 39. Correlation analysis between IgG antibodies mediated virion capture and neutralisation. Spearman r correlation analyses were performed between the proportions of virions captured and the percentage neutralisation for the panel of IgG antibodies (10 μ g/mL). The panel of 12 IgG bNAbs comprised: b12, CH31, VRC01 and VRC03 (gp120 CD4bs-specific), 2F5 and 4E10 (gp41 MPER-specific), PG16 and PG9 (gp120 V1/ V2-specific), 447-52D, 2G12 and PGT136 (gp120 V3/glycans-specific) and the polyclonal HIVIG. VCA and TZM-bl neutralisation assay were repeated three times for each IgG of the panel and in triplicate samples. N=number of IgG antibody, r = coefficient of correlation and P = exact p -value calculated with 95%CI.

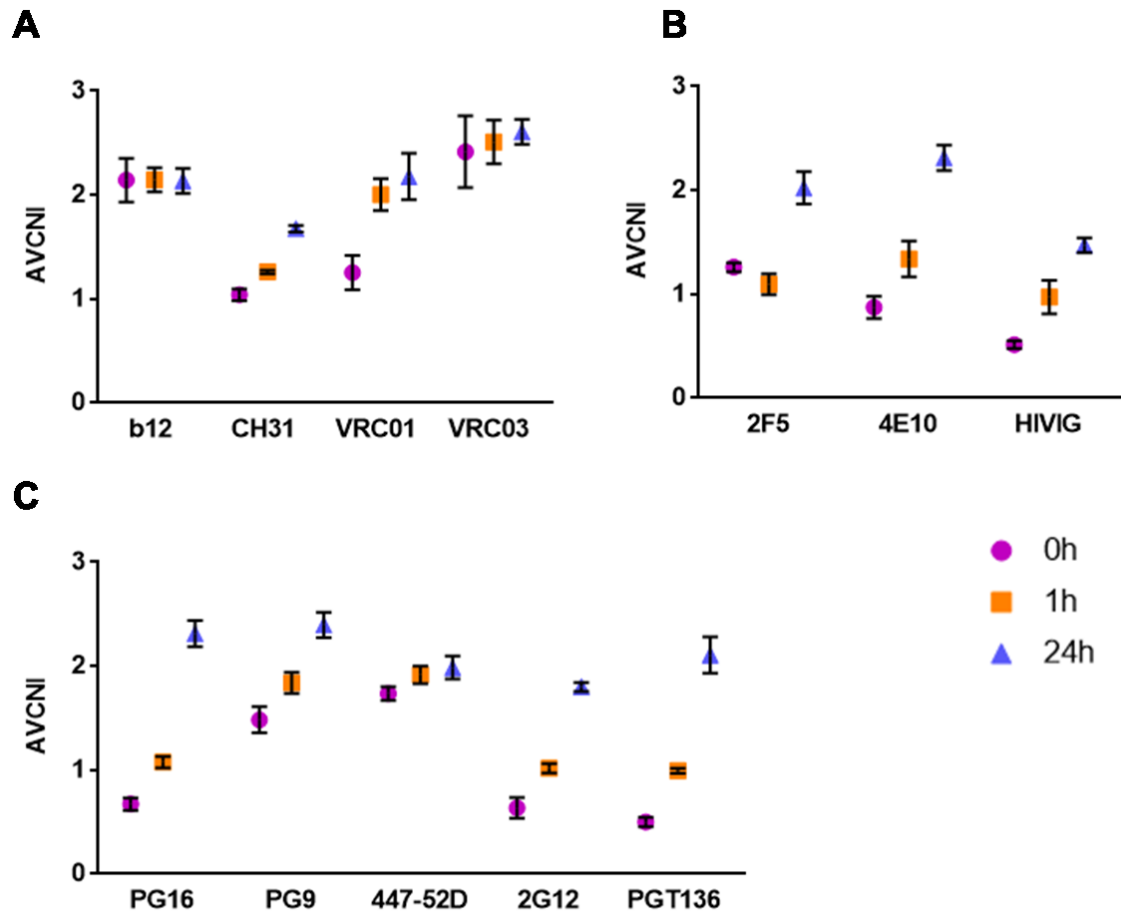


Figure 40. Impact of incubation time on IgG antibodies AVCNI. AVCNI values for the panel of IgG antibodies was calculated using the ratio neutralisation/capture after no pre-incubation (0h), 1 and 24h of incubation with HIV-1_{BaL}. The panel of IgG antibodies tested were grouped according to their Env epitope specificity: (A) gp120 CD4bs directed, b12, CH31, VRC01 and VRC03, and (B) gp41 MPER-specific 2F5 and 4E10, and the polyclonal HIVIG, (C) gp120 V1/ V2-specific PG16 and PG9 and V3/glycans directed 447-52D, 2G12 and PGT136.

The preceding results show that the impact of antibody-virus incubation time on antiviral function might be influenced by epitope specificity. Here, the objective was to investigate whether the impact of antibody-virus incubation on antiviral function differs according to the antibody isotype and structure. For this purpose the proportion of virions captured and the neutralisation activities of the panel of b12, CH31, 2F5 and 7B2 IgG, mIgA₂ and dIgA₁₋₂ were compared for 0h, 1h and 24h pre-incubation.

The data show that there were no changes in the proportions of virions captured by the panel of antibodies the longer the antibody-virus incubation time (Figure 41). Therefore, this indicates that antibody isotype and structure did not influence how time impacted on antibody ability to capture virions.

Comparison of the neutralisation curves obtained for the panel of b12, CH31 and 2F5 IgG, mIgA₂ and dIgA₁₋₂ bNAbs after 0, 1 and 24h of incubation time with HIV-1_{BaL} appeared to be shifted towards the right of the neutralisation curve overtime (Figure 42). This shift was particularly apparent for IgG antibodies and less for their IgA counterparts. Moreover, the comparisons of the percentages of neutralisation achieved by the panel of antibodies (for 10µg/mL) indicate that while the neutralisation activity of b12 IgG (anti-gp120 CD4bs) remained the same the longer the antibody-virus incubation time, b12 mIgA₂ neutralised significantly more virions after 1h (71.5±2.5%) and 24h (76.1±2.8%) incubation than after no-incubation (63.7±2.4%, both $p<0.0001$), (Figure 43A). Similarly, b12 dIgA₂ neutralised higher proportion of virions after 1h and 24h (99.6±0.4%) pre-incubation than without pre-incubation (89.3±1.4%, both $p<0.0001$).

In contrast, a greater neutralisation activity after 0 and 24h antibody-virus incubation were observed for both IgG and IgA isotypes of CH31 (anti-gp120 CD4bs) (Figure 43C). Nonetheless, while the neutralisation activity of CH31 IgG increased by about 20% between $t=0h$ and $t=24h$, it only increased by about 10% for CH31 mIgA₂ (21.3±0.9% vs 31.4±0.5%, $p<$) as well as for CH31 dIgA₂ (30.9±5.4% vs 40.4±3.5%).

Similarly, the increase in neutralisation activity overtime was more prominent for 2F5 IgG than with its IgA counterparts. Whereas the percentage of neutralisation of 2F5 IgG increased by about 30% between $t=0h$ and $t=24h$, it increased by less than 10% for 2F5 mIgA₂ (19.1±4.3% vs 36.5±4.2%, $p<0.05$) and by about 15% for 2F5 dIgA₂ (22.1±1.9% vs 39±2.1%, $p<0.005$).

These results indicate that extended incubation increased the ability of b12, CH31 and 2F5 IgG, mIgA₂ and dIgA₁₋₂ to neutralise virions. However, the increase in antibody-virus time impacted differently on IgG and IgA (mIgA₂ and dIgA₁₋₂) antibodies targeting the same epitope. A greater increase in neutralisation activity was observed overtime with IgG antibodies compared to IgA antibodies. This indicates that antibody isotype influenced the changes in neutralisation potency overtime. Moreover, the results show that there were no

differences of the effects of antibody-virus incubation on the ability to neutralise HIV-1_{BaL} virions between mIgA₂ and dIgA₁₋₂ presenting the same epitope specificity. This implies that antibody structure (monomeric vs. dimeric) was not a contributing factor. Furthermore, the results show that while the proportions of virions remained stable the longer the antibody-virus incubation time, the neutralising activities increased overtime for both IgG and IgA antibodies. This further suggests that extended antibody-virus incubation caused an increase of iVirions captured and/or neutralisation of iVirions captured, resulting in greater neutralisation activities overtime.

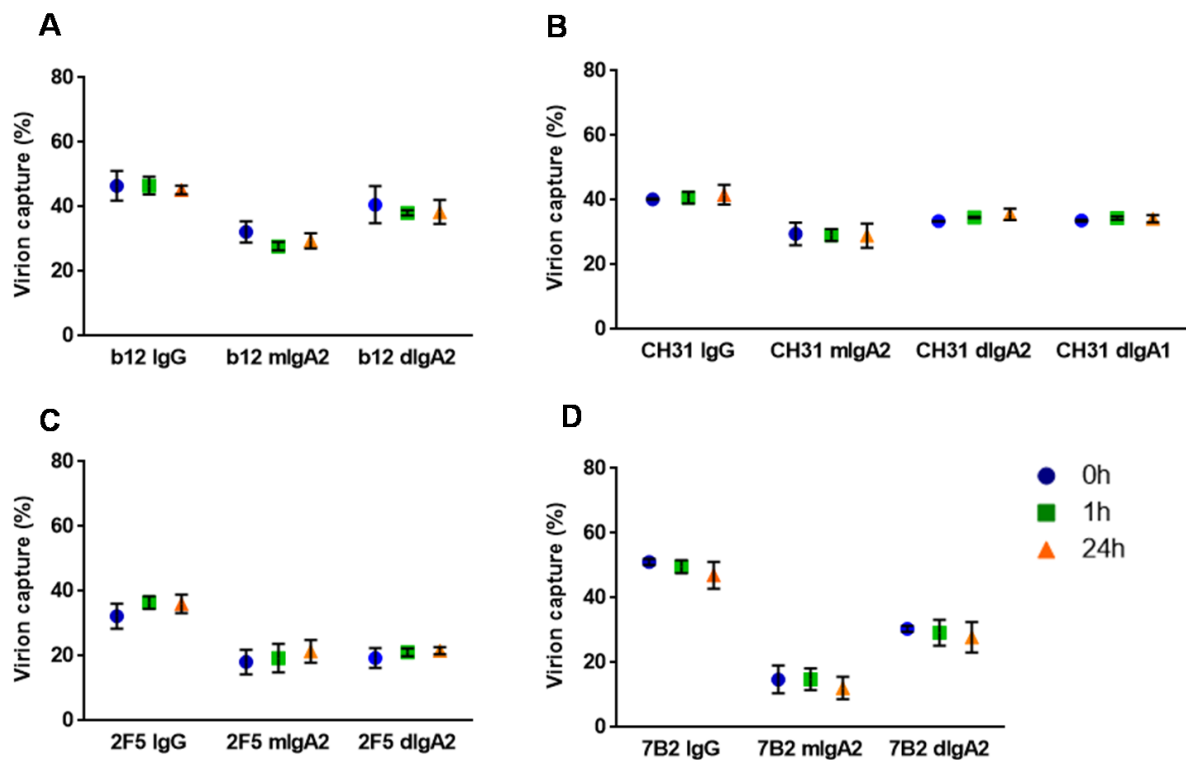


Figure 41. Effect of incubation time on virus capture by different antibody isotypes. The quantity of virions captured by each individual b12, CH31, 2F5 and 7B2 IgG, mIgA₂ and dIgA₁₋₂, following five minutes (0h), 1h and 24h incubation with purified HIV-1 virions was assessed in the VCA. Although differential trends in the amount of virions captured were observed they were not significant with $p < 0.05$.

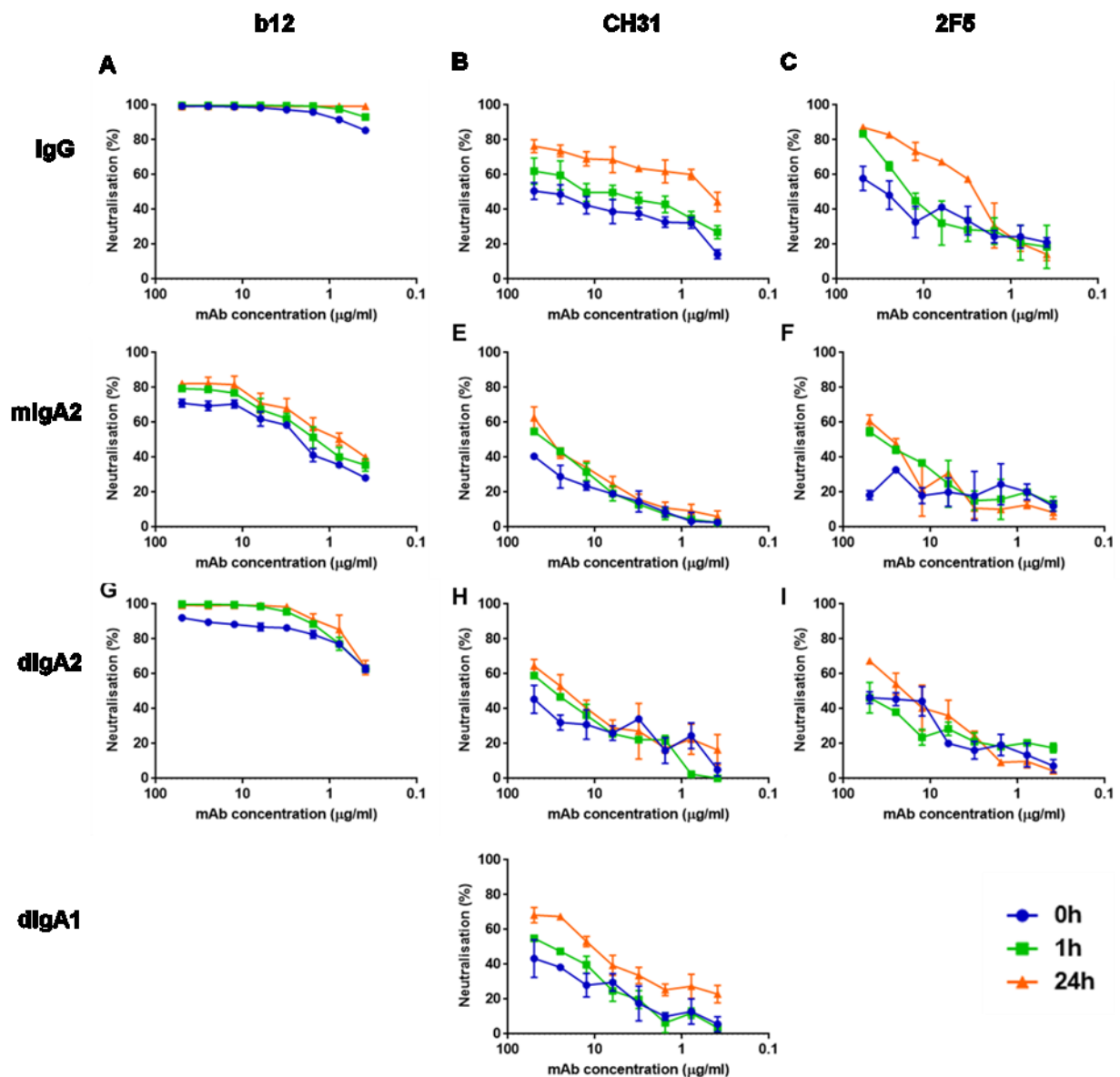


Figure 42. Neutralisation by IgG and IgA antibodies recognising the same epitope. The curves show the neutralisation activity of b12, CH31 and 2F5 IgG, mIgA₂, dIgA₂ and dIgA₁ after incubation with HIV-1_{BaL} virions, less than 5 min (0h) (blue), 1h (green) or 24h (orange), respectively, prior to the addition of TZM-bl cells. Neutralisation activity was measured by TZM-bl assay and the data presented are obtained from three independent experiments and each sample was tested in triplicate.

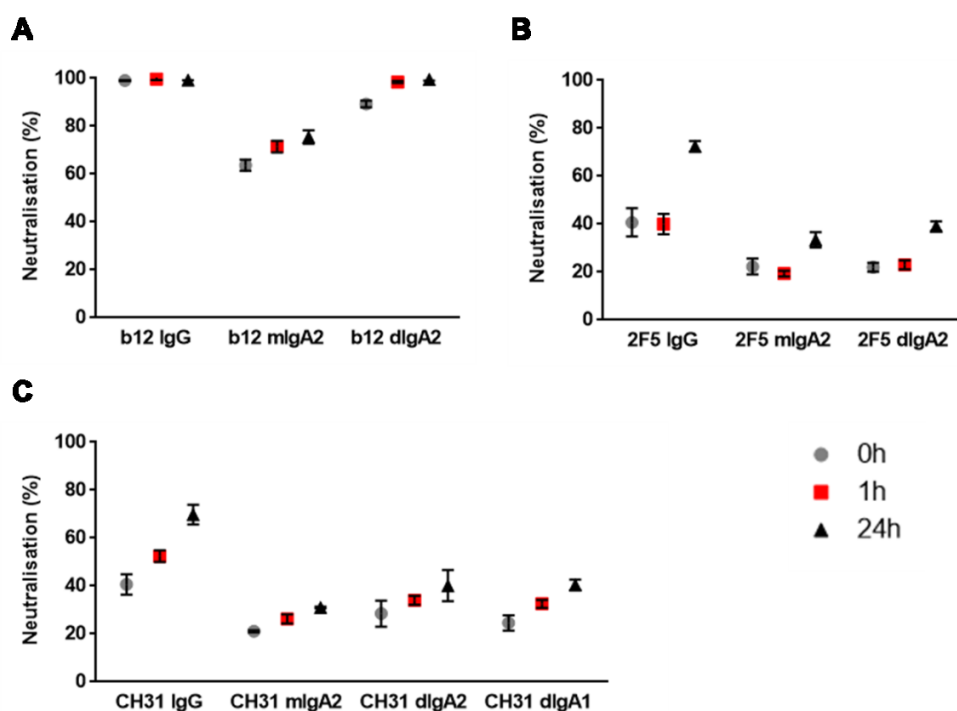


Figure 43. Impact of incubation time and antibody isotype on neutralisation. Graphs showing the percentages of neutralisation for 10 μ g/mL of b12 (A), 2F5 (B) and CH31(C) IgG, mlgA₂ and dIgA₁₋₂ antibodies, after 0, 1 and 24h incubation with HIV-1_{BaL}, and prior to addition of the TZM-bl cells.

It was observed that virion capture significantly correlated with neutralisation for the panel of IgG, mlgA₂ and dIgA₂ antibodies (Figure 44). However, the analysis revealed that the longer the antibody-virus pre-incubation time, the weaker the correlation between virion capture and neutralisation. This suggests that the longer the incubation time the greater, the differences in antiviral outcomes between IgG and IgA antibodies. This results in a weaker correlation between virion capture and neutralisation overtime.

Conversely, the comparisons of the AVCNI for the panel of IgG and IgA antibodies indicate that changes observed might not be influenced by the isotype (Figure 45). No changes in AVCNI were observed the longer the incubation time for b12 IgG, mlgA₂ and dIgA₂ gp120 (gp120 CD4bs-specific) and significant changes were observed with CH31 bNAbs (gp120 CD4bs-specific) and 2F5 bNAbs (gp41 MPER). The changes observed were comparable for IgG, mlgA₂ and dIgA₂ of identical specificity. Together, these results indicate that the capacity of both HIV-1 Env-specific IgG and IgA bNAbs to efficiently neutralise iVirions captured and/or to capture iVirions increases with time.

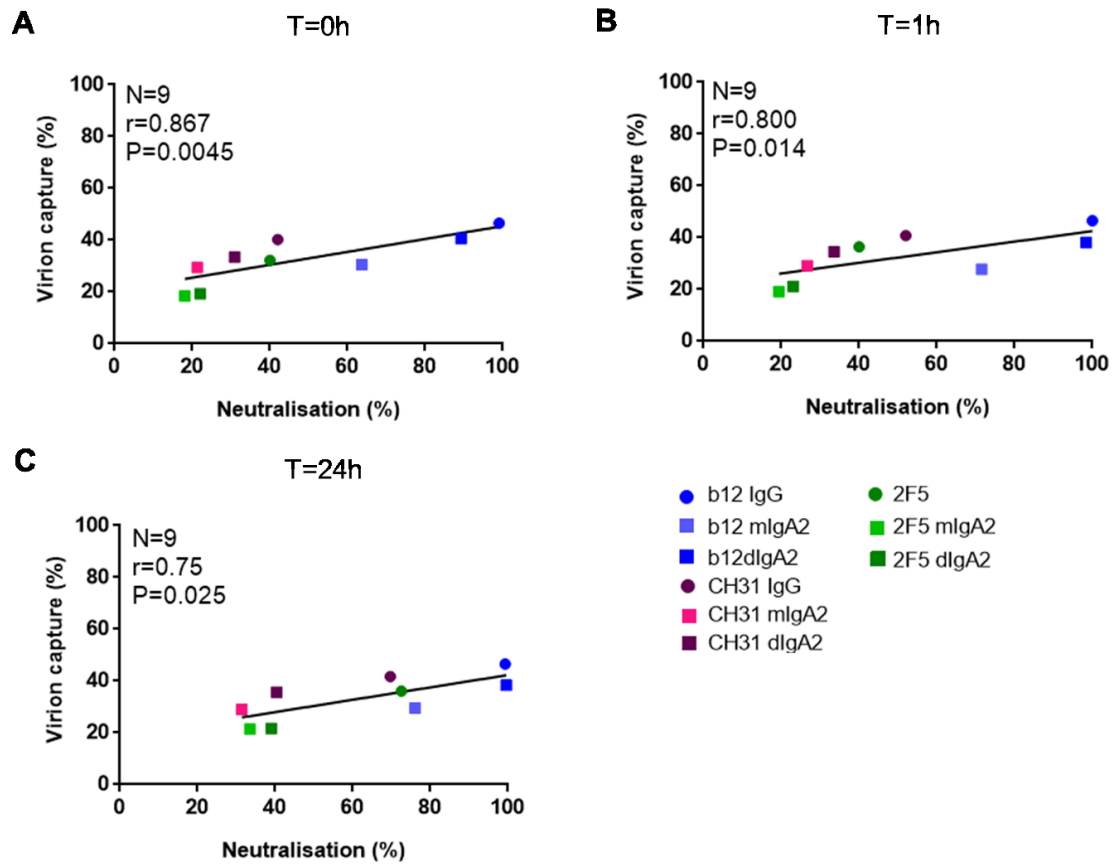


Figure 44. Correlation analyses between virion capture and neutralisation. Spearman r correlation analyses between the percentages of virion capture and neutralisation, after 0, 1 and 24h of antibody-virus incubation were performed for the panel of b12, CH31 and 2F5 IgG, mIgA₂, dIgA₂ antibodies. VCA and TZM-bl neutralisation assay were repeated three times for each IgG and in triplicate samples. N=number of neutralising IgG, r = coefficient of correlation and P= exact p-value calculated with 95%CI.

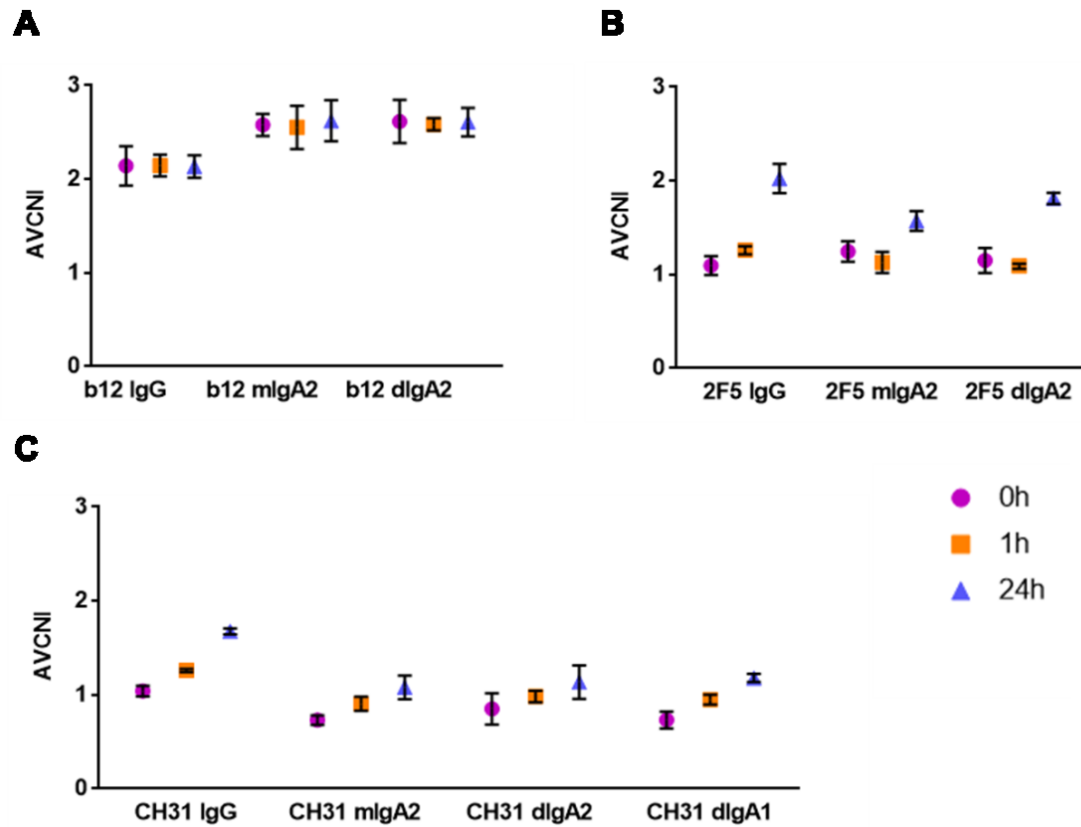


Figure 45. Impact of incubation time on the AVCNI of a panel of IgG and IgA antibodies. AVCNI values for the panel of b12, CH31 and 2F5 IgG, mIgA₂, dIgA₁₋₂ antibodies were calculated using the ratio neutralisation/capture, after no pre-incubation (0h), 1 and 24h of incubation with HIV-1_{BaL}. This was done prior the measurement of the percentages of virions captured and the percentages of neutralisation, for 10µg/mL of antibody. The panel of IgG and IgA antibodies tested were grouped according to the motif targeted: (A) the gp120 CD4bs directed b12, (B) the gp41 MPER-specific 2F5 and (C) the gp120 CD4bs, CH31.

3.3 Discussion

Several studies have indicated that both NAb and nNAb can capture iVirions. However, only NAb can neutralise iVirions captured ^[428, 453, 454]. The mechanisms and specificities underlying these antiviral properties remain poorly understood. In light of these observations, the first objective of this chapter was to identify the epitope features that mediate efficient antibody virion capture and potent neutralisation. It was hypothesised that the epitope characteristics required to capture a high level of virions differ from those needed to efficiently neutralise.

The findings of this study indicate that, generally, IgG antibodies of different specificities but targeting the same Env sites presented comparable ability to capture virions (Figure 19). Notably, it was observed that IgG antibodies directed against gp120 V1/V2 and V3/glycans, and gp41 cluster I presented greater capture capacities than IgG antibodies recognising gp120 CD4bs and gp41 MPER, (Figure 19). These results might be explained by the external position of the gp120 V1/V2 and V3/glycans located in the outer domain of the HIV-1 Env spike. This renders these sites readily accessible to bulky antibodies and might facilitate virion capture by the antibodies targeting this site (Figure 18). Studies describing HIV-1 Env structures using electron tomography and cryoelectron microscopy demonstrated that V1/V2 is located in the apex of the Env trimer ^[64, 459] and that the V3 loop is underneath V1/V2 with its arm directed externally ^[50, 75, 460]. Moreover, the HIV-1 Env gp120 subunit is almost entirely covered at its external surface by a shield of glycans ^[64, 264, 461] and epitope mapping studies indicated that 2G12 and PGT136 recognise high-mannose glycans located in the outer domain of gp120 ^[261, 438, 450, 462]. Conversely, the gp120 CD4bs, which plays a crucial role in viral attachment to target cells, is embedded within the core of gp120 ^[64, 459] which reduces antibody access to this epitope (Figure 18). Likewise, X-ray crystallography analyses demonstrated that the gp41 MPER is poorly exposed and hardly accessible at the viral surface ^[257, 429].

Taken together, these steric constraints might lessen the ability of IgG antibodies targeting the gp120 CD4bs and gp41 MPER to effectively capture virions compared to those directed against gp120 V1/V2 and V3/glycans.

Interestingly, it was observed that the nNABs, 7B2 and 4B3, captured higher to comparable levels of virions to most IgG antibodies of the panel (Table 4). This, although 7B2 and 4B3 interact with gp41 cluster I, which is occluded by gp120, and hardly accessible on the native trimer ^[259, 463]. Epitope mapping studies have demonstrated that 7B2 and 4B3 recognised non-conformational motifs of the immunodominant region of gp41 that are mostly accessible on non-functional Env forms including gp41 monomers and/or “gp41 stumps” or in the post-fusion Env spike ^[253, 437, 464]. Therefore, a possible explanation for these results might be that 7B2 and 4B3 captured relatively high proportions of virions because they may interact preferentially with non-functional forms of Env which exposed the gp41 cluster I ^[253, 454]. Overall, these data suggest that for IgG antibodies, epitope accessibility is the main feature that mediates efficient virion capture.

The comparisons of the quantities of virions captured by the panel of IgA antibodies suggest that antibody structure (monomeric vs dimeric) and epitope accessibility impacted on IgA antibodies ability to capture virions (Figure 20). It was observed that dIgA antibodies binding to the gp120 CD4bs (b12 and CH31) and the gp41 cluster I (7B2) captured significantly greater proportions of virions than their mIgA counterparts. In contrast, the results indicate that 2F5 dIgA which binds to the gp41 MPER captured comparable amount of virions to its mIgA₂ equivalent. This implies that the four binding sites and the dimeric conformation of dIgA molecules might enable the formation of cross bivalent interactions, and confer advantages for capturing more virions at a time compared to monomeric structures. However, these results also infer that the advantages for capturing virions conferred by the dimeric conformation might be dependent on the epitope accessibility. For instance, the gp41 MPER sites recognised by 2F5 dIgA₂ may not enable dIgA₂ to form bivalent interactions due to high level of steric constraints for this epitope. This suggests that for IgA antibodies both epitope accessibility and antibody structure play a major on the virion capture function.

Contrary to the results observed with the VCA, analysis of the neutralisation for the panel of IgG antibodies show that IgG antibodies recognising the same Env sites present different abilities to neutralise virions (Figure 22). Additionally, the gp41 cluster I-specific 7B2 and 4B3 which presented the ability to capture virions, failed to neutralise. Besides, it was observed that b12 IgG which binds to the CD4bs, and 447-52D which recognises the

gp120 V3 site neutralised virtually all the iVirions present. This indicates that epitope specificity rather than accessibility is the major factor influencing neutralisation activity by IgG antibodies. A possible explanation for this might be related to the key role played by the CD4bs in viral attachment to the susceptible cells ^[86, 287]. Likewise, a recent study has demonstrated that the deletion of the gp120 V1/V2 V3 abolished virus entry to target cells^[465].

Additionally, the data show that the gp41 MPER directed 4E10 and 2F5 IgG antibodies presented the lowest abilities to neutralise virions within the panel. These findings were similar to the results of a study which investigated the phenomenon of incomplete neutralisation of bNAbs; and classed a large panel of bNAbs including b12, PG9, 2G12, PG16, 2F5 and 4E10, according to their neutralisation activity against >100 HIV-1 virus in a U87 target cell assay ^[466]. Indeed, this study also found that 2F5 and 4E10 presented the lowest neutralisation activity of their panel and their ranking of the above bNAbs was the same ^[466]. Furthermore, these findings are in accord with a study that used a cocktail of 2G12, 4E10 and 2F5 for the treatment of HIV-1 infected patients following cessation of ART; and found that greater control of HIV-1 infection appeared to correlate with higher plasma concentrations of 2G12 which suggested that 2G12 present greater antiviral capacity than 4E10 and 2F5 ^[425].

The results of the IgA neutralisation assay indicate that b12 mIgA₂ and b12 dIgA₂ neutralised over three times more iVirions than their CH31 equivalents, respectively, while they all recognised the CD4bs. This also shows that epitope specificity plays a major role in IgA neutralisation. Moreover, it was observed that only b12 dIgA₂ presented a greater ability to neutralise virions than its mIgA₂ equivalent. Therefore, the higher virion capture of b12 dIgA₂ compared to its mIgA₂ equivalent correlated with greater neutralisation activity, whilst for CH31 dIgA it did not correlate. A probable explanation for this result might be related to the overall lower neutralisation activity of CH31 IgA compared to b12 IgA. Moreover, although it was not significant CH31 dIgA₁₋₂ appeared to neutralise slightly more virions than CH31 mIgA₂. Additionally, the differences in virion capture observed between CH31 dIgA₁₋₂ and CH31 mIgA₂ were relatively low and might not be reflected by the neutralisation. In total, these data suggest that although structure might influence IgA neutralisation, epitope specificity might be the principal factor impacting on IgA neutralisation.

Altogether, these results indicate that for IgG which only exists as a monomer, epitope accessibility is the predominant parameter influencing virion capture ability; while for IgA which can be found as a monomer or a dimer, both epitope accessibility and antibody structure are major elements impacting on the capacity to capture virions. Conversely, the findings of this study suggest that epitope specificity is the predominant parameter influencing ability of both IgG and IgA antibodies to neutralise iVirions. Therefore, these results support the concept that the epitope and antibody features that mediate efficient virion capture differ from those needed for high neutralisation potency. Moreover, these data imply that dIgA which is the first line of defence against pathogens invasion at mucosal surfaces may have advantages for capturing HIV-1 virions compared to monomeric antibodies. These findings may have important implications for vaccine research as the mucosal compartment is the main portal of HIV entry.

The second objective of this chapter was to study the relationship between virion capture and neutralisation. It was hypothesised that although virion capture has been reported to occupy its own immunological space, ability of IgG and IgA antibodies to capture virions would correlate with their neutralisation activities. Correlation analysis revealed that the more IgG or IgA antibodies captured virions the more they neutralised iVirions. However, although significant, the correlation between these two antibody functions was low. This result might be explained by the presence in great quantity of dVirions. Previous research has indicated that HIV-1 infected cells produced dVirions abundantly and that this might be a mechanism to blur antibody responses ^[448, 449]. It can be conjectured that most NAbS of the panel may also bind to dVirions which may explain the poor correlation between virion capture and neutralisation. Another explanation might be that some NAbS might capture iVirions without effectively neutralising them. Nevertheless, these findings are in agreement with those of Lui et al. which also found that virion capture correlated with neutralisation ^[453]. However, while their results indicated that virion capture of the MN HIV strain correlated with neutralisation, they did not find a significant correlation with the NL4-3 or CM244 viral strains ^[453]. This suggests that the relationship between antibody ability to capture virions and neutralise iVirions may be affected by the viral strain.

To assess the ability of NAbS to neutralise iVirions captured and/or to selectively capture iVirions over dVirions, an AVCNI was devised and calculated using the ratio

neutralisation (%)/virion capture (%). The analysis revealed that b12 IgG and b12 dIgA₂ were the most potent bNAbs of the panel of IgG and IgA antibodies, respectively. They were able by capturing the lowest amount of virions to neutralise virtually all the iVirions present (Table 6, 7, 8 and 9). These findings were in agreement with the fact that b12 was isolated from a phage display library derived from a clade B HIV-1-infected patient and presents a high neutralisation activity against clade B viruses such as HIV-1_{BaL} ^[289, 442]. Moreover, the results indicate that epitope specificity was the principal factor influencing the AVCNI. This might be explained by the fact that neutralisation is a better correlate of reduction of infectivity compared to virion capture and is predominantly influence by epitope specificity ^[262]. Thus, these results imply that the devised AVCNI may be an effective tool to select bNAbs that present the best potential to prevent HIV-1 infection. The use of this novel tool has implications for the selection of bNAbs for passive immunisation studies.

Lastly, the data of this study indicate that antibody isotype may impact on antibody function as IgG and IgA antibodies presenting the same epitope specificity appeared to capture and neutralise different proportions of virions. Furthermore, it was observed that depending on the epitope accessibility dIgA antibodies presented enhanced antiviral function compared to their mIgA counterparts. Based on these observations and the pivotal role of IgA in mucosal immunity, the objective of the subsequent analysis was to determine the impact of isotype on antibody function. This research aimed to improve the understanding of the potential role of IgA in prevention of HIV infection and to assess how this may differ from IgG. Moreover, this investigation may offer a better understanding of the relationships between antibody-virus interaction and antibody function.

In this second part of this chapter, the main objective was to examine the influence of antibody isotype on antiviral function by comparing a panel of IgG, mIgA and dIgA antibodies presenting the same epitope specificity. It was proposed that isotype would impact on antibody ability to capture virions and to neutralise iVirions. Particularly, it was anticipated that dIgA antibodies with their four binding sites may have enhanced antiviral function compared to monomeric IgG and IgA antibodies recognising the same epitope.

Interestingly, the results of this study indicate that the gp120 CD4bs-specific, b12 and CH31, the gp41 MPER-specific 2F5, and the gp41 cluster I-specific, 7B2 IgG, all presented greater abilities to capture virions than their mIgA₂ and dIgA₁₋₂ equivalents of identical specificity (Figure 27). Likewise, it was observed that b12, CH31 and 2F5 IgG bNAbs neutralised significantly more iVirions than their mIgA₂ and dIgA₁₋₂ counterparts (Table 10). This implies that isotype influences antibody ability to capture virions and neutralise iVirions. The findings of this study are consistent with those of Peachman et al. which indicate that antibody isotype plays a crucial role in HIV-1 VCA and in TZM-bl neutralisation assay ^[467]. Particularly, they demonstrated that 2F5 IgM captured a significantly lower amount of HIV-1 clades A through AE and was less efficient at neutralising them than 2F5 IgG. A possible explanation for these results might be related to the binding kinetics of antibody-virus interaction ^[467]. Cooper LJN et al. identified apparent specificity differences among streptococcal IgG expressing identical variable (V) domains and suggested that isotype class switching altered the kinetic constants of antibody-antigen interaction ^[468].

Based on these data, it can be postulated that IgG and IgA antibodies may present distinct kinetic parameters of interaction with virions which lead to differential abilities between IgG and IgA antibodies to capture and neutralise virions. A research study that compared IgG₁ and IgA₁ bNAbs and their Fab (Fragment-antigen binding), of identical specificity for tubulin, demonstrated that they all interacted with significant differences in affinity ^[469]. Additionally, Tomaras et al. reported that HIV-1 Env-specific IgA₂ and IgG₁ bNAbs with the same V region presented considerably different binding affinities against HIV-1 Env ^[238]. It can be hypothesised that the panel of HIV-1 Env IgG tested might have captured higher proportions of virions and neutralise more iVirions than their IgA counterparts because they present higher affinities for iVirions than the IgA of identical specificities. Generally, these results indicate that isotype impact on antibody function and that this might be related to their binding kinetics to antigen.

Most strikingly, the comparisons of the AVCNI show that b12 and CH31 IgG and IgA antibodies of identical specificity (CD4bs-specific) presented significantly distinct AVCNI, while 2F5 IgG and 2F5 IgA had comparable AVCNI (Table 12). This suggests that epitope specificity in a close relationship with antibody isotype may influence antibody ability to efficiently neutralise iVirions captured and/or to select iVirions over dVirions. As mentioned

above, these results might be explained by the influence of the Fragment constant (Fc) domain on the binding of Fab to antigen ^[238, 469].

Both IgG and IgA can be found at the mucosal portal of viral entry and may play a role in the prevention of HIV-1 infection ^[205, 470]. However, very little is known about how IgG and IgA may interact and how this may impact on antiviral outcomes. Based on the observations that the panel of HIV-1 Env IgG and IgA antibodies presented different capacities to capture virions and neutralise iVirions, subsequent analyses aimed to determine how the combination of IgG and IgA isotypes of identical specificities might impact on antiviral function. It was anticipated that as IgG of the panel presented greater abilities to capture and neutralise HIV-1 virions than their IgA equivalents, the mixture of IgG and IgA recognising the same epitope would attenuate these IgG effector functions due to competition between IgG and IgA antibodies for the same Env-binding sites.

The findings of this investigation indicate that the mixtures of b12, CH31, 2F5 and 7B2 IgG with their mIgA counterparts, at the ratio 1:1, diminished the overall amount of virion captured compared to IgG alone; and resulted in a proportion of virions captured in between the amount of virions captured by IgG and mIgA taken separately (Figure 29). This suggests that mIgA antibodies competed with their IgG equivalents for capturing virions which reduced IgG antibodies virion capture. These results were in agreement with the competitive VCA which demonstrated that b12, CH31, 2F5 and 7B2 mIgA₂ antibodies were able to inhibit virion capture by their IgG equivalents (Figure 31). Conversely, the mixtures of the IgG antibodies with their dIgA₂ counterparts captured equivalent quantity of virus to IgG alone, the exception being the combination of b12 IgG and b12 dIgA₂. The latter led to a reduction in the amount of virions captured compared to b12 IgG to the same level as b12 dIgA₂ taken separately (Table 14).

These results might be explained by the fact that mIgA antibodies of the panel have lower ability to capture virions than their dIgA counterparts which leads to a greater negative impact on virion capture outcome. Thus, the results of this study indicate that IgG and IgA recognising the same epitope compete for capturing HIV-1_{BaL} virions and that this competition may be more pronounced between IgG and mIgA₂ than between IgG and dIgA₂ antibodies, due to the lower ability of mIgA₂ compared to dIgA₂ to capture virions. Generally, these data suggest that the ability of a given antibody to inhibit virion capture by

another antibody isotype of identical specificity might not only be dependent on the isotype, but also the structure (monomeric vs dimeric). Furthermore, the results of this investigation suggest that the combination of IgG and IgA recognising the same epitope do not enhance antiviral function and in contrast might alleviate antiviral outcomes.

Overall, the findings of this study are consistent with post hoc analyses of the RV144 vaccine trial which reported that vaccine-induced HIV-1 Env-specific mIgA may have attenuated IgG protective responses through competition for the same HIV-1 Env binding sites^[238, 340].

On the contrary, consecutive analyses, indicate that the combinations of b12 and CH31 (gp120 CD4bs-specific) IgG, mIgA₂ and dIgA₂ bNAbs with their 2F5 (gp41 MPER-specific) counterparts of the same isotype resulted in an increase in the proportion of virions captured (Figure 32). However, the extent of this increase was influenced by the antibody isotype and specificity, with a greater increase in virions captured observed with IgA antibodies compared to IgG and with 2F5 and b12 compared to b12 and CH31. This suggests that the impact was bigger the lower the ability of antibodies to capture virions. These results were in agreement with those of Lui et al. which reported that the combination of HIV-1 Env BNabs increases iVirions capture^[428].

Interestingly, it was observed that rise in virions captured did not automatically correlate with enhance neutralisation. Although, b12 and 2F5 IgG, mIgA₂, and dIgA₂ mixtures captured significantly more virions when combined than separately (Figure 32), they did not neutralise more iVirions when mixed, compared to b12 bNAbs alone (Figure 33). These results might be explained by the considerable greater capacities of b12 bNAbs to capture virions and neutralise iVirions compared to 2F5 bNAbs. It can be supposed that when mixed with b12 bNAbs, 2F5 bNAbs did not provide additional benefits to the potency of b12 bNAbs mediated neutralisation. Conversely, the combinations of CH31 and 2F5 bNAbs might have led to increases in the amounts of virions captured and of iVirions neutralised due to the lower antiviral activities of CH31 bNAbs compared to b12 bNAbs. Moreover, the additive effects on neutralisation of CH31 and 2F5 mixtures was more pronounced for IgG antibodies than IgA which indicate that isotype influences the impact of combination on antiviral outcomes. Furthermore, these strengthen the hypothesis that the potential impact on antiviral activity is greater the lower the ability of the bNAbs which are mixed to capture and neutralise iVirions.

Collectively, these results support the statement of Lui et al. which stipulated that “although capture of infectious HIV-1 correlated with other humoral immune responses, the extent of variation between these humoral responses and virion capture indicates that virion capture antibodies occupy unique immunological space” ^[453].

Following these observations, the impact of mixture of a bNAb with a nNAb on antiviral outcomes was investigated. Both bNAbs and nNAbs might be produced in response to HIV-1 infection during natural infection. Nonetheless, it is not clear how bNAbs and nNAbs may interact to prevent or control HIV-1 infection. It was proposed that depending on the epitope specificities and isotype the combination of bNAbs with nNAbs may lead to increased virion capture and neutralisation.

The results of this investigation indicate that whereas the combination of b12 (CD4bs-specific) bNAbs and 7B2 (gp41 cluster I) nNAbs, at the ratio 1:1, resulted in an increase in virion capture compared to b12 bNAbs or 7B2 nNAbs taken separately (Figure 34); it did not affect b12 bNAbs neutralisation (Figure 35). These results suggest that 7B2 nNAbs did not compete with b12 for capturing virus and did not interfere with b12 neutralisation. Similarly, these data show that while CH31 IgG and 7B2 IgG mixture captured considerably more virions than each assessed separately, the combination of CH31 and 7B2 IgG antibodies did not neutralise significantly more iVirions than CH31 IgG alone. This also suggests that 7B2 IgG did not compromise CH31 IgG neutralisation ability. Strikingly, it was observed that the combination of CH31 IgA and 7B2 IgA captured more virions than each of those taken separately and that this correlated with an increase in neutralisation. A possible explanation for these results might be related to CH31 IgA AVCNI. The results indicate that CH31 IgA presented an AVCNI of about 1 which suggests that they did not efficiently neutralise iVirions that they captured and/or that they had no selectivity and interact indistinguishably with iVirions or dVirions. Moreover, a recent study has shown that 7B2 captured preferentially non-infectious virions ^[428]. Thus, it can be hypothesised that by capturing mostly dVirions 7B2 IgA might have facilitated CH31 IgA capture of iVirions over dVirions thereby increasing neutralisation.

Interestingly, the findings of this study indicate that the mixture of 7B2 nNAbs (gp41 cluster I-specific) with 2F5 bNAb (gp41 MPER-specific) did not lead to an increase in the quantity of virions captured and abolished 2F5 bNAbs mediated neutralisation. Although

previous studies, suggest that 7B2 interacts only with non-functional Env forms, a recent study has demonstrated that 7B2 can capture iVirions^[428]. Yet, another recent study has indicated that a proportion of iVirions exposed both functional and non-functional Env forms^[454]. Therefore, it can be presumed that 7B2 nNABs might bind to a proportion of functional Env and that by binding to the gp41 cluster, 7B2 nNABs might cause steric hindrance and prevent 2F5 bNABs binding to the adjacent gp41 MPER, abrogating 2F5-mediated antiviral function. This would also imply that 7B2 interacts with higher affinity to iVirions than 2F5. Overall, these results are in accord with a previous research which indicated that HIV-1 has evolved a non-neutralising dominant epitope to enable nNABs to bind with stronger affinities and to block adjacent neutralising epitopes and particularly the gp41 MPER^[305, 458].

Thus, these data suggest that both antibody isotype and structure impact on antibody ability to capture and neutralise virions. However, antibody isotype might be the predominant antibody feature that influences antibody function with IgG presenting consistently greater abilities to capture and neutralise virions than their mIgA and dIgA equivalents independently of epitope specificity or accessibility. Yet, in addition to these epitope and antibody characteristics, the time of antibody-virus incubation might impact on antiviral outcomes.

The ability of an antibody-based vaccine to prevent HIV-1 infection might be strongly dependent on its capacity to act rapidly^[366, 404]. Nonetheless, the impact of antibody-virus incubation time on antiviral function and how this impact might be affected by other parameters such as epitope specificity and isotype is still not well understood. Based on these observations, the aim of the third part of this chapter was to determine the effect of antibody-virus incubation time on virion capture and neutralisation. In addition, it sought to evaluate the extent to which time influenced the impact of antibody characteristics (epitope specificity, accessibility, isotype, structure, AVCNI) on function. It was speculated that by increasing the chance of antibody-virus encounter, extended incubation time would lead to enhance antibody function.

Interestingly, the results of this investigation indicate that increase in antibody-virus incubation time did not lead to a rise in virion capture as observed with the panel of IgG antibodies (Figure 37). The data show that the quantities of virions captured were stable

overtime for the vast majority of the IgG antibodies tested, except the gp120 V1/V2-specific PG9 and the gp120 V3/glycans-specific 2G12 and PGT136 for which significant declines in virion capture were observed overtime. A possible explanation for these results might be related to the epitope specificities of PG9, 2G12 and PGT136. PG9 is known to be a glycan-dependent human antibody that preferentially binds to the HIV-1 Env glycoprotein trimer and 2G12 and PGT136 are glycans-specific bNAbs ^[261, 298, 440, 451]. Therefore, it can be postulated that Env glycans might undergo structural changes overtime or become less stable with time which might affect their recognition.

Furthermore, the results show that greater antibody-virus incubation time prior addition of the susceptible cells correlated with a higher neutralisation potency for all of the assessed IgG bNAbs (Figure 37). Notably, 4E10 (gp41 MPER-specific), 2G12 and PGT136 (gp120 V3/glycans-specific) presented the largest increases in the neutralisation activity among IgG antibodies of the panel. Therefore, while the proportion of virions captured remained stable overtime or even significantly decreased overtime for PG9, 2G12 and PGT136, the neutralisation of iVirions increased with the time.

Notwithstanding, the viral samples used in these experiments are likely to have contained both dVirions and iVirions as produced by infected cells during natural infection ^[448, 449]. Moreover, previous studies indicate that although bNAbs capture both iVirions and dVirions, they predominantly capture iVirions ^[428]. a plausible explanation for these results might be that the increases in antibody-virus incubation time may lead to heat-mediated viral degradation as previously reported ^[471]. Another explanation may be that extended incubation causes an increase in iVirions captured, on other terms, a shift towards the capture of iVirions over dVirions, as the total amount of virions captured remained stable while the neutralisation activity increased. This is supported by the general increase in AVCNI observed overtime with IgG antibodies, which indicates a higher neutralisation of captured iVirions (Figure 40). Moreover, it was observed that the strength of the correlation between virion capture and neutralisation increased with time (Figure 39). This might be due to the fact that neutralisation involves the capture of iVirions and that the amounts of iVirions captured overtime appear to increase.

Alternatively, these results might be related to the mechanisms of antibody-mediated neutralisation. Ruprecht et al. have recently demonstrated that antibodies

recognising the CD4bs, V3 loop, and the MPER can induce gp120 shedding, and have detected shedding activity with high frequency in plasma from patients infected with divergent genetic HIV-1 subtypes ^[472]. Thus, it can be hypothesised that by increasing the chance of antibody-virus encounter, extended antibody-virus incubation time might cause higher amount of iVirions to be irreversibly inactivated by antibody-mediated shedding of gp120 and this prior addition of the TZM-bl cells. However, a study has recently reported that VRC01 does not induce gp120 shedding from gp41 while it was observed here that its neutralisation activity increased with time ^[473]. Furthermore, a study of Upadhyay et al. have recently shown that V3 and V2 epitopes are subject to conformational masking and become more accessible to antibody by prolonged antibody-virus interaction. Together, this implies that prolonged incubation might induce conformation changes in Env molecules which are bound to antibody and these changes might enhance neutralisation of iVirions captured.

Results obtained with the IgG panel were similar to those observed with the panel of IgG and IgA antibodies recognising the same epitopes, and also indicate that extended antibody-virus incubation time did not generally lead to increase in virions captured, but enhanced neutralisation. Nevertheless, it was observed that the rise in neutralisation activity overtime was overall more important for IgG than IgA, which implies that isotype influence the changes in neutralisation potency overtime. However, the increases in neutralisation activities were comparable between mIgA and dIgA antibodies of identical specificities which indicate that the effect of time on neutralisation was not influenced by the antibody conformation (monomeric vs dimeric). Therefore, these results suggest that the effect of the time on the neutralisation potency is predominantly influenced by the epitope specificity and antibody isotype. This was also the case when assessed by AVCNI.

3.4 Conclusion

The objective of this research was to improve the understanding of antibody epitope and structural features that influence antiviral function. This study supports previous research which indicates that although virion capture and neutralisation might correlate in some extent, they are two distinct antiviral functions ^[428, 453]. Our data suggest that

accessibility may be the predominant epitope characteristic influencing antibody ability to capture virions while specificity may be the major epitope parameter impacting on antibody neutralisation potency: antibody interacting with readily accessible epitopes presented enhanced virion capture and the anti-CD4bs b12 and the anti-V3 447-52D were the most potent neutralising bNAbs within the panel.

Moreover, these findings challenged the old dogma which stipulates that an antibody is a bifunctional molecule with a variable (V) and a constant (C) domain exerting distinct functions ^[474]. Indeed, this research infers that the V and C domains of an antibody might be structurally and functionally be linked which may impact on antiviral function as observed with IgG presenting enhanced antiviral function compared to mIgA and dIgA with identical V domains, and with dIgA displaying improved antiviral function compared to their mIgA equivalents. These findings have significant implications for HIV research as dIgA is known to play a key role in mucosal immunity and that IgG is also present at the mucosal surfaces where HIV is mainly transmitted.

However, the conclusions of this chapter are limited by the number and specificities of the bNAbs tested and the fact that only a single virus was used. Further research should be undertaken to investigate the impact of specificity, accessibility, isotype and structure on the kinetic parameters of antibody-HIV-1 interaction. Such research may improve the understanding of the relationship between antibody-virus interaction and antiviral function. In addition to these epitope and antibody features, the results of this research indicate that extended antibody-virus incubation time increases neutralisation activity without in general impacting on virion capture.

Future work is needed to determine whether the findings of this study are due to a greater affinity of NAb for iVirions compared to dVirions, to more antibody-mediated shedding of gp120, heat-induced virus degradation, or to antibody-induced Env conformation changes overtime. Overall, this research may offer a better understanding of the mechanisms of antibody-mediated neutralisation and provide insights for the selection of bNAbs for passive immunisation studies and the design of antibody-based vaccines.

Chapter 4

Kinetic constants of interaction of HIV-1 envelope-specific antibodies

4.1 Introduction

The vast majority of successful vaccines strategies against viral pathogens involve strong antibody responses. In the context of HIV vaccine research, a number of studies have suggested that nNABs and bNABs present antiviral functions that could prevent HIV-1 transmission. These include Fab-mediated function such as capture and neutralisation of virions and FcR mediated function including antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cell-mediated virus inhibition (ADCVI) and antibody dependent cellular phagocytosis (ADCP). All of these are predicated on the recognition and binding of specific antibodies to the HIV-1 Env glycoprotein.

One of the main functions of an antibody is to bind pathogens present in the serum or other body fluids such as mucus and milk and to label them for destruction by immune cells. This involves the non-covalent binding of the Fab of the antibody molecule to antigen and of its Fc domain to FcRs expressed on adaptive immune cells. The existence of these two Ab domains was highlighted, in the late 1950s, by the Nobel Prize-winning work of Rodney R. Porter which demonstrated that the antibody molecule could be cleaved by proteolytic digestion into two fragments: Fab and Fc^[475].

Furthermore, the elucidation of the mechanism of antibody diversity revealed that the antibody molecule was encoded by two distinct genes which underwent rearrangements in the genome. These genes were shown to encode for a variable (V) domain and a constant (C) domain of the antibody molecule^[476]. It was also found that during antibody responses, the V regions evolve and adapt to antigen while the C regions maintained their structure and that isotype class switching resulted in new effector functions while preserving antibody specificity^[477]. Moreover, several X-ray crystallographic studies of Fab and Fc fragments provided structural evidence that the V region and the first domain (CH1) of the C region were separated by a flexible hinge polypeptide chain which both isolates and ties the two regions of an antibody molecule^[478].

Altogether, these findings supported the idea that the V and C domains of an antibody molecule could be cleaved into a Fab and Fc domains which are structurally and functionally independent. The V region was defined as being responsible for antibody specificity and affinity for antigen, while the C portion was thought to confer antibody

isotype, regulate antibody half-life and mediate effector functions such as complement activation, opsonisation and Fc-receptor function ^[479].

The findings of the previous chapter challenge some of the existing dogma regarding the bifunctionality of the antibody molecule and indicate that isotype and structure influence antiviral function with HIV-1 Env-specific IgG and IgA sharing identical V domains presenting different capacity to capture virions and neutralise iVirions. These results suggest that the constant domains of an antibody molecule might impact on the way the Fab domain interacts with antigen resulting in different antiviral properties between antibody presenting the same Fab and distinct Fc portions. These findings extend a growing body of evidence which indicate that the C and V portions of an antibody molecule may be functionally linked ^[467, 468, 474, 480].

However, the influences of antibody isotype and structure (monomeric vs polymeric) on the kinetic constants of antibody-antigen interaction are not well understood. Elucidating antibody features that impact on antibody-antigen interaction may have important implications for HIV research. In particular, little is known about how isotype and structure impacts on antibody interaction with HIV-1 Env, responsible for viral entry into susceptible cells and a major target in HIV vaccine research. Moreover, the impact of Env epitope specificity and accessibility on the binding kinetics of antibodies to HIV-1 Env is still unclear.

In this context, greater understanding of the constraints governing antibody-antigen interaction may offer novel insights into the rational design of antibody-based vaccines and immunotherapies against HIV-1 infection. This may also help to better understand the impact of the constant domains on Fab-mediated function, and provide further evidences challenging previous dogma around antibody bifunctionality. The overall aim of the present chapter is to study the influence of antibody characteristics on the kinetic parameters of antibody interaction with HIV-1 Env.

The kinetics of antibody-antigen interactions are described by the rates of antibody-antigen complex formation called association constant rate (on-rate or K_{on}) and the rates of dissociation of antibody-antigen complexes in two species (antibody and antigen) named dissociation constant rate (off-rates or K_{off}). The K_{on} represents how quickly an antibody binds to antigen and has the dimension of $M^{-1} s^{-1}$ and the K_{off} represents how fast an antibody dissociates from antigen and has for unit s^{-1} . The binding affinity (the so called dissociation constant or K_D) corresponds to the ratio off-rate to on-rate, and has the physical

dimension of a concentration (M) and represents the strength of attraction between antibody (A) and antigen (B):

$$KD = \frac{K_{off}}{K_{on}} = \frac{[A] \cdot [B]}{[AB]}$$

To study the kinetic parameters of antibody-HIV-1 Env interaction, a soluble form of the trimeric HIV-1 Env exposed on the viral surface, called gp140 was used. On the viral surface, HIV-1 Env spike forms a trimer of a heterodimer consisting of a transmembrane glycoprotein (gp41) and a surface glycoprotein (gp120). Soluble versions of trimeric HIV-1 Env have been developed for over a decade to serve as immunogens in HIV vaccine research and to improve the understanding of the mechanisms of antibody-mediated neutralisation [459, 481]. However, currently no HIV-1 Env vaccine immunogen has elicited bNAbs in clinical trials [407, 415]. This may reflect the fact that previously tested soluble Env proteins imperfectly mimic the functional native Env, and are potentially unstable *in vivo* [482]. Indeed, the processes of oligomerisation and cleavage of the HIV-1 gp160 precursor into gp120 and gp41 modifies antigenicity and destabilised Env structure, the prefusion trimer being in a metastable conformation [464]. To avoid potential problems with stability, we used SOSIP HIV-1_{BaL} gp140 trimer [483], which is a cleaved, solubilised version of the trimeric HIV-1_{BaL} Env spike comprising stabilising mutations (SOSIP) [75, 459, 482]. These types of Env proteins have been shown to be good mimics of functional native Env proteins exposed on the HIV surface [75, 482].

The objective of this chapter was to examine the antibody features which influence the kinetic constants of antibody interaction with HIV-1_{BaL} gp140. It was hypothesised that epitope specificity and accessibility would impact on HIV-1 Env-specific antibody binding kinetics and particularly on antibody association constant rates, with antibody targeting readily accessible epitopes presenting higher on-rates than those subject to steric constraints. Furthermore, it was proposed that antibody isotype (IgG vs IgA) and structure (monomeric vs dimeric) would influence the kinetic parameters of antibody-gp140 interaction with IgG, mIgA and dIgA antibodies recognising the same epitopes presenting different binding kinetics.

This chapter comprised five aims:

- Aim 1: to develop an antibody binding assay using Bio-Layer Interferometry (BLI) that enables measurement of the kinetic parameters of antibody-antigen interaction.
- Aim 2: to examine the impact of epitope specificity and accessibility on the kinetic constants of antibody-gp140 interaction using a panel of 14 IgG and 9 IgA antibodies which recognise a range of Env epitopes.
- Aim 3: to assess the impact of antibody isotype and structure on antibody-gp140 interaction by comparing the kinetic constants of IgG, mIgA and dIgA antibodies.
- Aim 4: to determine whether antibodies which present identical epitope specificity and distinct isotype compete for interaction with gp140.
- Aim 5: to assess the feasibility of measuring HIV-1 Env IgG and IgA antibodies interaction with HIV-1_{BaL} virions using the BLI system.

The first part of this chapter describes the development of the BLI binding assay to measure antibody interactions with HIV-1_{BaL} gp140 and examines the influence of epitope specificity and accessibility on the kinetic parameters of this interaction. The second part investigates the impact of antibody isotype and structure on binding kinetics by comparing HIV-1 Env IgG, mIgA and dIgA antibodies of identical epitope specificity, and studies how these antibody characteristics affect binding competitiveness. The last part of this chapter evaluates the use of the BLI system to measure antibody binding to HIV-1 virions.

4.2 Results

4.2.1 BLI binding assay to measure antibody-HIV-1_{BaL} Env gp140 interaction

The first aim of this chapter was to establish a novel antibody binding assay that allows measuring the kinetic parameters of interaction between antibody and HIV-1_{BaL} Env gp140 (BaL gp140) using the BLI instrument. The BLI system was chosen for this purpose because it enables the rapid determination of binding kinetic constants, it was accessible and easier to implement compared to other techniques such as Surface Plasmon Resonance (SPR) or ELISA.

The BLI binding assay consisted of capturing gp140 proteins with a lectin called *Galanthus nivalis agglutinin* (GNA) which binds to α 1-3 and α 1-6 linked high mannose structures present on the HIV and SIV Env spikes. GNA lectin is commonly used in antibody assays to capture HIV and SIV Env proteins (e.g. affinity purification chromatography and ELISA) due to its high affinity, avidity, and broad selectivity for Env proteins.

Here, a new approach using a streptavidin (SA) biosensor and GNA conjugated with biotin (biotin-GNA) was developed (Figure 46). The interaction between SA and biotin is one of the strongest non-covalent interaction ever measured in Nature, and for this reason SA was used to immobilise biotinylated GNA onto the biosensor tip^[484]. After a washing step to remove unbound biotin-GNA proteins, BaL gp140 was captured by immobilised biotin-GNA on the SA biosensor. To choose the best concentration to use for the next step of the assay different concentrations of BaL gp140 were tested. The binding signal to biotin-GNA of BaL gp140 increased with the concentration of gp140 proteins indicating a specific and concentration-dependant binding (Figure 46B).

The top concentration of 50 μ g/mL of BaL gp140 was selected for the following step to enable the highest amount of antibody to bind. Examination of the binding kinetics indicated that BaL gp140 hardly dissociated from biotin-GNA and bound with a very strong affinity to it ($K_D < 1.0$ pM). This demonstrated that biotin-GNA was able to capture BaL gp140 proteins and to maintain a strong interaction even after washing. To determine whether it was possible to detect antibody binding to captured BaL gp140, CH31 IgG (gp120 CD4bs-specific) was used at different concentrations for the assay development (Figure 47).

Hence, the final assay protocol was constituted of three steps: (1) biotin-GNA immobilisation onto SA biosensor, (2) biotin-GNA capture of HIV-1_{BaL} gp140 and (3) antibody binding to captured gp140 antibody interaction with captured BaL gp140 was measured by the BLI instrument as a shift in the interference pattern overtime. From this, the association constant rate (k_{on}), dissociation constant rate (k_{off}) and binding (K_D) affinity constants were derived. GNA, BaL gp140 and antibody concentrations used were optimised for the assay.

To generate a full kinetic profile of antibody-BaL gp140 interaction and obtain the kinetic parameters aforementioned, different antibody concentrations were measured. In this assay, at least three antibody concentrations were tested starting from 2 μ M with a two-fold serial dilution to 0.5 μ M and each measurement was performed independently and repeated three times (Figure 47). To calculate correct binding constants, the amount of time allotted to the association and dissociation phases were long enough to observe a curvature of the binding response followed by decay in the response (360s each), (Figure 47). The amplitude of the binding signal increased with the concentration of antibody applied to the biosensor chip indicating that CH31 bound to captured BaL gp140 in a concentration-dependant manner. The kinetic parameters: K_D , k_{on} and k_{off} , of CH31 were calculated using the 1:1 global analysis model which fitted the data by generating nonlinear regression curve-fits. The analysis demonstrated that CH31 bound to BaL gp140 with a relatively high affinity, in the upper nanomolar range ($K_D=636\text{nM}\pm42.8\text{nM}$).

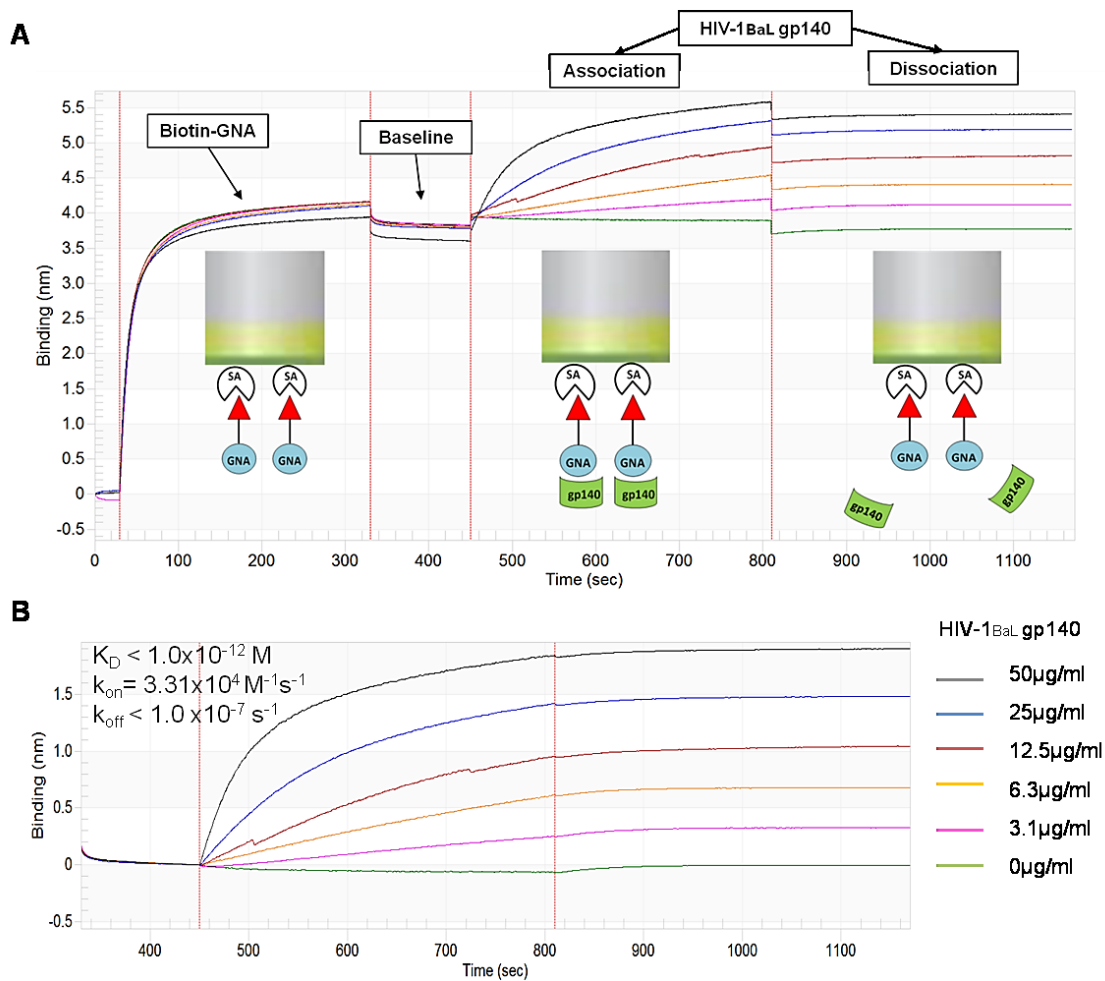


Figure 46. Development of the BLI binding assay: biotin-GNA capture of BaL gp140. (A) Successive steps of the BLI binding assay: initial baseline (30s), biotin-GNA loading onto streptavidin biosensor (300s), capture of HIV-1_{BaL} gp140 (360s) and dissociation phase (360s). The illustrations under the interaction curves indicate each binding step. (B) Enlargement of the association and dissociation stages with shift correction applied at the beginning of the dissociation phase. HIV-1_{BaL} gp140 binding to biotin-GNA is measured for the different gp140 concentrations ranging from 0 (buffer only negative control) to 50 µg/mL. Each measurement was repeated three times and kinetic parameters were calculated using the 1:1 global analysis model with all the repeated measurements. The resulting kinetic constants of biotin-GNA and BaL gp140 interaction are shown: the binding affinity (K_D), association constant rate (k_{on}) and dissociation constant rate (k_{off}).

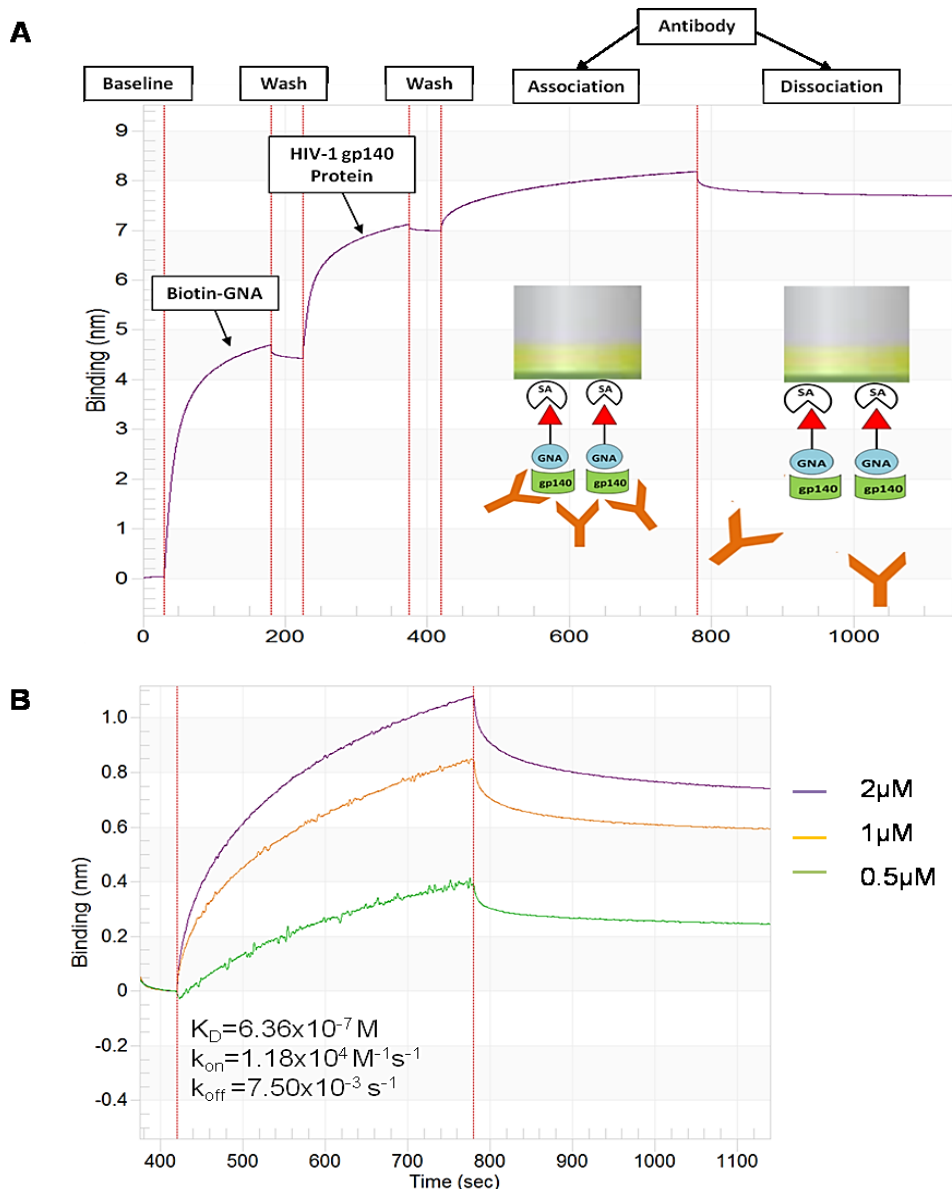


Figure 47. Measurement of antibody-BaL gp140 interaction using the BLI instrument. The figure (A) shows the successive steps of the BLI binding assay. (1) A baseline is established and (2) biotinylated GNA (3.85µM) is immobilised on SA biosensor which results in a binding signal. (3) The biosensor is washed to remove non-immobilised biotin-GNA. (4) HIV-1_{BaL} gp140 (357nM or 50µg/mL) is captured by immobilised biotin-GNA. (5) The biosensor is washed to remove unbound gp140 proteins. (6) The antibody (here CH31 IgG at a concentration of 2µM) binds to gp140 while the association constant rate (k_{on}) is measured. (7) Then, CH31 IgG unbinds from gp140 while the dissociation constant rate (k_{off}) is determined. The figure (B) displays an enlargement of the association and dissociation phases of CH31 IgG interaction with BaL gp140, separated by a vertical red line. The processed data curves of the three concentrations tested are shown in purple (2µM), orange (1µM) and green (0.5µM) and measurements were repeated three times. The data were processed and fitted using the 1:1 global analysis model to calculate the kinetic parameters: K_D , k_{on} k_{off} .

4.2.2 Impact of epitope specificity and accessibility on IgG binding to BaL gp140

To study the influence of epitope specificity and accessibility on the kinetic parameters of antibody- BaL gp140 interaction, the same panels of HIV-1 Env-specific IgG and IgA used in Chapter 3 (Figure 19) were tested in this assay.

The first panel comprised 14 IgG which were divided into six groups according to their Env epitope specificities: (1) gp120 CD4bs, (b12, CH31, VRC01 and VRC03), (2) gp120 V3/glycans (2G12, PT136 and 447-52D), (3) gp120 V1/V2 (PG9 and PG16), (4) gp41 Membrane Proximal External Region (MPER), (2F5 and 4E10), (5) gp41 cluster I (7B2 and 4B3) and (6) multiple epitope-specific (HIVIG). HIVIG is a polyclonal NAb, 7B2 and 4B3 are nNAbs and the 11 other antibodies are bNAbs. The second panel included 9 IgA antibodies: the mIgA₂ and dIgA₂ isoforms of b12, CH31, 2F5 and 7B2 and the dIgA₁ isoform of CH31 (Figure 19). Separate panels of IgG and IgA antibodies were used in this assay, as it was thought that antibody isotype might influence antibody binding kinetics, and that the focus on the first part of this chapter was on the impact of epitope specificity and accessibility on the kinetic parameters of antibody-BaL gp140 interaction.

Comparisons of the binding kinetics of the panel of IgG antibodies demonstrated that IgG antibodies from the same epitope specificity group, presented generally similar binding affinities but interacted with different on-rates and/or off-rates (Figure 48). Notably, no significant differences were observed between the K_D values of the gp120 V1/V2 directed PG16 and PG9 (55.7 ± 0.9 nM and 25.4 ± 0.8 nM). Similar observations were made with the gp120 V3/glycans 2G12, PGT136, and 447-52D, the gp41 MPER-specific 2F5 and 4E10, and the gp41 Cluster I directed 4B3 and 7B2. However, the gp120 CD4bs-specific IgG antibodies b12, CH31, VRC01 and VRC03 interacted with HIV-1_{BaL} gp140 with significantly different affinities. Particularly, b12 and VRC01 (19 ± 2.7 nM and 23 ± 2.4 nM, respectively) bound with a one-log higher affinity to BaL gp140 than CH31 and VRC03 (636 ± 34.4 nM and 734 ± 49 nM, respectively) (all $p < 0.0001$). Remarkably, it was observed that b12, VRC01, PG16, PG9, 447-52D, 2G12, PGT136, 7B2 and 4B3, all presented comparable binding affinities for BaL gp140 which were significantly greater than reported with CH31, VRC03, 2F5, 4E10 and HIV-IG (Figure 48) (all $p < 0.05$).

This indicated that although epitope specificity and epitope accessibility influence IgG affinity for BaL gp140, epitope accessibility might be the predominant factor impacting

on IgG binding affinity, with IgG antibodies interacting to readily accessible epitopes presenting superior affinities than those targeting epitopes subject to steric constraints (e.g. gp41 MPER and some gp120 CD4bs epitopes as mentioned in the preceding chapter).

Conversely, significant differences in on-rates and/or off-rates could be observed between IgG antibodies recognising the same Env site. Particularly, b12 bound significantly more quickly to BaL gp140 ($6.2 \pm 0.1 \times 10^4 \text{M}^{-1}\text{s}^{-1}$) compared to CH31, VRC01 and VRC03 (1.2 ± 0.3 , 1.8 ± 0.2 and $97 \pm 4 \times 10^4 \text{M}^{-1}\text{s}^{-1}$, respectively) ($p < 0.001$); and VRC01 interacted more rapidly than VRC03 ($p < 0.05$). Nonetheless, no significant differences in on-rates were observed between CH31 and VRC01 and between CH31 and VRC03 (Figure 48). Also, CH31 and VRC03 presented comparable off-rates (7.5 ± 1.5 and $7.1 \pm 1.2 \text{ms}^{-1}$, respectively) and both dissociated more rapidly from BaL gp140 than b12 and VRC01 (1.2 ± 0.14 and $0.41 \pm 0.008 \text{ms}^{-1}$, respectively) (all $p < 0.0001$).

Similarly, the gp120 V1/V2-specific PG16 and PG9 presented significantly different on- and off- rates. PG9 bound faster to BaL gp140 than PG16 (72 ± 2.6 and $85 \pm 3.0 \times 10^4 \text{M}^{-1}\text{s}^{-1}$, respectively) and dissociated less rapidly (4.0 ± 0.1 and $2.2 \pm 0.05 \text{ms}^{-1}$, respectively) (all $p < 0.0001$). As well, the gp120 V3/glycans-specific 2G12 interacted more rapidly with BaL gp140 than 447-52D which also bound faster than PGT136 (93 ± 2.9 , 60 ± 1.1 and $50 \pm 5 \times 10^4 \text{M}^{-1}\text{s}^{-1}$, respectively) (all $p < 0.0001$). In contrast, 2G12 dissociated faster from BaL gp140 ($1.8 \pm 0.04 \text{ms}^{-1}$) than 447-52D ($0.94 \pm 0.14 \text{ms}^{-1}$, $p < 0.05$) and PGT136 ($0.77 \pm 0.01 \text{ms}^{-1}$, $p < 0.01$).

Interestingly, there were no significant differences in all three kinetic constants between the gp41 MPER-directed, 2F5 and 4E10. On the contrary, differences were observed between the gp41 Cluster I-specific, 7B2 and 4B3. Particularly, 4B3 bound more rapidly to BaL gp140 than 7B2 (38 ± 1.5 and $30 \pm 2.8 \times 10^4 \text{M}^{-1}\text{s}^{-1}$, respectively) ($p < 0.001$) and remained bound longer than 7B2 (0.49 ± 0.03 and $1.42 \pm 0.24 \text{ms}^{-1}$, respectively) ($p < 0.001$).

Furthermore, it was observed that IgG antibodies targeting gp120 V1/V2, the gp120 V3/glycans and gp41 Cluster I sites interacted significantly more rapidly with BaL gp140 than those targeting the gp41 MPER, multiple Env sites, and the gp120 CD4bs except b12 (all $p < 0.0001$) (Figure 48B). This indicates that IgG antibodies binding to readily accessible Env epitopes bound more rapidly than those directed against occluded Env epitopes which suggests that epitope accessibility might be the major factor influencing IgG association constant rate.

The gp120 CD4bs-specific b12 and VRC01, the gp120 V3/glycans-specific 447-52D, 2G12 and PGT136 and the gp41-specific 7B2 and 4B3, all presented similar off-rates and remained bound longer to BaL gp140 than the other IgG antibodies in the panel (all $p < 0.05$) (Figure 48C). This suggests that epitope accessibility also influences IgG off-rates, however, to a lesser extent than observed with the gp120 V1/V2-specific PG16 which presented a similar off-rate to the gp41 MPER-directed 2F5 and 4E10. Therefore, this indicates that epitope specificity might play a more important role in IgG off-rate than in IgG binding affinity and on-rate for Env.

In total, these results indicate that both epitope specificity and accessibility influence the kinetic parameters of IgG-HIV-1_{BaL} gp140 interaction. However, these data suggest that epitope accessibility is the predominant factor affecting IgG on-rate and binding affinity to Envgp140. IgG antibodies directed against easily accessible Env epitope may have enhanced ability to bind rapidly and with high affinity than those directed against obstructed Env epitopes.

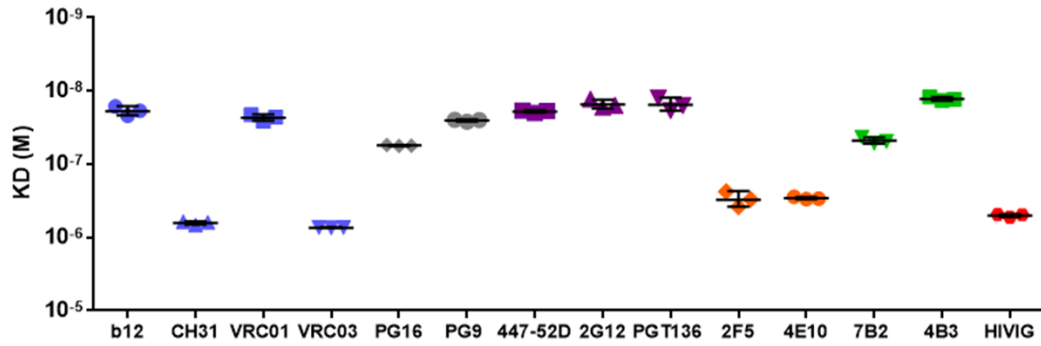
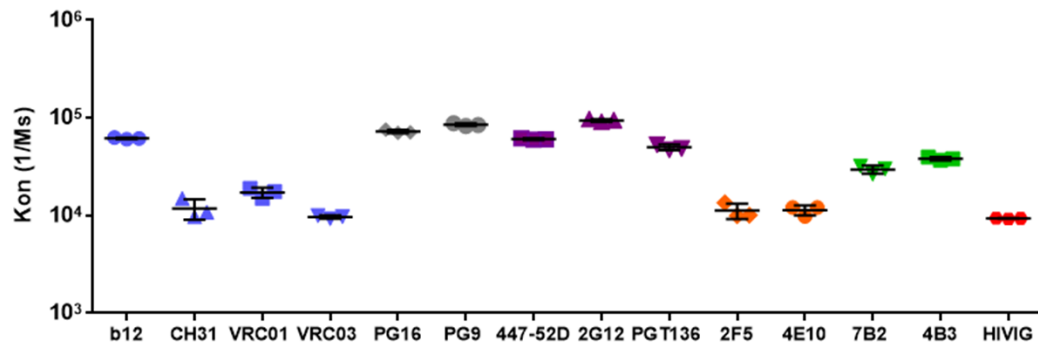
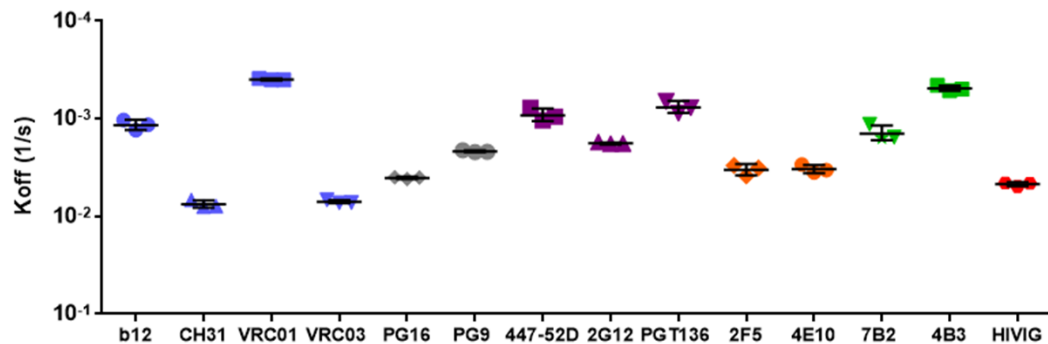
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Figure 48. HIV-1 Env-specific IgG antibodies binding kinetic parameters. The kinetics parameters of a panel of IgG antibodies were measured using the BLI system. IgG antibodies were grouped into six categories according to their epitope specificities: gp120 CD4bs (blue), gp120 V1/V2 (grey) gp120 V3/glycans (purple), gp41 MPER (orange), gp41 linear epitopes (green) and several epitopes (red) for the HIVIG polyclonal antibody. The binding affinity (K_D), association constant rate (K_{on}) and dissociation constant rate (K_{off}) are displayed as means $\pm$ SEM ($n=3$) and comparison analyses were performed with two-way ANOVA-Tukey's MCT with 95%CI.

4.2.3 Kinetic parameters of a panel of HIV-1 Env-specific IgA antibodies

As previously performed for the IgG isotype panel, here, the binding kinetic parameters for the panel of IgA antibodies were compared to evaluate the impact of epitope specificity and accessibility on IgA-HIV-1_{BaL} gp140 interaction. b12, CH31, 2F5 and 7B2 monomeric and dimeric IgA antibodies were compared in this assay.

Remarkably, no significant differences in the kinetic constants: K_D , K_{on} and K_{off} between the mIgA₂ and dIgA₂ forms of b12, 2F5 and 7B2 antibodies recognising the same epitope were observed. This was also the case for CH31 dIgA₂ and CH31 dIgA₁ (Figure 49). However, although CH31 mIgA₂, dIgA₂ and dIgA₁ presented similar on-rates and off-rates, CH31 mIgA₂ bound with a lower affinity to HIV-1_{BaL} gp140 than CH31 dIgA₂ ($p < 0.01$) and dIgA₁ ($p < 0.05$) (461 ± 20 nM, 260 ± 33 nM and 291 ± 30 nM, respectively). Similar to the results obtained with their IgG counterparts, b12 IgA and 7B2 IgA bound with comparable binding affinities to BaL gp140, and with greater affinities than CH31 and 2F5 IgA antibodies. However, unlike their IgG counterparts CH31 IgA and 2F5 IgA presented similar binding affinities. Overall, b12 IgA and 7B2 IgA bound more quickly and dissociated more slowly compared to CH31 IgA and 2F5 IgA (Figure 50).

Thus, these data show that in general mIgA and dIgA antibodies of identical epitope specificity present comparable kinetic constants of interaction with HIV-1_{BaL} gp140. This suggests that the IgA structure (monomeric vs dimeric) might not be a dominant factor affecting binding kinetics. Moreover, the results observed with the panel of IgA antibodies were relatively similar to those obtained with the panel of IgG antibodies, and further indicate that epitope accessibility more than epitope specificity might be a major factor influencing HIV-1 Env-specific antibodies binding kinetics.

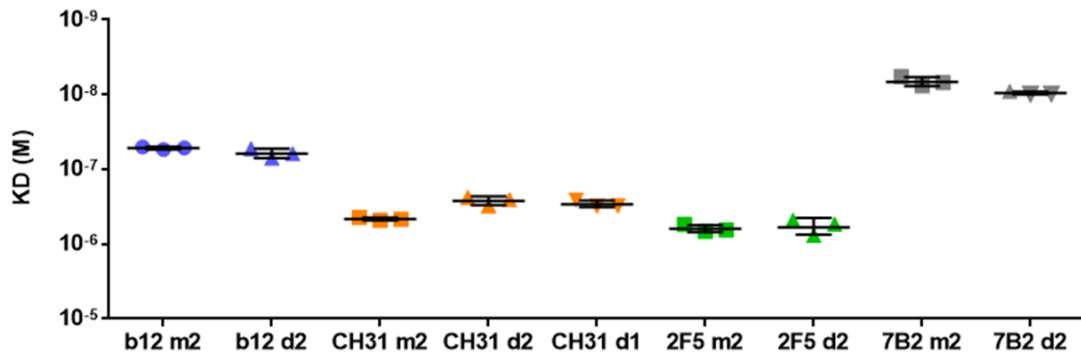
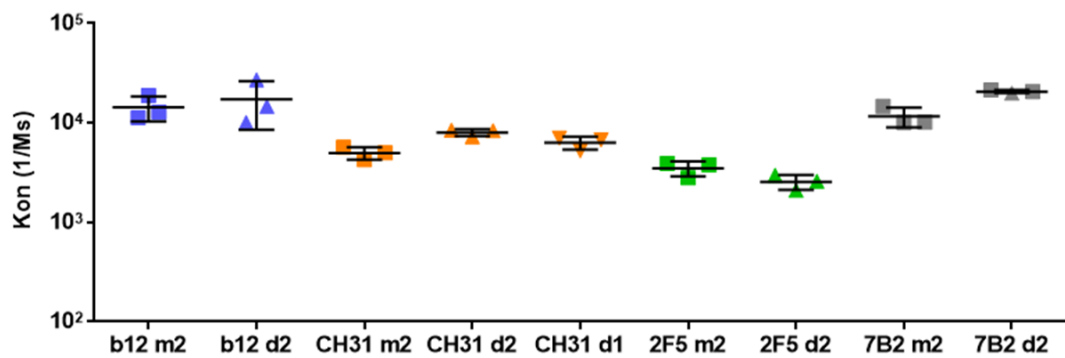
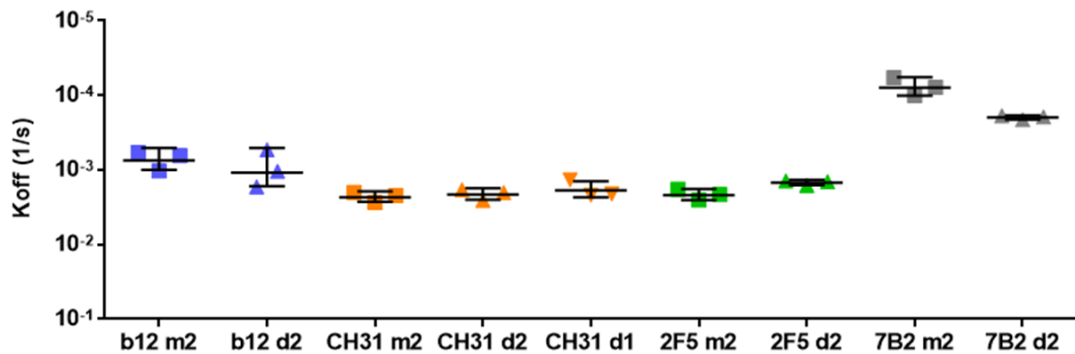
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Figure 49. HIV-1 Env-specific IgA antibodies binding kinetic parameters. The kinetic parameters of the panel of monomeric and dimeric IgA forms of b12 (blue), CH31 (orange), 2F5 (green) and 7B2 (grey) were measured using the BLI system. MIgA₂, dIgA₂ and dIgA₁ were labelled m2, d2 and d1, respectively. b12 and CH31 are specific for gp120 CD4bs; 2F5 recognises the gp41 MPER, and 7B2 a gp41 linear epitope. The binding affinity (K_D), association constant rates (k_{on}) and dissociation constant rates (k_{off}) are shown as means $\pm$ SEM ($n=3$) and comparison analyses were performed using two-way ANOVA-Tukey's MCT with 95%CI.

4.2.4 Influence of antibody isotype on binding kinetics to BaL gp140

To assess the influence of antibody isotype on the kinetic parameters of antibody-HIV-1_{BaL} Envgp140 interaction, the IgG₁, mIgA₂ and dIgA₁₋₂ forms of b12, CH31, 2F5 and 7B2 were compared using the devised BLI binding assay.

Comparison of the binding signals of the panel of bNAbs to BaL gp140 revealed that IgG, mIgA₂ and dIgA₁₋₂ bNAbs targeting the same epitope presented distinct binding kinetic profiles (Figure 50). Notably, the amplitude of the binding signal was the lowest for IgG followed by mIgA₂, then dIgA₁₋₂ bNAbs. Moreover, differences in the amplitude of the binding signals were observed between b12, CH31, 2F5 and 7B2 from the same isotype. The binding signal amplitudes for 2F5 bNAbs were two-fold lower than those of b12 bNAbs, and three-fold lower than those of CH31 and 7B2 bNAbs. Remarkably, the shape of the binding signals also varied according to the epitope targeted. Altogether, these results indicate that bNAbs binding profiles varied according to the antibody isotype and epitope specificity.

Binding kinetic analysis indicated that bNAbs from the panel presented high binding affinities for HIV-1_{BaL} gp140 with K_D values within the nanomolar range (10^{-7} to 10^{-9}) (Figure 51). However, significant differences between bNAbs targeting distinct Env epitopes were observed, with 7B2 and b12 bNAbs binding to BaL gp140 with higher affinities than CH31 and 2F5 bNAbs. Furthermore, for all bNAbs tested, significant differences in the kinetic parameters of interaction with BaL gp140 were reported between the IgG and IgA isotypes. Particularly, b12 IgG and 2F5 IgG bound with higher affinities to BaL gp140 (19 ± 2.7 nM and 303 ± 73 nM) than their monomeric (50 ± 2.1 nM and 623 ± 66 nM) and dimeric IgA₂ (62 ± 8.8 nM and 595 ± 149 nM) counterparts, respectively (all $p < 0.05$). In contrast, CH31 IgG and 7B2 IgG bound with lower affinities (636 ± 43 nM and 48 ± 5 nM) than their mIgA₂ (461 ± 20 nM and 6.8 ± 0.95 nM) and dIgA₂ (260 ± 36 nM and 9.5 ± 0.41 nM) equivalents (all $p < 0.05$). Besides, CH31 mIgA₂ presented a lower affinity for BaL gp140 than its dimeric counterpart (both $p < 0.01$). Conversely, no significant difference in K_D values were observed between CH31 dIgA₁ and CH31 dIgA₂ (291 ± 30 nM and 260 ± 36 nM).

Comparison analysis of the association and dissociation constant rates of the panel of bNAbs demonstrates that IgG antibodies interacted systematically more rapidly with BaL gp140 than mIgA and dIgA antibodies of identical specificity, which presented comparable on-rates (Figure 51). Conversely, mIgA₂ and dIgA₂ recognising the same epitopes presented

faster off-rates than their IgG equivalents. Additionally, while 7B2 IgG, mIgA₂ and dIgA₂ presented a one-log differences in off-rates values (1.42 ± 0.025 , 0.079 ± 0.022 and 0.198 ± 0.012 ms⁻¹, respectively), no significant differences in dissociation constant rates were reported between the mIgA₂ and dIgA₁₋₂ forms of b12, 2F5 and CH31.

Therefore, comparison of the kinetic parameters of the panel of HIV-1 Env-specific IgG, mIgA₂, and dIgA₁₋₂ bNAbs imply that antibody isotype impacts on the binding kinetics to HIV-1_{BaL} gp140 as observed with IgG, mIgA₂, and dIgA₁₋₂ of identical specificity presenting different kinetic parameters. Most importantly, the findings of this study suggest that bNAbs of the IgG isotype have enhanced capacity to bind rapidly to gp140 compared to those of the IgA isotype. Furthermore, the results of this investigation indicate that IgA oligomeric state (monomeric vs dimeric) may not influence IgA association constant rate as observed with mIgA and dIgA antibodies recognising the same epitope presenting similar on-rates.

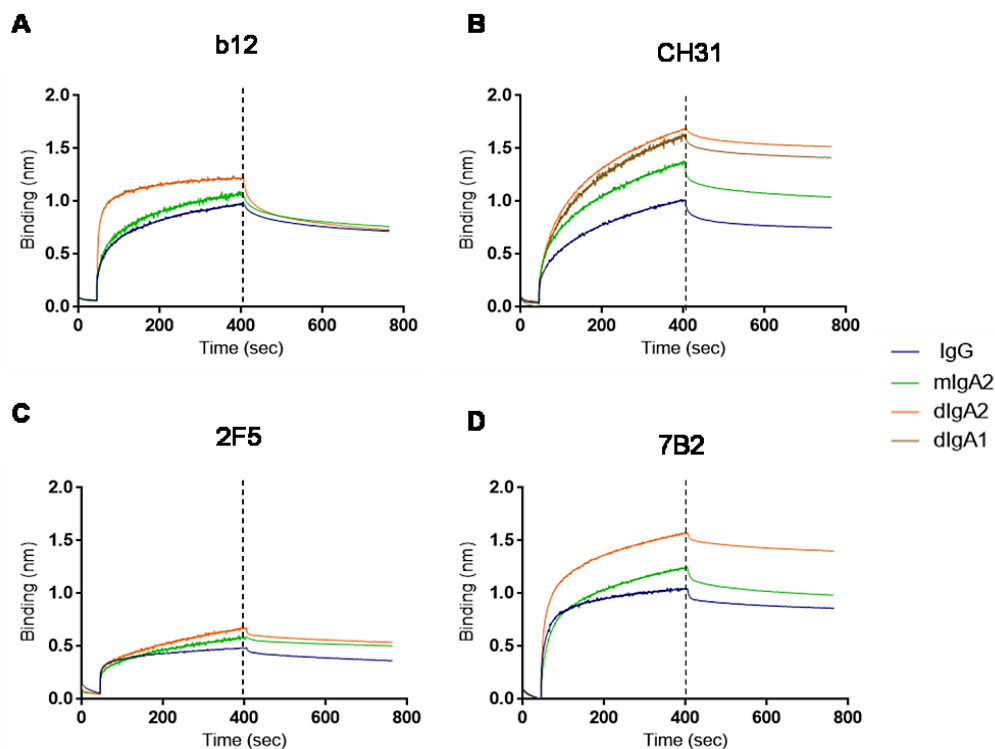


Figure 50. Binding kinetic profiles of IgG and IgA targeting the same epitope. The interaction between HIV-1_{BaL} gp140 and a panel of 13 IgG₁, mIgA₂ and dIgA₁₋₂ bNAbs recognising four distinct epitopes: (a) b12, (b) CH31, (c) 2F5 and (d) 7B2 was measured using BLI assay. Only the binding signals of the antibody association and dissociation phases at a concentration of 2 μ M are shown.

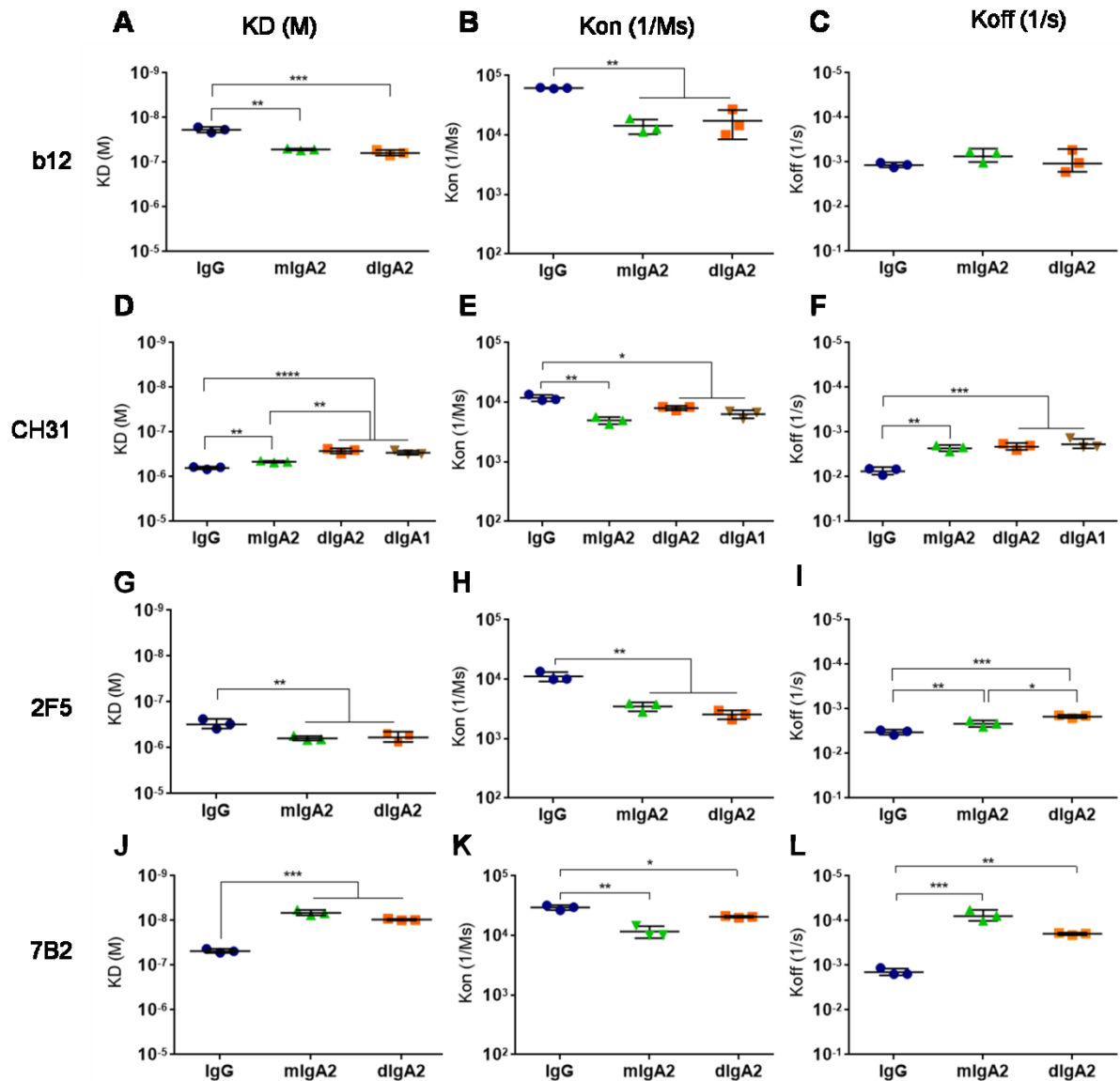


Figure 51. Impact of antibody isotype on binding kinetics to BaL gp140. The binding affinity (K_D), on-rate (K_{on}) and off-rate (K_{off}) of b12, CH31 2F5 and 7B2 IgG, mIgA₂, dIgA₁₋₂ were measured with the BLI system. The measurement of the kinetic parameters of each mAb was repeated three times with at least three different Ab concentrations (2, 1 and 0.5 μ M). K_D , K_{on} and K_{off} values were derived by a global fit 1:1 of all data for each experiment. Standard deviations of repeated measures were calculated (black errors bars). Two-way ANOVA was performed to compare the kinetic parameters of the different mAb isotypes of identical specificity. Tukey's MCT using 95%CI was used and p-value results were presented as * $p<0.05$, ** $p<0.01$, *** $p<0.005$, **** $p<0.001$.

4.2.5 IgG and IgA antibodies recognising the same epitope compete to bind to BaL gp140

Based on the observations of the previous chapter that HIV-1 Env-specific IgG and IgA (mIgA and dIgA) antibodies recognising the same epitope compete for capturing HIV-1_{BaL} virions, it was conjectured that this was due to competition for binding to the same Env sites. A competitive ELISA was developed and performed to determine whether IgG, mIgA₂ and dIgA₂ presenting the same epitope specificity compete for binding to HIV-1_{BaL} Envgp140 (Figure 52). For this purpose, b12, CH31, 2F5 and 7B2 IgG were incubated with BaL gp140 either alone or with their mIgA₂ or dIgA₂ counterparts. A total of five different ratios of IgG/mIgA₂ and IgG/dIgA₂ were tested while the concentration of BaL gp140 was the same in all assays. To assess the ability of mIgA₂ and dIgA₂ to inhibit the binding of their IgG equivalents to BaL gp140, antibody binding detection was measured for IgG only using a goat α -Human IgG HRP. The percentage of inhibition of IgG binding to BaL gp140 due to the presence of unlabelled mIgA₂ or dIgA₂ was calculated for each ratio. Likewise, the capacity of IgG to block mIgA₂ or dIgA₂ interaction with BaL gp140 was determined by detecting antibody binding using goat α -Human IgA HRP with or without IgG recognising the same epitope. Then, the percentage of inhibition of the binding to HIV-1_{BaL} gp140 of mIgA₂ or dIgA₂ antibodies due to the presence of IgG antibodies with the same epitope specificity was determined by comparing the binding signals obtained with or without IgG.

Analysis demonstrated that b12, CH31, 2F5 and 7B2 IgG antibodies and their mIgA₂ and dIgA₂ counterparts competed for interaction with HIV-1_{BaL} gp140 (Figure 52). In general, the greater the ratios IgG/mIgA₂ and IgG/dIgA₂, the more extreme were the percentage of binding inhibition. Differences in levels of binding inhibition were observed between IgG and mIgA₂ and between IgG and dIgA₂ antibodies of identical specificities. When used at the same concentration of IgG and mIgA₂ (ratio 1:1), CH31 mIgA₂ inhibited about three times more CH31 IgG binding than the other way around ($54 \pm 16\%$ vs $14 \pm 2\%$, respectively, $p < 0.01$) and 2F5 IgG blocked over eight times more 2F5 mIgA₂ interaction with BaL gp140 ($51 \pm 6\%$ vs $6 \pm 2\%$, respectively, $p < 0.0001$). Conversely, b12 IgG and b12 mIgA₂ presented comparable inhibition of each other ($28 \pm 10\%$ vs $22 \pm 7\%$, respectively). Likewise, 7B2 IgG and 7B2 mIgA₂ displayed similar inhibition ($32 \pm 3\%$ vs $44 \pm 6\%$, respectively). Additionally, at an IgG/dIgA₂ ratio of 1:1 CH31, 2F5 and 7B2 IgG and dIgA₂ weakly and equally inhibited each other,

whereas b12 dIgA₂ reduced by half b12 IgG binding ($54\pm 2\%$) and b12 IgG reduced b12 dIgA₂ binding by only $10\pm 2\%$ ($p<0.0001$).

The results show that IgG antibodies presented greater ability to inhibit the interaction of their mIgA₂ counterparts than their dIgA₂ counterparts at an unequalled ratio. Notably, at a ratio of IgG/mIgA₂ of 10:1, b12, CH31, 2F5 and 7B2 IgG antibodies reduced the binding of their mIgA₂ equivalents by $97\pm 2\%$, $62\pm 1\%$, $84\pm 1\%$ and $37\pm 2\%$, respectively, whereas for the same ratio of IgG/dIgA₂, they inhibited the binding of their dIgA₂ counterparts by $17\pm 4\%$, $8\pm 1\%$, $4\pm 1\%$ and $5\pm 2\%$, respectively. However, b12 IgG and CH31 IgG poorly inhibited the binding of their dIgA₂ equivalents at any ratio tested and the percentage of binding inhibition induced seemed to be independent of the ratios tested.

These data indicate that IgG and IgA (mIgA or dIgA) bNAbs recognising the same motif compete for interaction with HIV-1_{BaL} gp140. Furthermore, these findings demonstrate that antibody isotype influences the outcome of the binding competition between IgG and IgA antibodies recognising the same epitope, and that the competition is more pronounced between IgG and mIgA₂ antibodies than between IgG and dIgA₂ antibodies. This suggests that dIgA₂ antibodies present greater ability to resist to IgG binding inhibition than their mIgA₂ counterparts. Moreover, these data highlight the greater capacity of b12 dIgA₂ to inhibit b12 IgG binding to HIV-1_{BaL} gp140 compared to the other dIgA₂ antibodies tested. Thus, these results imply that the capacity of an antibody to inhibit the binding of its IgG or IgA counterpart is dependent on the antibody epitope specificity, isotype and structure.

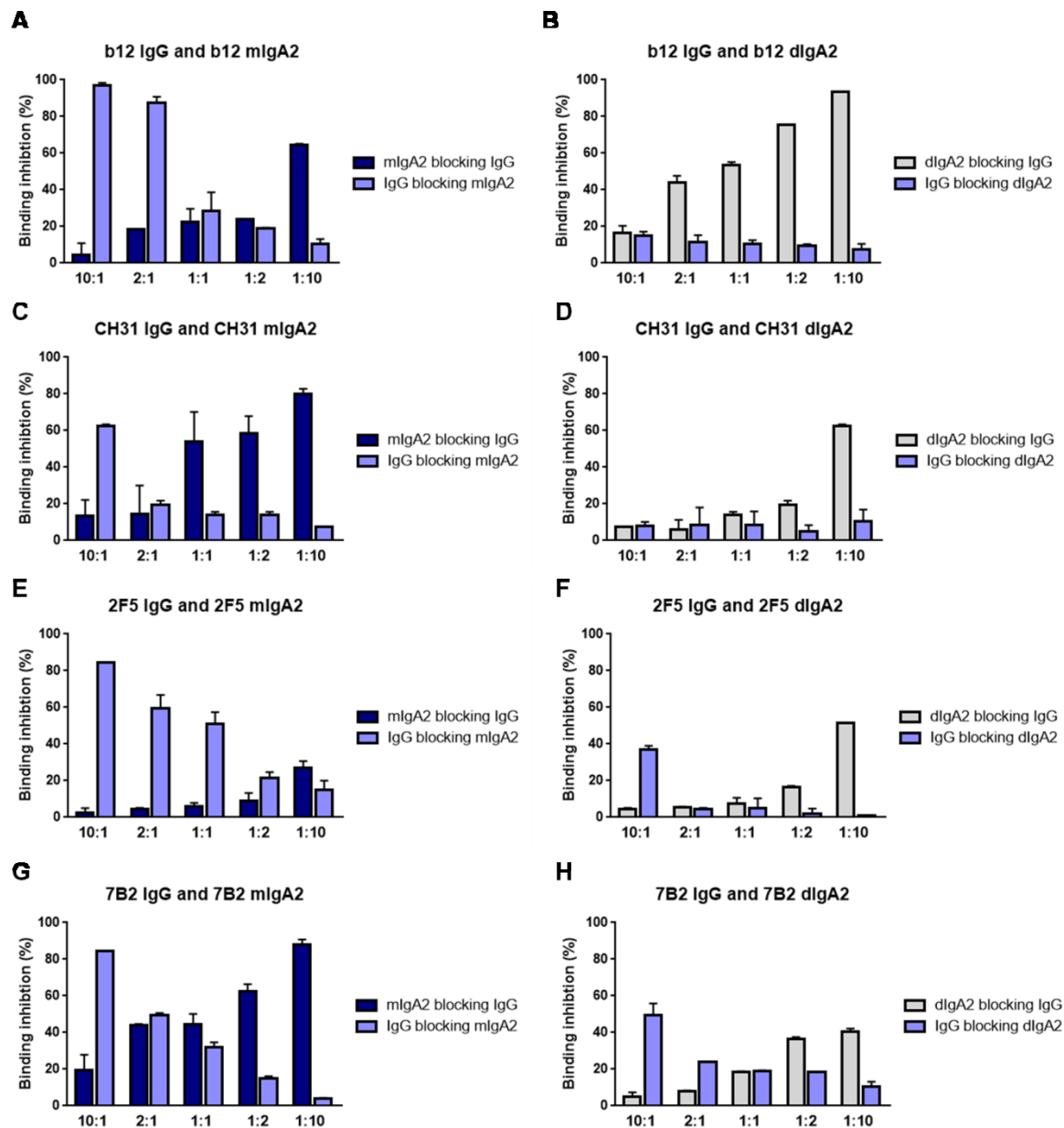


Figure 52. Competitive ELISA between IgG and IgA antibodies targeting the same epitope. The bar charts (A), (C), (E) and (G) shows the comparison of IgG and mIgA₂ antibodies; and (B), (D), (F) and (H), of IgG and dIgA₂ antibodies capacities to block the binding to BaL gp140 of their isotype counterparts presenting the same epitope specificity, and this for b12, CH31, 2F5 and 7B2, respectively. The y-axis presents the percentage of inhibition of IgG binding by mIgA₂ (dark blue), of mIgA₂ binding by IgG (light blue) and of dIgA₂ binding by IgG (grey) compared to antibody isotype alone. The x-axis presents the ratio of IgG/mIgA₂ or IgG/dIgA₂ tested which vary from ten times more IgG than IgA (10:1) to ten times more IgA than IgG (1:10).

4.2.6 BLI assay development to measure antibody binding to HIV-1 virions

The preceding BLI approach was designed to measure the interaction of HIV-1 Env-specific IgG and IgA antibodies with HIV-1_{BaL} gp140. Here, a similar methodology was tested to determine whether it was possible to measure IgG and IgA antibodies binding to whole HIV-1_{BaL} virions using the BLI system.

Biotin-GNA molecules were first immobilised on SA biosensor tip, then different concentrations of HIV-1_{BaL} virions were measured (Figure 53). The amplitude of the signal for HIV-1_{BaL} virions binding to biotin-GNA increased proportionally with the concentration of virions added (Figure 53B). The binding signal of HIV-1_{BaL} to biotin-GNA started to decrease at the beginning of the dissociation phase before stabilising after around 60s. Analysis of the kinetic parameters shows that HIV-1_{BaL} virions interacted with biotin-GNA with very strong affinity, in the lower picomolar range ($K_D=46\pm\text{pM}$). While HIV-1_{BaL} virions bound quickly to biotin-GNA ($K_{on}=33.8\pm\times 10^6\text{M}^{-1}\text{s}^{-1}$), they hardly dissociated from it ($K_{off}=30.4\pm\mu\text{s}^{-1}$).

Thus, these data indicate that immobilised biotin-GNA on SA biosensor was able to efficiently capture HIV-1_{BaL} virions and to maintain a strong interaction with them, even after washing. This was a prerequisite for the measurement of antibody interaction with captured virions, on the SA biosensor, in the next step of the assay.

The last step of the assay aimed to determine the kinetic constants of antibody-HIV-1_{BaL} virions (Figure 54). Contrary to the result obtained with HIV-1_{BaL} gp140, CH31 IgG binding to HIV-1_{BaL} virions was very low and the kinetic parameters of this interaction could not be measured. The entire panel of antibodies was tested to evaluate whether it was the method used or CH31 IgG which was responsible for this result. However, no increase over 0.1 nm for the binding signal, during the association phase, was observed with any of the antibodies from the panel, while their interaction with HIV-1_{BaL} gp140, as mentioned above, could be measured accurately.

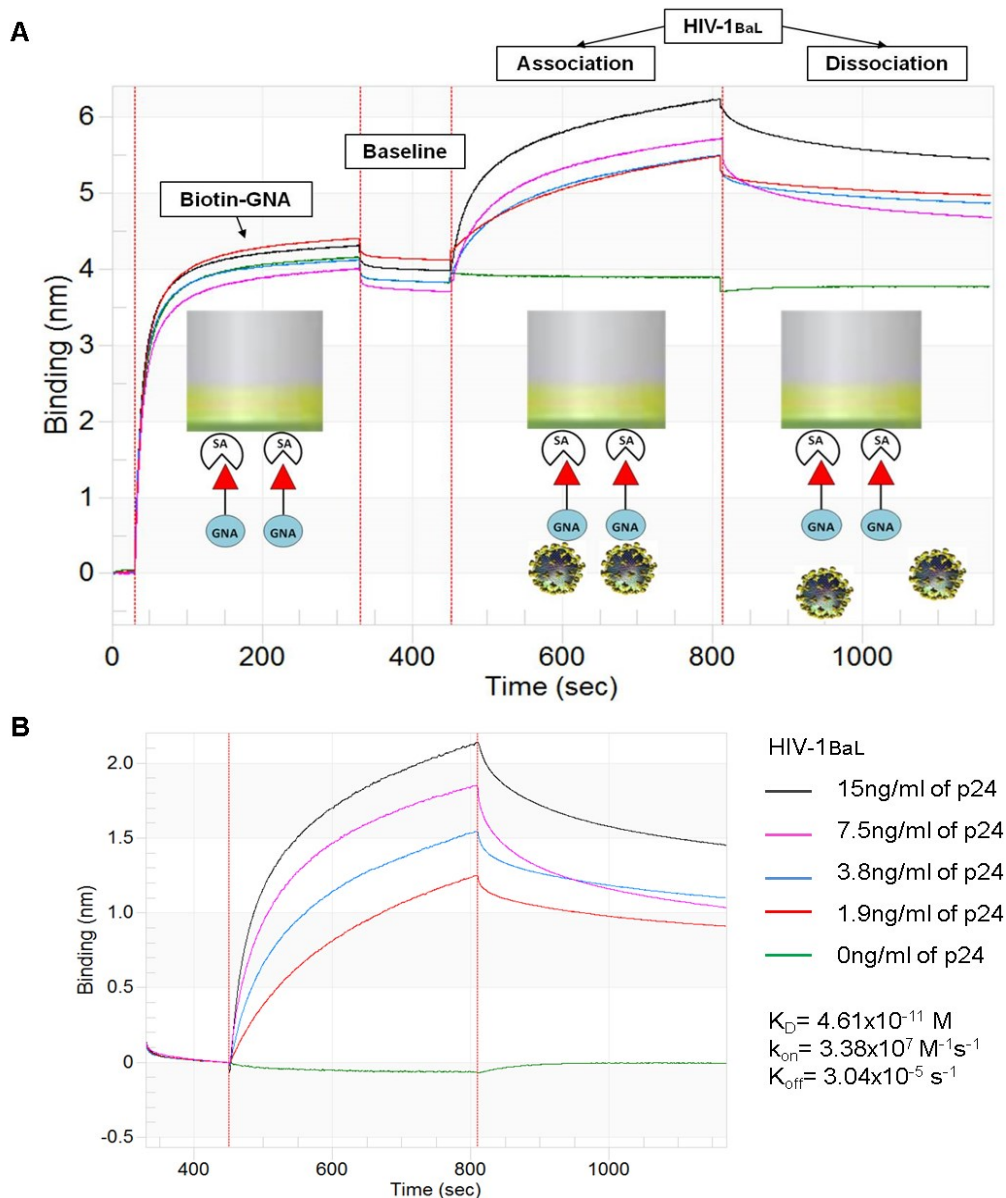


Figure 53. Development of the BLI binding assay: biotin-GNA capture of BaL virions. The first figure (A) illustrates the early steps of the BLI binding assay: initial baseline (30s), biotin-GNA loading onto the SA biosensor (300s), association and dissociation phases of HIV-1_{BaL} virions (360s each). The drawings under the interaction curves depict each binding step. The second figures shows (B) an enlargement of the association and dissociation stages, with correction applied at the beginning of the association step. The binding affinity (K_D) of HIV-1_{BaL} virions to GNA was measured for different concentrations 15, 7.5 3.8 and 1.9ng/mL of HIV-1_{BaL} virions, displayed in black, pink, blue and red respectively. The green line corresponds to the buffer only negative control. Each measurement was repeated three times and kinetics parameters were calculated using the 1:1 global analysis model with all the repeated measurements. The resulting kinetic parameters of biotin-GNA and HIV-1_{BaL} interaction are shown: the K_D , the association constant rate (K_{on}) and dissociation constant rate (K_{off}).

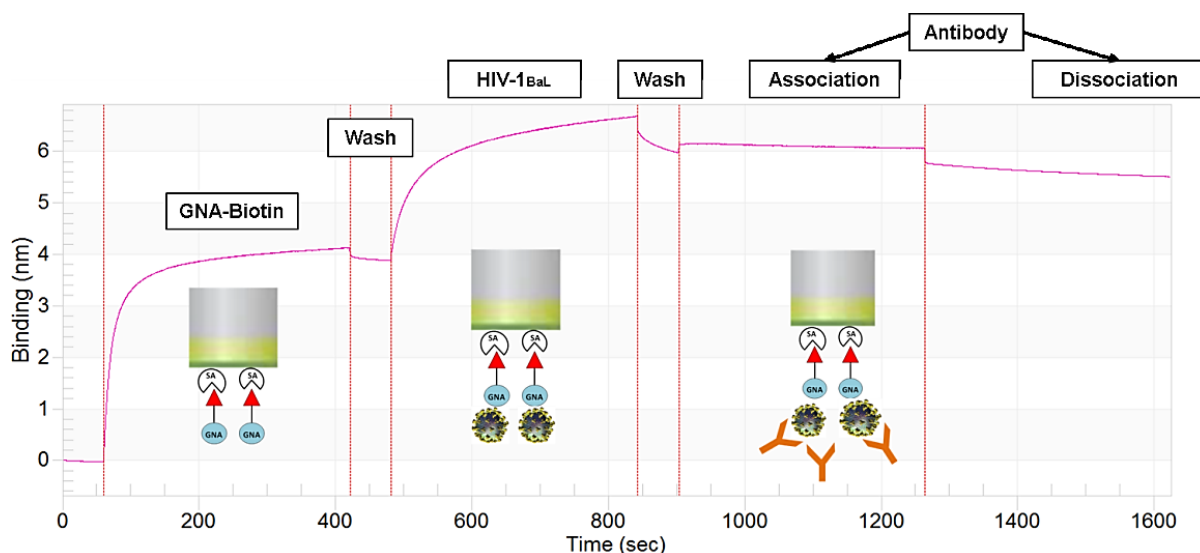


Figure 54. BLI binding assay development to measure antibody-HIV-1_{BaL} virion interaction.

The figure shows the successive steps of the BLI binding assay to assess antibody binding to HIV-1_{BaL} virions captured by biotin-GNA on the SA biosensor tip. (1) A baseline was first established and then (2) biotinylated GNA (3.85 μ M) was immobilised on the streptavidin biosensor tip which resulted in a binding signal. (3) The biosensor tip was washed to remove non-immobilised biotin-GNA. (4) HIV-1_{BaL} virions (357nM) were captured by biotin-GNA. (5) The biosensor tip was washed to remove unbound HIV-1_{BaL} virions. (6) The antibody (here CH31 IgG at 2 μ M) binding signal during the association and (7) dissociation steps was recorded.

To facilitate the potential detection of anti-gp41 antibody binding to HIV-1_{BaL} virions, the virus sample was heated to cause dissociation of gp120 from Env trimer and to expose gp41, as previously reported in the literature ^[471]. However, the heat treatment did not result in an increase of gp41 bNAbs binding to immobilised virions. This indicates that it may not be possible to detect the binding of IgG and IgA to HIV-1_{BaL} virions using this method.

Based on these findings, an alternative assay format to detect antibody binding to virions was evaluated. The first step of this novel binding assay was to immobilise antibody of interest directly onto a biosensor tip, and to measure antibody binding to virions in the second step of the assay. As there were no available biosensors capable of capturing both IgG and IgA antibodies, two different strategies had to be established to measure immobilised IgG and IgA antibodies binding to HIV-1 virions.

IgG antibodies were immobilised on a protein A (PA) biosensor tip to assess IgG antibodies binding to HIV-1_{BaL} virions (Figure 55). PA which is a surface protein from

Staphylococcus aureus recognising the Fc region of human IgG with high affinity was selected to immobilise IgG^[444]^[444]. Moreover, the feasibility of this approach was supported by the fact that affinity measurements of macromolecules binding to immobilised IgG on PA biosensors have been previously described in earlier studies^[444]. However, to the best of our current knowledge, this method has not been tested with HIV-1 virions. In the first stage of the assay, 4E10 IgG (gp41 MPER-specific) was immobilised on the PA biosensor and after a baseline (washing step), HIV-1_{BaL} was applied during the association phase which was followed by a dissociation phase (Figure 55).

The kinetic parameters of 4E10 IgG interaction with HIV-1_{BaL} were measured using the 1:1 global analysis model of the BLItz Pro™ Software (Figure 55B). Analysis demonstrates that 4E10 bound with a very high affinity to HIV-1_{BaL} in the picomolar range ($K_D = 2.9 \pm 0.19 \text{ pM}$). Remarkably, 4E10 binding affinity for HIV-1_{BaL} virions was over 4 logs greater than for HIV-1_{BaL} gp140 ($0.29 \pm 0.02 \mu\text{M}$). Furthermore, HIV-1_{BaL} virions interacted 1,000 times faster with 4E10 than 4E10 interacted with BaL gp140 ($8.6 \pm 0.4 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ vs $1.1 \pm 0.13 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$) and HIV-1_{BaL} virions dissociated about 10 times more slowly from 4E10 than 4E10 detached from BaL gp140 ($25 \pm 0.2 \text{ ms}^{-1}$ and $3.3 \pm 0.4 \text{ ms}^{-1}$, respectively). This suggests that the assay format used impacted on the binding kinetics, and/or the use of HIV-1 virions compared to HIV-1 gp140.

Once the IgG BLI binding assay protocol was established, the ability to detect the binding of the entire panel of IgG antibodies to HIV-1_{BaL} virions was assessed. Notably, HIV-1_{BaL} virions binding to the panel of IgG antibodies was solely observed with 4E10, PG16, b12, 2F5 and PG9 (Figure 56). However while the kinetic parameters of PG9 interaction with HIV-1_{BaL} Envgp140 could be measured, PG9 binding to HIV-1_{BaL} virions was too weak to enable the determination of the K_D , K_{on} and K_{off} values. Moreover, the results show that 4E10, PG16, b12 and 2F5 interacted with HIV-1_{BaL} virions with very high affinities, within the picomolar range (10^{-12} M), about five logs greater than observed with HIV-1_{BaL} gp140 which were in the nanomolar range (10^{-7} M to 10^{-8} M) (Table 22). In addition, whereas b12, PG16 and PG9 presented greater affinities (one-log higher) for BaL gp140 (19 ± 3 , 25 ± 8 and $56 \pm 9 \text{ nM}$, respectively) than 2F5 and 4E10 ($303 \pm 73 \text{ nM}$ and $295 \pm 18 \text{ nM}$); here, 2F5 and 4E10 presented slightly stronger, but comparable binding affinities for HIV-1_{BaL} virions ($5.8 \pm 0.4 \text{ pM}$ and $2.9 \pm 0.2 \text{ pM}$) than b12 and PG16 ($6.3 \pm 0.3 \text{ pM}$ and $9 \pm 0.6 \text{ pM}$).

Overall, significant differences of kinetic parameters were observed between IgG antibodies interactions with HIV-1_{BaL} virions compared to HIV-1_{BaL} gp140, with stronger and faster binding of HIV-1_{BaL} to IgG antibodies than observed with BaL gp140, and also with the detection of only five out of fourteen IgG antibodies binding to HIV-1_{BaL} virions. Together, these results suggest that IgG antibodies binding to HIV-1_{BaL} virions measured in this experiment might not be mediated by Env spikes.

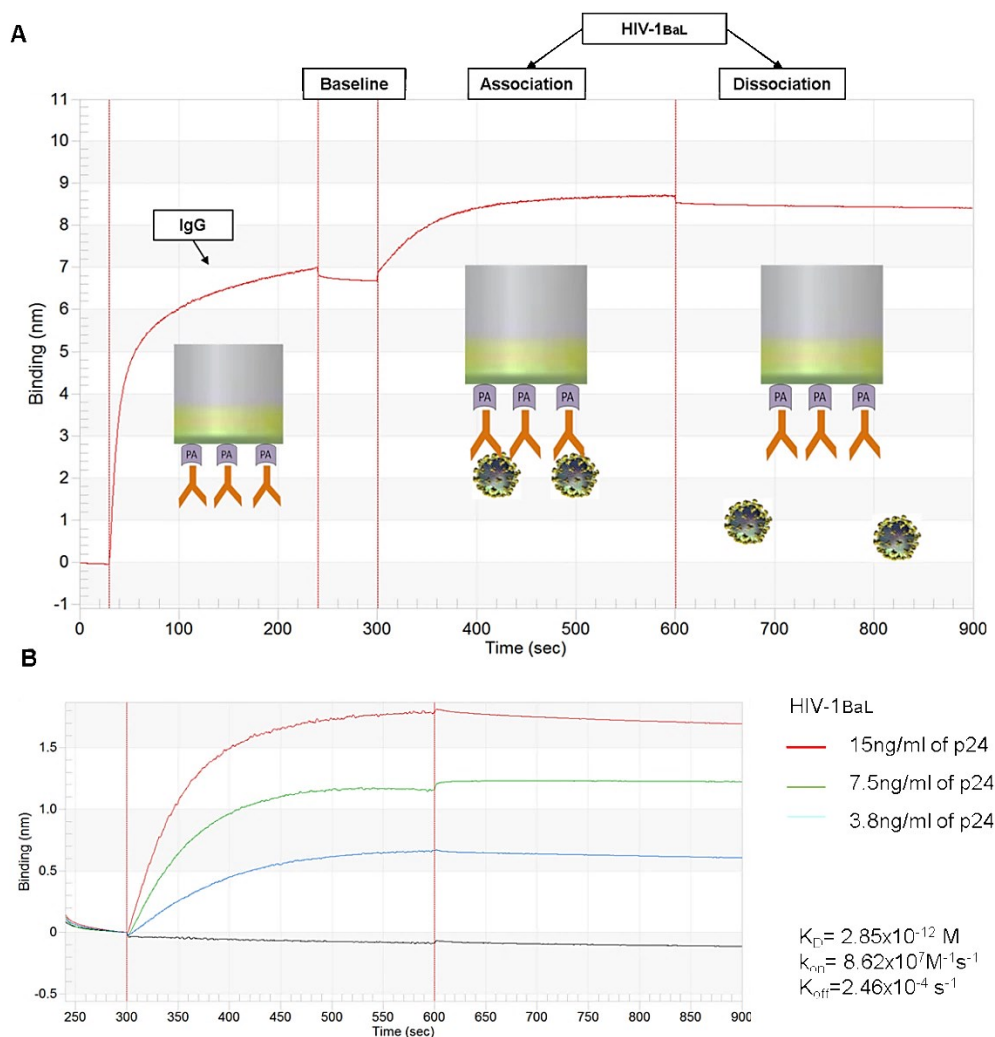


Figure 55. Measurement of IgG antibodies-HIV-1_{BaL} virion interactions with the BLI system.

Graph A shows the successive steps of the IgG BLI binding assay: (1) A baseline was first established and then an IgG antibody (here b12 IgG at 50 µg/mL) was immobilised on the PA biosensor tip which resulted in a binding signal. (2) The PA biosensor was washed to remove non-immobilised IgG antibodies. (3) HIV-1_{BaL} virions were captured by IgG antibodies. (4) The PA biosensor was washed to remove unbound HIV-1_{BaL} virions. (5) HIV-1_{BaL} virions bind to immobilised IgG antibodies while the K_{on} was measured. (6) HIV-1_{BaL} virions dissociates from IgG antibodies while the (K_{off} was determined. Graph B depicts an enlargement of HIV-1_{BaL} binding and dissociation from immobilised b12 IgG. Four concentrations of HIV-1_{BaL} were tested 15, 7.5, and 3.75, 1.9 ng/mL of p24, shown in red, green and blue, respectively. No interaction between the IgG isotype negative control (50 µg/mL) and HIV-1_{BaL} (15 ng/mL of p24) was detected (black line). Each measurement was repeated three times and the kinetic parameters were calculated using the 1:1 global analysis model.

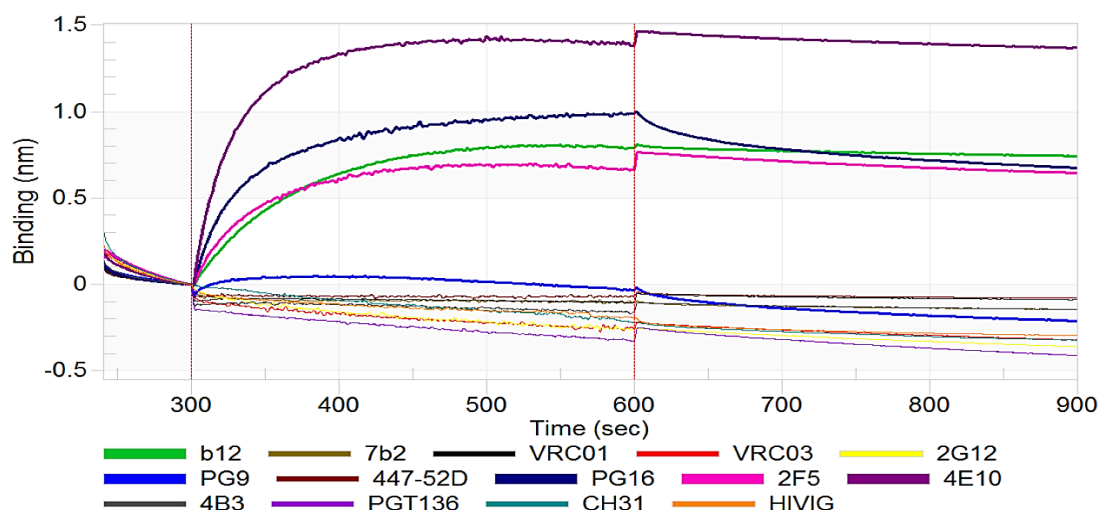


Figure 56. Binding of a panel of IgG antibodies to HIV-1_{BaL} virions using the BLI system. The ability to detect the binding of a panel of 14 HIV-1 Env-specific IgG antibodies to HIV-1_{BaL} virions using a developed IgG BLI binding assay was evaluated. For each antibody, measurements were repeated three times and different dilutions of virions were tested. Here, only the binding signals obtained with the top concentration of HIV-1_{BaL} (15ng/mL of p24) are depicted. HIV-1_{BaL} virions binding to the panel of IgG antibodies was observed for 4E10 (purple), PG16, (dark blue), b12 (green) and 2F5 (pink). A very low binding signal was also detected with PG9 (blue) while binding of the other IgG antibodies from the panel failed to be detected. The first red line separates the initial baseline from the association phase and the second red line, the association from the dissociation phases of IgG antibodies-HIV-1_{BaL} virions interaction.

Table 22. Comparison of the kinetic parameters of IgG interaction with BaL virions and BaL gp140.

Antibody	HIV-1 _{BaL}			HIV-1 _{BsL} Env gp140		
	K _D (10 ⁻¹² M)	K _{on} (10 ⁷ M ⁻¹ s ⁻¹)	K _{off} (10 ⁻⁴ s ⁻¹)	K _D (10 ⁻⁷ M)	K _{on} (10 ⁴ M ⁻¹ s ⁻¹)	K _{off} (10 ⁻³ s ⁻¹)
4E10	2.9±0.19	8.62±0.39	2.46±0.24	2.9±0.18	1.1±0.13	3.3±0.43
2F5	5.75±0.35	1.95±0.14	1.12±0.18	3±0.73	1.1±0.20	3.3±0.12
b12	6.28±0.25	8.25±0.21	5.19±0.36	0.19±0.03	6.2±0.12	1.2±0.14
PG16	8.98±0.57	1.46±0.55	1.31±0.13	0.25±0.08	5±0.33	2.2±0.05
PG9	ND	ND	ND	0.56±0.09	7.2±0.30	4±0.11

ND, Not determined (binding to low).

To investigate whether the interaction observed between 4E10, PG16, b12, 2F5, PG9 and HIV-1_{BaL} virions was mediated by HIV-1_{BaL} Env present on the viral surface; the ability of these IgG bNAbs to bind to ΔEnvHIV-1 pseudovirus lacking Env protein was assessed (Figure 57). The same protocol used for measuring IgG antibodies binding to HIV-1_{BaL} was applied to measure IgG antibodies binding to ΔEnvHIV-1 pseudovirus. Analysis revealed that all five IgG antibodies were able to bind ΔEnvHIV-1 pseudovirus with comparable kinetic parameters to

HIV-1_{BaL} virions (Table 23). As with HIV-1_{BaL} virions, PG9 interacted with ΔEnvHIV-1 pseudovirus with low affinity and the kinetic parameters could not be measured accurately. Therefore, these findings indicate that the interaction between 4E10, PG16, b12, 2F5, PG9 and HIV-1_{BaL} virions was not mediated by Env protein exposed on the virion surface. Consequently, IgG antibodies binding to HIV-1_{BaL} virions could not be detected using this method, with the BLI instrument.

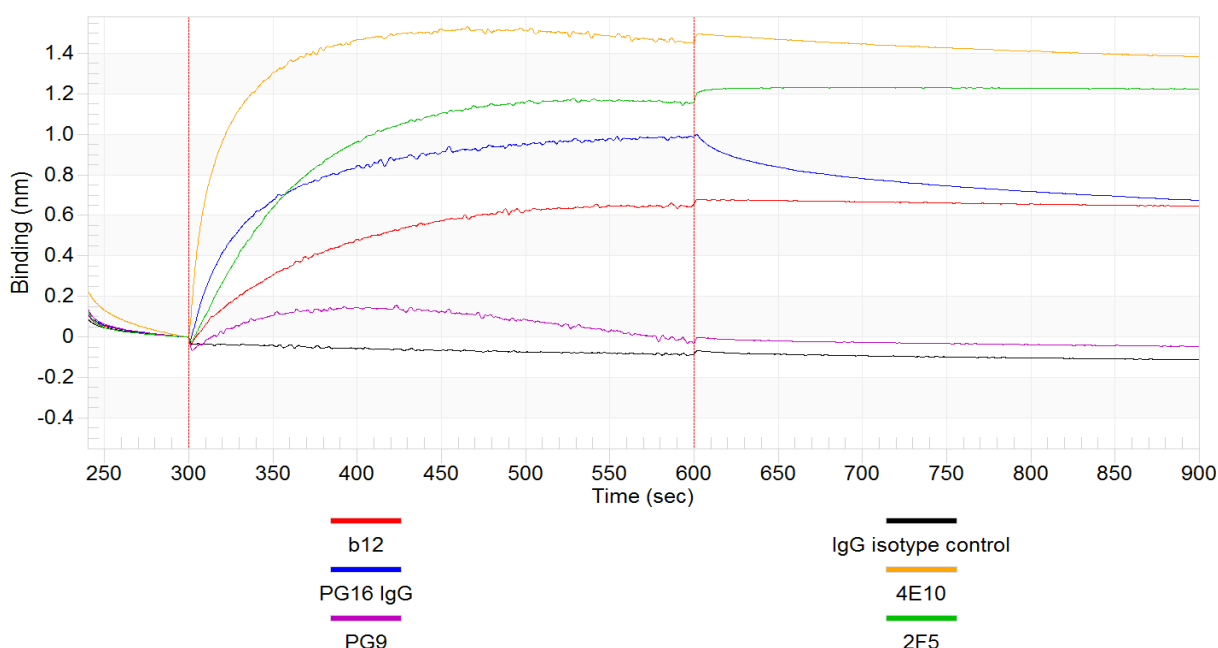


Figure 57. HIV-1 Env-specific IgG antibodies binding to pseudovirus devoid of Env protein. To determine whether IgG antibodies binding to HIV-1_{BaL} observed using the BLI system was mediated by Env exposed on virion surface, HIV-1 Env-specific IgG antibodies binding to pseudovirus lacking Env (15ng/mL of p24) was tested. Binding signal to pseudovirus was detected for 4E10 (orange), 2F5 (green), PG16 (blue), b12 (red) and PG9 (purple) which indicated that the interaction of these IgG antibodies with HIV-1_{BaL} previously observed was not mediated by Env. The interaction curve obtained with the IgG isotype negative control is shown in black.

Table 23. IgG antibodies binding to ΔEnv pseudovirus kinetic constants.

IgG	K_D (10^{-12} M)	K_{on} (10^7 M ⁻¹ s ⁻¹)	K_{off} (10^{-4} s ⁻¹)
4E10	1.8±0.16	7.1±0.22	1.3±0.33
2F5	2.8±0.23	8.1±0.15	2.3±0.11
b12	5.1±0.31	6.4±0.47	3.2±0.29
PG16	9.7±0.62	1.5±0.03	1.5±0.09
PG9	ND	ND	ND

ND, Not determined (binding to low).

To measure IgA antibodies interaction with HIV-1_{BaL} virions, biotinylated-anti IgA antibody was immobilised on a SA biosensor, as there was no available biosensors to directly immobilised IgA antibodies (Figure 58). After immobilisation of the biotinylated anti-IgA on the SA biosensor and a washing step, CH31 mIgA₂ was applied, and prior HIV-1_{BaL} binding step, a second washing step was performed. Virions association and dissociation to captured CH31 mIgA₂ was measured in the last step of the assay. No significant binding signal was observed with CH31 mIgA₂ as well as with any antibodies of the panel of IgA antibodies tested. Therefore, as with IgG antibodies, IgA antibodies binding to HIV-1_{BaL} virions could not be detected with the BLI system. Thus, the results of this study indicate that the BLI instrument was not sensitive enough to enable the measurement of IgG and IgA antibodies interaction with HIV-1_{BaL} but instead non-specific bindings of some IgG antibodies were detected with HIV-1_{BaL} and ΔEnvHIV-1 pseudovirus.

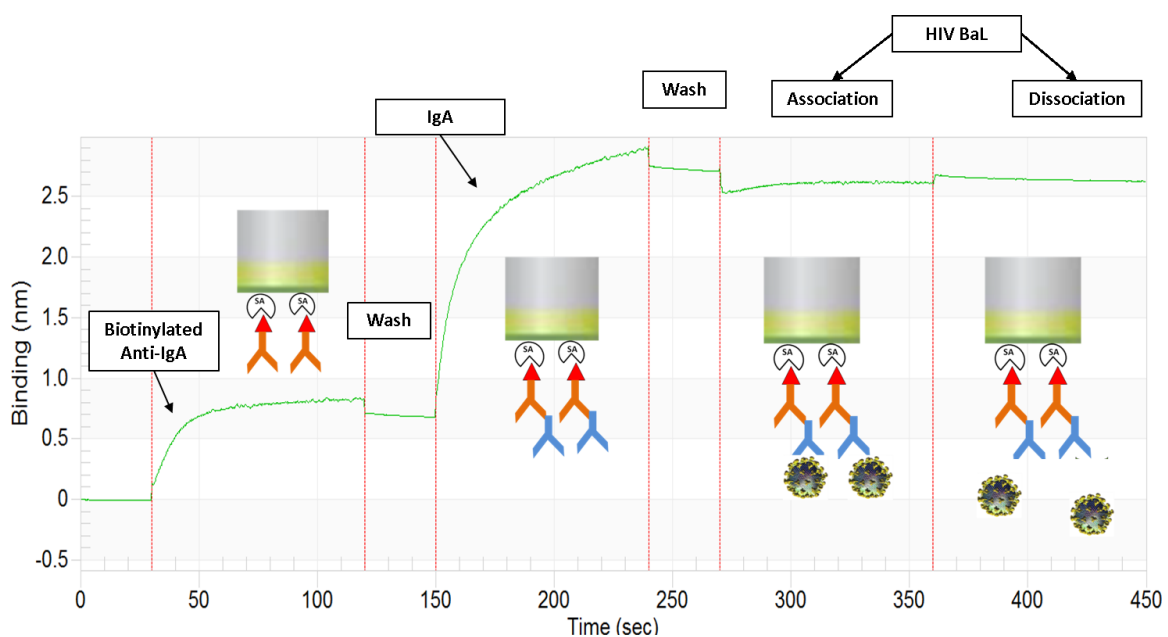


Figure 58. BLI binding assay to measure IgA antibodies-BaL virions interaction. Biotinylated anti-IgA antibody was loaded onto a streptavidin biosensor for 150s. CH31 mIgA₂ was then captured by biotinylated anti-IgA antibody (150s). Subsequently, HIV-1_{BaL} virions binding to CH31 mIgA₂ was recorded (360s for each association and dissociation phases). No significant binding signal could be detected and the kinetics parameters of this interaction could not be measured.

4.3 Discussion

Both IgG and IgA isotypes are present at genital and rectal mucosal surfaces, where HIV-1 infection is initiated in the vast majority of the cases. Nonetheless, very little is known about how antibody isotype influences HIV-1 virion binding and which of the IgG or IgA isotype provides the most effective mechanism to block HIV-1 infection at sexual mucosal surfaces. Thus, the aim of this chapter was to investigate the possibility that antibody isotype and structure along with epitope specificity and accessibility impact on the binding kinetic parameters of HIV-1 Env-specific antibodies.

A BLI binding assay was developed to measure the binding affinity, on-rate and off-rate of IgG, mIgA₂ and dIgA₁₋₂ antibodies interaction with HIV-1_{BaL} gp140. This method was taken from a protocol established in the early 1990's that utilises GNA to capture Env glycoprotein of HIV and SIV for ELISA experiments ^[443]. The present study shows that biotinylated-GNA immobilised on the SA biosensor efficiently captured HIV-1_{BaL} gp140 with a very strong affinity below the picomolar range (Figure 46). The high affinity of GNA for BaL gp140 was mainly due to its low off-rate which implies that BaL gp140 hardly dissociated from biotin-GNA during the washing step preceding the antibody binding step. This indicates that antibody off-rates calculated in the last step of the assay, which are in the 10^{-3} to 10^{-5} s⁻¹ range, are virtually entirely due to antibody dissociation from BaL gp140 and therefore that BaL gp140 dissociation from biotin-GNA can be considered as negligible (Figure 47-48).

For the first study of antibody-HIV-1_{BaL} gp140 interaction, CH31 IgG (gp120 CD4bs-specific) which is a VRC01-like antibody that presents cross-clade reactivity against Clade A, B and C HIV-1 strains was tested (Figure 47). The results show that CH31 IgG binding affinity for HIV-1_{BaL} gp140 is comparable to data found in the literature for VRC01-like antibodies which are within the nanomolar range ^[293, 482]. Therefore, the results demonstrate that the devised BLI binding assay enables to efficiently measure antibody binding kinetic constants of interaction with gp140 captured on a biosensor.

This novel BLI binding assay was then utilised to evaluate the influence of epitope specificity and accessibility on the kinetic parameters of antibody-HIV-1_{BaL} gp140 interaction, using a panel of 14 IgG and 9 IgA antibodies. All IgG and IgA antibodies tested interacted with HIV-1_{BaL} gp140, which concurs with other studies that have shown their

ability to bind to different HIV Env clades, including clade B Env such as BaL gp140^[280, 434, 435, 485] (Figure 48).

The results of this research demonstrate that IgG antibodies within the same epitope specificity group presented, in general, similar binding affinities, and interacted with different on-rates and/or off-rates (Figure 48). This was particularly true for antibodies directed against gp120 V3/glycans (2G12, PGT136 and 447-52D), gp120 V1/V2 (PG9 and PG16), gp41 MPER (2F5 and 4E10) and gp41 Cluster I (7B2 and 4B3).

Taken together, these findings are broadly consistent with the literature. 2G12 and PGT136 have been reported to bind to high-mannose glycans located in the outer domain cluster of gp120 with strong affinity and PGT136 was shown to compete for interaction with 2G12^[297, 486]. This suggests that they have overlapping epitopes and might explain why they present comparable binding affinity for HIV-1_{BaL} gp140. Additionally, PGT136 and 2G12 recognise glycans on the gp120 V3 loop which might explain why they interact with similar affinity to 447-52D^[297, 450, 462]. Moreover, Davenport et al. showed that the gp120 V1/V2 directed PG9 and PG16 interacted with the monomeric and trimeric gp140 forms with identical binding affinities^[481]. Likewise, a study that compared the binding properties of 2F5 and 4E10 to JRFL gp140 (Clade B) demonstrated that these bNAbs interacted with moderate and comparable affinities in the upper nanomolar to micromolar range^[303]. Moreover, the non-neutralising antibodies 4B3 and 7B2 share several characteristics and recognise gp41 Cluster I linear epitopes which are close from each other which may explain their comparable binding affinities^[436, 437].

Additionally, the findings of this research imply that epitope accessibility might be the predominant factor influencing antibody binding affinity, with IgG antibodies targeting readily accessible epitopes presenting similar and higher affinities for BaL gp140, compared to those directed against occluded epitopes (Figure 48). The latter included the gp120 CD4bs directed CH31 and VRC03, the gp41 MPER-specific 2F5 and 4E10 and the polyclonal HIVIG, which as mentioned in the previous chapter recognised Env sites subject to steric constraints limiting bulky antibodies access.

In all, the results of this study are consistent with previous reports which found that antibody binding affinity is influenced by epitope accessibility. Notably, earlier studies

demonstrated that the binding affinity of HIV-1 Env glycan-specific antibodies could be enhanced by increasing accessibility to their epitopes^[487, 488].

Furthermore, other studies showed that greater access to the MPER following treatment with soluble CD4 led to better binding of the MPER-specific 2F5 and 4E10 to HIV-1 virions^[472, 489]. In all, these data support the idea that epitope accessibility plays a significant role in antibody binding affinity to Env. Moreover, the findings of the present study extend these results and indicate that epitope accessibility is the predominant factor influencing antibody binding affinity for Env and that this has more of an impact than epitope specificity.

Nevertheless, some exceptions were observed for example b12 and VRC01, which although binding to the CD4bs, subject to steric hindrance, interacted with HIV-1_{BaL} gp140 with similar affinities than antibodies targeting readily accessible epitopes and with one-log higher affinities than their analogous gp120 CD4bs directed CH31 and VRC03. Similar to these results, Zhou et al. have found that though VRC01 and VRC03 were isolated from the same patient and are very similar antibodies, VRC03 reacted with HIV-1 gp120 with 10-fold lower affinity compared to VRC01^[293, 490]. Furthermore, it has been reported that the VRC01-like antibody CH31 contains, as VRC03, large indels which might explained why CH31 and VRC03 interact with comparable and lower binding affinities with BaL gp140 compared to VRC01^[490]. Additionally, both VRC01 and b12 bind to the outer domain of gp120 which may explain why they present comparable and high binding affinities^[293].

Subsequent analysis also demonstrated that epitope accessibility plays a fundamental role in IgG association constant rate, with in general IgG antibodies interacting with readily accessible epitopes binding more rapidly than those targeting obstructed epitopes. This is not surprising and implies that the more accessible is an epitope within Env, the more quickly antibodies bind to Env. This finding is in agreement with the definition of antibody association constant rate which translates how fast an antibody binds to its target. However, this study suggests that the influence of epitope accessibility on the off-rate of IgG antibodies was relatively less important. Indeed, antibodies binding to readily accessible Env epitopes (e.g. Envgp120 V1/V2) presented similar off-rates to those targeting the Envgp41 MPER. An explanation for these results might be that antibody off-rate is dependent on the strength of antibody-antigen interaction rather than on the epitope accessibility^[444].

Overall, these findings were similar to those obtained with the panel of mIgA and dIgA antibodies which implies that epitope accessibility is also critical in IgA antibodies binding kinetics (Figure 49). In addition, the results of this research indicated that the oligomeric state of IgA (monomeric vs dimeric) does not generally influence IgA kinetic parameters as observed with mIgA and dIgA antibodies of identical specificity presenting comparable kinetic constants.

In the second part of this chapter, the impact of antibody isotype and structure on binding kinetics was investigated using the panel of IgG, mIgA₂ and dIgA₁₋₂ forms of b12, CH31, 2F5 and 7B2. Analysis indicated that the antibody binding profile obtained with the BLI instrument varied according to the isotype and epitope targeted. The lowest binding signals were observed with IgG followed by mIgA₂ and dIgA₁₋₂ bNAbs which reflects that antibody binding signal increased with antibody mass which is of 150, 160 and 385KDa for IgG, mIgA₂ and dIgA₁₋₂, respectively ^[226, 235] (Figure 51). This suggests that the amplitude of the binding signal is influenced by the mass of the antibody.

Moreover, the data revealed that each antibody induced a unique binding signal and that 2F5 bNAbs presented the lowest binding signal from the panel of bNAbs tested (Figure 50). These results might be explained by the poor accessibility of the gp41 MPER recognised by 2F5 bNAbs and the absence of the viral membrane which is known to be important for 2F5 binding ^[457]. This further emphasises the role of epitope accessibility on antibody binding to Env. Additionally, these findings can be explained by the principles of the BLI system, for which the binding of molecules to the surface of the biosensor induces a shift in the interference patterns; and the extent of this shift is directly proportional to the amount of molecules bound to the biosensor surface ^[491].

In summary, these data indicate that the magnitude of the binding signal is dependent on the antibody molecular weights and therefore on the antibody isotype as well as of the epitope accessibility, which all impact on the amount of antibody binding to the biosensor surface.

Furthermore, the results of this study demonstrate that b12 IgG and 2F5 IgG bound with higher affinities to BaL gp140 than their IgA counterparts while 7B2 IgA and CH31 IgA interacted with higher affinities than their IgG equivalents (Figure 52). These findings are in line with the higher magnitude of the binding signals observed with CH31 and 7B2 IgA

antibodies compared to b12 and 2F5 IgA antibodies; which suggest that greater amounts of CH31 IgA and 7B2 IgA bound to BaL gp140 immobilised on the biosensor chip compared to b12 IgA and 2F5 IgA.

In total, these results support the idea that antibody isotype impacts on antibody-HIV Env interaction, as observed with IgG, mIgA₂ and dIgA₁₋₂ of identical specificity presenting different binding affinity. Furthermore, the results of this research are in accord with previous studies which suggest that the C domain of an antibody which defines antibody isotype impacts on the way the V domain of an antibody, which confers epitope specificity, interacts with an antigen. Notably, a study comparing the binding properties of bNAbs detected in the serum from a lymphoma patient, reported that IgA₁ and IgG₁ presenting identical V regions manifested significant differences in affinity^[482]. Precisely, they found that tubulin-specific IgG₁ had a significantly lower affinity than its IgA₁ equivalent as measured by SPR^[482]. Another group studying human anti-HIV-1 Env bNAbs demonstrated that IgA₂ and IgG₁ sharing the same epitope specificity presented significantly different binding affinities against HIV-1 Env by SPR analysis. They have demonstrated that an IgA₂ mAb specific for the C1 region of HIV-1 Env interacted with different HIV-1 Env strains with 2.9-fold to 40-fold higher magnitude and affinity than its IgG equivalent^[238].

Most strikingly, the findings of this research showed that all the IgG from the panel bound more quickly to BaL gp140 than their monomeric and dimeric IgA₁₋₂ counterparts (Figure 51). Conversely, CH31, 2F5 and 7B2 IgA antibodies dissociated significantly less rapidly from Env than their IgG equivalents. A previous study looking at the Phl p7 grass pollen allergen have also reported significant differences in binding rates between a full panel of human IgG and IgA isotypes using SPR. They observed up to three-fold differences in both on- and off-rate constants between IgG and IgA recognising the same epitope.

Together, these results extend previous research on HIV-1 Env-specific antibody binding kinetics and signpost that isotype influences antibody binding affinity, association and dissociation constant rates, and therefore that antibody isotype along with epitope accessibility play a significant role in the antibody binding function.

Successively, the ability of IgG to inhibit the binding to HIV-1_{BaL} gp140 of their mIgA₂ and dIgA₂ equivalents recognising the same epitope and *vice versa* was analysed in a

competitive ELISA assay using five different ratios of IgG/IgA (mIgA₂ or dIgA₂) (Figure 52). The greater capacity on binding inhibition of the gp120 CD4bs-specific CH31 mIgA₂ compared to CH31 IgG for a same antibody concentration (ratio 1:1) (Figure 52C) might be explained by the higher affinity of CH31 mIgA₂ for HIV-1_{BaL} gp140 compared to CH31 IgG, (Figure 52D). Similarly, the gp41 MPER directed 2F5 IgG might have significantly inhibited 2F5 mIgA₂ binding to BaL gp140 (Figure 52E) due its greater affinity for HIV-1_{BaL} gp140 compared to 2F5 mIgA₂, (Figure 51G). However, although b12 IgG presented a higher affinity for BaL gp140 than b12 mIgA₂, (Figure 51A), they demonstrated comparable capacity of binding inhibition, (Figure 52A). Likewise, although 7B2 mIgA₂ interacted with BaL gp140 with higher affinity than 7B2 IgG, (Figure 51J), they were equally efficient at blocking their respective binding (Figure 52G).

Overall, the findings of this study indicate that the outcome of the binding competition between IgG and mIgA₂ antibodies recognising the same epitope may be influenced by antibody affinity and epitope accessibility. Both, CH31 and 2F5 bNAbs bind to epitopes subject to high levels of steric constraints. Therefore, a probable explanation for these findings may be that when IgG and mIgA₂ antibodies of identical specificity target epitopes subject to steric constraints, it is the antibody isotype which presents the greatest affinity for BaL gp140 which wins the binding competition; whereas without steric hindrance, antibody binding affinity did not impact on the outcome of the binding competition and IgG and mIgA₂ antibodies of identical specificity.

The results of the binding competition between IgG and dIgA₂ antibodies differ from those observed between IgG and mIgA₂ antibodies, which suggest that antibody structure (monomeric vs dimeric) impacts on the outcome of the binding competition. Although, b12 dIgA₂ bound with a lower affinity to BaL gp140 than its IgG counterpart (Figure 51A), b12 dIgA₂ was resistant to b12 IgG inhibition at all ratios of IgG/dIgA₂ tested and was capable of efficiently blocking b12 IgG binding where the level of inhibition correlated with the increase in dIgA₂ concentration (Figure 52B). In contrast, while CH31 dIgA₂ presented higher affinity than CH31 IgG (Figure 51D), they showed comparable binding inhibition, except at the ratio IgG/dIgA₂ 1:10 where IgA considerably inhibited CH31 IgG (Figure 52D). Interestingly, the IgG and dIgA₂ isotypes of the gp41 directed bNAbs 7B2 and 2F5, presented comparable capacities of inhibition.

These results indicate that dIgA₂ antibodies presented greater capacity to resist to IgG antibodies binding inhibition than mIgA₂ antibodies. This was due to their dimeric conformation and their four binding sites and possible ability to form cross bivalent interactions. Moreover, these results suggest that the superior capacity of b12 dIgA₂ to resist to b12 IgG competition and to inhibit b12 IgG binding may be due to; the greater accessibility of the epitope recognised by b12 compared to CH31, 2F5 and 7B2 epitopes, and greater ability to form cross bivalent interactions compared to the other dIgA₂ antibodies in the panel. This is in agreement with the fact that b12 recognises the outer domain of the CD4bs and is less subject to steric hindrance compared to the epitopes targeted by CH31, 2F5 and 7B2 bNAbs ^[434, 457]. Furthermore, the results of this study are in accord with those of the competitive capture assay (see Chapter 3, part 2, Figure 29) and imply that IgG and dIgA antibodies recognising the same epitope compete for binding and capturing virions. Moreover, these data show stronger competition between IgG and mIgA antibodies than between IgG and dIgA antibodies of identical specificities and further highlight the impact of structure on antiviral function and the enhanced abilities conferred by dimeric structure.

Thus, the results of this research indicate that the capacity of IgG antibodies to inhibit the binding to BaL gp140 of their mIgA₂ counterparts and *vice versa* is dependent on the affinity and epitope accessibility. In contrast, IgG antibodies inhibition of dIgA₂ antibodies and *vice versa* is independent of the affinity but dependent on the epitope accessibility and the capacity of the dIgA₂ molecule to form cross bivalent interactions.

After measuring the kinetic parameters of antibody-HIV-1 Env interaction that provided a fundamental two species system (antibody-antigen), work was undertaken to develop a BLI binding assay and to study antibody binding to virions, as this could provide a better overview of the interaction in a complex system (antibody-virus). In the first approach, the same methodology developed for investigating antibody-HIV-1 Env interaction was tested for the study of antibody-virus interaction. Similar to that observed for HIV-1_{BaL} gp140, the results suggest that GNA captured HIV-1_{BaL} virions using the same recognition mechanism to that for HIV-1_{BaL} gp140, and that GNA capture of HIV-1_{BaL} virions was likely mediated by membrane bound HIV-1_{BaL} Env. However, unlike HIV-1_{BaL} gp140, subsequent binding of antibody to GNA-captured HIV-1_{BaL} virions was very low, and did not

allow the measurement of the kinetics parameters of antibody-virus interaction (Figure 54). A possible explanation for these results might be that the binding of antibody to virions did not induce a shift in the interference pattern high enough to be detectable, as the mass of an antibody is negligible compared to the mass of a virion. Moreover, X-ray crystallography studies have shown that each HIV-1 virion contains a small number of Env glycoprotein (14 ± 7) embedded in the viral membrane ^[49, 50]. This indicates that there were probably a significantly lower number of HIV-1_{BaL} Env in the virus preparation, compared to the sample containing HIV-1_{BaL} gp140, which may limit the detection of antibody binding to virions.

Furthermore, Zhu et al. have discovered that Env spikes on virions surfaces were often organised in a cluster suggesting that they might have constraints to diffuse in the viral membrane ^[50]. This implies that GNA might capture HIV-1_{BaL} virions by binding to cluster of Env spikes limiting the access to antibody, and compromising antibody binding detection using this method. Consequently, to overcome this issue an alternative strategy which involved the direct immobilisation of antibody on the biosensor surface followed by the binding of virions was tested (Figure 55). Two approaches were developed, one for IgG antibodies and the other for IgA antibodies. The first method allowed the detection of virions binding to immobilised IgG antibodies on the PA biosensor. However, only five IgG bNAbs (4E10, PG16, PG9, b12 and 2F5), out of the fourteen from the IgG panel, could efficiently capture HIV-1_{BaL} virions (Figure 56), while the binding of these IgG antibodies with HIV-1_{BaL} gp140 had been previously detected in the antibody-antigen BLI assay (Figure 48).

These findings suggest that the interactions observed here between these five IgG antibodies and HIV-1_{BaL} virions might not have been mediated by HIV-1_{BaL} gp140 but rather via antibody binding to lipids/molecules present in the viral membrane. Indeed, these five IgG antibodies exhibit broad cross-clade neutralising activities against HIV-1 and some researchers have postulated that bNAb development might be limited by immune tolerance controls and that some bNAbs shared characteristics with autoantibodies and might react with component of the viral membrane which derived from the infected cell ^[303, 492]. Those bNAbs include the gp41 MPER-specific 4E10 and 2F5 bNAbs, which the binding to phospholipid antigens present in the viral membrane have been reported in several studies ^[303, 457].

Additionally, a recent study performed by Bonsignori et al. has reported the occurrence of anti-dsDNA reactivity by an HIV-1 CD4bs bNAb in an individual with systemic

lupus erythematosus (SLE) ^[493]. This study investigated the possibility that HIV-1 infected persons with autoimmune disease and deficient immune tolerance mechanisms may produce bNAbs more easily than those without autoimmune diseases. Based on these observations, they suggested that some bNAbs and SLE-associated autoantibodies arise from similar pools of B cells ^[478]. Therefore, this raised the possibility that the gp120 CD4bs-specific b12, and the Envgp120 V1/V2 PG16 and PG9 might also share characteristics with autoantibodies and originated from similar pools of B cells, and thus might bind to lipids/molecules present in the viral membrane. These findings are in agreement with a growing number of studies which have demonstrated that bNAbs can bind to other self and/or foreign antigens (i.e. polyreactivity).

To further investigate this possibility, the ability of 4E10, 2F5, b12, PG16 and PG9 to bind to Δ Env HIV-1 pseudovirus lacking Env protein was tested (Figure 57). Interestingly, all five bNAbs were able to bind to Δ Env HIV-1 pseudovirus with similar affinities to those observed for wild type HIV-1_{BaL} virions; this corroborated the hypothesis that the observed antibody binding was not mediated by Env protein. In all, these findings suggest that the BLI was not sensitive enough to detect IgG antibodies binding to HIV-1 Env protein present on the viral surface most likely due to a low number of Env spike on the viral surface. Likewise, the alternative strategy developed to detect IgA antibodies binding to HIV-1_{BaL} virions was also unsuccessful, probably for the same reason (Figure 58).

Therefore the findings of this research agree with previous studies showing that the gp41-directed 2F5 and 4E10 can interact with virions in a non-Env mediated manner. Furthermore, this study is the first to the best of our knowledge to highlight the ability of the gp120 CD4bs-specific b12 and the gp120 V1/V2 PG9 and PG16 to bind to HIV-1 virions in a non-Env mediated manner. Additionally, the results of this research indicate that the BLI may not be adapted to the of study antibody-HIV-1 virions interaction, likely due to the low number of Env spikes exposed on the viral surface.

4.4 Conclusion

This chapter has investigated whether IgG, mgA₂ and dlG_{A2} sharing the same epitope specificity differ in their ability to bind to HIV-1_{BaL} Envgp140 and present different binding kinetics parameters. For this purpose, a novel BLI binding assay that enables the measurement of the kinetic constants of antibody-BaL gp140 was successfully developed. The results of this investigation indicate that isotype influences HIV-1 Env-specific IgG and IgA antibodies interaction with BaL gp140, with in general IgG antibodies binding faster to Env than mgA₂ and dlG_{A2} antibodies recognising the same epitope. Additionally, these findings imply that epitope accessibility affects the antibody on-rate, while the strength of antibody-antigen interaction may have an effect on the antibody off-rate. This research also shows that dlG_{A2} antibodies present greater ability to resist to IgG antibodies inhibition compared to mlgA₂ antibodies likely due to their aptitude to form cross-bivalent interaction providing their higher avidity. Taken together, these findings provide additional evidence, with respect to previous studies, that the isotype of an antibody and therefore the Fc portion of an antibody may play a role in the function of antigen binding.

The present research also aimed to develop a method for measuring antibody binding to whole virions using the BLI system. However, the BLI instrument was not sensitive enough to detect antibody binding to virions mediated by Env viral surface proteins and instead five bNAbs binding to lipids/molecule present on a pseudovirus lacking Env have been identified. The findings of this study agree with previous studies which indicated that 2F5 and 4E10 interact with molecules on the viral membrane ^[303, 457]. However, to the best of our knowledge this study is the first to demonstrate that b12, PG16 and PG9 can also interact with HIV-1 virions via a non-HIV-1 Env mediated mode of recognition. The identification of the molecules recognised by these bNAbs would be a fruitful area for further work. In the meantime, the study of the relationship between antibody binding kinetic parameters and viral capture and neutralisation functions may improve the understanding of the impact of antibody-virus interaction on antiviral function against HIV-1; and this is the objective of the next chapter.

Chapter 5

Correlation analyses between antibody binding kinetics and antiviral function

5.1 Introduction

The unique mechanisms of HIV-1 immune evasion and the apparent incapacity of the immune system to mount effective immune responses against it have hampered HIV vaccine development ^[254, 494]. Throughout the past 30 years, the field of HIV vaccine research has attempted to elucidate the mechanisms that induce bNAbs responses ^[495]. Investigators have tried various vectors (e.g. DNA, protein), routes of administration (e.g. intramuscular injection, subcutaneous) and adjuvants (e.g. toll-like receptor agonists, alum) in the hope to elicit antibodies with high neutralisation potency and breadth. Nonetheless, all the vaccine candidates tested so far have been unsuccessful and the developments of novel immunogens and vaccine strategies capable of inducing this type of antibody seem difficult to achieve soon ^[377].

The quest for eliciting bNAbs has been fuelled by several passive administration studies in non-human primate models which have demonstrated that the infusion of bNAbs conferred protection against transmission of a pathogenic HIV-1/SIV chimeric virus ^[279, 353, 421]. These studies suggest that a vaccine that induces bNAb responses may confer sterilising immunity against HIV-1 infection, at mucosal surfaces, the main portals of viral entry. This research also indicates that passive administration of bNAbs into humans may help to treat and potentially achieve sustained remission in HIV-1 infected individuals. Indeed, a recent study has demonstrated for the first time that a single infusion of an experimental antibody called 3BNC117 significantly reduced HIV viral load in infected patients for nearly a month ^[496]. There are few other Phase I trials of therapeutic passive immunisation that are being conducted and one large efficacy trial in Sub-Saharan Africa (The AMP study). However, to ensure the efficiency and the usefulness of these trials, a substantial number of questions remain to be addressed. Particularly, it is unclear how passive immunisation trials can inform vaccine design and how to select bNAbs or combinations of bNAbs to test in clinical studies to inform vaccine design ^[497].

Consequently, research that aims to better understand the mechanisms that mediate antibody function including the ability of antibody to capture virion and to neutralise infectious viral particles (iVirions) might provide critical insights for the selection of potent bNAbs for immunotherapy and for the design of immunogens for vaccine research.

In the first part of this thesis, the ability of panels of HIV-1-specific IgG, mIgA and dIgA antibodies to capture HIV-1_{BaL} virions and to neutralise HIV-1_{BaL} iVirions was assessed using a novel virion capture assay (VCA) and a TZM-bl neutralisation assay. The findings of this research indicate that epitope accessibility might be the predominant factor influencing antibody virion capture while epitope specificity might be the major factor impacting on antibody neutralisation. Furthermore, data from this study show that IgG antibodies present greater ability to capture virions and to neutralise iVirions than mIgA and dIgA antibodies recognising the same epitope which implies that antibody isotype influence antiviral function.

As the prerequisite to antibody antiviral function is the ability of antibody to recognise and bind to virus, it was thought that studying the binding kinetics of antibody-HIV-1 Env interaction might offer an additional understanding of the mechanisms that mediate antiviral function by Env-specific antibody. Consequently, in the second part of this thesis, the kinetic parameters of the interaction between the panels of HIV-1 Env-specific IgG, mIgA and dIgA antibodies and a soluble form of HIV-1_{BaL} Env (gp140) were measured using Bio-Layer Interferometry (BLI). The kinetic constants measured were: the association constant rate or on-rate constant (K_{on}), which indicates how quickly an antibody binds to antigen, the dissociation constant rate also known as off-rate (K_{off}) which translates how rapidly an antibody detaches from antigen, and the affinity, the intrinsic equilibrium constant for formation of antibody-antigen complexes, equal to the ratio of on-rate to off-rate constants.

The findings of this study suggest that antibodies that bind the most quickly and with the highest affinity to Env gp140 recognise readily accessible epitopes, while antibodies that dissociate the most slowly may present tighter antibody-antigen interaction. Additionally, it was observed that IgG antibodies presented higher on-rates than their mIgA and dIgA equivalents. Based on these observations, it was hypothesised that the ability of HIV-1 Env directed IgG and IgA antibodies to capture virions would correlate with their association constant rates. Conversely, it was posited that the neutralisation activity would not correlate with the on-rate, but with the epitope specificity and binding affinity.

Furthermore, as the findings of Chapter 3 indicate that prolonged antibody-virus incubation time resulted in enhanced neutralisation without generally impacting on virion

capture, it was postulated that the time of incubation would impact on the strength of the correlation between binding kinetic parameters and antibody antiviral function.

Thus, the overall objective of this chapter was to investigate the relationship between the kinetic constants of antibody-HIV-1 Env interaction and antibody antiviral function. For this purpose, this chapter was divided into four interlinked aims:

Aim 1: To study the relationship between antibody kinetic constants and the proportion of virions captured by different panels of IgG, mIgA and dIgA antibodies.

Aim 2: To examine the association between antibody kinetic parameters and antibody-mediated neutralisation.

Aim 3: To assess the impact of antibody-virus incubation time on the strength of the correlation between binding kinetics and antibody function.

Aim 4: To evaluate the influence of antibody factors (e.g. epitope specificity and accessibility, antibody isotype and structure) on the association between kinetic constants (K_D , K_{on} and K_{off}) and antiviral activities (virion capture and neutralisation).

The results and discussions sections of this chapter are divided into two parts; the first part focuses on virion capture and the second on neutralisation. In both sections, the relationship between antibody antiviral functions and kinetic constants was examined as a function of the antibody-virus incubation time using four different IgG and IgA antibodies panels.

5.2 Results

In all correlation analysis between kinetic constants and antibody antiviral function was carried out for the four panels of bNAbs tested. The first panel comprised twelve bNAbs of the IgG isotype recognising a range of Env sites: gp120 CD4bs (b12, CH31, VRC01 and VRC03), gp120 V1/V2 (PG9 and PG16), gp120 V3/glycans (2G12, PGT136 and 447-52D), gp41 MPER (2F5 and 4E10), and multiple epitopes for the polyclonal HIV-IG. The second panel included seven IgA bNAbs: the mIgA₂ and dIgA₂ forms of b12, CH31 and 2F5 and the dIgA₁ of CH31. The third panel contained ten IgG and IgA antibodies: b12, CH31 and 2F5 IgG, mIgA₂

and dIgA₂ and the dIgA₁ form of CH31. The last panel comprised all the IgG and IgA antibodies aforementioned, so 19 bNAbs in total. This enabled the study of whether the relationship between the kinetics constants of antibody-HIV-1 Env interaction and antiviral function differ according to the antibody isotype. Spearman *r* correlation analyses were performed for 0, 1 and 24h antibody-virus pre-incubation time, which allowed the determination of whether the association between antibody binding kinetics and antiviral function changes with the time.

5.2.1 Relationship between kinetic constants and antibody virion capture

The first aim of this chapter was to investigate the influence of epitope specificity and accessibility, on the relationship between bNAbs binding kinetics to HIV-1_{BaL} Env_{gp140} and capacity to capture HIV-1_{BaL} virions. It was hypothesised that IgG and IgA antibodies binding to easily accessible epitopes would have a greater ability to capture virions than those targeting occluded epitopes and that bNAbs on-rate would positively correlate with virion capture.

The results indicate that while IgG antibodies on-rate was the main determinant of HIV-1 Env-specific IgG ability to capture virions in the absence of any pre-incubation period (T=0h) (Figure 59A), however, after 1h (Figure 59D) and 24h (Figure 59H) incubation the binding affinity was the major indicator of IgG antibodies capacity to capture virions. Moreover, the data demonstrate that after 1h and 24h of antibody-virus incubation, the slower IgG antibodies dissociated from Env, the more effectively they captured virions (*p*=0.024 and *p*=0.04, respectively). Furthermore, it was observed that the longer the antibody virion incubation time, the stronger the correlation between the *K_D* and *K_{off}* of the panel of IgG antibodies and virion capture, whereas the association between *K_{on}* and virion capture weakened with the time of incubation (Figure 59).

Overall, these results show that initially the more rapidly IgG antibodies bind to virions, the more they captured virions. After 24h incubation, the more IgG antibodies interacted with high affinity and dissociated slowly, the more they captured virions. This implies that the time of IgG antibodies incubation with virus impacted on the relationship between IgG antibodies kinetic constants and capacity to capture virions.

In the absence of any pre-incubation ($T=0h$), the tested antibodies segregate into two groups with different binding characteristics (Figure 59B). Six IgG bNAbs cluster on the left hand side of the graph with slow K_{on} values: the polyclonal HIV-IG, the anti-gp120 CD4bs VRC03, VRC01 and CH31, and the gp41 MPER-specific 4E10 and 2F5. The second group of bNAbs cluster on the right side of this graph and display faster K_{on} values and include the anti-gp120 V3/glycans 2G12, PGT136 and 447-52D, the anti-gp120 V1/V2 PG16 and PG9 and the CD4bs-specific b12. Comparison of the epitope specificities of the two groups of IgG bNAbs indicate that the first group recognised mainly external Env epitopes (gp120 V1/V2 V3/glycans) which are readily accessible whereas the second group interacts with Env epitopes that may be more obstructive for IgG binding. This is particularly true for MPER-specific antibodies 2F5 and 4E10 that bind to gp41 epitopes close to the viral membrane and elicit a change in envelope conformation. It has also been reported that CD4bs antibodies may require a degree of conformational flexibility with respect to envelope to access the CD4 binding site^[64]. The obvious exception in this case is the CD4bs-specific bNAbs b12; the assumption being that b12 bNAbs binding is unimpeded by any conformational masking^[251, 434].

Interestingly, the observed segregation of two groups of IgG bNAbs is also seen in the correlation plot between K_D and the proportion of virions captured when virus is pre-incubated with antibody for 1h and 24h (Figure 60 D, H). These two groups were similar to the previous ones, except that VRC01 (gp120 CD4bs-specific) joined the first group. In sharp contrast, no distinctive clusters of IgG bNAbs could be identified in the correlation plot between K_{off} and virion capture at any time point tested (Figure 59 C, F, I). Nevertheless, a similar pattern was observed when evaluating dissociation constant rate (Figure 59 C, F, I).

Study of the relationship between the panel of IgA bNAbs and the kinetic constants of antibody-HIV-1_{BaL} Envgp140 interaction indicate that without antibody-virus pre-incubation ($T=0h$), the K_{on} presented the strongest association with virions capture ($p=0.088$) compared to the K_D ($p=0.167$) and K_{off} ($p=0.661$), (Figure 60). However, none of these parameters significantly correlated with IgA-mediated virion capture. A similar pattern was observed after 1h incubation. However, after 24h incubation, significant correlations were observed between IgA-mediated virion capture and IgA on-rate ($p=0.024$) as well as IgA binding affinity ($p=0.048$), (Figure 60 G-H). Thus, in contrast to the panel of IgG bNAbs,

the longer the antibody-virus incubation time, the stronger the correlation between K_D , K_{on} and virion capture. Furthermore there was no correlation with the K_{off} . In addition, unlike the panel of IgG bNAbs, the on-rate remained the strongest indicator of IgA bNAbs ability to capture virions after 24h incubation, while for IgG bNAbs binding affinity was the strongest indicator. This suggests that antibody isotype might impact on the relationship between kinetic constants and virion capture.

Evaluation of the relationship between binding kinetics and virion capture for the panel of IgG and IgA bNAbs, recognising the same epitopes, reveals that the K_{on} was the only kinetic parameter which significantly correlated with virion capture by both antibody panels, irrespective of the incubation time (Figure 61). This indicates that the faster IgG and IgA bNAbs interact with virions, the more efficiently they captured viral particles. Moreover, it was observed that the longer the antibody-virus incubation time, the stronger the correlation between on-rate and virion capture. These data imply that the greater ability of IgG bNAbs to capture virions than their IgA counterparts is due to their ability to bind more rapidly to the virus. Additionally, correlation studies with the whole panel of IgG and IgA bNAbs (N=19) demonstrated that although binding affinity significantly correlated with virion capture, the association constant rate was the best determinant of bNAbs ability to capture virions, at any time point tested (Figure 62). Furthermore, two distinct clusters of antibodies could also be observed in the correlation plots between on-rates and virion capture capacity (Figure 61 B, E, and H), with on the right side the six IgG bNAbs mentioned before (Figure 61 H) and on the left side the rest of the antibodies of the panel.

Taken together these results imply that the more quickly bNAbs bound to virus the more they captured virions, and that this ability might be related to epitope accessibility and antibody isotype, with bNAbs binding to readily accessible epitopes presenting enhanced virion capture, and with IgG bNAbs exhibiting improved virion capture over IgA bNAbs.

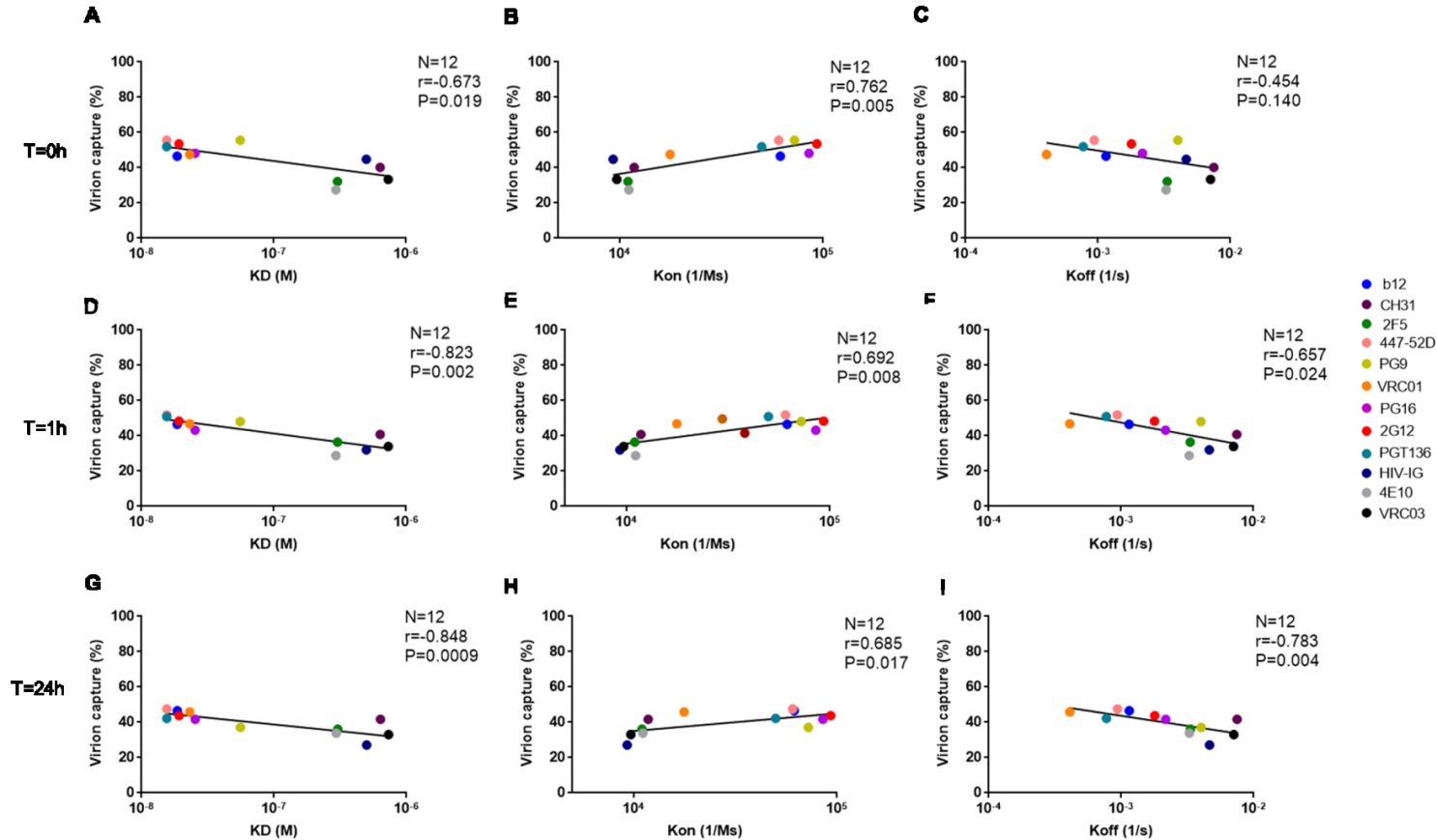


Figure 59. Correlation analysis between IgG binding kinetics and virion capture. The relationship between the amounts of HIV-1_{BaL} virion captured and the binding kinetic parameters to HIV-1_{BaL} gp140, for a panel of 12 HIV-1 Env-specific IgG bNAbs was evaluated using Spearman's r correlation analysis. The panel of IgG bNAbs used recognise a range of Env sites: gp120 CD4bs (b12, CH31, VRC01 and VRC03), gp120 V1/V2 (PG9 and PG16), gp120 V3 (447-52D), gp120 glycans (2G12 and PGT136), gp41 MPER (2F5 and 4E10) and multiple Env epitopes (polyclonal HIV-IG). The kinetic parameters measured were the K_D , K_{on} K_{off} . The analyses were performed for each antibody-virus incubation time tested: 0, 1 and 24h. The results of the Spearman correlation coefficients (r) and the p-values (P) are indicated for each analysis as well as the number (N) of bNAbs tested.

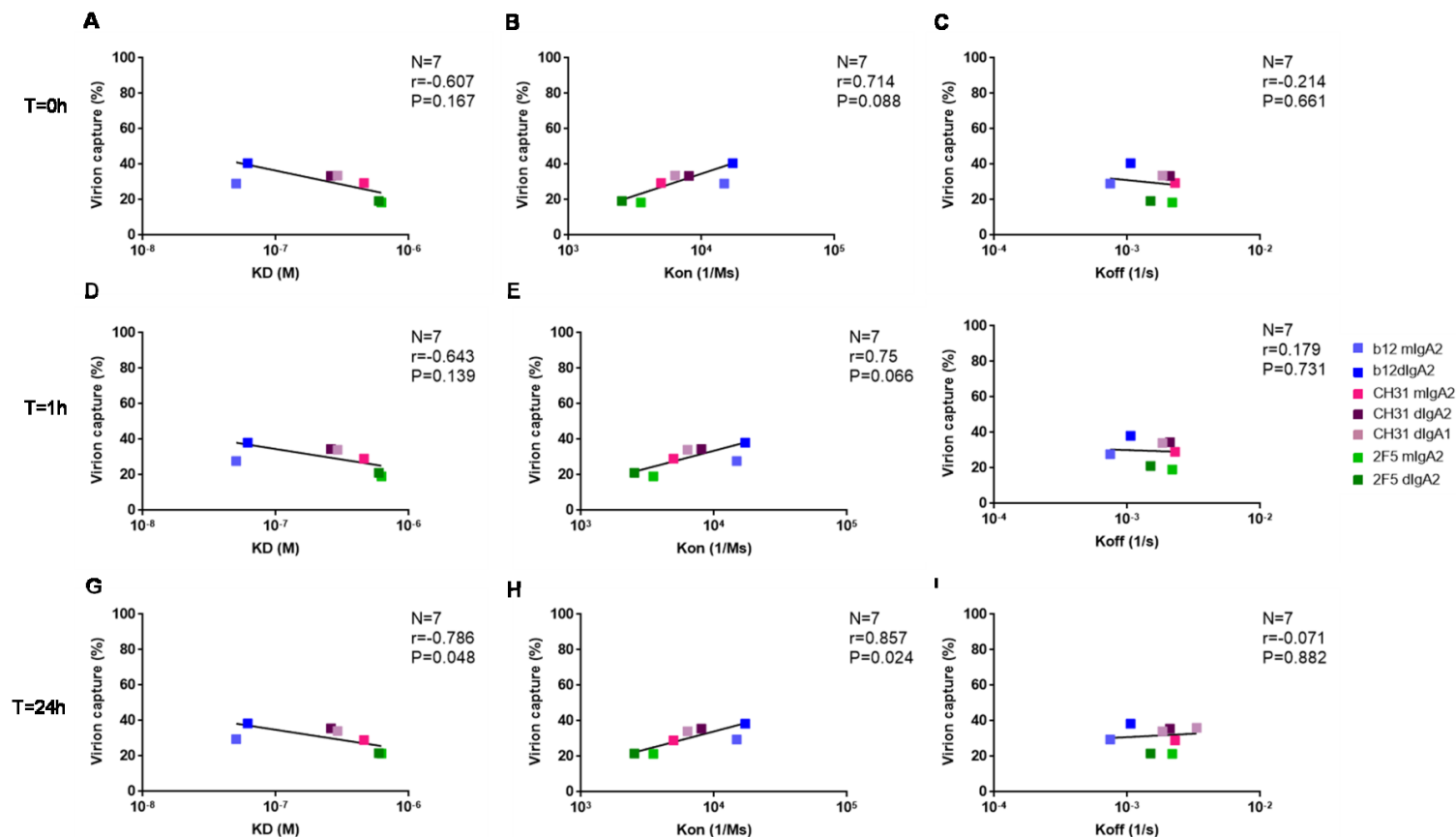


Figure 60. Correlation analysis between IgA antibodies binding kinetics and virion capture. The association between the proportion of HIV-1_{BaL} virions captured and the kinetic constants of a panel of 7 HIV-1 Env-specific IgA bNAbs was measured using Spearman's r correlation analysis. This panel includes the mlgA₂ and dlga₂ forms of b12, CH31 and 2F5 bNAbs and the CH31 dlga₁ isotype of CH31. b12 and CH31 recognise the gp120 CD4bs, and 2F5, the gp1 MPER. The parameters assessed were the binding affinity (K_D), the association constant rate (K_{on}) and the dissociation constant rates (K_{off}). The analyses were performed for each antibody-virus incubation time tested: 0, 1 and 24h. The results of the Spearman correlation coefficients (r) and the p-values (P) and the number (N) of bNAbs are presented for each comparison.

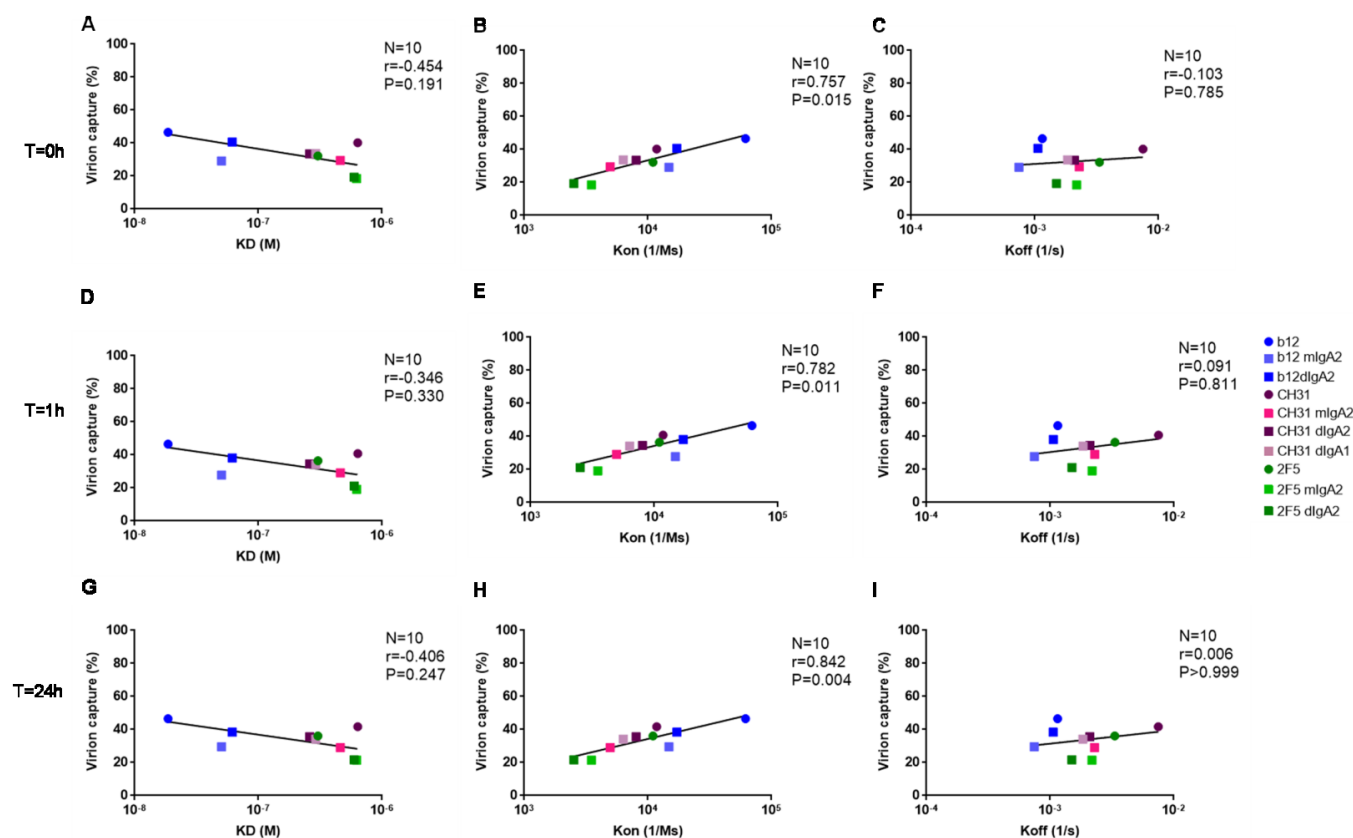


Figure 61. Correlation analysis between kinetic constants and virion capture of IgG and IgA antibodies targeting the same epitope. The relationship between the quantities of HIV-1_{BaL} virion captured and the kinetic constants of interaction with HIV-1_{BaL} p140 for a panel of 10 of bNAbs was measured by Spearman's r correlation analysis. The panel comprised the IgG, mIgA₂ and dIgA₂ forms of the gp120 CD4bs-specific b12, CH31 and the gp41 MPER-specific 2F5 bNAbs. The kinetic constants compared were the K_D , K_{on} and K_{off} . The analyses were performed for each antibody-virus incubation time tested: 0, 1 and 24h. The Spearman correlation coefficients (r) and the p-values (P) and the number (N) of bNAbs are indicated for each correlation analysis.

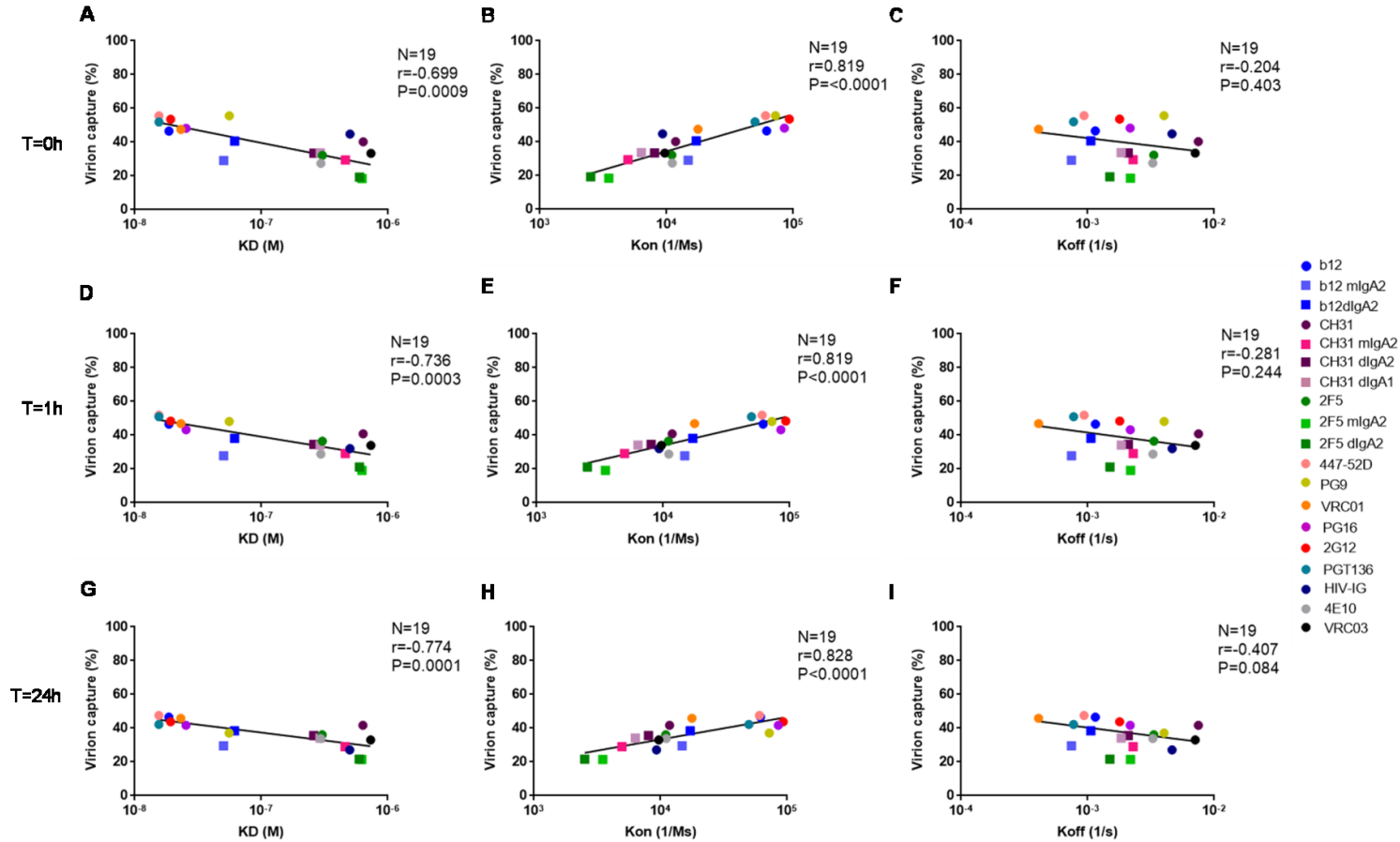


Figure 62. Correlation analysis between kinetic constants and virion capture of a panel of IgG and IgA antibodies. The relationship between the amounts of HIV-1_{BaL} virion captured and the binding kinetic constants to HIV-1_{BaL} gp140 for a panel of 19 IgG and IgA bNAbs was measured using Spearman's r correlation analysis. The kinetic parameters assessed were the binding affinity (K_D), the association constant rate (K_{on}) and the dissociation constant rates (K_{off}). The analyses were performed for each antibody-virus incubation time tested: 0, 1 and 24h. The Spearman correlation coefficients (r) and the p-values (P) and the number (N) of bNAbs are indicated for each analysis.

5.2.2 Association between kinetic constants and neutralisation by HIV-1 Env specific antibodies

In the second part of this chapter, the relationship between antibody kinetic constants of interaction to HIV-1_{BaL} gp140 and antibody-mediated neutralisation against HIV-1_{BaL} virus was investigated using the same panels of IgG, mIgA and dIgA bNAbs. It was posited that antibody epitope specificity and affinity for HIV-1_{BaL} gp140 would correlate with neutralisation potency, and that antibody isotype would influence the association between binding kinetics and neutralisation.

Correlation analysis using the panel of IgG bNAbs revealed that none of the kinetic parameters correlated with neutralisation without antibody-virus pre-incubation or after 1h incubation (Figure 62). However, following 24h incubation with virus, binding affinity and dissociation constant rate significantly and inversely correlated with neutralisation activity. Indeed the stronger the affinity and slower the dissociation constant rate the more potent was IgG-mediated neutralisation. Correlation with neutralisation was slightly more robust with IgG binding affinity ($r=0.679$, $p=0.015$) than with IgG K_{off} ($r=0.532$, $p=0.038$).

As observed previously, two groups of IgG bNAbs could be distinguished in the correlation plot for affinity and neutralisation (Figure 63G). On the left side of Figure 63G, the group of IgG bNAbs, which presented the strongest neutralisation activity and the highest affinity, was identical to those that presented the highest virion capture and affinity following 24h incubation (Figure 59G). This was unsurprising as virion capture significantly correlated with neutralisation and the strength of this association increased with antibody-virus incubation time (Figure 25). Conversely, no distinctive groups of IgG bNAbs could be seen in the correlation plot between K_{off} and neutralisation activity which further indicated that IgG off-rate is most likely influenced by epitope specificity rather than accessibility. Additionally, these results indicated that between 0 and 1h incubation with virus, the neutralisation potency of the panel of IgG bNAbs was influenced by factors other than their affinity, on-rate or off-rate for HIV-1 Env. Nevertheless, after a prolonged incubation with virus (>1h), the ability of IgG bNAbs to maintain a strong (high affinity) and a stable interaction (slow off-rate) with HIV-1 virion was necessary to achieve high neutralisation activity.

Interestingly, IgA bNAbs kinetic constants correlated with their neutralisation activity (Figure 64). However, the impact of time on the strength of the correlation between kinetic constants and neutralisation activity for the panel of IgA bNAbs was not consistent with the lowest p-values observed for K_D and K_{on} at 1h incubation with virus (both $p=0.003$) and for the K_{off} after 24h incubation ($p=0.034$). The inconsistencies of these results indicate that the number of IgA bNAbs and the specificities tested might not be sufficient to study the relationship between IgA binding kinetics and neutralisation. In addition, these data indicate that isotype might influence antibody mechanisms of neutralisation with IgA and IgG bNAbs presenting different relationship between binding kinetics and neutralisation capacity.

To further investigate the impact of antibody isotype on neutralisation and binding kinetics, correlation analyses were performed for the panel of b12, CH31 and 2F5 IgG, mIgA and dIgA bNAbs (Figure 65). It was observed that the neutralisation activity of the panel strongly and positively correlated with the on-rates, for all three antibody-virus incubation times tested (all $p<0.0001$) (Figure 65 B, E, H). The results also show that IgG and IgA bNAbs binding affinity significantly correlated with neutralisation (Figure 65 A, D, G); however to a lesser extent than observed with bNAbs on-rate ($p=0.027$ vs $p<0.0001$, respectively at 24h incubation), (Figure 65 G-H). Moreover, comparison of the relationship, between virion capture and kinetic constants and between neutralisation and kinetic constants, indicated that IgG and IgA bNAbs association constant rate was a better determinant of their ability to neutralise iVirions than to capture iVirions ($p=0.004$ vs $p<0.0001$, respectively $T=24h$). Likewise, comparisons of the combined panel of IgG and IgA bNAbs (19 bNAbs) also demonstrated that although affinity correlated with neutralisation, the K_{on} was the kinetic parameter that best predicted the ability of bNAbs to neutralise virions (Figure 66).

In summary, these results suggest that IgG bNAbs presented a greater capacity to capture and neutralise iVirions than their IgA counterparts due to their ability to bind more quickly to virions than bNAbs of the IgA isotype. Moreover, the findings indicate that bNAbs binding to readily accessible epitope presented enhanced antiviral function compared to those targeting occluded epitopes. Furthermore, these data imply that the rapid-binding capacity of HIV-1 Env-specific bNAbs is the greatest determinant of the ability to capture and neutralise iVirions, and thus the on-rate might be the best predictor of bNAbs antiviral function.

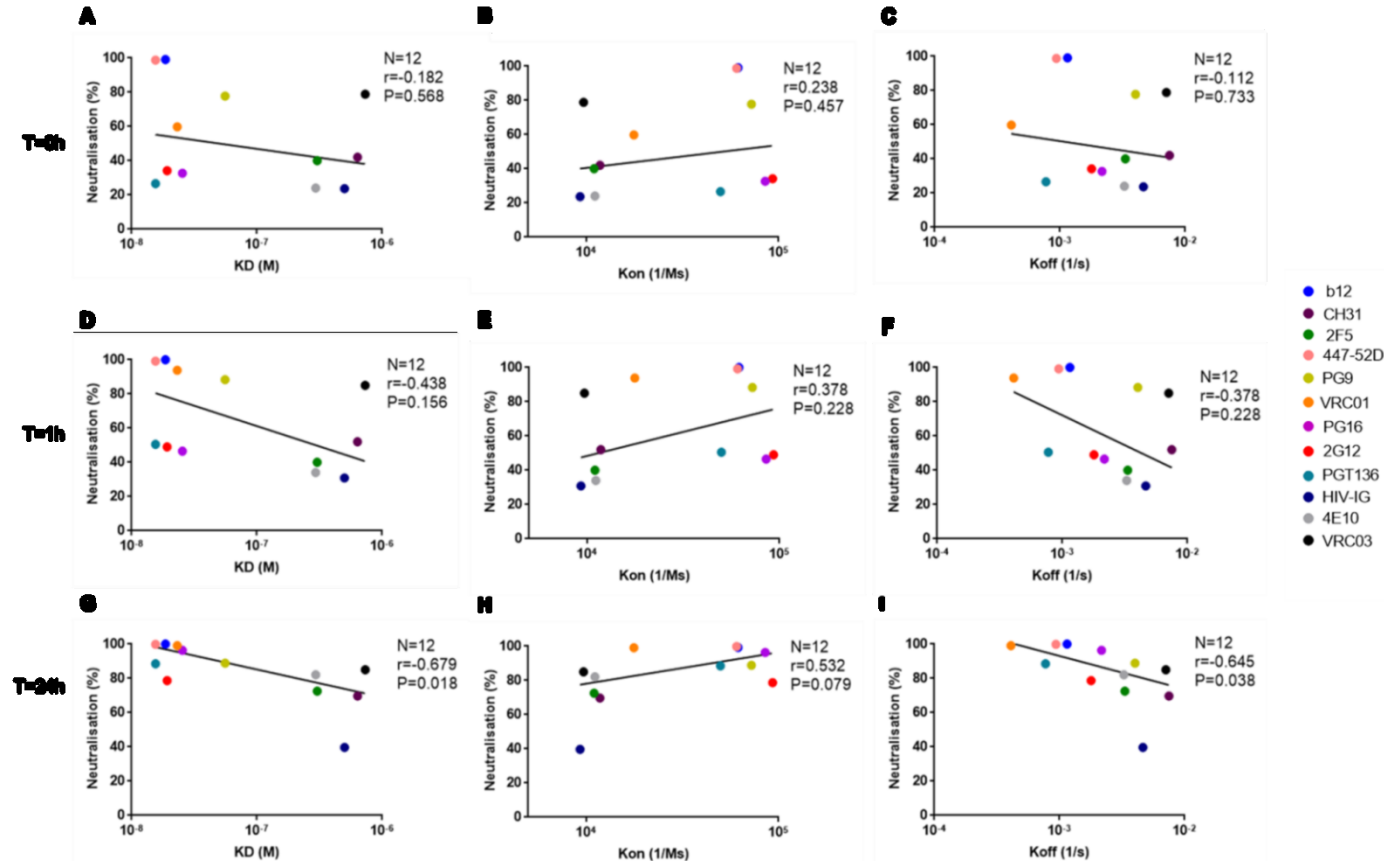


Figure 63. Correlation analysis between IgG antibodies kinetic constants and neutralisation. The relationship between the neutralisation potency against HIV-1_{BaL} and the binding kinetic parameters to HIV-1_{BaL} Envgp140 for a panel of 12 HIV-1 Env-specific IgG bNAbs was assessed, using Spearman's r correlation analysis. The panel of IgG bNAbs bind to a range of Env sites: gp120 CD4bs (b12, CH31, VRC01 and VRC03), gp120 V1/V2 (PG9 and PG16), gp120 V3 (447-52D), gp120 glycans (2G12 and PGT136), gp41 MPER (2F5 and 4E10) and multiple Env epitopes (polyclonal HIV-IG). The kinetic parameters compared were K_D , K_{on} and K_{off} . The analyses were performed for each antibody-virus incubation time tested: 0, 1 and 24h. The results of the Spearman correlation coefficients (r) and the p-values (P) and the number (N) of bNAbs are presented for each analysis.

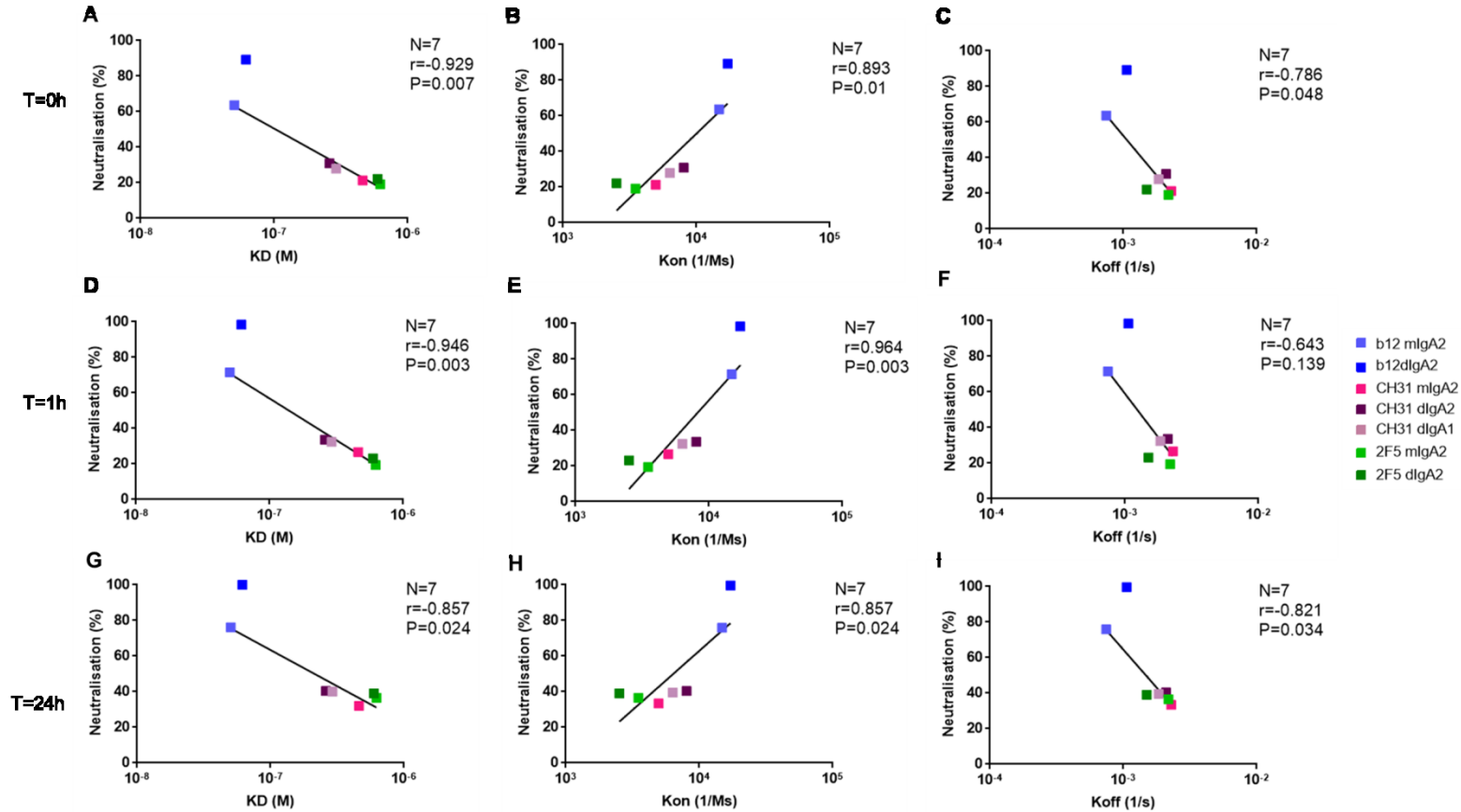


Figure 64. Correlation analysis between IgA antibodies kinetic constants and neutralisation. The association between the neutralisation potency against HIV-1_{BaL} and the binding kinetic constants to HIV-1_{BaL} gp140 for a panel of 7 HIV-1 Env-specific IgA bNAbs was assessed using Spearman's r correlation analysis. The panel comprised the IgG, mlgA₂ and dlgA₂ forms of the gp120 CD4bs-specific b12, CH31 and the gp41 MPER-specific 2F5 bNAbs. The kinetic parameters compared included the K_D , K_{on} and K_{off} . The correlation analyses were performed for each antibody-virus incubation time tested: 0, 1 and 24h. The Spearman correlation coefficients (r) and the p values (P) and the number (N) of bNAbs are presented for each comparison.

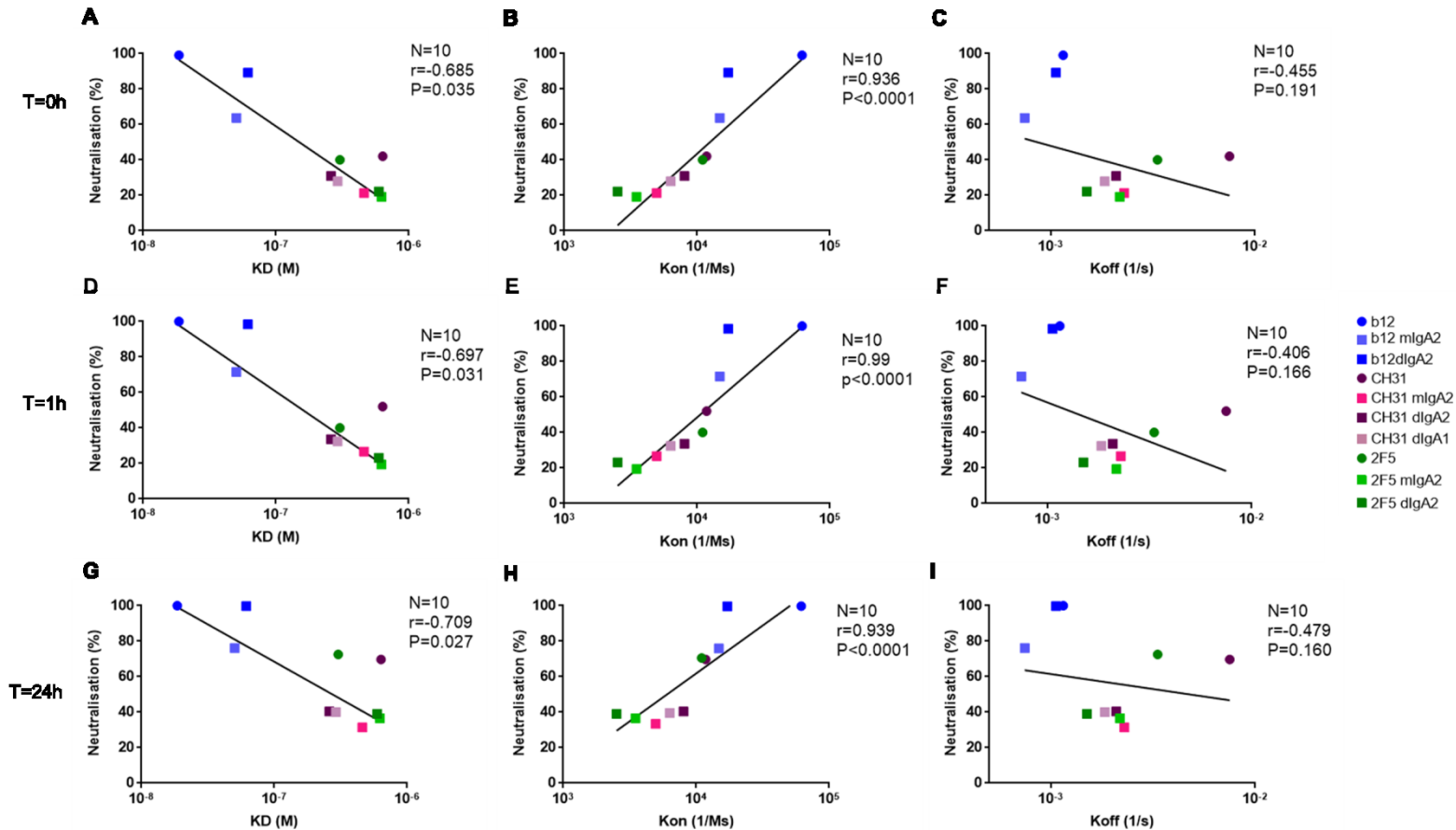


Figure 65. Correlation analysis between kinetic parameters and neutralisation of a panel of IgG and IgA antibodies recognising the same epitope. The relationship between the neutralisation activity and the kinetic constants of interaction with HIV-1_{BaL} gp140 for a panel of ten HIV-1 Env-specific IgG and IgA bNAbs was evaluated by Spearman's r correlation analysis. The parameters measured comprised the K_D , K_{on} and K_{off} . The correlation analyses were performed for each antibody-virus incubation time tested: 0, 1 and 24h. The Spearman correlation coefficients (r) and the p -values (P) and the number (N) of bNAbs are presented for each comparison.

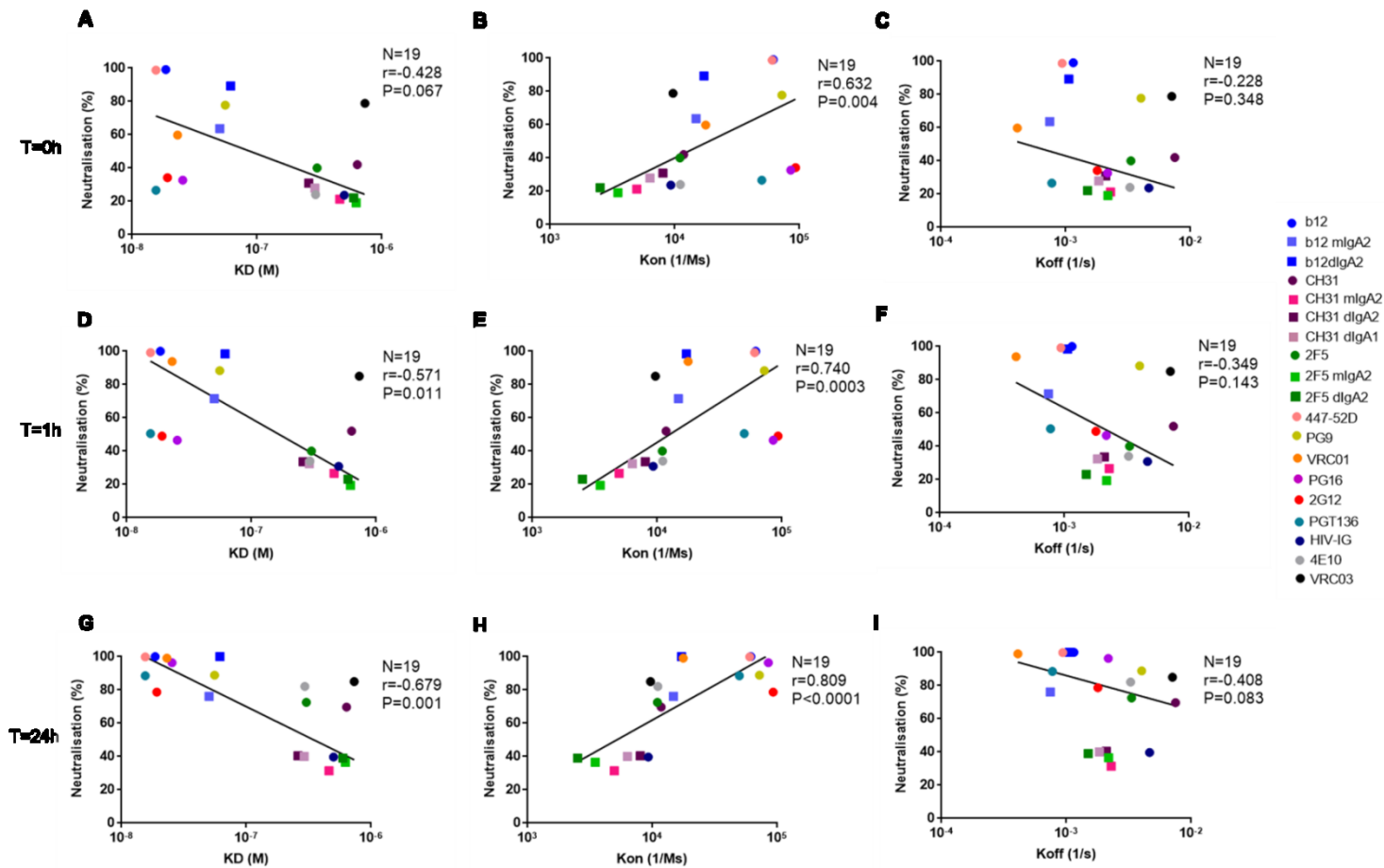


Figure 66. Correlation analysis between kinetic parameters and neutralisation of a panel of 19 IgG and IgA antibodies. The association between the HIV-1_{BaL} neutralisation potency and the binding kinetic constants to HIV-1_{BaL} Envgp140 of a panel of 19 IgG and IgA bNAbs was measured using Spearman's r correlation analysis. The kinetic parameters assessed were the K_D , K_{on} and K_{off} . The analyses were performed for each antibody-virus incubation time tested: 0, 1 and 24h. The Spearman correlation coefficients (r) and the p-values (P) and the number (N) of bNAbs are indicated for each analysis.

5.3 Discussion

The major precondition for most antibody antiviral functions is the ability of antibody to bind specifically and with high affinity to viral antigen. The current research presents correlation analyses between the binding kinetics of HIV-1 Env-specific IgG, mIgA and dIgA bNAbs and their capacity for virion capture and neutralisation.

Based on observations in the preceding chapters that antibody that bound to readily accessible Env epitopes captured higher proportions of virions (Chapter 3), and that these antibodies interacted more quickly with HIV-1_{BaL} gp140 than those targeting occluded Env sites (Chapter 4), it was predicted that antibody on-rate would positively correlate with the amount of HIV-1_{BaL} virion captured by antibody. Moreover, the previous results indicate that, generally, extended antibody-virus pre-incubation time resulted in enhanced neutralisation activity without impacting on the amount of virions captured (Chapter 3). Consequently, it was hypothesised that the strength of the correlation between kinetic constants and antibody function would be affected by antibody-virus pre-incubation time. Furthermore, the findings of the preceding chapters have demonstrated that IgG bNAbs presented greater ability to capture and neutralise virions and bound more rapidly to soluble Env than their IgA equivalents, recognising the same epitope. As a result, it was anticipated that when assessing both IgG and IgA panels, the association constant rate would be the predominant indicator of antibody ability to capture and neutralise virions.

Remarkably, the findings of the present study imply that IgG bNAbs directed against easily accessible Env epitopes may have the ability to bind quickly to Env and to capture virions. Indeed, gp120 V1/V2 V3/glycans epitopes are located on the external surface of Env and are easily reached by bulky antibodies ^[64, 290]; and b12 which binds to the CD4bs is known to be highly active against clade B virus such as HIV-1_{BaL} ^[434]. Taken together, these results suggest that the faster the kinetics of binding, and by implication the accessibility of the epitope, the higher the level of virion capture. Similarly, Yasmeen et al. suggested that increasing epitope accessibility may lead to higher antibody on-rates constants and also pointed out the importance of epitope accessibility on antibody capacity to bind rapidly ^[482].

However, after a sufficiently long period of time, here 1h, the ability of IgG bNAbs to bind rapidly to easily accessible epitopes may no longer be the major factor impacting on virion capture, and the capacity to maintain a strong interaction (affinity) becomes the dominant parameter influencing virion capture. These findings are in agreement with those of a previous study which showed that the binding magnitude and avidity of IgG antibodies from RV144 vaccinees to HIV-1 MN gp120 and to Env ConS gp140 (clade B) significantly correlated with virion capture. However, in this study they also found no correlation with magnitude and avidity using Env proteins from HIV-1 CM244 (clade E) and NL4-3 (clade B) [453]. This supposes that the relationship between the kinetic constants and the virion capture function is influenced by the virus strain, where the epitope and/or conformation may differ. Importantly, the findings of the present research are the first to demonstrate a clear correlation between IgG on-rate (K_{on}) and binding affinity (K_D) to HIV-1_{BaL} gp140 with virion capture.

After investigating the relationship between IgG kinetic constants and virion capture, the same study was performed using a panel of IgA bNAbs to determine whether antibody isotype impacted on this relationship. Interestingly, unlike that observed with the IgG bNAbs panel, for IgA bNAbs, antibody on-rate and binding affinity only correlated with virion capture following 24h incubation (Figure 60). Moreover, while for the panel of IgG bNAbs binding affinity was most closely correlated with virion capture following 24h incubation, for IgA bNAbs the association constant rate demonstrated the strongest correlation. A possible explanation for these differences might be related to the numbers of antibody tested (12 IgG vs 7 IgA) and the restricted range of distinct epitope specificities evaluated (12 for IgG and 3 for IgA). These factors might have affected the strength of the correlation between antibody kinetic constants and virion capture.

Therefore, the results obtained with the panel of IgA bNAbs must be interpreted with caution as they might be biased. Another explanation for these results might be related to the differences in on-rates reported between IgG and IgA bNAbs of identical specificity. As IgA bNAbs presented slower on-rates than IgG bNAbs, after 24h incubation with virus, IgA ability to bind rapidly to the targeted epitope might still be the predominant factor influencing the capacity to capture virions. This presupposes that, as observed with IgG

bNAbs, after a sufficient period of antibody-virus incubation time, IgA binding affinity might also become the major parameter impacting on virion capture.

Together, these data suggest that antibody isotype may impact on the relationship between binding kinetic constants and virion capture, with IgA bNAbs binding more slowly to virions than corresponding IgG bNAbs.

These suggestions are strengthened by the observation that for IgG, mIgA and dIgA bNAbs, recognising the same epitopes, the association constant rate was the only kinetic parameter which significantly correlated with virion capture and that the longer antibody-virus incubation, the stronger the correlation (Figure 61). Again this indicates that IgG bNAbs presented a greater capacity to capture virions than mIgA and dIgA bNAbs of identical specificity due to their ability to bind more rapidly to HIV-1 Env exposed on viral surface than their IgA counterparts. Furthermore, comparisons of the combined panel of IgG and IgA bNAbs (7 IgA and 12 IgG bNAbs) directed against various Env sites indicate that both antibody binding affinity and on-rate impacted on virion capture capacity (Figure 62). Although the strength of the correlation between the kinetic parameters and virion capture increased with the incubation time, the on-rate was the kinetic constant which best predicted the efficiency of virion capture at any time point tested.

Thus, these results indicate that HIV-1 Env-specific bNAbs ability to bind rapidly to virion (on-rate) may be the initial limiting factor influencing their capacity to capture virions. Then, after sufficient time, once antibody binding to HIV-1 virions reaches equilibrium, the strength of antibody-virus interaction (antibody binding affinity) becomes the predominant limiting factor. These data also provide further evidence that antibody isotype influences on-rate and as a consequence antiviral function ^[467, 469, 479]. This suggests that IgG-isotype structure may be more adapted to interact quickly with HIV-1 Env than IgA-isotype with the same specificity. As mentioned previously (see Chapter 3, Discussion, Part 2), the heavy chains of IgG and IgA differ by their sequence, including the length of their hinge region, the number and location of interchain disulphide bonds, of attached oligosaccharide moieties and of C domains ^[498]. These differences probably impact on the binding kinetics and result in differences in antiviral abilities between IgG and IgA bNAbs of identical specificity. An additional caveat is that the IgA bNAbs tested are not natural antibodies but have been constructed molecularly. This means that they have not gone through any selection process

in the germinal centre. Therefore, it is plausible that natural IgA could be generated and selected through somatic hypermutation with high K_{on} values and that the molecular switched into an IgG backbone might make them bind less efficiently and impact on their capacity to capture virions.

In the second part of this chapter the relationship between kinetic constants and antiviral function was further investigated by studying the influence of binding kinetics on neutralisation potency. The findings of the preceding chapters indicated that epitope specificity might be the main factor influencing antibody neutralisation; accordingly, it was proposed that neutralisation activity would not correlate with antibody on-rates but with other factors such as antibody specificity and affinity. Additionally, as observed with virion capture function, it was anticipated that the relationship between kinetic constants and neutralisation activity would be influenced by antibody-isotype.

Correlation analyses between HIV-1 Env-specific IgG, mIgA and dIgA bNAbs binding kinetics and neutralisation potency revealed further differences between virion capture and neutralisation function. Notably, the results indicate that binding kinetic constants were not the leading factors impacting on bNAbs ability to neutralise virions with no or 1h incubation. However, after 24h incubation with virus, the strength (K_D) and the duration of IgG-virion interaction (K_{off}) became dominant parameters influencing neutralisation potency (Figure 63).

The findings of this research are in line with those of a recent study which has demonstrated that antibody fast dissociation constant rate correlated with greater persistent fraction of infectious virus ^[482]. Likewise, Vancott et al. found that anti-gp120 V3 bNAbs off-rate was predictive of their capacity to neutralise a clade B HIV-1 virus (IIIB, MN) ^[499]. Currently, there are two dominating models of antibody-mediated neutralisation of HIV-1 virions. The first called the “occupancy model” postulates that neutralisation by antibody is determined by antibody occupancy of Env sites and affinity for Env spikes on virion and requires a threshold level of antibody binding to prevent infectivity, independently of epitope specificity ^[308, 317]. The second model proposes that antibody-mediated neutralisation is only dependent of antibody qualitative characteristics such as epitope specificity and binding affinity ^[345, 500]. The findings of this study support a combination of the two models and suggest that with no or short (1h) incubation, the level

of antibody occupancy of Env sites are the dominant determinants impacting on neutralisation: as observed by the lack of correlation between kinetic constants and neutralisation activity. Furthermore, the results of this study indicate that after 24h incubation, once IgG bNAbs may have reached threshold levels of Env sites occupancy, the ability to maintain a strong (high affinity) and stable (slow off-rate) interactions with virus become the predominant parameters influencing neutralisation.

Subsequent analysis investigated the relation between binding kinetics and neutralisation using a panel of IgA bNAbs (Figure 64), to determine whether this differed from the results obtained with the panel of IgG bNAbs (Figure 63). These data indicate that for the evaluated panel of IgA bNAbs, binding affinity and on-rate were the factors that best predicted the ability to neutralise virions. In particular, the results suggest that the ability of b12 IgA to interact more rapidly and with a higher affinity with BaL gp140 than CH31 IgA and 2F5 IgA correlated with greater virion capture and neutralisation capacities. This further emphasises the importance of epitope specificity, affinity, and on-rate for neutralisation potency. Nonetheless, the results obtained with IgA bNAbs present some inconsistencies and cannot be extrapolated as a general rule for the IgA isotype. Additionally, as mentioned previously the IgA bNAbs tested are molecular products and do not originate from natural selection *in vivo*. Thus, although these results should be interpreted with caution, the data obtained with the panel of IgA bNAbs also support the proposed model of antibody-mediated neutralisation.

Analysis of the combined antibody panels (IgG and IgA bNAbs) differ from those observed with the IgG panel and indicate that when comparing panels of bNAbs comprising HIV-1 Env-specific IgG and IgA, the association constant rate is the parameter which best predicts bNAbs virion capture and neutralisation (Figure 65-66). This implies that the ability of a panel of IgG and IgA bNAbs to bind rapidly to HIV-1 virions is the predominant factor influencing virion capture and neutralisation functions. Furthermore, this indicates that IgG bNAbs present a greater capacity to capture virions and to neutralise iVirions than IgA bNAbs due to their ability to bind more rapidly to HIV-1 virions than IgA bNAbs.

Thus this study enables to better understand the mechanisms of antibody-mediated neutralisation, however *in vivo* antibody that takes 24h to neutralise effectively HIV-1 virions are unlikely to prevent the infection. Overall, these results suggest that the most

desirable characteristic of a bNAb to block HIV-1 transmission might be its ability to interact rapidly with virions.

The findings of this research are in accord with those of a recent study which using mathematical models has indicated that HIV-1 Env-specific bNAbs with the highest on-rates should offer greater neutralisation activity *in vivo*. Moreover, this study suggests that to prevent HIV-1 infection bNAbs must accumulate at sufficient concentrations to neutralise HIV quicker than virions penetrate the cervicovaginal mucus (CMV) and reach the target cells ^[501]. Additionally, these findings corroborates those of a recent study which has found that dose-response curve slope is a critical predictor of both bNAbs activity and breadth and may predict the relationship between bNAb epitope structure and *in vivo* efficacy ^[502]. Furthermore, the findings of the present research are in accord with those of an earlier study which demonstrated that although affinity correlates with neutralisation of vesicular stomatitis virus (VSV), the correlation with on-rate was statistically stronger ^[503]. This implies that antibody rapid binding to virus might be as important as the strength of antibody-antigen interaction in preventing infection.

5.4 Conclusion

The relationship between HIV-1 Env-specific IgG, mIgA and dIgA bNAbs binding kinetic constants and virion capture and neutralisation was investigated by Spearman *r* correlation analysis. This research aimed to improve the understanding of the parameters of antibody-virus interaction that predict antiviral activity against HIV-1. The findings of this investigation indicate that HIV-1 Env-directed bNAbs, that interact the most rapidly with virions, present the strongest potential to capture virions and to neutralise iVirions *in vitro*. Furthermore, the results of this research suggest that bNAb on-rate is influenced by antibody epitope accessibility and isotype with bNAbs targeting readily accessible epitopes and of the IgG-isotype compared to IgA-isotype, presenting enhanced antiviral function against HIV-1.

Additionally, this research provides some evidence for a third model of mechanism of antibody-mediated neutralisation, and proposes that antibody capacity to block infectivity might be, in the first instance, determined by the levels of Env sites occupancy, and once threshold levels of occupancy are reached, it might be influenced by antibody

epitope specificity and capacity to bind with high affinity and with a slow dissociation rate to HIV-1 Env on the viral surface. Overall, the current investigation affords important insight for the selection of bNAbs for passive immunisation studies, providing a greater understanding of the mechanism of antibody-mediated viral neutralisation, as well as of the relationship between binding kinetics and antibody antiviral function, and of the parameters that impact on this relation. However, only one virus strain was used in these assays, and a previous study has suggested that viral strain may affect the relationship between antibody binding parameter and antiviral function^[453]. Thus additional research is needed to confirm that these findings are generalisable to other viral strains.

Future research should also be undertaken to test these bNAbs *in vivo* via passive immunisation studies in non-human primate models. Such research may validate the superior efficacy of rapid-binding bNAbs against HIV-1 infection. This would have important implications for the field of immunotherapy and would require the association rate constant, rather than the affinity, to be adopted as the major criteria for antibody selection. This would represent a major change in emphasis where to date binding affinity has been generally considered as the major determinant of antibody capture and neutralisation^[453, 500, 503].

Chapter 6

Summary and General Conclusion

This chapter describes how the thesis contributes to research and links with the current literature. It also outlines the thesis limitations and describes the future research needed in the field. Finally, the implications for vaccine research and passive immunisation studies are discussed.

6.1 Thesis' contribution to research

The overall focus of this thesis was to identify and analyse the parameters of antibody-virus interaction which influence antiviral function.

In the first part of this thesis, the impact of epitope specificity and accessibility and of antibody isotype and structure on antiviral activities were investigated. Study of the amount of virions captured and the neutralisation activity, of panels of IgG and IgA antibodies, indicated epitope accessibility as the major factor determining virion capture, while epitope specificity was the dominant factor in determining neutralisation potency. Subsequent, comparisons of IgG, mIgA and dIgA bNAbs isotypes recognising the same epitope demonstrated that antibody isotype and structure influence antiviral function. Notably, antibodies of the IgG isotype were found to have enhanced antiviral capacities compared to those of the IgA isotype, independently of epitope specificity. Moreover, the dimeric structure conferred advantages to dIgA antibodies to capture and neutralise virions compared to mIgA antibodies, depending on the epitope targeted.

Subsequent analysis of the impact of antibody mixtures on antiviral function demonstrated that combinations of IgG and IgA antibodies recognising the same epitope lessen IgG effector function depending on the epitope specificity. The results suggest that this was due to competition for the same Env sites and the lower antiviral activity of the IgA-isotype compared to the bIgG-isotype. Conversely, mixtures of antibodies, neutralising or non-neutralising and binding to distinct Env sites, resulted in an increase in virions capture and neutralisation. This increase was dependent on the epitope specificity and occurred in the absence of competition for the same Env binding sites. This provided proof-of concept for the potential use of mixtures of antibodies recognising different epitopes in passive protection studies and immunotherapies against HIV infection.

In addition to these epitope and antibody features that influence antiviral function, it was found that antibody-virus interaction time impacted on the outcome of the VCA and TZM-bl neutralisation assay. While prolonged antibody-virus pre-incubation did not usually

affect the quantity of virions captured, it generally led to increase in neutralisation activity. This provided insights into the understanding of the mechanisms of antibody-mediated neutralisation. Further research indicated that the amount of virions captured significantly correlated with neutralisation for the same antibody concentration, with greater neutralisation activity, the higher the proportion of virions captured by the bNAb.

To compare the capacity of bNAbs to neutralise captured iVirions, an antibody Virion Capture Neutralisation Index (AVCNI), which corresponds to the ratio of neutralisation to virion capture, was devised. The findings of this thesis indicate that the AVCNI is influenced by antibody epitope specificity and isotype, and by antibody-virus interaction time. It was observed that bNAbs directed against Env_{gp120} CD4bs presented the highest AVCNI values and that IgG and IgA antibodies recognising the same epitope may present distinct AVCNI values, as a function of the epitope specificity. Moreover, the data indicate that the AVCNI increased with time. This reflected the fact that, in general, the neutralisation activity was augmented overtime while the amount of virions captured remained relatively stable. However, the data suggest that the AVCNI is not influenced by antibody oligomeric structure (monomeric vs dimeric) with mIgA and dIgA bNAbs of identical specificity presenting comparable AVCNI. Thus, these findings imply that bNAbs with the highest AVCNI value present the greatest ability to neutralise iVirions captured and that the AVCNI might be used as a tool to select bNAbs with the best ratio neutralisation/capture capacity.

To identify the kinetic parameters of antibody-virus interaction that impact on antiviral function, the second part of this thesis examined the influence of epitope and antibody characteristics on the binding kinetics for the panels of IgG, mIgA and dIgA antibodies. For this purpose, an antibody binding assay using the Bio-Layer Interferometry (BLI) system that enables measurement of the binding affinity, on-rate and off-rate for the panels of HIV-1 Env-specific antibodies was developed. The findings of this study showed that IgG antibodies binding to readily accessible Env epitopes presented higher on-rates than those targeting occluded Env epitopes and that HIV-1 Env-specific IgG interacted more rapidly with HIV Env than their mIgA and dIgA equivalents. Although different strategies were tested, the BLI instrument did not enable measurement of antibody interaction with Env exposed on the surface of HIV-1_{BaL} virions. Furthermore, the binding of some IgG antibodies to both HIV-1_{BaL} and ΔEnv HIV-1 pseudovirus (lacking Env protein) was detectable

using the BLI instrument, suggestive of non-specific binding. This demonstrates that the Envgp120 MPER-specific 4E10 and 2F5, the Envgp120 CD4bs-specific b12 and the Envgp120 V1/V2-specific PG16 and PG9 bNAbs of the IgG-isotype could interact with virions in a non-Env mediated manner. Further, this suggested that the BLI system was not sensitive enough to detect antibody binding to HIV-1 Env protein present on the viral surface probably due to the low number of Env spikes ^[50].

To determine how antibody antiviral capacity observed in the first part of this thesis relates to the binding kinetics to BaL gp140 measured in this second part, correlation analysis between kinetic constants and antiviral function were performed in the last section of this thesis. Analysis revealed that the association constant rate is the predominant kinetic parameter influencing IgG and IgA bNAbs s antiviral function. Most strikingly, it was found that IgG bNAbs directed against easily accessible epitopes had a greater capacity to capture and neutralise virions compared to those targeting epitopes with steric constraints. Furthermore, the greater capacity of IgG compared to IgA bNAbs to capture and neutralise virus significantly correlated with higher on-rates to HIV-1_{BaL} gp140.

Thus, the findings of this thesis indicate that the more rapidly bNAbs interact with virions, the more they capture virions and neutralise iVirions. Furthermore, this thesis demonstrates that antibody association constant rate is influenced by epitope accessibility and antibody isotype. Taken together, these data imply that association constant rate may be an important guide in the selection of bNAbs for passive protection and immunotherapy.

6.2 The thesis in context with published literature

The findings of this thesis challenge the old dogma of over half a century which suggests that the V (binding site) and C (isotype) domains of an antibody molecule are structurally and functionally independent ^[474-476]. This study provides further evidence to recent research which have suggested that antibody isotype influences the kinetic parameters of antibody-antigen interaction and impacts on antibody function ^[238, 468]. Additionally, in accord with previous research this thesis emphasises the importance of epitope accessibility on antibody capacity to bind quickly to antigen ^[482]. Furthermore, this thesis is the first study to demonstrate that antibody association constant rate could be

used to predict the capacity of HIV-1 Env-specific IgG and IgA bNAbs to capture and neutralise iVirions. This thesis suggests that antibody association constant may have greater predictive utility than antibody affinity, currently used as the primary kinetic indicator of antibody antiviral *in vivo* efficacy ^[345, 453].

The results of this thesis are also in line with previous studies which have indicated that prolonged antibody-virus interaction increases neutralisation activity due to antibody-mediated gp120 shedding of Env on viral surface and/or antibody induced conformational changes in Env molecules ^[472, 487]. However, increase virus degradation at 37°C with the time could also be a contributing factor leading to increase neutralisation potency ^[471].

In addition, this thesis proposes a third model for the mechanisms of neutralisation by antibody, which is a combination of the “occupancy model” ^[317] and the model of neutralisation based on antibody qualitative features ^[345] (i.e. affinity, specificity, etc.). This thesis suggests that antibody capacity to block infectivity is, in the first instance, determined by the rate of antibody association: influenced by epitope accessibility, and antibody isotype and structure. Once Env occupancy has reached equilibrium, neutralisation potency is further influenced by the strength (affinity) and duration (off-rate) of antibody-virus interaction; depending on the epitope specificity. This thesis also provides proof-of concept that polyclonal binding of a mixture of bNAbs with nNAbs may enhance the capacity to capture and neutralise infectious virions. In agreement, with previous research this thesis demonstrates that certain bNAbs may interact with virions in a non-Env mediated manner, by binding to the lipids or host proteins in the viral membrane ^[303, 457]. This potential reactivity against host derived membrane is further supported by recent research indicating that bNAbs development may be restricted by immune tolerance controls and the demonstration that some bNAbs share characteristics with autoantibodies reacting with components of the viral membrane, derived from the infected cell ^[303, 492, 493, 504].

In total, this thesis identified parameters that influence HIV-1 Env-specific antibody antiviral function and provides insights into the understanding of the relationship between antibody-virus interaction and the mechanisms of antibody-mediated virion capture and neutralisation.

6.3 Thesis limitations

The findings of this thesis are limited by the number of IgG, mIgA and dIgA antibodies evaluated and their specificities. Additionally, work was only performed with a single viral isolate (HIV-1_{BaL}). Therefore, the results of this study may not be applicable to all HIV-1 Env antibody specificities and HIV-1 strains. Moreover, binding kinetics studies were performed with HIV-1_{BaL} Envgp140 which although is a good soluble mimic of Env exposed on the viral surface, is not a perfect mimic of Env spike embedded in a viral membrane and containing the full cytoplasmic tail. Other limitations of this thesis are the use of molecularly constructed IgA antibodies rather than naturally occurring versions. Moreover, the IgM isotype which is also present at the mucosal surfaces was neither investigated, nor other IgG isoforms (IgG₂₋₄). Furthermore, only the ability of antibody to bind, capture and neutralise virions was evaluated, other antiviral functions which could be critical to prevent HIV-1 infection such as ADCP, ADCC, and ADCVI were not explored.

6.4 Future work and implications for vaccine research and passive immunisation studies

Further work is required to evaluate the impact of epitope specificity, accessibility and antibody isotype and structure on the control and prevention of HIV-1 infection *in vivo*, in particular through passive immunisation studies with bNAbs in animals' models. Such research will enable the field to assess the superior efficacy of rapid-binding bNAbs *in vivo*. This would confirm if the association constant rate alone or in combination with antibody affinity is an important predictor of *in vivo* efficacy. Another aspect would be to evaluate the ability of rapid-binding bNAbs to limit viral escape as this may have a critical impact in controlling HIV-1 infection. Moreover, future passive immunisation studies using combination of bNAbs compared to a single bNAb such as the on-going clinical trial led by the Nussenzweig group would determine whether mixtures of potent bNAbs provide enhanced antiviral outcomes against HIV-1 infection *in vivo*.

The work of this thesis suggests that design of immunogens that induce rapid-binding bNAbs responses could be critical to vaccine research efforts. In this regard, more research is needed to determine why some bNAbs presenting high on-rates fail to capture and neutralise iVirions. It would be interesting to determine whether this is linked to their capacity to capture non-infectious virions or to other factors. Furthermore, subsequent

research that aims to elucidate the causes of the increase in neutralisation activity, the longer the antibody-virus interaction time, might help to improve the understanding of the mechanisms of antibody-mediated neutralisation.

In further study, the use of a greater panel of IgG, mIgA and dIgA antibodies recognising the same epitopes, as well as of a broader range of viruses, would confirm whether bNAbs of the IgG isotype present greater ability than those of the IgA isotype to block HIV-1 infection. Moreover, studying naturally occurring anti-HIV-1 IgA antibodies obtained via isolation and cloning as well as other IgG subclasses (IgG₂₋₄) would be crucial to better decipher the impact of antibody isotype on antiviral function against HIV-1 infection. Further, these future studies might examine other antiviral functions than those tested in this thesis including AVCNI and ADCC. Together, this research might have important implications for the field of HIV prevention as IgA plays a pivotal role in mucosal immunity. Nevertheless it should be noted that IgG is the predominant isotype in the lower genital tract where HIV-1 is mostly transmitted, thus better understanding of the IgG role in mucosal protection is also of high importance.

Overall, the findings of this thesis provide insights for future vaccine research and passive immunisation studies against HIV infection and may have additional implications for other viral infections (e.g. Influenza, RSV and HSV).

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Publications arising from this thesis

- SG Okala, D King, and R Shattock. Impact of antibody isotype and association constant rate on antiviral functions against HIV-1. *Journal of the International AIDS Society*. Vol 19, Suppl 5. (2016). Oral presentation at AIDS conference 2016, Durban, South Africa.
- Okala SG, King DF, Shattock RJ. Comparison of HIV-1 envelope specific IgA and IgG antiviral ability to prevent HIV-1 infection: additive, inhibitory and synergistic effects, *Journal of the International AIDS Society*, Vol: 18, ISSN: 1758-2652. (2015). Oral presentation at IAS conference 2015, Vancouver, Canada.
- Okala SG, King DF, Rogers PM, Shattock RJ. antibody Isotypes Differ in their Capacity to Bind, Capture and Aggregate HIV-1 Virions. *AIDS research and human retroviruses*. 30 Suppl 1. (2014). Oral presentation at HIVR4P conference 2014, Cape Town, South Africa.