

Exploring The Potential Of Engineered Antibodies To Prevent HIV Cell-Free And Cell-Cell Transmission

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Declaration

I *Rebecca Toumi van Dorsten* declare that this Thesis is my own, unaided work. It is being submitted for the Degree of *PhD of Philosophy* at the University of the Witwatersrand, Johannesburg.

It has not been submitted before for any degree or examination at any other University.



(Signature of candidate)

25th day of May 2021, in Johannesburg.

Dedication

“True education is a kind of never ending story — a matter of continual beginnings, of habitual fresh starts, of persistent newness.”

J.R.R. Tolkien

Presentations and publications

Publications

van Dorsten, Rebecca T., Bronwen E. Lambson, Constantinos Kurt Wibmer, Marc S. Weinberg, Penny L. Moore, and Lynn Morris. 2020. Neutralization Breadth and Potency of Single-Chain Variable Fragments Derived from Broadly Neutralizing Antibodies Targeting Multiple Epitopes on the HIV-1 Envelope. *Journal of Virology* 94(2).

Presentations

Talks

- 1) Presentation at MBRT Research Day 2015, Engineering single-chain variable fragments (scFv) of broadly neutralizing antibodies to HIV. Rebecca van Dorsten, Bronwen Lambson, C. Kurt Wibmer, Mashudu Madzivhandila, Stuart Ali, Salim Abdool Karim, Harold Marcotte, Penny L. Moore, Marco Weinberg, and Lynn Morris.
- 2) Oral presentation at the Faculty of Health Sciences Research Day 2016: Potency and breadth of single-chain variable fragments (scFv) of broadly neutralizing HIV antibodies. Rebecca T van Dorsten, Constantinos Kurt Wibmer, Bronwen Lambson, Marco Weinberg, Penny L. Moore, Lynn Morris.
- 3) Oral presentation at Faculty of Health Sciences Research day 2020: Combinations of scFv made from HIV broadly neutralizing antibodies demonstrate high potency and coverage against a multiclade panel of viruses. Rebecca T van Dorsten, Penny L Moore, Lynn Morris.

Posters

- 1) Poster at Research for Prevention Conference, Chicago: Potency and breadth of single-chain variable fragments (scFv) of broadly neutralizing HIV antibodies, Rebecca T van Dorsten, Constantinos Kurt Wibmer, Bronwen Lambson, Marco Weinberg, Penny L. Moore, Lynn Morris.
- 2) Poster at antibody engineering meeting 2017 San Diego: Breadth and potency of single-chain variable fragments (scFv) of broadly neutralizing HIV antibodies (bNAbs) in free-virus and cell-cell neutralization of HIV, R.T. van Dorsten, L.M. Reh, C.K. Wibmer, B.E. Lambson, M. Weinberg, A. Trkola, P.L. Moore, L. Morris.

- 3) Poster at the Faculty of Health Sciences Research Day 2018: Combinations of single-chain variable fragments (scFv) of broadly neutralizing HIV antibodies (bNAbs) demonstrate exceptional breadth and potency. Rebecca T. van Dorsten, Bronwen E. Lambson, Constantinos K. Wibmer, Marco Weinberg, Penny L. Moore, Lynn Morris.

Abstract

Broadly neutralizing antibodies (bNAbs), isolated from some HIV-infected individuals, can prevent infection in animal models and are being evaluated for the prevention and treatment of HIV infection in humans. To further explore the potential of bNAbs, we designed and expressed single-chain variable fragments (scFv) that artificially link the variable regions of antibody heavy and light chains and which may have advantages due to their smaller size. We tested individual scFv derived from bNAbs targeting the major sites of vulnerability on the HIV-1 envelope glycoprotein, namely CAP256-VRC26.25 (V2 apex), PGT121 (N332-supersite), 3BNC117 (CD4bs), and 10E8v4 (MPER) using a pseudovirus neutralization assay. Each scFv retained good neutralizing activity against a large multi-subtype virus panel, although there was variable loss of function compared to the parental IgG antibodies. For CAP256-VRC26.25 (hereafter CAP256.25), there was a significant loss of potency (138-fold), in part related to differential interaction with charged amino acids in the V2 epitope at residues 166 and 169. There was also a reduction in potency for the 3BNC117 scFv (13-fold) among viruses lacking the N276 glycan. Similarly, a reduction in potency for the PGT121 scFv (4-fold) was found among viruses lacking an N332 glycan and in viruses with a longer V1 loop. This variation at key residues affected scFv neutralization more than the matched IgGs, suggesting that scFv interacted with their epitopes in subtly different ways. Remarkably, the scFv of 10E8v4 maintained breadth of 100% in our panel with only a minor reduction in potency. Overall, these scFv of clinically relevant bNAbs retained significant neutralizing activity, indicating that they may be suitable for passive immunization to prevent HIV-1 infection.

HIV envelope diversity represents a significant challenge to the efficacy of bNAbs and it is widely acknowledged that double or triple combinations will be needed to ensure complete coverage. To assess whether this would also apply to scFv, we tested five scFv of bNAbs in equimolar combinations against a small multiclade panel of viruses. This included the four bNAbs described above plus 8ANC195, an antibody that targets the gp120-gp41 interface. Similar to IgG, experimental combinations of two and three scFv showed a significant improvement in potency and breadth compared to single scFv. Using the Loewe additive model, combination titres were predicted using single scFv data and compared to the experimental combination data. Most combinations followed this additive model of potency and no significant antagonism was observed for any combination of two or three scFv. Low levels of synergy were observed within several combinations specifically those containing

CAP256.25 and 10E8v4 scFv. This was shown by the improved potency of the experimental titres compared to the predicted titres based on this model. These data and model were used to extrapolate and predict neutralization titres for a larger 45-virus panel. At therapeutic levels (1µg/mL), 100% coverage was reached for one of the dual and three of the triple combinations. These combinations always contained 10E8v4 scFv but never 8ANC195. Moreover, the geometric mean potency for the best triple combination consisting of CAP256.25, 10E8v4, and 3BNC117, was significantly improved to 0.047µg/mL compared to the most potent single scFv (0.12µg/mL, $p < 0.0001$). These results show that combinations of scFv generally follow an additive model of potency, with modest levels of synergy and that their breadth and potency are significantly improved when applied in combinations.

Transmission of HIV through cell-associated HIV particles may play an important role in sexual and perinatal transmission. bNAbs have been shown to have reduced activity in this mode of transmission, which could limit their ability to prevent infection. Furthermore, cell-associated virus is thought to play an important role in maintaining HIV infection by establishing and maintaining the HIV reservoir. To explore the ability of smaller molecules to prevent this mode of transmission, we tested the scFv and IgG of four bNAbs, namely CAP256.25, PGT121, 3BNC117, and 10E8v4, in a cell-free and cell-cell transmission assay. We found that the IgG lost potency against cell-associated virus compared to the neutralization of free virus, with a 9-fold geometric mean potency difference for all IgG. This potency loss was most pronounced in PGT121 IgG (19-fold) and 3BNC117 IgG (15-fold) but was also noted for CAP256.25 IgG (3.4-fold) and 10E8v4 IgG (4.9-fold). However, the scFv neutralized both cell-free and cell-associated viruses with similar potency (geometric mean potency difference between 1.3 and 2.3 fold). Thus, despite the differential potency losses between IgG and scFv for three of the four bNAbs in the free virus assay, scFv retain an advantage in blocking this mode of transmission. The net result was that 3BNC117 and PGT121 IgG and scFv neutralized cell-associated virus with similar potency, though the CAP256.25 scFv was slightly less potent than the IgG (13-fold). In contrast, 10E8v4 was slightly better at preventing cell-cell transmission as an scFv than its IgG counterpart (2-fold), possibly due to the location of its epitope close to the cell membrane. These data demonstrated that scFv may show potential for the prevention of cell-associated virus neutralization, as their small size likely allows them to better diffuse between cells as well as into the mucosal tissues where cell-cell transmission occurs.

In conclusion, the scFv of HIV-directed bNAbs retained much of their function in both cell-free neutralization and cell-cell neutralization assays. Furthermore, they showed the expected level of activity when tested in combination and in some cases displayed modest synergy. Due to their smaller size, scFv are more easily incorporated into novel expression vectors and could be particularly useful for vectored immunoprophylaxis (VIP) for the prevention of HIV infection. Taken together, the findings from this thesis support the further research and development of scFv derived from HIV bNAbs for potential use in clinical applications.

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Abbreviations

	10X	10 times
A	a.a.	Amino acid
	AAV	Adeno Associated Virus
	Abs	Antibodies
	Ad5	Adenovirus type-5
	ADCC	Antibody-Dependent Cellular Cytotoxicity
	AIDS	Acquired Immune Deficiency Syndrome
	AMP	Antibody-Mediated Protection
	ART	Anti-retroviral therapy
	ARV	Anti-retroviral
	APC	Antigen-presenting cells
	APS	Ammonium persulfate
B	BCR	B-cell receptor
	bNAbs	Broadly neutralizing antibodies
	bp	base pair
C	C1-C5	Constant regions of HIV envelope 1-5
	CA	Capsid
	CAP256	CAPRISA donor 256
	CAP256.25	CAP256-VRC26.25
	CAP256_SU	CAP256 donor, superinfecting Virus
	CAPRISA	Centre for the AIDS Program of Research in South Africa
	CCR5	Chemokine (C-C motif) receptor 5
	CD	Cluster of differentiation
	CD4bs	CD4 binding site
	CDC	Centre for Disease Control
	cDNA	Complementary DNA
	CDR	Complementary-determining regions
	CDRH	Complementarity-determining region of the heavy chain
	CDRL	Complementarity-determining region of the light chain

	CH 1-3	Constant heavy chain 1-3
	CHR	C terminal heptad repeat
	CMV	Cytomegalovirus
	CMV/R	CMV based vector
	CL	Constant light chain
	CO ₂	carbon dioxide
	CT	Cytoplasmic tail
	C-terminus	Carboxyl-terminus
	CXCL	Chemokine (C-X-C motif) ligand
	CXCR	Chemokine (C-X-C motif) receptor
D	DC	Dendritic Cells
	D-chain	Diversity chain (antibody recombination)
	DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
	DDT	dithiothreitol
	DEAE-dextran	Diethylaminoethyl dextran
	dh ₂ O	distilled H ₂ O, (water)
	DMEM	Dulbecco's Modified Eagle Medium
	DNA	Deoxyribonucleic acid
	D-Loop	loop in HIV envelope
E	EDTA	Ethylenediaminetetraacetic acid
	ELISA	Enzyme-linked immunosorbent assay
	Env	Envelope
	ER	Endoplasmic reticulum
	EtoH	Ethanol
	Ext Coeff.	Extinction Coefficient
F	Fab	Fragment of antigen-binding
	FBS/ FCS	Fetal bovine serum/ Fetal Calf Serum
	Fc	Fragment crystallisable
	FP	Fusion peptide
G	G ₂	Phase 2 of the cell cycle

	Gag	Group-specific antigen
	GDP	Gross Domestic Product
	Geomean	Geometric mean
	gp120	Glycoprotein 120 kDa
	gp160	Glycoprotein 160 kDa
	gp41	Glycoprotein 41 kDa
	Group M	HIV-1 major group
	Group N	HIV-1 non-M, non-O group
	Group O	HIV-1 outlier group
	Group P	HIV-1 group pending
H	HCL	Hydrochloric acid
	His	Histidine
	HEK	Human embryonic kidney cells
	HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
	HIV-1/2	Human Immunodeficiency Virus type-1/2
	HR	Heptad repeat
	HRV-3C	Human Rhino Virus 3C protease site
	HTLV	Human T- cell leukaemia virus
	HVTN	HIV Vaccine Trials Network
I	IC ₅₀	50% inhibitory concentration
	ICAM-1	Intercellular Adhesion Molecule 1
	IFN	Interferon
	Ig	Immunoglobulin
	IGHG	Immunoglobulin heavy chain gene
	IL	Interleukin
	iMab	ibalizumab
	IN	Integrase
J	J chain	Joining chain
L	L1-3	Linker 1-3
	LAV	Lymphadenopathy associated virus
	LANL	Los Alamos National Lab

	LB	Lysogeny broth
	LC	Light chain
	LFA-1	Lymphocyte function-associated antigen
	LTR	Long terminal repeat
M	MA	Matrix
	mAb	Monoclonal antibody
	MHC	Major Histocompatibility Complex
	mRNA	messenger RNA
	MOPS	3-(N-morpholino)propanesulfonic acid)
	MPER	Membrane-proximal external region
	MSM	Men who have sex with men
	MTCT	Mother to Child Transmission
N	Nabs	Neutralizing Antibodies
	NaCl	Sodium Chloride
	NaOAc	Sodium Acetate
	NC	Nucleocapsid
	Nef	Negative factor
	NFL	Native Flexibly Linked
	NHP	Non-human primate
	NHR	N terminal heptad repeat
	ni-NTA	Nickel nitrilotriacetic acid resin
	NIH	National Institute of Health
	NIAID	National Institute of Allergy and Infectious Diseases
	NK	Natural Killer cell
	NL4-3inGLuc	NL4-3 based backbone with a Gaussia Luciferase reversed reporter
	NNRTIs	nonnucleoside reverse transcriptase inhibitors
	NRTIs	Nucleoside/Nucleotide reverse transcriptase inhibitors
	nt	Nucleotide
	N-terminus	Amino-terminus
O	ON	Over Night

	OptiMEM	Reduced Optimal Minimal Essential Medium
P	p6	Protein 6
	PBMCs	Peripheral blood mononuclear cells
	PBS	Phosphate-buffered saline
	pcDNA	Vector for expression
	PCP	Pneumocystis carinii pneumonia
	PCR	Polymerase chain reaction
	PDB	Protein Data Bank
	PEI	Polyethylenimine
	PI virus	Primary infecting virus from donor CAP256
	Pol	Polymerase
	PR	Protease
	PrEP	Pre-exposure prophylaxis
	Rev	Regulator of expression of viral proteins
R	R5 viruses	CCR5 tropic viruses
	RNA	Ribonucleic acid
	RNase H	Ribonuclease H
	RT	Reverse transcriptase
	at RT	at Room temperature
S	scFv	Single-chain variable fragment
	SDS-page	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
	SHM	Somatic Hypermutation
	SHIV	SIV and HIV chimaera
	SIV	Simian Immunodeficiency Virus
	SIVcpz	Simian Immunodeficiency Virus chimpanzees
	SP1/2	Spacer Peptide 1/2
	SOC	Super Optimal broth with Carbolyte repression
	SU virus	Super infecting virus from donor CAP256
T	T/F	Transmitted/founder virus
	TAE	Tris-acetate-EDTA

	Tat	Trans-activator
	TdT	Terminal deoxynucleotidyl transferase
	TEMED	N,N,N',N'-tetramethyl ethylene-diamine
	Tris	tris(hydroxymethyl)aminomethane
	Tris-Cl	Tris(hydroxymethyl)aminomethane-chloride
	TMD	transmembrane domain
	TNT	open-ended nanotubes
	TZM-BL	cell line
U	UNAIDS	United Nations program on HIV/AIDS
	USA	United States of America
V	V1-5	Variable loops 1-5
	VDJ	(V) variable (D) diversity (J) joining genes
	V-gene	Variable gene (antibody recombinations)
	VH	Variable heavy chain
	VHH	Single Domain Variable heavy chain / variable heavy chain of a heavy chain only antibody
	Vif	Viral infectivity factor
	VL	Variable light chain
	Vpr	Viral protein R
	Vpu	Viral protein unique
W	wk	Week
	WHO	World Health Organization

Prefixes

k	Kilo	10^3
c	Centi	10^{-2}
m	Milli	10^{-3}
μ	Micro	10^{-6}
n	Nano	10^{-9}
p	Pico	10^{-12}

SI Units

°C	Degree Celsius
gr	grams(s)
Kg	Kilograms
m	Meter
mol	Mole
s	Second (sec)
m ²	Square meter

Non SI units

Å	Angstrom
bp	Base pair
Da	Dalton
g	Standard gravity
L	Litre
M	Molar (mol)
min	Minute
pH	Cologarithm of hydrogen
RLU	Relative light units
rpm	Revolutions per minute
U	International unit
V	Volt

Symbols

3', 5'	Three prime, five prime
α	Alpha
~	Approximately
β	Beta
Δ, δ	Delta
ϵ	Epsilon
γ	Gamma
κ	Kappa
λ	Lambda
μ	Mu

Amino Acids

A	Ala	Alanine
R	Arg	Arginine
N	Asn	Asparagine
D	Asp	Aspartate
C	Cys	Cysteine
E	Glu	Glutamate
Q	Gln	Glutamine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
L	Leu	Leucine

K	Lys	Lysine
M	Met	Methionine
F	Phe	Phenylalanine
P	Pro	Proline
S	Ser	Serine
T	Thr	Threonine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
V	Val	Valine

Nucleotides

A	Adenine	H	A, C, or T
C	Cytosine	R	A or G
G	Guanine	S	G or C
T	Thymine	W	A or T
U	Uracil	Y	C or T

1 Introduction

1.1 The HIV epidemic

1.1.1 *Status of the epidemic*

Vaccines have successfully eradicated smallpox, and have reduced the impact of many childhood diseases. However, the global emergence of HIV, new strains of influenza, widespread antibiotic resistance, Ebola epidemics, and increasing resistance to antimalarials has demonstrated that infectious diseases are in a constant state of evolution. HIV has been a particular challenge as it has been difficult to halt the spread of this pandemic. Currently, about 36.9 million people are living with HIV, of whom 1.8 million are children <14 years with about 1.8 million people becoming newly infected each year (United Nations, 2019). A total of 77.3 million people have been infected since the start of the epidemic. HIV has spread around the world and caused a significant loss of quality and quantity of life. This resulted in a decreased life expectancy in sub-Saharan Africa from 65.9 years in 1990 to 52.6 years in 2004 and which recently recovered to 63.8 years in 2019 (United Nations, 2019). The infection has hit hardest among the world's poorest populations in sub-Saharan Africa, with 27 million infected people currently living in this region (Dwyer-Lindgren *et al.*, 2019). Moreover, HIV/AIDS had much larger effects on a socioeconomic scale than was first assumed, causing loss of income, continued stigmatization, and loss of quality of life with a 2-4% decrease in GDP across sub-Saharan Africa (Dixon, 2002). Women have been particularly affected by the HIV epidemic and account for 54% of the infected people in this region. In particular, young women (15-24 years) are at high risk with over 75% of new infections in the sub-Saharan region. Abuse and violence correlate with a 50% higher risk of HIV infection with over 35% of women experiencing physical or sexual abuse in their lives (Jewkes *et al.* 2010; World Health Organization 2019).

South Africa has the biggest per capita epidemic in the world with 7.1 million people living with HIV and a prevalence of 18.9% in 2017 (Statistics South Africa, 2018). The HIV epidemic caused a significant drop in life expectancy in South Africa from 62.2 in 1992 to 52 in 2002. The epicentre of this epidemic lies in KwaZulu-Natal province. The prevalence in this area is exceptionally high among women (44.1%), with a prevalence of 22% in young women (15-24 years) and peaking at 66.4% among women aged 35-39 years (Kharsany *et al.*, 2018). This

group is particularly vulnerable due to age-disparate sexual partners (Kharsany *et al.*, 2018; Maughan-Brown *et al.*, 2018; Tanser *et al.*, 2018). Moreover, a high incidence of vaginal bacteriosis and inflammation increases the risk of HIV transmission (Barnabas *et al.*, 2018; Maughan-Brown *et al.*, 2018). These statistics highlight the fact that despite the significant progress made in education, and access to treatment and preventative measures, there remains an urgent need for more effective interventions such as a vaccine.

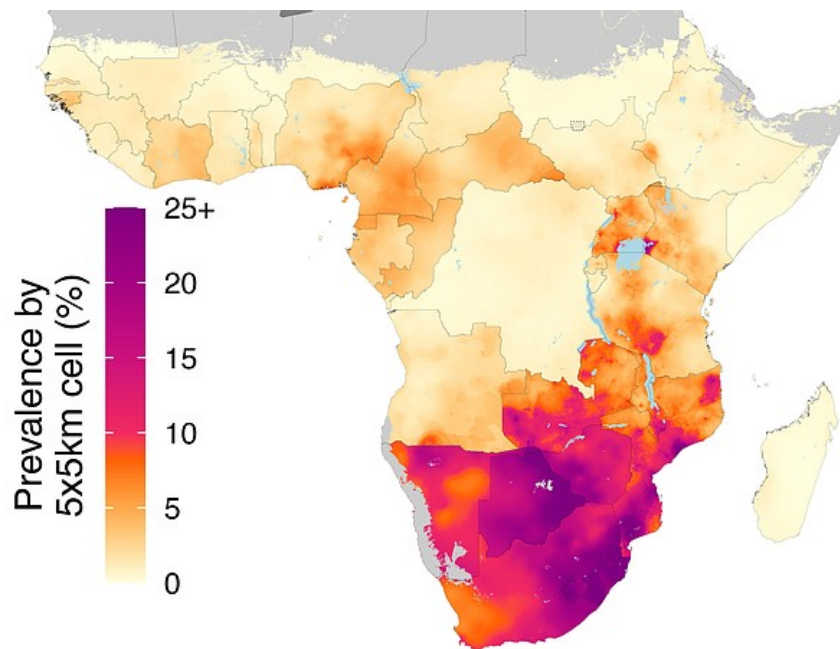


Figure 1.1 HIV prevalence in sub-Saharan Africa.

The map shows the prevalence of HIV in sub-Saharan Africa in a 5x5km cell percentage with the darker colors indicating a higher prevalence. The highest prevalence is in countries in southern Africa with hotspots of >25% in Botswana, Mozambique and KwaZulu-Natal, South Africa (Dwyer-Lindgren *et al.* 2019).

1.1.2 History and origins of HIV

HIV was first recognized in the 1980s but there is evidence that it entered the human population much earlier (Worobey *et al.*, 2008; Faria *et al.*, 2014). The epidemic in the USA likely started in the mid to late 1970s with the first cases being documented in 1981 when 5 young homosexual men presented with a rare lung infection called *Pneumocystis carinii pneumonia* (PCP) and multiple reports were made of cases of Kaposi Sarcoma, an unusually aggressive cancer that is rarely observed in immune-competent individuals (Masur *et al.*, 2010). Similar cases of PCP were documented in intravenous drug users. In the same year, 270 cases of severe immune deficiency were reported in the USA with 121 men dying (Centers for Disease Control (CDC), 1981; Hymes *et al.*, 1981; Masur *et al.*, 2010). The virus causing

Acquired Immune Deficiency Syndrome (AIDS) was identified independently by research groups in the USA and France in 1984 (and was called HTLV-III, LAV, and ARV) and was later renamed Human Immunodeficiency Virus (HIV) (Popovic *et al.*, 1984; Ratner *et al.*, 1985; Barré-Sinoussi *et al.*, 2004).

HIV first arose in humans after a zoonotic event, which allowed the retrovirus to cross species (Sharp *et al.*, 2011; Faria *et al.*, 2014). A simian strain called SIVcpz (Simian Immunodeficiency Virus) found in chimpanzees is closely related to HIV-1 and can infect both chimpanzees and humans. It is believed that this virus adapted to enable human transmission on several occasions creating the different groups M and N (Bailes *et al.*, 2003). HIV-1 Groups O and P are thought to have crossed species from gorillas (Van Heuverswyn *et al.*, 2006). Group O is mainly endemic in Cameroon (1-2% prevalence) and neighbouring countries, and groups N and P are rare outside this region. The majority of infections worldwide are caused by group M, which is further classified into subtypes A, B, C, D, F, G, H, J, and K and recombinant versions of these subtypes (Hemelaar *et al.*, 2006; LANL, 2015). The geographical distribution of these subtypes is subject to a founder effect making certain subtypes dominant in different parts of the world. Thus, subtype B dominates in Western Europe, the USA, and Australia, whereas subtype A occurs mainly in Eastern Europe, Central Asia, and East Africa. Recombinant viruses account for an increasing number of infections (18%) and CRF01-AE is dominant in South East Asia. Subtype C is most dominant in southern Africa and India and accounts for over 50% of all infections worldwide (Hemelaar *et al.*, 2006; Bbosa *et al.*, 2019). HIV diversity is highest in west and central Africa, a reflection of the origins of the virus in this area (Hemelaar *et al.*, 2006; Buonaguro *et al.*, 2007). Moreover, samples from 1959 and 1960 from Kinshasa, Congo show great diversity indicating that HIV-1 M spread and diversified in the Congo area from the beginning of the 20th century. The second major strain of HIV, HIV-2 accounts for far fewer infections in humans, and mainly occurs in West Africa (Mali, Guinea-Bissau, Gambia, Senegal, Nigeria, and Sierra Leone) (van Tienen *et al.*, 2018). The cross-species transmission of HIV-2 likely occurred from a separate zoonotic event from a Sooty Mangabey. HIV-2 is less transmissible, and as it takes much longer for infected individuals to progress to AIDS, it is a lesser contributor to the global epidemic (Bailes *et al.*, 2003; Sharp *et al.*, 2011).

1.1.3 Natural History of HIV infection and disease pathogenesis

Those at higher risk of HIV infection include sex workers, men who have sex with men (MSM), intravenous drug users, prisoners, and transgendered people. The distribution of risk groups

varies per geographical region. HIV infection occurs where there is a breach of the epithelial or mucosal barrier, as happens with unclean needles (risk of 0.63% per event) or in MSM due to the fragile lining of the rectum (1.4%). As mentioned previously women in heterosexual relationships in sub-Saharan Africa form another major risk category, although the risk per individual sexual encounter is relatively low (0.08%). Infants and newborns of mothers who have untreated HIV are also at high risk (23%), however, targeted interventions to prevent Mother to Child Transmission (MTCT) have significantly reduced infection in this group. Similarly, due to the screening of blood products, although the infection risk after receiving contaminated blood products is 93%, the number of people infected through transfusion has dropped to near zero (Patel *et al.*, 2014; Ghosn *et al.*, 2018).

After HIV infects a person there is an initial eclipse phase where the virus spreads through the lymph nodes and a viral reservoir is established. During this phase, HIV also establishes itself in the gut lymphocytes causing progressive but massive depletion of the lamina propria CD4+ T-cells (Robb and Ananworanich, 2016; Cicala *et al.*, 2019). CD4+ T-cells are the main target cell for HIV, however other cells are also infected contributing to the reservoir (Robb and Ananworanich, 2016; Zerbato *et al.*, 2016; Clayton *et al.*, 2017). During the acute phase, there are “flu-like” symptoms of infection for about 2 weeks (Robb, Eller, *et al.*, 2016). During this phase, HIV has already spread through the body, and the viral load increases rapidly to its peak up to 10^6 - 10^7 copies per ml of blood. This causes high levels of inflammation and widespread immune activation (Chang *et al.*, 2010). Infected individuals rapidly develop adaptive immune responses and through an HIV-specific CD8 cytotoxic lymphocytic response and the interplay between host and virus, a viral set point is reached about 4-5 weeks after peak viremia (Robb, Eller, *et al.*, 2016). This viral set point and the immune-specific response are indicators for disease progression with lower setpoints resulting in slower progression to AIDS (McPhee *et al.*, 2019). The effects of HIV on the immune system are complex causing the death of CD4+ T-cells through direct cytopathic and indirect mechanisms (Doitsh *et al.*, 2016). Thymic infection leads to accelerated loss of function impairing the regeneration of T-cells. In the secondary lymph nodes, a rapid decline of CD4+ T-cells is observed, causing immune deficiency (Okoye *et al.*, 2013). This increased inflammatory state promotes damage to the lymphoid system by lymphatic fibrosis and blunting of T-cell function. Moreover, there is a sustained dysfunctional activation of the immune system in HIV infection, such as

hypergammaglobulinemia. This contributes to the later immune deficiency causing the progression to AIDS (Chang *et al.*, 2010; Pace *et al.*, 2011; Seitz, 2016; Ghosn *et al.*, 2018).

The HIV reservoir is established early in infection. Macaque models of Simian Immune Virus (SIV) infection demonstrate the establishment of an HIV reservoir as early as 3 days post-infection (Nixon *et al.*, 2017). Long-lived naïve CD4 cells of monocyte and macrophage lineages and memory CD4+ T-cells, which have self-renewal capacity, are maintained long-term supplying this latent reservoir (Nish *et al.*, 2017). Anti Retroviral Therapy (ART) is unable to eliminate these cells and can only prevent new infections. Moreover, treatment interruption results in the re-emergence of the virus and active replication, necessitating lifelong treatment of those infected. It is estimated that for the reservoir to decay naturally, it would take multiple decades even with complete viral suppression (Pace *et al.*, 2011; Chavez *et al.*, 2015; Churchill *et al.*, 2016).

The time frame for progression to AIDS (3-10 years) depends on several factors including age at the time of infection, viral set point, host immune factors, and viral fitness factors (Robb and Ananworanich, 2016). Elite controllers, for example, exhibit a combination of host/immune factors and, in some cases, less fit viruses allow them to control viral replication even in the absence of ART (Okulicz *et al.*, 2011; Ackerman *et al.*, 2016). The infecting virus also plays an integral role in the immune system's ability to control the virus as superinfection can contribute to the loss of viral control in Elite controllers (Caetano *et al.*, 2019). AIDS is characterized by <350 CD4+ T-cells per μL of blood, which is correlated with an increased risk for opportunistic infections such as varicella-zoster virus, bacterial infections, and tuberculosis. In advanced disease, defined as a CD+ T-cell count of <200 cells/ μL and high viral loads, severe immune deficiency increases the risk of opportunistic infections and invasive cancer. Over the course of the infection, HIV causes irreversible damage to many organs due to the high inflammatory state, causing cardiovascular disease, hepato-renal dysfunction, neurological dysfunction, and other comorbidities (Schacker *et al.*, 2002; Deeks *et al.*, 2013). If left untreated, AIDS usually causes mortality in under 3 years (Zwahlen *et al.*, 2006). Babies and infants have an especially high mortality rate and short life expectancy if HIV is left untreated (Goetghebuer *et al.*, 2009; Desmonde *et al.*, 2011). Even under successful ART suppression, HIV patients remain at high risk for co-morbidities as both the virus and the treatment have damaging effects (Ghosn *et al.*, 2018). Early initiation of ART can reverse and limit the immunological failure but not the damage already caused by the virus (Hunt, 2017;

Deleage *et al.*, 2019). Moreover, late initiation of ART allows the virus to cause irreparable damage to the immune system resulting in immunological failure and non-recovery even though viral suppression is achieved (J.C. *et al.*, 2012; Hileman *et al.*, 2017). The current guideline for new HIV diagnosis is to immediately start lifelong ART treatment. This is thought to limit the damaging effects of HIV -reducing the morbidity and mortality-, but it is also expected to reduce transmission (Ferry *et al.*, 2009; INSIGHT START Study Group *et al.*, 2015; TEMPRANO ANRS 12136 Study Group, 2015). However, it is estimated that in South Africa about 25% of people who initiate ART, default, giving rise to resistant HIV strains, and increased risk for comorbidities (Miller *et al.*, 2010).

1.2 Structure and genome of HIV

1.2.1 HIV genome

HIV is a retrovirus, which are RNA viruses that transcribe their genetic material into DNA and then integrate it into the host genome (Seitz, 2016). A hallmark of retroviruses is that they have reverse transcriptase and integrase enzymes, which allow for the transcription from RNA to DNA for genetic integration. Retroviruses have a membrane enveloping a capsid in which the genetic material encoding *gag*, *pol*, and *env* genes are carried. HIV is also categorized as a lentivirus which is a subtype of retrovirus that possesses regulatory *tat* and *rev* genes and accessory genes such as *vpu*, *vif*, *nef*, and *vpr* to enable infection of non-dividing cells.

The HIV virion is about 100nm in diameter and consists of a viral Envelope that is a host-derived lipid membrane, which is studded with HIV-derived trimeric proteins (Figure 1.2). The matrix protein is closely associated with the HIV membrane. The cone-shaped Capsid core, contains the viral enzymes Integrase, Reverse Transcriptase, Protease, and the Tat protein. The Capsid also contains the Nucleocapsid proteins, which has two Zinc fingers that associate with the two strands of viral RNA. HIV-1 has a 9.2kb unspliced genomic transcript and is effective in its genome usage as it employs three reading frames with some genes overlapping. Moreover, through differential RNA splicing, it is able to express nine genes through three mRNA transcripts.

The major genes, *gag*, *pol*, and *env* encode for the main structural proteins of HIV, namely the viral enzymes (Protease, Reverse Transcriptase, and Integrase) and the Envelope proteins gp120 and gp41. The other genes encode for regulatory proteins Tat, Rev, and the accessory

proteins Vif, Vpu Vpr, and Nef. The accessory proteins regulate the host cell environment to ensure replication and transmission and to restrict cellular antiviral activity.

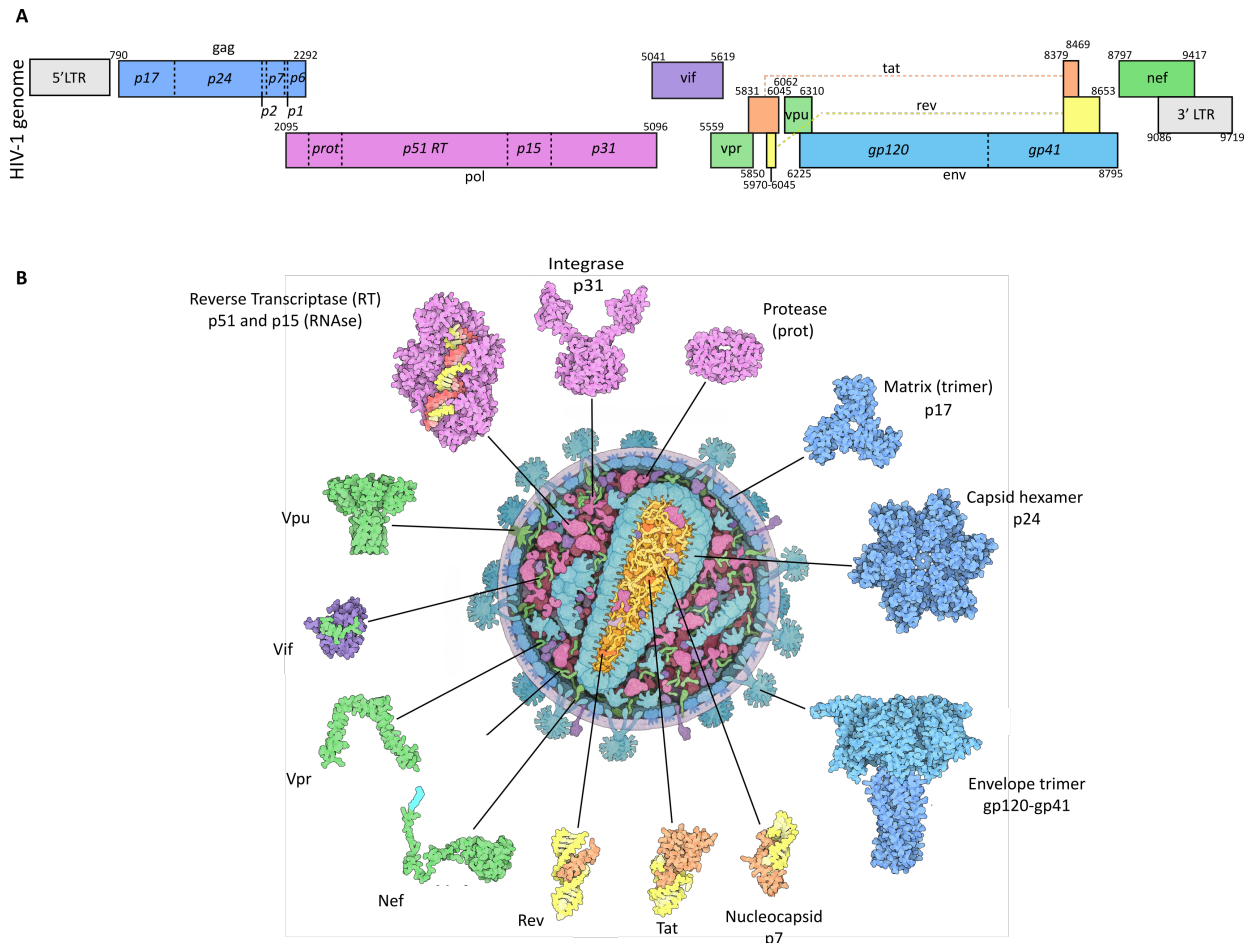


Figure 1.2 The HIV genome and proteome.

(A) A schematic representing the HIV-1 genome consisting of 3 overlapping transcripts. The Long Terminal Repeats on the 5' and 3' end mediate insertion of the HIV genome into the host chromosome. The LTR on the 5' also serves as a HIV promoter and the LTR on the 3' as a polyadenylation signal. The gag gene in blue encodes for the Matrix (p17), the Capsid (p24) and the Nucleocapsid (p7) and the P6 protein. The pol gene in lilac codes for Protease (prot), Reverse Transcriptase (p51), RNase (P15) and Integrase (p31). The Envelope gene (in blue) encodes for gp120 and gp41, which form the Envelope trimer. Accessory genes are represented in green (Vpu, Vpr and Nef) and purple (Vif). In orange and yellow are the two regulatory genes tat and rev which are encoded for by spliced introns, with regulatory components in the exon. The nucleotide identifiers are indicated in small numbers at the edges of each gene. **(B)** The structure of an HIV virion is represented. The HIV virion consists of a bi-lipid membrane with Envelope trimers expressed on its surface. A Matrix protein is closely associated with this membrane and Envelope protein. This encases a capsid, which contains the nucleocapsid and the HIV RNA. Structural proteins are colored in blue, whereas the HIV enzymes are colored lilac, the accessory proteins green and dark purple and regulatory proteins are colored yellow-orange. The figure is adapted from HIV LANL and the HIV virion structure on PDB101.

The *gag* gene encodes the precursor Gag protein (p55, 55kDa) which during further processing through viral protease is cleaved into Matrix (MA) protein (p17), Capsid (CA) protein (p24), Nucleocapsid (NC) protein (p7), and p6 protein. There are also 2 spacer peptides (p1 and p2) which are required for ordered assembly of the viral particles and are part of cleavage sites of Gag (Kräusslich *et al.*, 1995; Hill *et al.*, 2002). Gag is synthesized in the cytoplasm from viral genomic RNA and targeted to the plasma membrane by a motif in the Matrix domain but the exact mechanism is unclear. It assists in the assembly process of HIV by recruiting so-called lipid rafts, which assist in the viral assembly and incorporation of the Envelope proteins into the HIV membrane. These lipid rafts also play a role in the cell-cell transmission of HIV and are important in the formation of virological synapses.

The *pol* gene encodes for the viral enzymes Reverse Transcriptase, Protease, RNase H, and Integrase. Reverse transcriptase reverse transcribes the viral RNA into DNA and is inhibited by two HIV drug classes, Nucleoside/nucleotide reverse-transcriptase inhibitors (NRTIs/ NtRTIs) and Non-nucleoside reverse-transcriptase inhibitors (NNRTI). Protease processes the uncleaved HIV proteins and is essential for the functionality of the Gag precursor protein. Integrase integrates the viral DNA into the host genome and RNase H hydrolyses the RNA strand of the RNA/DNA duplex after DNA synthesis so a second strand of DNA can be generated. Integrase can be inhibited by an ART drug class names integrase inhibitors. Pol forms part of Gag-Pol (160kDa) and is transcribed as a polyprotein. It is transcribed in much lower quantities than Gag as a frameshift is needed for expression (GagPol is expressed at a 5% level of Gag). High levels of Gag are needed for the packaging of viral proteins and RNA.

The HIV genome also encodes for two regulatory proteins, Tat and Rev. Tat regulates the reverse transcription of HIV whereas Rev is needed for the proper translation of HIV proteins. HIV also codes for the accessory proteins Nef, Vpr, Vif, and Vpu, which increase the virulence of HIV and have many additional functions. Vpr aides in the nuclear translocation of the viral DNA, and ensures that the host cell is in the G2 phase of cell division in order to integrate the viral DNA. Vpu binds CD4 and causes downregulation through ubiquitin-mediated proteasome degradation. Nef and Vif play various roles in infectivity (Vogt, 1997; Turner *et al.*, 1999; Watts *et al.*, 2009; Los Alamos National Laboratory, 2017).

The *env* gene encodes for the Envelope protein, gp160, which is further cleaved into gp120 and gp41. Gp120 and gp41 combine to form the Envelope hetero-trimers. The Envelope trimer

enables HIV to bind CD4+ cells and fuse with the target cell's membrane (Watts *et al.*, 2009; Seitz, 2016). The Envelope trimer is the only target for neutralizing antibodies, a major focus of vaccine design, and its functions are described in more detail below.

1.2.2 HIV assembly and release

The viral RNA after transcription is transported to the cytoplasm, where the spliced RNA is translated. Two strands of single-stranded unspliced dimerized RNA are packaged into each viral particle through association with the Nucleocapsid chaperone. Through multimerization of the Capsid protein, Gag and Gag-Pol are assembled into the HIV membrane. The hexa/pentameric Capsid and the trimeric Matrix together form a protein lattice which after budding is cleaved by Protease (Borman, 2009; Pornillos *et al.*, 2009; Chimukangara *et al.*, 2019). This allows HIV to rearrange into the mature infective HIV particle characterized by the cone-shaped Capsid lattice (Checkley *et al.*, 2011; Lingappa *et al.*, 2014). One of the HIV drug classes targets protease and therefore inhibits this final maturation step.

1.2.3 HIV attachment and entry

Viruses that initiate or establish infections are named transmitted/founder viruses (Joseph *et al.*, 2015; Wagh, Kreider, *et al.*, 2018). Due to compartmentalization in the donor and due to the mucosal barriers there is the formation and the transmission of a subsection of the clonal virus population (Boeras *et al.*, 2011; Tincati *et al.*, 2016; Mabvakure *et al.*, 2019). Transmitted founder viruses are characterized by being CCR5 tropic, and containing a high amount of Env trimer in their envelopes. Moreover, their binding to Dendritic Cells (DC) is also enhanced compared to circulating viruses (Ashokkumar *et al.*, 2018). There seems to be a bias towards viruses with higher replication rates, and there is evidence that if less fit escape mutants are transmitted there is a quick return to the consensus sequence for those sites (Joseph *et al.*, 2015; Ashokkumar *et al.*, 2018).

HIV-1 targets CD4 positive immune cells, which also express the viral co-receptors, normally CXCR4 or CCR5 depending on HIV's tropism. When HIV encounters a host cell, the initial attachment can be non-specific through charge-driven interactions between Env and negatively charged heparin sulfate proteoglycans (Wilén *et al.*, 2012). The HIV particle uses host cellular transport pathways to move to a suitable fusion location. The interaction with the $\alpha 4\beta 7$ integrin or receptors such as the DC-SIGN on dendritic cells is more specific (Clapham *et al.*, 2001; Dale *et al.*, 2013; Izquierdo-Useros *et al.*, 2014; Chen, 2019; Cicala *et al.*, 2019).

These interactions bring the Envelope trimer into close proximity to CD4, and the co-receptors CXCR4 or CCR5. CD4 contact causes polarization of the trimer on the Envelope of the HIV particle, increasing the concentration of trimer at the cell contact site and improving infection (Hernandez *et al.*, 1996; Cohen *et al.*, 2004; Chien *et al.*, 2007, 2009). The Envelope glycoprotein trimer then binds to CD4, via its CD4 binding site. After binding, there is a rearrangement of the trimer, notably the V1/V2 variable loop and subsequently the V3 (Cardaci *et al.*, 2013). Moreover, a bridging sheet from two double-stranded β sheets is formed. The co-receptor binding of HIV is partly mediated by the V3-loop of the trimer, and this triggers the fusion cascade of the HIV particle with the host cell membrane. Most HIV-1 virus particles make use of the CCR5 co-receptor (R5 viruses), especially in the case of subtype C viruses. CXCR4 tropic HIV-X4 usually emerges later in infections and is often associated with a sharp decline in CD4+ T-cells (Ribeiro *et al.*, 2006; Goetz *et al.*, 2009).

The Envelope trimer mediates fusion through the exposure of the hydrophobic core of gp41 after the co-receptor binding. The gp120 units fold open and the gp41 fusion peptide (FP) is inserted into the host cell membrane (Figure 1.3 left). The gp41 protein folds into a hairpin structure formed of two heptad repeats in the gp41 protein, an N terminal (NHR) and a C terminal heptad repeat (CHR) forming a six-helix bundle. This brings the transmembrane domain (TMD) in the HIV membrane together with the fusion peptide (FP) in the host cell membrane (see Figure 1.3) (Chen, 2019).

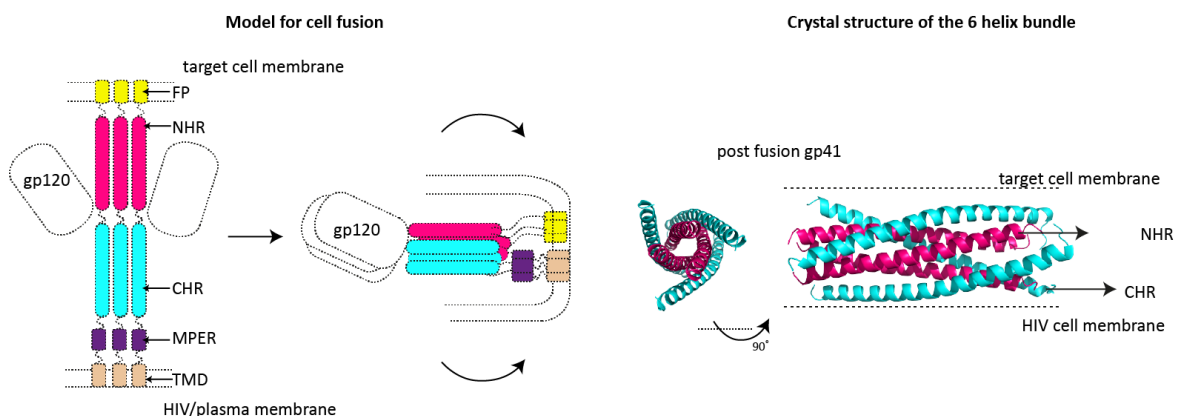


Figure 1.3 Cell fusion of HIV with the target cell.

A model for cell fusion by the gp41 protein. The fusion peptide (FP-yellow) inserts into the target cell membrane. The C-terminus Heptad Repeat (CHR-blue) fold over at a hinge and forms a 6-helix bundle with the N Terminal Heptad repeat (NHR-pink). The MPER is shown in purple and the transmembrane domain (TMD) in beige. On the right, a crystal structure of the 6-helix bundle model is shown in two orientations. Colors are as before (PDB: 27XR crystal structure was used to model the CHR and NHR in pymol).

1.2.4 HIV integration

After entry into the cytosol, the viral RNA is transcribed to double-stranded DNA and the RNA is digested by an RNase domain on the RT. Reverse transcriptase is a highly error-prone enzyme allowing for nucleotide changes, which contributes to extensive HIV diversification. Nucleoside/Nucleotide reverse transcriptase inhibitors (NRTIs) are analogues of nucleotides, which lead to the termination of DNA synthesis. While they block viral DNA synthesis, they also block some host cell DNA synthesis explaining their toxicity. NNRTIs or nonnucleoside reverse transcriptase inhibitors also inhibit reverse transcriptase by binding directly to the Reverse Transcriptase enzyme. These drugs are generally better tolerated than NRTIs but can display neurotoxicity or hepatotoxicity (Arts *et al.*, 2012; Maartens *et al.*, 2014; WHO, 2019).

After transcription, the DNA is trafficked to the nucleus of the cell as part of the preintegration complex. Integrase catalyses the integration of the viral DNA into the host cell's genome. Integrase inhibitors have been approved for HIV-1 treatment since 2007 and generally cause fewer side effects compared to older drugs. These drugs prevent the integration of HIV into the genome (Ali *et al.*, 2009; Ghosn *et al.*, 2018; WHO, 2019).

1.3 Cell-cell transmission

Cell-free transmission, the infection of target cells by free virus particles, is a relatively inefficient mode of transmission for viruses. Cell-cell transmission is about 100-1000 times more efficient *in vitro*, by concentrating viral particles at the transmission site (Del Portillo *et al.*, 2011; Kolodkin-Gal *et al.*, 2013; Anderson, 2014; Costiniuk *et al.*, 2014). Although its role *in vivo* is not entirely clear, cell-cell transmission may allow HIV to circumvent the antiviral immune system by hiding in non-lysosomal compartments or transmitting through virological and immunological synapses where access is limited (see below) (Carr *et al.*, 1999; Chen *et al.*, 2007). HIV exploits existing cell contacts such as the immunological synapse and by inducing cell contact with CD4+ cells through various mechanisms including the virological synapse.

1.3.1 The immunological synapse and the virological synapse

Cell-cell transmission can occur in two major ways. The first occurs as HIV hijacks existing pathways in the immune system. Dendritic cells (DCs) are antigen-presenting cells (APC) that are found in mucosal surfaces. Although DCs are not easily infected, HIV is able to use their antigen-capture receptors to facilitate transport to the lymph nodes. A DC can bind many virions through C-type lectins such as DC-SIGN or SIGLEC-1 and endocytose HIV particles into

non-lysosomal compartments. HIV increases the number of filopodia on the dendritic cell surface, which in turn increases binding to T-cells and increases the formation of the naturally occurring immunological synapse between the T-cell and the DC (Aggarwal *et al.*, 2012). HIV can use the immunological synapse and transform it into an infectious synapse and infect the T-cell. Similar molecules are recruited to the synapse as in the immunological synapse but in addition, HIV bound to C-type Lectins are also recruited to the site. The DC cell forms an enclosed space through its membrane protrusions and by binding between the T-cell receptor and the MHC peptide on the surface of the APC. This interaction is further stabilized through microtubules and adhesion molecules such as LFA-1 and ICAM-1 (Sanders *et al.*, 2002; Rodriguez-Plata *et al.*, 2013). The T-cell sends out sensory filopodia, which are CD4 enriched, into the DC compartment that contains the virions. The DC releases signal molecules through the synapse resulting in the recruitment of CD4, CCR5, and CXCR4 to these filopodia in the target cell. This and a high concentration of HIV particles significantly increases infection rates (Akiyama *et al.*, 2015). There is evidence that HIV induces the formation of the infectious synapse but it is unclear whether the formation of this synapse is Env induced as synapse formation as well as cell-cell transmission occurs even when Env is suppressed (Gousset *et al.*, 2008).

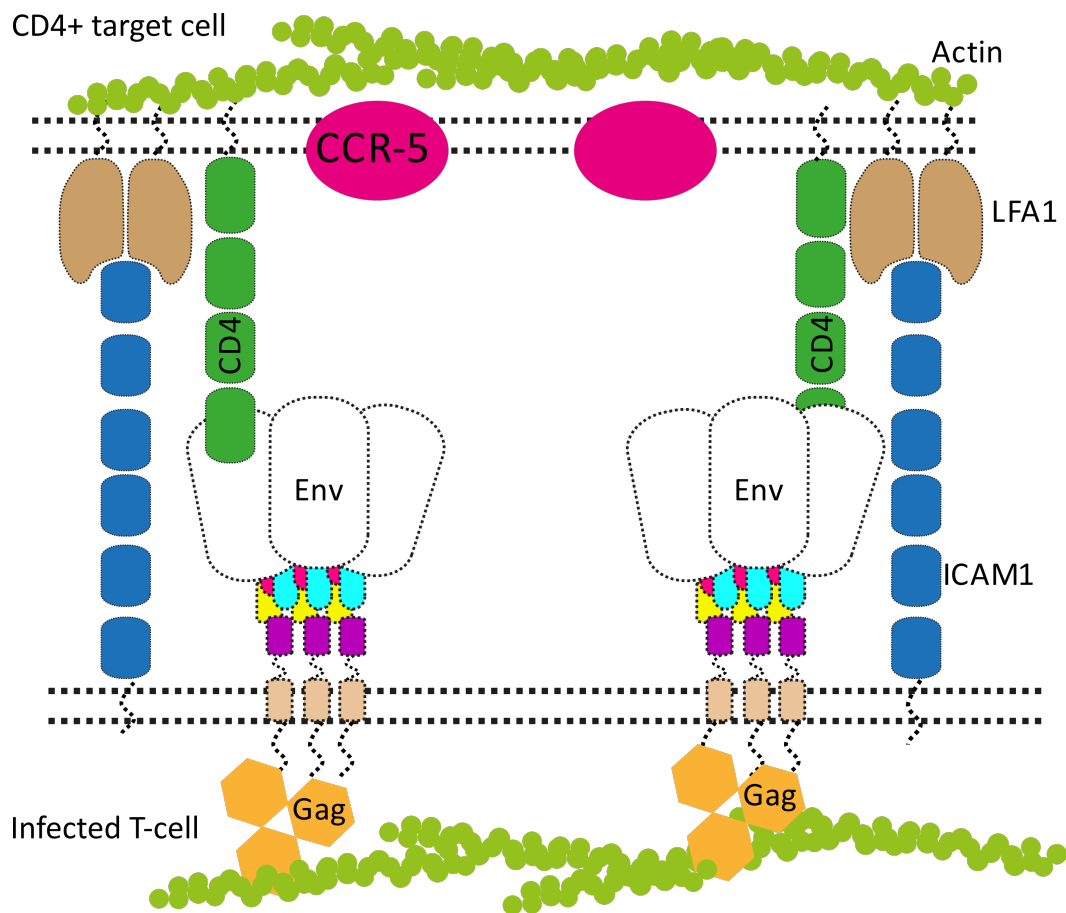


Figure 1.4. The virological synapse.

A model for cell-to-cell transmission is shown where Env engages CD4 on target cells. Due to the recruitment of ICAM1 (blue)-LFA1 (brown) stabilization, Actin (light green) remodeling and Gag (orange hexagon) engaging the cytoplasmic tail of Env, Fusion between the cells is actively inhibited. Viral assembly is activated and recruited to the contact site leading to transmission of immature viral particles into vesicles of the target cell. Based on the state of the cell and HIV regulation this results either in pyroptic death of the target cell or in induced latency.

HIV-1 can also directly induce a virological synapse, which forms between infected cells and uninfected cells expressing CD4 and the receptor CCR5 (or CXCR4) (Jolly *et al.*, 2004; Chen *et al.*, 2007). Virological synapses are well studied in T-cells and are very similar to the immunological synapses normally formed between APCs and T-cells. These synapses result in signal transduction and changes in both the donor and target cells and can form through close cell contact but also through filopodia (Bracq *et al.*, 2018). Envelope-mediated contact is established between infected cells, expressing gp120 on the cell surface, and CD4 on target cells (Len *et al.*, 2017). This triggers a rapid sequential recruitment process on the cell surface of lipid rafts, adhesion molecules, Env and Gag protein, and enhancement of viral assembly locally (Zhong *et al.*, 2013; Wang *et al.*, 2019). The interaction is stabilized by LFA1 and ICAM-

1 and HIV recruits tetraspanins, a type of membrane protein involved in cell adhesion and proliferation, and actin-membrane organizing proteins to the synapse. These interactions also prevent membrane cell fusion between the target and donor cells and stabilize the virological synapse (Jolly *et al.*, 2004, 2007). In cell-cell transmission, Envelope mediated fusion to the target cell is also inhibited. The immature Gag associated with the cytoplasmic tail of the Envelope protein further inhibits the fusion event. The immature HIV particles with uncleaved Gag also inhibit viral fusion between immature virion particles that are budding into the synapse and the uninfected target cell. Rather, than direct infection of the target cell by the immature virions, there is a CD4-dependent translocation into a trypsin-resistant intracellular compartment. The virions further mature within these compartments, which then fuse into the target cell. *In vitro* formation of viable syncytia is observed between infected and uninfected cells through gp120-CD4 mediated fusion, however, its biological relevance *in vivo* is unclear (Murooka *et al.*, 2012).

1.3.2 Other cell-cell mechanisms, nanotubes, and vesicles transport

Several other mechanisms of cell-cell transmission have been described. Different protrusions from cells such as filopodia and open-ended nanotubes (TNT) mediate transfer between different T-cell types directly and through vesicles such as organelles (Sowinski *et al.*, 2008). HIV makes use of the existing TNT structures formed between T-cells and or myeloid cells to rapidly spread through a T-cell network. Similarly, HIV transfer to target cells in intracellular vesicles from the endoplasmic reticulum (ER) and the Golgi has been reported in macrophages. HIV-1 increases the number of infectious vesicles formed, through Nef protein, which alters the organisation and expression of various scaffold and membrane proteins in the Clathrin pathway such as tetherin and MHC-1 (Elliott *et al.*, 2015; Buffalo *et al.*, 2019). HIV can infect macrophages, however, this is independent of CD4 and thus is unlikely to use the virological synapse. These macrophages can become infected through phagocytosis of HIV-1 infected cells by a yet unknown mechanism (Baxter *et al.*, 2014). Moreover, HIV is also able to transcytose through epithelial cells, which mediates HIV transfer through the epithelial barrier during mucosal transmission.

1.4 The role of cell-cell transmission in HIV infection

1.4.1 *Cell-cell transmission in primary infection*

Cell-cell transmission likely plays a vital role in HIV infection as infectious cell-associated HIV has been found in breastmilk and in genital secretions. However, whether HIV infects through cell-free or cell-cell transmission in primary infection remains unclear (Sallé *et al.*, 2010; Anderson *et al.*, 2014; Milligan *et al.*, 2014; Sagar, 2014). This is likely dependent on the transmission route as different barriers to infection are present. Cell-associated transmission could explain transmission in the presence of neutralizing antibodies (Chen *et al.*, 2007; Malbec *et al.*, 2013; Reh *et al.*, 2015; Li *et al.*, 2019). Moreover, cell-cell mediated spread is also likely responsible for ongoing replication despite ART virological suppression (Sigal *et al.*, 2011). In cell-cell assays of transmitted/founder viruses, higher levels of cell-cell transfer were observed in all clones which was independent of the quantity of Envelope surface expression (Li *et al.*, 2019). Although infection of the target cell was still dependent on the infectivity of the clone, cell-cell transmission significantly increases the chances of infection compared to cell-free infection (Li *et al.*, 2019).

1.4.2 *The role of cell-cell transmission in active infection and the HIV reservoir*

Cell-cell transmission targets a different sub-population of CD4⁺ cells (Agosto *et al.*, 2018). Cell-free virus preferentially infects activated permissive CD4⁺ cells, which represent about 5% of the total CD4⁺ cell population. Since free virus is not able to engage the innate immune response this results in a productive infection of activated CD4⁺ T-cells, which eventually die by apoptosis. Cell-to-cell transfer, however, infects naïve and resting CD4⁺ cells (Agosto *et al.*, 2018). This cell-cell infection is linked to the rapid CD4⁺ T-cell depletion seen in acute infection and the progressive CD4⁺ T-cell depletion seen in chronic disease and progression to AIDS (Galloway *et al.*, 2015). Recent studies demonstrate that cell-cell transmission is the driver for pyrotypic death of lymphoid tissue-derived T-cells (Galloway *et al.*, 2015). Moreover, these pyroptic CD4⁺ cells increase the migration of T cells into the lymph node and subsequently halts T-cells in the lymph node. Together cell-free and cell-cell infection drive chronic inflammation, causing progression to AIDS in the long term (Galloway *et al.*, 2015)

Memory CD4⁺ T-cells are also likely infected through cell-cell mediated contacts, which contribute to the formation of the reservoir (Tabler *et al.*, 2014). It is well established that cell-free virus can infect CD4⁺ cells that transition back to memory cells, a resting state that

prohibits viral production and transcription, however, this latency is easily reversible (Agosto *et al.*, 2007; Han *et al.*, 2007; Henderson *et al.*, 2012; Pace *et al.*, 2012; Chavez *et al.*, 2015; Sloan *et al.*, 2015; Zerbato *et al.*, 2016). Co-culture experiments with memory CD4⁺ cells and myeloid cells (CD14⁺) demonstrate that effective infection of these cells is only possible if cell-cell contact is established (Evans *et al.*, 2013). Cell-cell mediated infection of latent CD4⁺ T-cells was also observed through the formation of virological synapses between CD4⁺ T-cells. When HIV is transmitted to latent CD4⁺ T-cells through cell-cell mediated transfection, it inhibits productive infection and T-cell activation in these memory cells while simultaneously inducing latency (Agosto *et al.*, 2018; Kumar *et al.*, 2018; Rezaei *et al.*, 2018; Pedro *et al.*, 2019).

Whereas many of the current ARVs effectively inhibit free virus transmission, there are variable results for cell-cell transmission (Agosto *et al.*, 2014; Duncan *et al.*, 2014). Secondly, there is evidence for low-level replication despite ART (either cell-free or cell-cell) due to imperfect tissue penetration, especially in the brain, the lymph nodes, and other difficult to reach sites (Sigal *et al.*, 2011). Due to the likely role cell-cell transmission plays in both primary infection, the reservoir, and progression to AIDS, there is a need to find new approaches to prevent cell-cell transmission.

1.5 The HIV Envelope protein

HIV expresses the gp120-gp41 Envelope trimer on its membrane, which enables binding to and cell fusion with the CD4⁺ T-cells in cell-free transmission. This trimeric protein is made of three gp120 and three gp41 domains forming heterodimers (Julien, Cupo, *et al.*, 2013; J. H. Lee *et al.*, 2016). The Env glycoproteins are synthesized as a polyprotein precursor and through the ER signal peptide trafficked to the ER where the signal is removed (Checkley *et al.*, 2011). A stop-transfer signal in the transmembrane domain of gp41 prevents full release into the ER and keeps the cytoplasmic tail of gp41 in the cytosol (Wyss *et al.*, 2005). Oligomannose precursors are added during processing of the gp160 proteins in the ER and Golgi complex by recognition of an N(X)T/S (X not P) amino acid sequence (Stanley P, Taniguchi N, 2017). After glycosylation with N-linked and O-linked glycans, the gp160 oligomerises into trimers but also other formations such as dimers (Checkley *et al.*, 2011). After Furin proteases cleavage, which ensures maturation, the trimer is then transported to the plasma membrane where it is incorporated into the membrane of the virus (Checkley *et al.*, 2011).

1.5.1 Structure of the Envelope trimer

The structure of the gp120-gp41 trimer was elucidated in 2013 (Liu *et al.*, 2008; Julien, Cupo, *et al.*, 2013). Individual subunits and the gp120 core were previously studied but these provided little insight into the functionality of the trimer (Kwong *et al.*, 1998). The first sufficiently stable soluble trimer structure was BG505-SOSIP.664, which has several modifications to stabilize the Envelope which allowed the crystallization and subsequent elucidation of the structure (Julien, Cupo, *et al.*, 2013; Sanders *et al.*, 2013). These structures elucidated pre-fusion and post-fusion forms of the trimer allowing a better understanding of the cell-entry process. The structures also demonstrated that the trimer has a conserved quaternary protein structure with specific hyperflexible and or hypervariable regions (Julien, Cupo, *et al.*, 2013; Sanders *et al.*, 2013).

The gp120 protein includes five hypervariable loops interspaced with five constant areas. The loops are flanked by cysteines that form disulfide bridges (except for V5) and they are variable in length from 50-90 amino acids (a.a.) for V1/V2, 19-44 a.a. for V4, and 14-36 a.a. for V5. The length of V1/V2 and its glycosylation sites increases with the duration of the disease, indicating that this loop aids in immune evasion (Julien, Cupo, *et al.*, 2013; Moscoso *et al.*, 2014). Moreover, the variable regions exhibit high sequence variation, which also increases with disease progression. The V3 loop is essential in viral entry and its length is conserved across clades (Almond *et al.*, 2010; Xiang *et al.*, 2014). Although it displays a relatively conserved sequence in clade C viruses, it is highly variable in the other clades (Tian *et al.*, 2002; Patel *et al.*, 2008). Structurally, the trimer can also be divided into an inner and outer domain connected by a bridging sheet to the trimer association domain. The inner domains are buried within the trimer whereas the outer domain and the variable loops extend out from the trimer (Zolla-Pazner *et al.*, 2016).

The CD4 binding site (CD4bs) in the HIV trimer is hedged by glycans and is formed by sequentially non-contiguous regions of gp120 from constant regions (C), C1, C2 (D loop), and the V5, that are in close proximity in the quaternary structure (Duan *et al.*, 2018; Zhou *et al.*, 2019). The binding site is located in the inner domain of the trimer (McGuire *et al.*, 2013; Jardine *et al.*, 2016; Duan *et al.*, 2018).

The co-receptor binding site is formed by the V3 loop, the bridging sheet, and the stem of the V1/V2 region. After CD4 binding rearrangements result in the exposure of the co-receptor binding site, which facilitates binding to the CCR5 or CXCR4 co-receptor (Kumar *et al.*, 2017; Tanaka *et al.*, 2017).

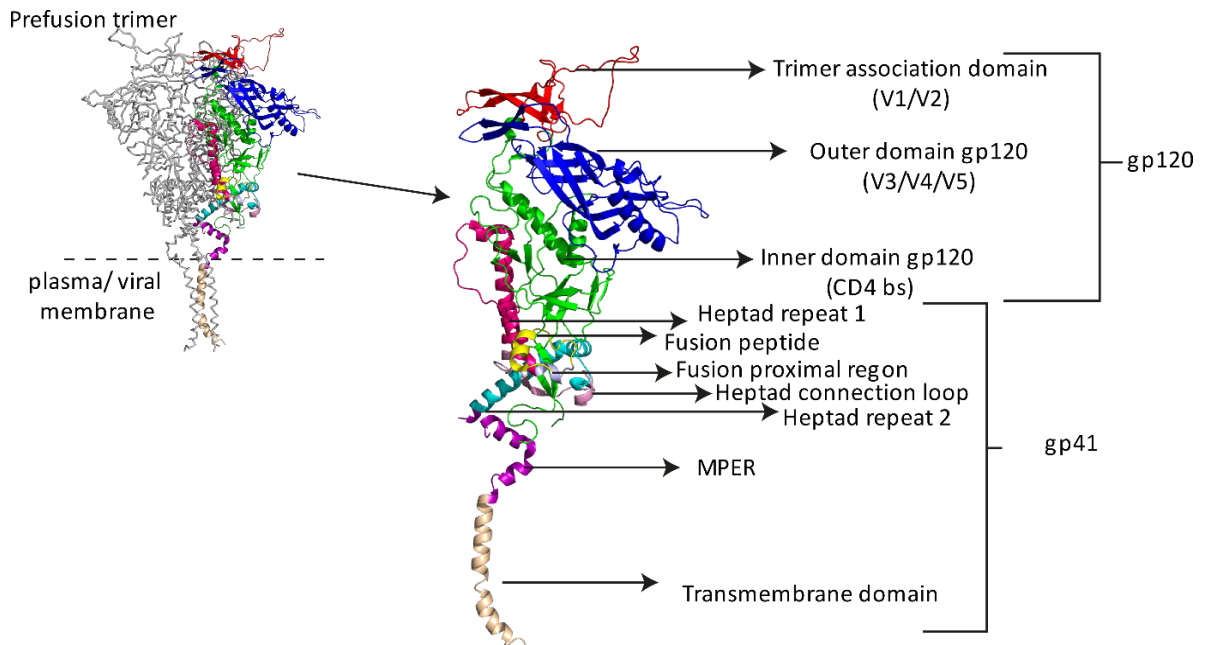


Figure 1.5 Crystal structure of the HIV envelope BG505.SOSIP.664 trimer.

The trimer consists of 3 units of gp120 and gp41 proteins. Individual subunits and structural units are indicated in different colors. Gp120 consisting of the trimer association domain (V1/V2) (red), the outer domain (V3, V4, and V5) (dark blue), the inner domain of gp120 which includes the CD4bs (green). The gp41 subunit consists of the heptad repeats (magenta, cyan) connected by the heptad connection (light purple), the fusion peptide (yellow) the fusion proximal region (lilac), the membrane-proximal region (purple), and the transmembrane domain (sand). (The model is based on the PDB 5v8l and 6e8w crystal and modelled with pymol)

In contrast to gp120, gp41 is more conserved likely due to its functionality in attaching the trimer to the HIV membrane and the viral entry mechanism. Gp41 envelops the N terminus and C terminus of the gp120 protomers with its hydrophobic core, consisting of four α -helices. The gp41 protein has three major domains, an extracellular domain, a transmembrane domain, and a cytoplasmic tail. The extracellular domain contains a fusion peptide at the N-terminal region which is hydrophobic, a polar region, two heptad repeats (HR1, HR2), and a tryptophan-rich region which forms the Membrane Proximal Region (MPER). The heptad repeats are connected through disulfide bridges and a flexible hydrophobic loop. The heptad repeats and the MPER region mediate viral fusion (Buzon *et al.*, 2010; Irimia *et al.*, 2016; J. H. Lee *et al.*, 2016).

The fusion peptide of the HIV Env is hidden within the interface region of the trimer until the conformational changes that occur during CD4 binding. After contact of CD4 and the co-receptor, the two heptad repeats unfurl themselves like a coil and the fusion peptide is inserted into the plasma membrane. The gp120 either is shed or moves out of the way during this process (Apellániz *et al.*, 2014).

The Membrane Proximal External Region (MPER) also plays a role in fusion and cell infection and is a highly conserved 24 amino acid region proximal to the cell membrane (Huang *et al.*, 2016; Kwon *et al.*, 2016; Soto *et al.*, 2016). The transmembrane domain anchors the Env into the lipid bilayer in a single α -helix and connects to a cytoplasmic tail (CT). This tail is about 150 a.a. in length and interacts with the Gag polyprotein during viral assembly and in cell-cell transmission in order to be incorporated into the virus particles. Moreover, mutations or truncations in this domain cause refolding in the gp120—gp41 trimer. The CT also mediates the clathrin-mediated endocytosis through a tyrosine-sorting domain located proximally to the cytoplasmic membrane. In the C-terminal region, three lentiviral lytic peptide sequences are present which also mediate membrane lysis and membrane incorporation (da Silva *et al.*, 2013; Narasimhulu *et al.*, 2018).

1.5.2 Immune evasion by the Envelope trimer

The highly sequence variable nature of the trimer makes it an evasive target for the immune system. Moreover, conformational changes described above, during binding and entry present various states to the immune system. In addition, the trimer also transitions between a closed mature state that evades antibody recognition to a more open state that is able to bind the CD4 receptor and the co-receptor to a fully post-fusion state (Bartesaghi *et al.*, 2013; Pancera *et al.*, 2014; Montefiori *et al.*, 2018; Wang *et al.*, 2018). A protective glycan shield covers the trimer. This glycan shield has a high level of organization and complexity, predominantly oligo-mannose, and is partly conserved across viral subtypes. Glycan shifting deletions and insertions present a challenge for the immune system (Doores, 2015; Panico *et al.*, 2016; Ferreira *et al.*, 2018; Ringe *et al.*, 2018). Glycans also contribute greatly to the molecular mass of the trimer, aid in protein folding and binding to the host cell surface (Doores, 2015; Behrens *et al.*, 2016; McCoy *et al.*, 2016; Montefiori *et al.*, 2018).

HIV also expresses non-native forms of the HIV Envelope on its surface that present distinct epitopes to the immune system. These induce non-neutralizing antibodies, which fail to inhibit

cell entry (Moore *et al.*, 2006; Gift *et al.*, 2017). There are only a limited number of epitopes on the HIV trimer that are conserved across virus strains. These conserved regions of the Envelope trimer can elicit broadly neutralizing antibodies (bNAbs) able to inhibit the majority of global strains. These bNAbs target the V1/V2 region, the 332 glycan on the V3 loop, the CD4bs, the fusion peptide, the MPER region as well as an epitope that spans both gp120 and gp41, called the interface. The rest of the trimer gives rise to non-neutralizing antibodies and non-broad antibodies. In the section below, we will further discuss the evolution of antibodies in HIV.

1.6 The structure of an antibody

Antibodies are “Y” shaped proteins produced by the adaptive immune system that target pathogens. They consist of two heavy (55kDa) and two light chains (24kDa) connected by disulfide bridges (cysteine–cysteine bridges) and a hinge region or regions that perform a hinge function (IgM, IgE). From a structural point of view, antibodies can also be divided into two Fabs (antibody binding fragment) and Fc (crystallisable fragment) regions (International Union of Immunological Societies, 2020).

1.6.1 The constant region and antibody isotypes

The heavy chain determines the isotype and subclass of an antibody. There are five constant regions (namely IgA, IgD, IgG, IgM and, IgE) which are encoded for by the α , δ , γ , μ , and ϵ genes. After primary infection, IgM expressed on the surface of B cells is the first isotype to appear and constitutes about 5-10% of the total antibodies in the sera. Antibodies then undergo class switching and IgG appears as a soluble antibody and is the most common isotype (70-85%), seen in secondary or reinfection with pathogens presenting the same epitopes. The other isotypes are present in the mucosa (IgA), or more often seen in allergic responses and in response to parasitic infections (IgE). IgD is mainly present on the surface of B cells but its exact function is somewhat unclear. IgG has the longest half-life and has four subclasses which all mediate different effector functions and have variable hinge lengths. The

light chain consists of one constant region and one variable region and is either a κ - or λ -chain (Janeway *et al.*, 2001).

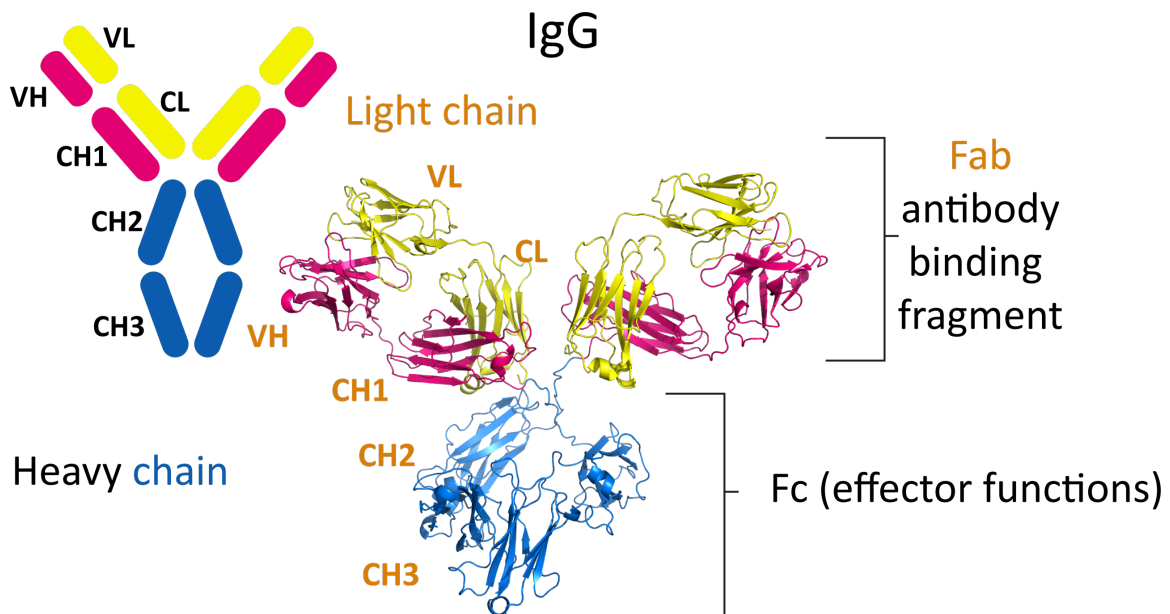


Figure 1.6 The basic structure of an IgG antibody.

The Fc fragment in blue, and the 2 Fab fragments consisting of the heavy chain in pink and the light chain in yellow. The heavy chain consists of 4 domains, the variable heavy chain (VH) and the constant heavy chain (CH1) in the Fab and the two constant regions in the Fc (CH2 and CH3). The light chain consists of a constant region (CL) and the variable light chain (VL) both part of the Fab. In the left corner is a drawn representation of the IgG and the crystal structure is shown as well. (Figure adapted in pymol from the 1IGY full IgG structure in PDB)

1.6.2 The variable region of antibodies

The variable region of the heavy and light chains are made up of three hypervariable complementarity determining regions (CDR) and four framework regions, which are less variable. Together these form the epitope binding region or the paratope. The variability within CDRs occurs first by recombination of the V, D, and J genes, mediated by VDJ recombinase giving rise to antibody genes. The heavy chain gene on chromosome 14 consists of 44 Variable (V) segments, 27 diversity (D) segments, and 6 Joining (J) segments. Light chains κ (Chromosome 2) and λ (Chromosome 22) do not have D gene segments but have 40 V segments and 5 J segments. In addition, there are insertions and deletions (indels) into the CDR3 which increases variability in length and sequence (Bowers *et al.*, 2014). This results in a very high level of antibody diversity $>10^{11}$ (Janeway *et al.*, 2001). Diversity in antibody genes is also generated through somatic hypermutation (SHM) which causes variation in the

antibody genes of individual B cells and is part of the maturation process of antibody-producing cells. This further fine-tunes the binding of the antibody and the epitope. In lymph nodes and the thymus, there are several checkpoints to prevent autoreactivity of the antibodies that might have developed after recombination and SHM events (Mesin *et al.*, 2016).

The strength of the interaction between paratope and epitope (affinity) is determined by the complementarity between target and antibody and influences the potency of the antibody. The higher the affinity of an antibody, the faster the antibody binds to the target, and the stronger this bond is maintained under unfavourable conditions. Affinity is a balance between the on- and off-rate of antibodies and affinity maturation of the antibody increases this bond by increasing on rate and decreasing off rate. However, several other factors can influence the overall binding between antibody and antigen, such as avidity or the strength of the bond between the antibody and epitope can also affect the potency of an antibody (Klasse, 2016).

1.6.3 The antibody response to HIV infection

Within two to three weeks of infection, HIV-infected patients develop a strong humoral response to the Envelope consisting mainly of binding antibodies to gp41, and the V3 region (Tomaras *et al.*, 2009). However, these antibodies do not exert pressure on the virus, as they are unable to neutralize the virus. After about 12 to 20 weeks of infection neutralizing responses develop, which are strain-specific for the infecting viruses (Li *et al.*, 2006; Tomaras *et al.*, 2008). These neutralizing antibodies target highly variable regions on the HIV env such as the V1/V2, V3, and V4 regions (Moore *et al.*, 2008, 2009). Rapid escape from these neutralizing antibodies ensues with likely little fitness cost to the virus (Bar *et al.*, 2012; Moore, 2018). Although all infected people mount these potent strain-specific, broadly neutralizing responses, described in more detail, below, bNAbs only arise in a small subset of people (Gray *et al.*, 2007, 2011; Tomaras *et al.*, 2008; Corti *et al.*, 2010).

1.7 Broadly neutralizing antibodies

First-generation neutralizing antibodies (4E10, b12, 2F5, and 2G12) were neither very broad nor very potent and some were found to be autoreactive (Kumar *et al.*, 2018). Identification of donors with broad plasma responses as well as improvements in B cell culture and sorting techniques have allowed the HIV vaccine field to isolate bNAbs against several major epitopes on the HIV trimer. These epitopes on the trimer span from the apex down to the membrane,

including the V2 region, the V3 supersites, the CD4 binding site, the gp120-gp41 interface, and the MPER region. Recently antibodies targeting a new epitope, the fusion peptide have also been isolated (Kong *et al.*, 2016; Xu *et al.*, 2018).

1.7.1 bNAb epitopes

V2 antibodies target a quaternary epitope, the apex of the trimer, which is only present on

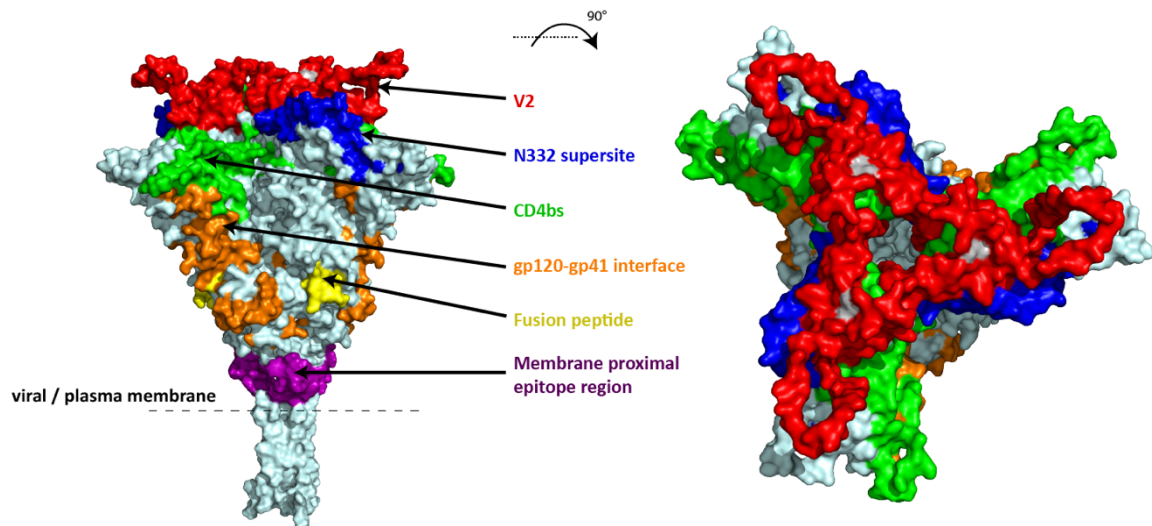


Figure 1.7 Epitopes on the gp120-gp41 HIV trimer.

Major bNAb epitopes are represented on the crystal structure of the BG505.SOSIP.664 trimer. The V2 region (red) is located at the apex of the trimer. The N332 supersite region including the V3 loop (blue) is located between close to the V2 region. The CD4 binding site (CD4bs) is indicated in green. Two more recently identified epitopes are the gp120-gp41 interface (orange) including the fusion peptide (yellow). The MPER (purple) is located close to the viral membrane. The figure is based on the 5v8l structure on the protein data bank (PDB) and made with Pymol.

the prefusion functional trimer. The epitope includes two conserved glycans at positions N156 and N160 and a small motif at positions 165-173. These residues are relatively conserved among clades accounting for the breadth of antibodies targeting this epitope (Bricault *et al.*, 2019). Most bNAbs targeting this site share structurally similar strategies of targeting this epitope through a long CDRH3, (26-39 a.a) which reaches through the glycan shield and engages key glycans. For example, PG9 binds two N160 glycans on different gp120 proteins making it specific for the trimer (Pejchal *et al.*, 2010; McLellan *et al.*, 2011; Doria-Rose, Georgiev, *et al.*, 2012; Julien, Lee, *et al.*, 2013; Pancera *et al.*, 2013; Wang *et al.*, 2017). Other V2 type antibodies such as the CAP256-VRC26 lineage are less dependent on the N160 glycan and primarily rely on binding to residues 165-171 (Doria-Rose *et al.*, 2014; Bhiman *et al.*, 2015; Kong *et al.*, 2016). Viral resistance is mediated by specific charge and residue changes within this loop or the loss of the 156 or 160 glycans (Doria-Rose, Georgiev, *et al.*, 2012; Moore *et al.*, 2013; Andrabi *et al.*, 2015; Doria-Rose *et al.*, 2015; Julg, Tartaglia, *et al.*, 2017).

The N332 supersite epitope is adjacent to the V2 apex. Similarly, to the V2 apex, antibodies to this site depend on a short relatively conserved peptide stretch between 323 and N332, in particular, the GDIR motif. Moreover, many also depend on the glycan at N301 (Krumm *et al.*, 2016; Caskey *et al.*, 2017). Escape from these antibodies occurs through glycan deletion at 332 or shifting to the 334 position. Moreover, due to the proximity of the V1/V2 region, viral escape can also be modulated through elongated V1 loops that cover the epitope (van den Kerkhof *et al.*, 2016; Anthony *et al.*, 2017; Silver *et al.*, 2020). Members of the PGT121 and PGT128 antibody lineages have evolved to adapt to these escape mechanisms. For example, PGT128 can neutralize these viruses (with glycan shifts or V1 elongation) when glycans at N301 and N295 are present (Krumm *et al.*, 2016). N332 supersite antibodies are mostly PGT121/PGT128 like, using similar mechanisms to target this site albeit at slightly different angles and with the involvement of other glycans such as N295, N137, and N156 (Garces *et al.*, 2015). The PGT135 bNAb is independent of the GDIR motif and targets additional glycans at positions N339 and N392. Moreover, it targets the base of the V4 loop as well as approaching the N332 glycan from a different angle (van den Kerkhof *et al.*, 2016). The most potent N332 supersite antibody to date is BG18, which was isolated from a paediatric donor (Barnes *et al.*, 2018).

Table 1.1 bNAb epitopes and characteristics.

<i>Epitope</i>	<i>Important residues</i>	<i>Escape/resistance</i>	<i>Antibody characteristics</i>	<i>bNAb examples</i>
V1/V2	N130, N156, N160, 165-173	Charge changes, loss of glycans	CDRH3 elongation	PG9, CAP256.25 (Burton <i>et al.</i> , 2009; Doria-Rose <i>et al.</i> , 2015)
V3	323-332, N301, N332. supporting: N156, N295, N339, N386, N392	Loss of N301 or N332 glycans V1 elongation/O-linked glycan enrichment of V1	Mainly CDRH3 based interaction, however with variations.	PGT121, PGT128, PGT135 (Walker <i>et al.</i> , 2011; Kong <i>et al.</i> , 2013)

<i>CD4bs</i>	Lacking N276 glycan, G458, Loop D, CD4-binding loop, V5	N276, G458Y, N234 glycan, 279-282 mutations, N461, D-loop, β 23/V5/ β 24 mutations	Mimic CD4, short CDRL3, long CDRH3	VRC01, VRC07, 3BNC117 (X. Wu <i>et al.</i> , 2010; Scheid <i>et al.</i> , 2011; Rudicell <i>et al.</i> , 2014)
<i>Interface</i>	N234, N276, R456, N611, N637, C5 region	N276 glycan loss	Framework elongation	8ANC195 35O22, PGT151 (Falkowska <i>et al.</i> , 2014; Huang <i>et al.</i> , 2014; Scharf <i>et al.</i> , 2014),
<i>Fusion peptide</i>	512-520, N88			VRC034.01 (Kong <i>et al.</i> , 2016)
<i>MPER</i>	C-terminal region of gp41 671-683	W672L, F673L, W680G, N671T	CDRH3 loop Hydrophobic	10E8 4E10

CD4 binding site targeting antibodies such as the VRC01 class often mimic CD4 (Scheid *et al.*, 2011). These antibodies tend to have a short CDRL3 loop to avoid clashing with the N276 glycan and have residues that have similar functions as CD4 residues. The formation of these antibodies is complex in that their elicitation requires the specific absence of the highly conserved N276 glycan, but subsequent evolution to tolerate this glycan (McCoy *et al.*, 2016; Duan *et al.*, 2018). Therefore, these antibodies are a relatively rare subclass of antibodies in patients (Lynch *et al.*, 2012). The second class of CD4bs antibody uses long CDRH3 loops however, due to the high glycosylation of the trimer in this area, these antibodies need to approach the trimer at the same angle as the VRC01 class antibodies (Zhou *et al.*, 2010).

The N234 and N276 glycans are also targets for gp120-gp41 interface antibodies, which can use an elongated framework region to contact R456 (Scharf *et al.*, 2014; Liu *et al.*, 2019). Moreover, the CDRH3 contacts a β sheet of gp120 that interacts with the gp41 region. 8ANC195, the first described interface bNAb, targets both the open and the closed trimer but can also bind gp120 alone through heavy chain interactions. The light chain of 8ANC195 makes significant contacts with the gp41 and the N637 glycan (Chuang *et al.*, 2013; Scharf *et al.*, 2014, 2015). Other interface antibodies, however, bind gp120 and gp41 simultaneously, such as 35O22, which also binds the β strand through an elongated framework (Doria-Rose *et al.*,

2014). Both 8ANC195 and 35O22 are dependent on the glycosylation of the trimer, requiring for example glycans at positions N234 and N276. PGT151 is more dependent on the N611 and N637 glycans, with a propensity for more complex glycans and also binds the end of gp120 in the C5 region (Blattner *et al.*, 2014; Falkowska *et al.*, 2014; Huang *et al.*, 2014). Interface antibodies often interact with the fusion peptide which forms a sub-epitope that has recently been described following the isolation of VRC34.01 (Kong *et al.*, 2016). This antibody targets 8 conserved residues (512-520) in the fusion peptide and the N88 glycan on gp120 which is in structural proximity to this site (Kong *et al.*, 2016; Wibmer *et al.*, 2017). VRC34.01 inhibits the structural conformational changes needed for fusion to the cell membrane. However, fusion peptide accessibility varies among HIV trimers, with some being exposed such as on the BG505 SOSSIP trimer and others being sequestered (Ananthaswamy *et al.*, 2019).

The MPER epitope is a highly conserved epitope forming an alpha-helix between the transmembrane domain and the ectodomain of gp41. 10E8 and 4E10, two very broad bNAbs bind the C terminal region (residues 671-683) of this alpha-helix, which is hydrophobic and partially buried within the viral membrane (Irimia *et al.*, 2016). This highly conserved region facilitates fusion during viral entry. The long CDRH3 loops of these antibodies interact with the viral membrane, which is likely the reason for the autoreactivity of most MPER antibodies (excluding 10E8v4) (Kwon *et al.*, 2016; Zhang *et al.*, 2019). Only recently has the MPER region (with a transmembrane domain) been crystalized giving more insight into how antibodies targeting this site interact with the epitope (Huang *et al.*, 2012; Georgiev *et al.*, 2014; J. H. Lee *et al.*, 2016; Kwon *et al.*, 2016; Soto *et al.*, 2016; Irimia *et al.*, 2017).

1.7.2 HIV and antibody coevolution

Several factors are associated with the development of breadth such as viral load, viral diversity, and duration of infection, which all increase antigenic exposure (Gray *et al.*, 2011; Rusert *et al.*, 2016). Several studies that follow patients from infection to the development of breadth demonstrate the interplay between antibody and viral epitopes (Wibmer *et al.*, 2013; Doria-Rose *et al.*, 2014; Bhiman *et al.*, 2015; McCoy *et al.*, 2016; Soto *et al.*, 2016; Steichen *et al.*, 2016; Zhou *et al.*, 2017; Stamatatos *et al.*, 2017; Voss *et al.*, 2017; Mabvakure *et al.*, 2018; Wagh, Kreider, *et al.*, 2018; Sacks *et al.*, 2019). These studies indicate that early viral variants select for an antibody lineage with binding or neutralizing capacity. After viral escape, the antibody, in turn, matures to accommodate variations within the epitope, which leads to further viral diversification. This continuous co-evolution between the virus and the immune

system is one of the drivers for antibody development that target conserved epitopes and can tolerate viral diversity and indirectly become broad (Bhiman *et al.*, 2015; Bonsignori *et al.*, 2017). Not all maturation leads to breadth, however, perhaps partly explaining why a large group of chronically infected patients do not develop breadth. This is a challenge in vaccine design as host factors may impede the development of breadth with bNAbs often requiring a rarer B-cell lineage (Liao *et al.*, 2013; Jardine *et al.*, 2015, 2016; Julg, Tartaglia, *et al.*, 2017; Reh *et al.*, 2018; Wiehe *et al.*, 2018). Although bNAbs and NAbs do not halt disease progression, a multitude of passive immunization studies indicate they would be able to prevent infection making them a prime vaccine objective (Mascola *et al.*, 2000; Pegu *et al.*, 2014; Saunders *et al.*, 2015; Bar *et al.*, 2016; Bolton *et al.*, 2016; Landais *et al.*, 2018).

1.8 The search for an HIV vaccine

There is a dire need for an HIV vaccine, which can prevent the spread of HIV. Even though there has been significant progress made in the treatment of HIV, this is having little impact on HIV incidence (G. Williams *et al.*, 2011). There has been a 35-year ongoing search for an effective vaccine to prevent HIV transmission with over 270 vaccine trials and six Phase IIb and III trials. These efforts have focused both on neutralizing antibody vaccines and vaccines aiming to elicit protective cellular immunity. Vaccines tested to date elicited antibodies to non-neutralizing targets (Rubens *et al.*, 2015). Moreover, vaccines thus far have only elicited neutralization responses to so-called tier 1 viruses, which have a naturally more open structure compared to the more difficult to neutralize tier 2 and 3 viruses (Gust *et al.*, 2009). It is thought that a protective vaccine must be able to elicit at least tier 2 neutralizing antibodies (Seaman *et al.*, 2010; Montefiori *et al.*, 2018).

The AIDSVAX vaccine, for which results were published in 2003 mainly elicited antibodies to epitopes that did not confer protection or against gp120 monomer (The rgp120 HIV Vaccine Study Group, 2005). Similarly, the STEP trial (2007) which studied vaccines aimed at eliciting T-cell mediated responses did not show protection against HIV. In fact, this vaccine increased the risk of HIV due to responses to the adenovirus vector (Buchbinder *et al.*, 2008; Duerr *et al.*, 2012). The first trial that showed low-level efficacy was the RV144 trial, which combined the two approaches. This vaccine used a canarypox vector with three HIV genes (ALVAC-HIV) followed with a gp120 boost (AIDSVAX) targeting both cellular immunity and neutralizing antibody responses. This vaccine conferred 31.2% effectiveness; however, it failed to elicit

either CD8 responses or neutralizing antibodies (Rerks-Ngarm *et al.*, 2009; Haynes *et al.*, 2012; Karasavvas *et al.*, 2012). Protection was associated with V2 binding antibodies mediating Fc functions, such as antibody-dependent cellular cytotoxicity (ADCC) (Haynes *et al.*, 2012). The HVTN100 study used the same approach with subtype C based genes and proteins in South Africa. Similar antibody responses as the RV144 trial were observed in this study (Bekker *et al.*, 2018). However, a larger-scale efficacy trial (HVTN 702) failed to provide protection (National Institute of Allergy and Infectious Diseases, 2020). Other ongoing trials include the HVTN705 trial, which employs a different prime-boost using an adenovirus vector with mosaic proteins genes with a subtype C gp140 protein boost (HVTN, 2020). Current efforts focus on Native Flexibly Linked (NFL) trimers and SOSIP trimer based vaccines that mimic the Envelope trimer in its native state. These vaccines are in an early phase 1 trial stage (NCT03699241) (Sanders *et al.*, 2015; clinicaltrials.gov, 2018; Ringe *et al.*, 2018; Dubrovskaya *et al.*, 2019). A second strategy uses trimers or outer domains with affinity for the germline precursors of bNAbs such as PGT121 or VRC01 (Garces *et al.*, 2015; Jardine *et al.*, 2016; McCoy *et al.*, 2016; Steichen *et al.*, 2016; Duan *et al.*, 2018; Bricault *et al.*, 2019; Umotoy *et al.*, 2019).

1.9 HIV Prevention and treatment with antibodies

1.9.1 Use of bNAbs in treatment and prevention

The isolation of next-generation bNAbs has renewed interest in using antibodies in both the prevention and treatment of HIV. The more recently isolated bNAbs demonstrate both high breadth and potency and have shown protection in macaques (Pegu *et al.*, 2014; Gautam *et al.*, 2016; Hessel *et al.*, 2016; Rosenberg *et al.*, 2016; Calenda *et al.*, 2019; Shapiro *et al.*, 2020). The bNAbs 3BNC117, PGT121, VRC01-LS, VRC07, and/or 10-1074, when administered to macaques, were able to block SHIV infection. Currently, VRC01 is being assessed in a passive immunization trial for its protective qualities in primary HIV infection (AMP). This will inform whether a vaccine eliciting these antibodies could be protective.

Treatment with PG121, PGT128, 10-1074, 3BNC117, PG16, NIH45-46, all showed reduced viral loads in infected animals. In addition, a reduction in cell-associated HIV was observed (Mascola *et al.*, 2000; Parren *et al.*, 2001; Sallé *et al.*, 2010; Pegu *et al.*, 2014; Bolton *et al.*, 2016; Gautam *et al.*, 2016; Clayton *et al.*, 2017; Parsons *et al.*, 2017).

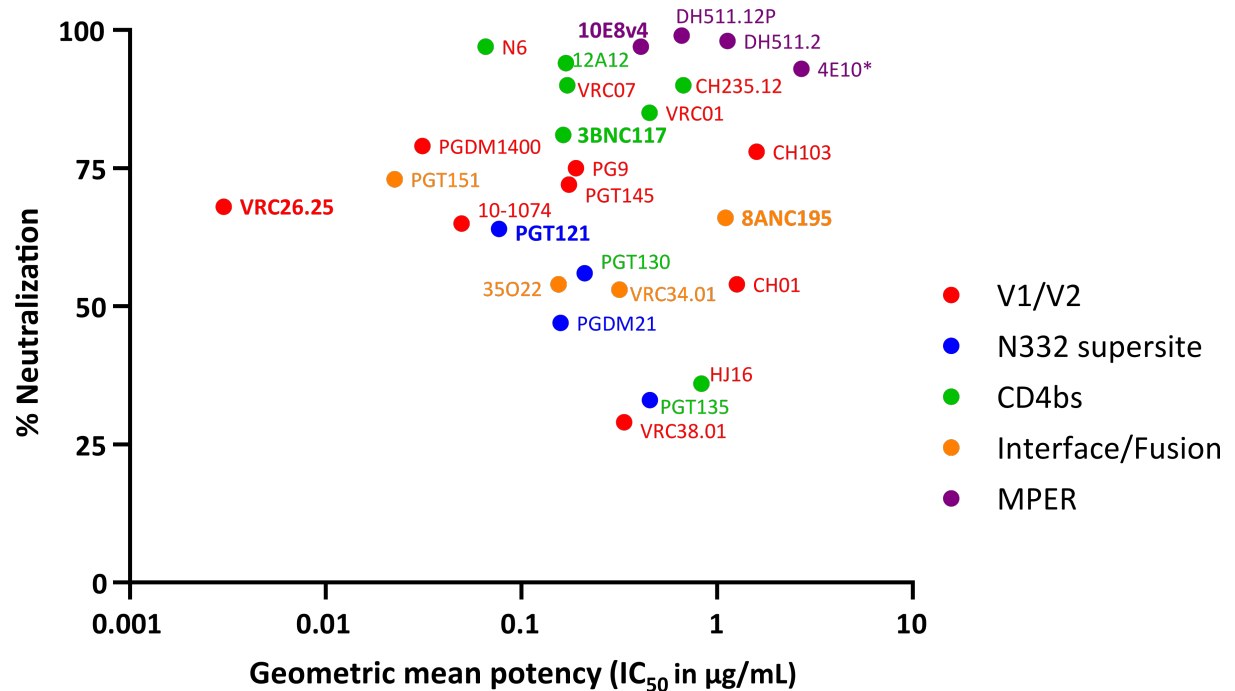


Figure 1.8 Breadth and potency of HIV bNAbs targeting all major epitopes.

Breadth and the potency of bNAbs targeting major sites on the HIV-1 envelope. bNAbs relevant to this thesis are **bolded**. Early generation bNAbs are indicated with a star. MPER antibodies (purple) demonstrate a high level of breadth. V2 antibodies (red) show very high potency but limited breadth. Data obtained on CATNAP (Yoon *et al.*, 2015) and figure inspired by (Sok *et al.*, 2018) Geometric mean only includes viruses neutralized and/or tested for that bNAb on average (between 118-800 viruses).

In human clinical trials, antibodies such as VRC01 and 3BNC117 (CD4 binding site) or 10-1074 (N332 supersite) have been tested in patients undergoing treatment interruptions and demonstrated viral suppression for 6-10 weeks during antibody treatment (Scheid *et al.*, 2016; Caskey *et al.*, 2017). bNAbs that were better at preventing cell-cell transmission such as 3BNC117 were associated with viral suppression and delayed rebound in patients. Antibodies, which were poorer in preventing cell-cell transmission such as VRC01, 10E8, however, did not achieve proper viral suppression in monotherapy (Reh *et al.*, 2015; Caskey *et al.*, 2017; Cheeseman *et al.*, 2017; Dufloo *et al.*, 2018; Mendoza *et al.*, 2018). Moreover, there are indications that bNAbs could reduce the viral reservoir (Klein *et al.*, 2012; Horwitz *et al.*, 2013; Barouch *et al.*, 2014; Halper-Stromberg *et al.*, 2014).

Combinations of antibodies outperform monotherapy, both in breadth and potency, in viral suppression studies and limit viral escape during treatment. Therefore a combination of antibodies will likely be needed to provide sufficient breadth and potency and to be able to target both cell-free and cell-cell transmission (Doria-Rose, Louder, *et al.*, 2012; Kong *et al.*, 2015; Wagh *et al.*, 2016; Wagh, Seaman, *et al.*, 2018). The breadth can be increased by using bNAbs with complementary neutralization profiles and, by using very potent antibodies, the overall potency could be increased. In addition, combinations of antibodies are likely to decrease HIV escape as multiple epitopes can be covered (Gruell *et al.*, 2018). In clinical trials, combinations of antibodies were able to maintain viral suppression for prolonged time periods (21 weeks median) compared to monotherapy. Moreover, no resistance mutations for the bNAbs were detected in these individuals, except for outgrowth of an already resistant virus present in the reservoir, as long as the plasma concentration remained above 10µg/mL during the trial (Mendoza *et al.*, 2018).

Two models describe the potential of antibody combinations. The first is the “Loewe Additive” model which assumes that the antibodies will act independently of each other and that their neutralization breadth and potency will provide an additive effect (Kong *et al.*, 2015). The “Bliss-Hill Independence” model assumes a similar premise, however, this model also takes into account the slope of the neutralization curves and can more accurately predict how combinations of bNAbs neutralize HIV (Wagh *et al.*, 2016; Wagh, Seaman, *et al.*, 2018). Combinations of three or more antibodies were shown to provide optimal coverage and sufficient potency for HIV prevention. Multiple antibodies covering the same viruses are needed moreover to prevent escape which could be achieved with three or more antibodies in the mixture (Wagh *et al.*, 2016; Wagh, Seaman, *et al.*, 2018). Combinations of antibodies targeting the same epitope, however, could show antagonism or competition. Antagonism can also be observed when the epitopes are in close proximity or when binding to the epitope causes conformational changes to the trimer preventing binding. For example, antibodies that induce opening of the trimer could compete with trimer-specific antibodies such as CAP256-VRC26.25 (CAP256.25) (Doria-Rose, Louder, *et al.*, 2012; Wagh *et al.*, 2016; Wagh, Seaman, *et al.*, 2018). Synergy can also be observed when one antibody increases the access of the other antibodies, something that can be observed with some MPER epitopes and CD4i antibodies (Ding *et al.*, 2016; Ferreira *et al.*, 2018). Further detailed analysis of this phenomenon is needed.

1.9.2 Delivery of bNAbs through AAV

Delivery of antibodies by recombinant Adeno-Associated Virus (AAV) is a promising approach to achieve passive immunity. Studies in chimpanzees and macaques with SIV and SHIV were able to demonstrate protection by using AAV mediated expression of bNAbs (Johnson *et al.*, 2009; Balazs *et al.*, 2012; Fuchs *et al.*, 2015; Saunders *et al.*, 2015). Moreover, similar strategies have shown protection from HIV in humanized mice (Fuchs *et al.*, 2016; Hay *et al.*, 2018). The first clinical trial used PG9 in an AAV-1 vector to express antibodies in patients (Priddy *et al.*, 2019). However, there were challenges with low antibody levels due to the formation of anti-AAV and anti-PG9 antibodies. Another trial testing VRC07, a CD4bs antibody (NCT03374202) delivered by AAV is currently underway (Saunders *et al.*, 2015) and antibodies such as CAP256.25 - have been expressed in AAV-8 and tested in mice models (van den Berg *et al.*, 2019). AAV are considered a safe gene delivery method however the platform has limitations in regards to genetic space on the vector and expression levels may vary. To improve expression, self-complementary vectors may be used, however, these are even more limited in genetic space. Smaller antibody-derived constructs, such as single-chain variable fragment (scFv) described below, have a much smaller genetic size compared to IgG (~800 base pairs vs ~2100 base pairs) and may, therefore more readily be expressed by AAV. Moreover, they may also be more readily expressed in combinations than their IgG counterparts in AAV (Naso *et al.*, 2017; Lin *et al.*, 2018).

1.10 Antibody Fragments

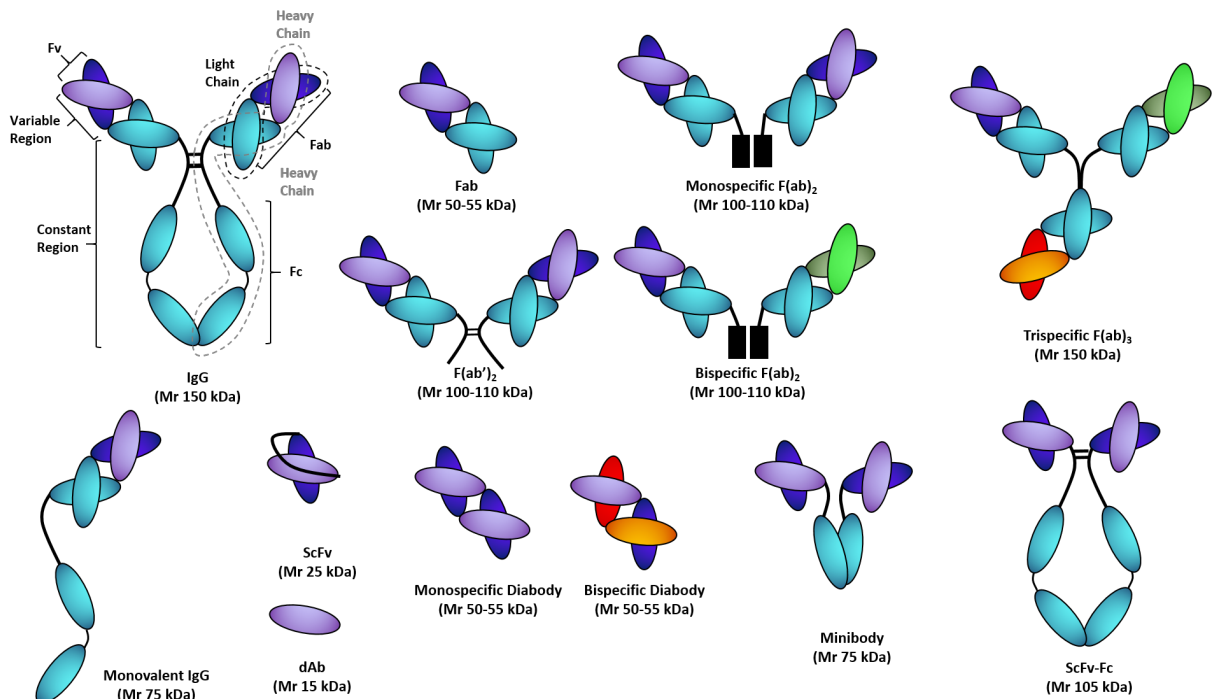


Figure 1.9 Antibody fragments and antibody constructs.

Constructs of antibodies have been engineered such as the Fab or various Fab based constructs. Similarly, scFv have been used in various antibody constructs as shown above (picture source: nicepng, 2020).

Several smaller fragments have been engineered from IgG (Figure 1.9). One of these fragments, scFv consists of the variable heavy and variable light chain connected by a flexible peptide linker and is one of the smallest fragments encompassing the entire paratope of the IgG. Most commonly, the linker is about 15 amino acids long, consisting of GGGGS repeats (Finlay et al., 2011). These scFv are about 30 kDa in size and have successfully been applied in various clinical settings both in treatment, imaging, and preventative measures (Figure 1.10) (West et al., 2012; Monnier et al., 2013). ScFv maintain the binding specificities of the parent antibodies and tend to diffuse into tissues more readily compared to the IgG (Colcher et al., 1998; Unverdorben et al., 2016). However, this comes at the cost of reduced half-lives associated with an absent Fc domain and increased kidney clearance (Kubetzko et al., 2006; Unverdorben et al., 2016). scFv are also proposed to be better against T-cell-cell transmission due to their smaller size and possibly reduced steric hindrance, a possibility that we explore in chapter 5 of this thesis.

1.10.1 scFv for HIV

Several HIV-directed scFv have been engineered from phage display libraries, and often target the CD4i epitope (Wang *et al.*, 2015; Kumar *et al.*, 2017; Tanaka *et al.*, 2017). These scFv tend to be more potent and broader compared to their IgG counterparts due to improved access to this recessed epitope (Khan *et al.*, 2017). However, only a few scFv have been developed from HIV bNAbs targeting epitopes on the closed trimer (West *et al.*, 2012; Wang *et al.*, 2015). PG9 has been expressed as an scFv and lost 40-fold of geometric mean potency, whereas various VRC01 and PG16 based fragments lost 10-20 fold geometric mean potency compared to the parental IgG. Moreover, the expression of VRC01 and PG16 scFvs were too low to test in neutralization assays (West *et al.*, 2012). m36, a heavy chain only antibody, and J3a, llama heavy chain only camelid antibody, were demonstrated to have both breadth and potency but were not compared to IgG based fragments. These antibodies have also been expressed on alternative vectors in for example lactobacillus, but have not yet undergone clinical testing (McCoy *et al.*, 2012; Marcobal *et al.*, 2016; Weiss *et al.*, 2019). scFv of bNAbs have also been produced in bacteria, however, almost all neutralizing activity was lost in these scaffolded scFv bNAbs (Guglielmi *et al.*, 2009; Wang *et al.*, 2015).

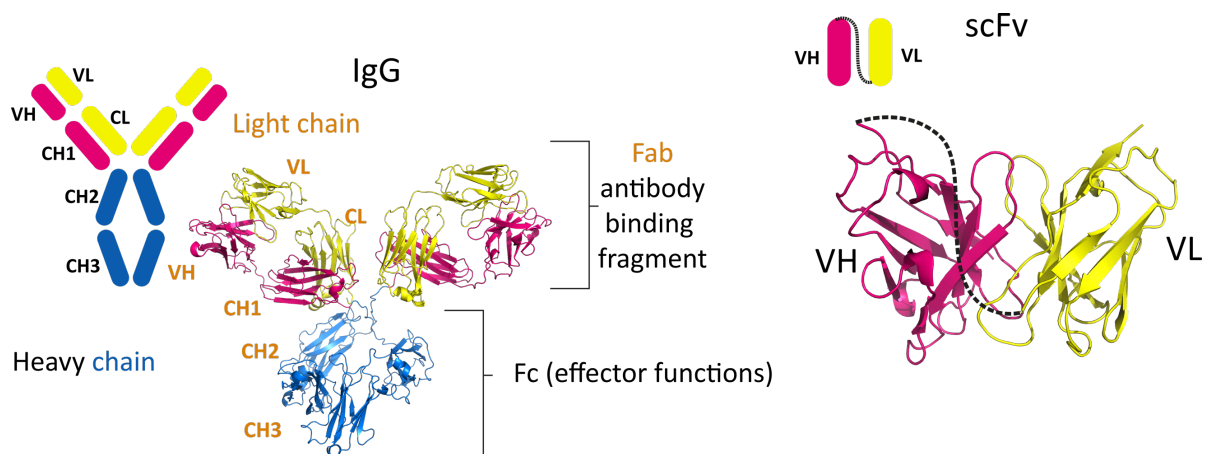


Figure 1.10 The structure of an scFv.

The scFv consists of the variable heavy (VH) in pink and the variable light (VL) chain in yellow of a full IgG. The two chains are connected by a glycine serine linker as demonstrated by the black dotted line. (Figure adapted in pymol from the 1IGY structure in PDB)

1.10.2 Multispecific scFv based and composite antibodies against HIV

Several bispecific antibodies have been developed, which can target two epitopes on the HIV-1 Envelope. These molecules demonstrated higher breadth compared to the single antibodies

(Khan *et al.*, 2018). Bispecific antibodies make use of a knob-in-hole and CrossMab strategy to ensure proper combination and heterodimerisation when synthesized (Klein *et al.*, 2016). This technique, engineers a knob or a hole in the CH3 region of two different antibody heavy chains and swaps the CL and CH1 chains. When the plasmids of two antibodies are thus co-expressed in one culture, they combine into a single heterodimeric antibody (Figure 1.11). Several dual specific bNAbs have been engineered using both or one of these techniques. For example, 3BNC117-PGT135, which targets both the CD4 binding site and the N332 supersite. Although an initial form of this antibody showed reduced potency, engineering in greater flexibility in the hinge region allowed for dual binding of the two arms, which improved its potency (Bournazos *et al.*, 2016). Another bispecific antibody used one of the broadest CD4bs antibodies, VRC07, on one arm with a variety of bNAbs targeting the V2 region, the N332 supersite, or the MPER on the other arm. This study found that VRC07 combined with PG9-16, a V2 antibody was the broadest (97%) and most potent (0.055g/mL) (Asokan *et al.*, 2015). The breadth and potency of dual specific antibodies depends on complementarity and the potencies of the parental antibodies. Further engineering by increasing flexibility and linker length between the scFv and or Fab components can allow for improved neutralization by allowing for the dual binding of both paratopes, either inter- or intratrimerically. For example, a bispecific antibody combining an scFv of CAP256.25 on one arm and N332 Fab antibody on the other arm demonstrated improved breadth and potency compared to either single antibody, likely by engaging both epitopes (Davis-Gardner *et al.*, 2020).

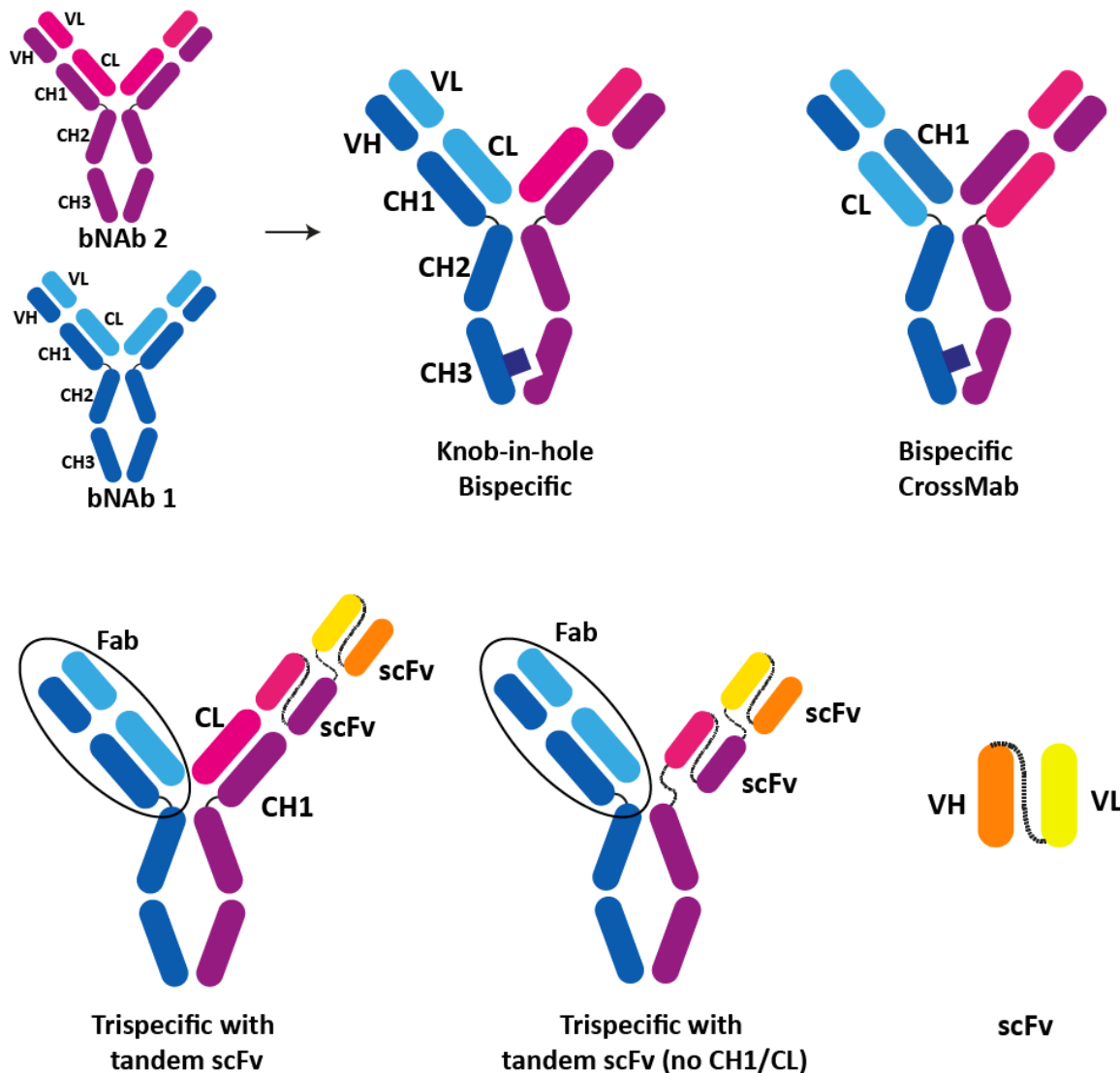


Figure 1.11 Bi and trispecific antibodies of HIV bNAbs.

Bi and tri specific antibodies make use of various strategies to combine multiple paratopes into one molecule. The Knob-in-hole engineers large amino acids into the CH3 region of one antibody and creates a space in the second antibody's CH3 region, which improves the portion of combined antibodies when expressed. By swapping the CH1 and CL regions proper light chain, heavy chain pairing is ensured. ScFv are also used in multispecific proteins by replacing one arm of the antibody with 1 or multiple scFv connected by glycine serine linkers. Sometimes the CH1, CL is removed in these antibodies or the hinges are further engineered to improve flexibility.

Trispecific antibodies display an even greater breadth and potency than bispecific antibodies. These antibodies often have one arm as Fab component and the other one with two scFv in tandem. Some trispecific antibodies also remove the CH1 and CL component of this arm. Most of the trispecific antibodies reach 100% breadth against large multiclade panels with extraordinary potency. Examples of these include a trispecific antibody with a 10E8 Fab (MPER) on one arm and a tandem of PGT121-VRC01 scFv on the other arm (V3-CD4bs)

(Steinhardt *et al.*, 2018). A second trispecific antibody that makes use of VRC01 Fab and 10E8v4 scFv in tandem with the V2 targeting PGDM1400 scFv is currently being tested in a phase 1 clinical trial. This trispecific antibody also reached 100% breadth, demonstrated protection from SHIV in macaque models, and was potent at a geometric mean of 0.3µg/mL (A5377, NCT03705169)(Xu *et al.*, 2017; ClinicalTrials.gov, 2020b).

A second strategy combines antibodies that target the HIV Env on one arm and either CD4 or CCR5 on the other arm using similar CrossMab or knob-in-hole technologies. A bispecific example of this is the 10E8-IMab, which combines the MPER targeting 10E8 antibody with a CD4 targeting antibody. This antibody is one of the most potent antibodies with a geometric mean potency of 0.002µg/mL with 100% breadth. A more soluble version using the 10E8v4 antibody is currently being tested in a phase 1 clinical trial (NCT03875209). This antibody had a 2.5 fold improvement in potency compared to the original antibody (Huang *et al.*, 2016; Padte *et al.*, 2018). Similarly, CAP256.25 has also been combined with the iMab, demonstrating an 11.4 fold increase in potency and improvement in breadth compared to the original antibody (Moshoeite *et al.*, 2019). Similarly, trispecific molecules have also been developed that target multiple sites on the HIV Env trimer along with CD4 and or CCR5. An example of this is 10E8Fab-PGDM1400scFv-PRO140scFv, with PRO140 being an antibody targeting CCR5 (Xu *et al.*, 2017; Steinhardt *et al.*, 2018)

1.11 Aims of this thesis

The potential of scFv of bNAbs has not fully been investigated for their use in passive immunization and or treatment. We, therefore, designed and tested the function of scFv of HIV bNAbs targeting five major epitopes on the HIV trimer. Since these antibodies are significantly smaller, we also examined whether differences in the epitope impacted the neutralization performance of the scFv compared to the IgG This is detailed in chapter 3 of this thesis, which was published in the Journal of Virology in 2020 (van Dorsten *et al.*, 2020).

Secondly, although combinations of antibodies have been tested for IgG and in multi-specific antibodies, this has not been done using scFv. scFv may show decreased steric hindrance and decreased antagonism, compared to IgG. Moreover, we wanted to assess the predictive models used for IgG to determine if they can be similarly applied to scFv, We, therefore, investigated the potential of scFv in combinations in chapter 4 of this thesis.

Lastly, we wanted to investigate the potential of scFv in preventing cell-cell transmission. As scFv are significantly smaller than IgG they could diffuse more readily into lymphoid tissues and mucosal tissues where cell-to-cell transmission likely occurs. In addition, their much smaller size could provide an advantage in accessing virological synapses compared to full-sized IgG. The potential of scFv compared to IgG is investigated in chapter 5 of this thesis.

The data in this thesis indicates that scFv engineered from bNAbs demonstrate remarkable retention of neutralization capacity compared to the IgG. Moreover, they have shown considerable breadth and potency when combined into double or triple combinations. Some of the combinations showed low levels of synergy and an overall improvement in neutralization potential. Lastly, we demonstrated that these scFv retain their function in cell-cell transmission assays in levels comparable to free virus neutralization.

2 Methods

2.1 Generation of scFv

2.1.1 *scFv cloning*

scFv constructs were made from seven different antibodies targeting five epitopes on the HIV trimer (CAP256.25-VRC26.25, PGT121, VRC01, 3BNC117, 8ANC195, 10E8, and 10E8v4). Of these, IgG versions were available in-house for five antibodies. Sequences for 3BNC117 and 10E8v4 scFv and IgG were ordered from GenScript (New Jersey, USA) (3BNC17 V_H-V_L L1 and 10E8v4 V_H-V_L).

scFv were cloned from IgG constructs using primers at the start (5') and (3') end of the variable heavy (V_H) and the variable light (V_L) regions (first round of PCR) (Figure 2.1). Primers matching the sequence of the heavy and light chain of the antibodies were designed (primers were ordered from Inqaba, South Africa). The iProof High-Fidelity DNA polymerase (Bio-Rad, South Africa) was used for all cloning PCRs following the kit instructions. The first antibody specific primer set amplified the variable heavy chain and contains a portion of the signal sequence and the Age1 cut site in the forward primer and an overlap with a portion of the linker in the reverse primer. For the light chain, the antibody specific primer pair contains an overlap of the linker in the forward primer and the HRV-3C cut site and part of the histidine (His) tag sequence in the reverse primer. A 15 amino acid (GGGGS₃) linker from an existing scFv in the lab was also amplified using a forward and reverse primer pair that included overlap into the variable heavy and light chains of the various scFv. This produced three PCR products. At this stage Dpn1 (NEB or FastDigest, Thermofisher) enzyme was added to digest the plasmid DNA, and the reactions were incubated for 30min at 37°C. Afterwards, the reactions were run on a 2% agarose gel (2% agarose, 1x TAE, 625ng/mL Ethidium Bromide) together with a 100bp ladder in 1x TAE at 80V for approximately 1hr, gel excised and purified using the QIAquick Gel Extraction Kit (Qiagen, Hilden Germany).

Next, an overlapping PCR was used to join each fragment into one V_H-V_L construct. A reverse primer (Reverse 2), which overlaps the histidine tag sequence and contains the BamH1 cut site, was combined with the forward primer 1 of the first reaction (Forward V_H 1) and the three PCR products of the variable heavy chain, the linker, and the variable light chain. To clone the V_L-V_H constructs the same methodology was used, however, the overlapping sections were

swapped, with the V_L being cloned with the signal sequence at the 5' end and an overlap in the reverse primer for the linker (3'). The V_H was cloned with an overlap in the 5' end into the linker and the 3' end into the HRV-3C cut site and the His-tag (see appendix 8.1 for full scFv and sequences including the various linkers used).

The pCMVR plasmid and final recombinant PCR product were digested with BamH1 and Age1 fast digest restriction enzymes for 1hr at 37°C using the FastDigest enzymes (Thermo Fisher Scientific, Massachusetts USA). The gel excised vector backbone, and the PCR product were cleaned using the QIAquick Gel Extraction and PCR Purification Kits (Qiagen, Hilden Germany). The vector backbone and the insert were ligated using the Rapid DNA Ligation Kit (Roche, Switzerland) and a molar ratio of insert to vector of 3:1, and incubated for 30min at 25°C.

The plasmids were transformed into JM109 Z-competent *E. coli* (Zymo Research, California USA) by adding 1μL of the ligation mixture to the JM109-Z cells at 4°C for 5min. Thereafter Super Optimal broth with Carbolyte repression (SOC) medium was added in a ratio of 5 volumes of SOC and 1 volume of cells and the cells were shaken at 150rpm for 1hr at 37°C. Then the bacterial cell mixture was spread on LB Agar plates and grown under kanamycin (50μg/mL) selection overnight (ON) at 37°C. Several colonies were picked, and grown ON with 5mL of LB broth with 50μg/mL kanamycin at 37°C. Subsequently, the plasmids were extracted using the QIAprep Spin Miniprep Kit (Qiagen, Hilden Germany) and sequenced with vector primers using 6pmol of primer, 1μL BigDye, 300ng DNA, 1.5μL buffer, and up to 10μL H₂O (BigDye™ Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Massachusetts USA). Sequencing reactions were cleaned up with 100% Ethanol (EtOH) to which Sodium Acetate (NaOAc, 3M, pH4.6) was added to a final concentration of 120mM. The reactions were spun for 30min at 2000g. Afterwards, the EtOH was removed, 70% cold (-20°C) EtOH was added and the reactions were spun for 5min at 2000g. Afterwards, this was removed as well and the reactions air-dried at 65°C for 3min. Sequencing reactions were run on the Applied Biosystems 3500xL Genetic Analyzer.

The sequences of plasmid inserts were compared to the reference sequences and validated minipreps were transformed into JM109-Z cells and grown overnight under kanamycin selection in 200mL of LB at 37°C, shaking at 150rpm in a baffled Erlenmeyer flask.

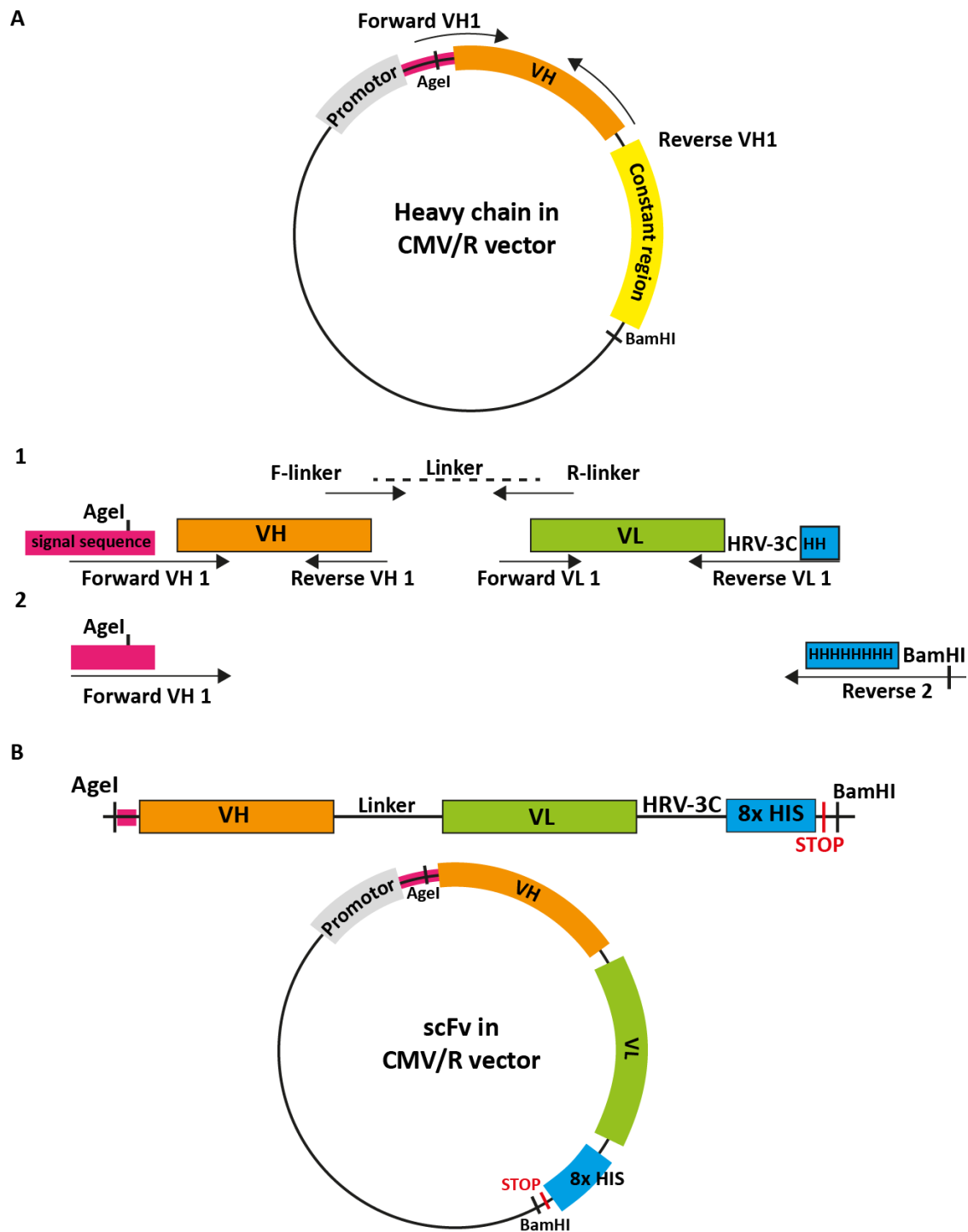


Figure 2.1 Cloning of scFv of HIV antibodies through overlapping PCR.

(A) The variable heavy (VH) and light (VL) chains were cloned from existing heavy and light chain vectors of the IgG in the pCMVR vector. The linker was cloned from an scFv in the pUC vector. For the first round of PCR (1), 3 sets of primers were used to create fragments with overlapping regions between the VH, the linker and the VL. The HRV-3C digestion site, the restriction enzyme site Agel and a portion of the signal sequence were also added by the primers. The second PCR (2) used the same forward primer (Forward VH 1) and the reverse primer containing the 8x HIS-tag and the BamHI restriction enzyme site (Reverse 2) to combine all 3 PCR products into a single amplicon. The HRV-3C site allows cleavage by an enzyme in order to remove the HIS tag. The 8x HIS-tag is used in downstream purification, and the enzyme restriction sites are used to clone the constructs into the vector. (B) The scFv construct in the VH-VL format both as a linear product and in the pCMVR vector.

Cultures were spun down at 1500g for 30min and the supernatants discarded. Plasmids were extracted using the QIAGEN Plasmid Plus Maxi Kit (Qiagen, Hilden Germany). Concentrations were measured on the Nanodrop at the DNA quantification setting and plasmid DNA was aliquoted and frozen.

A total of 6 constructs for each scFv was designed to incorporate three different linker lengths and multiple variable heavy-variable light chain and variable light-variable heavy chain orientations (L1, L2, and L3 in V_H - V_L and V_L - V_H). To create the different linkers, site-directed mutagenesis (QuikChange Lightning Site-Directed Mutagenesis Kit, Agilent Technologies) was used to introduce an additional GGGGS segment into Linker 2 (L2). Linker 3 scFv constructs were ordered from GenScript (New Jersey, USA) (CAP256.25, 8ANC195, PGT121). Amino acid sequences of the final scFv and linkers are shown in chapter 3.

2.1.2 scFv expression

scFv plasmid preparations (1000 μ g) were sterile filtered in 20mL of OptiMEM. Each plasmid was combined with 3mg of fresh linear Polyethylenimine HCl (PEI) max 40,000 in another 20mL OptiMEM and incubated for 20min at room temperature (RT). The transfection mixture was added to 960mL of 293F suspension culture at 1.5×10^6 cells ($P < 40$). The culture was grown in HEK293F freestyle media without additions at 10% CO₂, 70% humidity, and in a 150rpm shaking incubator for 6 days.

To optimize expression several additions or changes to the above protocol were tested. An addition of 100mL HyClone™ SFM4Transfx-293 Media (GE Lifesciences, Massachusetts USA) to the cells did not improve expression. Similarly, longer growth periods (>6 days) did not improve expression levels. In addition, the scFv were expressed in 293EXpi cells, which naturally grow to a higher confluence. However, no improvement in the final protein concentration was observed even though the total protein was increased.

2.1.3 scFv purification

After six days of culture, the suspension cultures were centrifuged at 1500g for 1hr at 10°C. The cells were discarded and the supernatant was filter-sterilized at 0.2 μ m and sodium azide was added to a final concentration of 0.02%. Ni-NTA columns were made using 10mL of Ni-Sepharose (containing 6% agarose beads charged with Nickel ions) (Sigma) in a gravity flow column (Biorad) and equilibrated with Phosphate Buffered Saline (PBS) resulting in a 5mL bed volume. The supernatant was applied to this Ni-NTA column at a speed of 0.5-1mL/min and

the beads were washed with 30mM of imidazole in PBS, pH7.5 with at least 10 column volumes and the protein was eluted with 20mL of 400mM of imidazole (in 1x PBS). The column was then cleaned with PBS and the supernatant reapplied, in the same manner, to make sure all protein was captured. When not in use, protein columns were stored in 70% ethanol (EtOH).

To optimize the final protein output and reduce aggregates, glycerol was added to the eluted protein to a final concentration of 2.5%-5%. The protein was concentrated to <5mL using the Vivapore tabletop concentrators or Vivaspin centrifuge concentrators and applied to a size exclusion column at 2.6mL/min (26/600 HiLoad Superdex or 16/600 HiLoad Superdex) on an Äkta Purifier liquid chromatography system. PBS based buffers were avoided for 8ANC195 scFv as this induced aggregation and PBS was replaced with a salt buffer containing 150mM NaCl and 3mM HEPES solution for all purification steps. The size of the protein was compared to a standard protein mixture. For the 16/600 Superdex (200pg), Thyroglobulin (670kDa), γ -globulin (158kDa), Ovalbumin (44kDa), Myoglobin (17kDa), and Vitamin B12 (1.35kDa) were used. The fractions corresponding to the scFv were collected and pooled (see Appendix 8.2, for data for scFv 10E8v4). The molecular weights of the scFv were calculated using the ExPASy ProtParam program: CAP256.25-VRC26.25 (31.349kDa), 10E8v4 (29.303kDa), 3BNC117 (28.471kDa) and PGT121 (28.889kDa). 8ANC195 (28.996kDa).

For the other four scFv (CAP256.25, 3BNC117, PGT121, and 8ANC195) the 26/600 Superdex (200pg) was used and compared to a low molecular weight protein mixture consisting of Carbonic anhydrase (29kDa), Ribonuclease A (13.7kDa), Ovalbumin (43kDa), and Aprotinin (6.5kDa), (see Appendix 8.2) This column is better at separating smaller molecular weight proteins, which ensures higher purity.

The size exclusion columns were equilibrated with 150mM NaCl, 3mM HEPES, and 2.5% glycerol for proteins that tended to aggregate (3BNC117 and 8ANC195) or 1x PBS with 13mM of NaCl added for the other proteins. The pH of the buffer was adjusted to pH6.5 due to the isoelectric points of scFvs being approximately 7.5. After size-exclusion, proteins were filter sterilized and concentrated to 1mg/mL for further experiments.

2.1.4 SDS-Page

An SDS-Page gel was run to assess the size of scFvs after expression. For the 10% resolving acrylamide gel, 2.5mL TRIS-HCL (1.5M pH 8.8) was mixed with 2.5mL of 40% acrylamide and

100µL of 10% SDS in 4.9mL dH₂O. Just before casting 100µL of 10% ammonium persulfate (APS) and 10µL of N,N,N',N'-tetramethyl ethylene-diamine (TEMED) were added.

This mixture was cast into a gel mould (Bio-Rad) and 100µL of isopropanol was added to the top to exclude air. After 30min at RT, the isopropanol was removed and the top of the gel washed with distilled water and dried for 1min.

The 4.8% acrylamide stacking gel was then prepared using 0.6mL of 40% acrylamide, 1.2mL of Tris-HCL (0.5M pH6.8), 50µL of 10% SDS and 3.1mL of dH₂O. Just before pouring, 50µL of 10% APS and 5µL of TEMED were added. After gently mixing, the stacking gel was poured on the resolving gel and a comb was inserted. The gel was incubated for 30min at RT and then removed from the stand but kept in the glass mould.

The scFv and IgG samples were prepared as follows: 10µg of protein was added to 2µL of 1M DDT (dithiothreitol) and 4µL of 4x sample stain buffer (NuPAGE LDS, Massachusetts USA), and made up to a maximum volume of 20µL. The samples were denatured at 95°C for 5min, cooled at RT for 5-10min, and loaded onto the gel. A protein multicolour ladder as a size reference was run (Novex Sharp prestained protein standard, Thermo Fischer, Massachusetts USA).

The inner chamber and outer chamber were filled with running buffer (10X Running buffer: 30gr of Tris base, 144g of glycine, and 10gr of SDS in 1000mL H₂O). The pH of the buffer was adjusted to pH 8.3. The gel was run at a constant voltage of 200V for 45min-1hr. Thereafter, the gel was carefully removed from the glass mould and washed three times with dH₂O. Coomassie blue stain (TaKaRa, Shiga Japan) was added and the gel was gently agitated at RT for approximately 1-2hrs. The gel was washed three times with dH₂O, destained for 1hr in dH₂O, and then photographed (see Figure 2.4).

Alternatively, precast Invitrogen NuPAGE Bis-Tris Gels 4-12% gradient gels were used which were run in MOPS (3-(N-morpholino)propanesulfonic acid) SDS buffer, (50mM MOPS, 50mM Tris Base, 0.1% SDS, 1mM EDTA, pH 7.7) at 200V (constant) for 50min.

2.2 IgG production

2.2.1 IgG expression

All heavy and light chains of the full antibodies (IgG) were cloned into the pCMVR vector (AIDS Reagent Program, Division of AIDS, NIAID, NIH). For the lambda chain of PGT121, the pBR322 based IG-lambda expression vector was used (AIDS Reagent Program, Division of AIDS, NIAID,

NIH). IgG plasmids were grown in JM109-Z bacterial cells and purified using the Qiagen mini and maxi kits as for the scFv plasmids. After purification, 200µg of heavy chain plasmid and 200µg of light chain plasmid was co-transfected with 1.2mg of PEI in 400mL of 1×10^6 /mL HEK293F cells. After 6 days, the supernatant was collected by centrifugation and filtered through a 0.22µm filter. Supernatants were applied to a Protein-A agarose column. The column was washed with 1x PBS and the proteins eluted using a 0.15M glycine buffer at pH 2.5. The eluted material was neutralized using 1M Tris, pH 8. Up to 5% glycerol was added to prevent aggregation. Afterwards, proteins were dialyzed into PBS pH 6.5 with 5% glycerol at 4°C. To prevent PGT121 from aggregating (which is especially prone to aggregation), the dialysis solution was altered to 150mM NaCl, 3mM HEPES, and 2.5-5% glycerol, and the protein dialyzed at RT. Proteins were concentrated using Vivaspin centrifuge concentrators or Vivapore tabletop concentrators as for the scFv. An extinction coefficient of 13.7 at 1% was used to measure IgG proteins on the Nanodrop. The molecular weights of the IgGs were also calculated using the ExPASy ProtParam program: CAP256.25 (150.71kDa), 10E8v4 (147.29kDa), 3BNC117 (146.24kDa), and PGT121 (146.2kDa).

2.2.2 Generation of pseudovirus stocks for neutralization assays

A total of 45 viruses from a well-described panel containing subtype A, B, and C viruses were grown. (Gray *et al.*, 2011) with the addition of CAP256_SU (Bhiman *et al.*, 2015) and BG505 N332. Plasmids containing cloned *env* genes in the pcDNATM3.1D/V5-His-TOPO[®] vector (Invitrogen, California, USA) were grown as described above, except ampicillin selection was used. Pseudovirus backbone pSG3^{Δenv} (AIDS Reagent Program, Division of AIDS, NIAID, NIH) (Wei *et al.*, 2002, 2003) was grown in Stbl2 cells (Invitrogen) under Ampicillin resistance (50µg/mL) at 30°C and purified using the endotoxin-free maxiprep kit (Qiagen, Hilden Germany). The backbone contains a defective *env* gene and truncated HIV proteins (CTAG in Env insertion and a defective *vpu* gene) which ensures single round infection in the target cells (Wei *et al.*, 2002, 2003).

HEK293T cells were grown overnight in a T75 flask to 50-80% confluence. 4µg of Env plasmid and 4µg of backbone plasmid were combined in 500µL of DMEM. This was mixed with 24µL of PEI max at 1mg/mL and left at room temperature (RT) for 30min. After 15min the transfection preparation was mixed again and carefully added to the HEK293T cells and incubated at 37°C, 5% CO₂ for 48-72hrs. The supernatant was then harvested, filtered through a 45µm filter, and the FBS concentration was adjusted to 20% total. 1mL aliquots were frozen at -80°C. Viral

stocks were tested for infectivity by dilution in TZM-bl cells using a luciferase assay. Pseudovirus levels were diluted to be at least 10-fold above cell background levels based on a titration assay.

2.2.3 Neutralization Assay

Each scFv and IgG were tested in a neutralization assay starting at 200µg/mL for the IgG and 50µg/mL for the scFv, accounting for their different sizes. Proteins were added in duplicate to growth media (DMEM 10% FCS, 25mM HEPES, 0.05mg/mL Gentamicin), and serial 3-fold dilutions were made by transferring 50µL to the next row of wells in a 96-well plate. Pseudovirus was then added to the protein mixture and incubated for 1hr at 37°C. A volume of 20µL of TZM-bl cells (grown in 10% FCS, 25mM HEPES, 0.05mg/mL Gentamicin) was added at 0.5×10^6 cells/mL with 14µL/mL of DEAE at 5mg/mL to the 96-well plate and incubated for 24hrs. After 24hrs, 130µL of media was added to each well to increase the total volume to 200µL. After a further 24 to 48hrs of incubation, 100µL of media was removed and replaced with the Bright-Glo firefly luciferase mixture (Promega, Wisconsin USA). After 2min incubation at RT, the media and luciferase were mixed by pipetting and 150µL transferred to a black 96 well plate. The plate was read on a Victor-X luminometer (Perkin Elmer, Massachusetts USA). Inhibitory concentrations (IC_{50} and IC_{80}) were calculated from the neutralization curves.

2.3 Data Analysis

2.3.1 Statistics

Statistics analyses were performed using the GraphPad 8 built-in software. Survival curves were used to draw the breadth potency curves and t-tests were performed to compare the breadth of IgG and scFv. A Wilcoxon matched-pair signed-rank test was used to assess differences between IgG and scFv within viral subtypes and to determine if potency differences between IgG and scFv were significant. Sequence alignments for viral analysis of IgG- scFv potency differences were made using the Analyze Align tool from the HIV LanL website (https://www.hiv.lanl.gov/content/sequence/ANALYZEALIGN/analyze_align.html).

3 Neutralization breadth and potency of single-chain variable fragments derived from broadly neutralizing antibodies targeting multiple epitopes on the HIV-1 envelope.

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Author contributions

- Rebecca van Dorsten: project and experimental design, experimental work, data analysis, manuscript and figure preparation.
- Bronwen E Lambson: advised and helped to troubleshoot scFv cloning and mutagenesis
- Constantinos Kurt Wibmer: helped design the first scFv and helped to troubleshoot protein expression issues.
- Marc S Weinberg: project design
- Penny L Moore: project/experimental design, data analysis, manuscript corrections/preparation
- Lynn Morris: project/experimental design, data analysis and manuscript corrections/preparation

3.1 Abstract

Passive administration of HIV-directed broadly neutralizing antibodies (bNAbs) can prevent infection in animal models, and human efficacy trials are underway. Single-chain variable fragments (scFv), comprised of only the variable regions of antibody heavy and light chains, are smaller molecules that may offer advantages over full-length IgG. We designed and expressed scFv of HIV bNAbs prioritized for clinical testing that target the V2-apex (CAP256-VRC26.25), V3-glycan supersite (PGT121), CD4 binding site (3BNC117), and MPER (10E8v4). The use of either a 15- or 18-amino-acid glycine-serine linker between the heavy- and light-chain fragments provided adequate levels of scFv expression. When tested against a 45-multisubtype virus panel, all four scFv retained good neutralizing activity, although there was variable loss of function compared to the parental IgG antibodies. For CAP256-VRC26.25, there was a significant 138-fold loss of potency that was in part related to differential interaction with charged amino acids at positions 169 and 170 in the V2 epitope. Potency was reduced for the 3BNC117 (13-fold) and PGT121 (4-fold) scFv among viruses lacking the N276 and N332 glycans, respectively, and in viruses with a longer V1 loop for PGT121. This suggested that scFv interacted with their epitopes in subtly different ways, with variation at key residues affecting scFv neutralization more than the matched IgGs. Remarkably, the scFv of 10E8v4 maintained breadth of 100% with only a minor reduction in potency. Overall, scFv of clinically relevant bNAbs had significant neutralizing activity, indicating that they are suitable for passive immunization to prevent HIV-1 infection.

3.1.1 Importance

Monoclonal antibodies have been isolated against conserved epitopes on the HIV trimer and are being investigated for passive immunization. Some of the challenges associated with full-sized antibody proteins may be overcome by using single-chain variable fragments (scFv). These smaller forms of antibodies can be produced more efficiently, may show fewer off-target effects with increased tissue penetration, and are more adaptable to vectored-mediated expression than IgG. Here, we demonstrate that scFv of four HIV-directed bNAbs (CAP256-VRC26.25, PGT121, 3BNC117, and 10E8v4) had significant neutralizing activity against diverse global strains of HIV. Loss of potency and/or breadth was shown to be due to increased dependence of the scFv on key residues within the epitope. These smaller antibody

molecules with functional activity in the therapeutic range may be suitable for further development as passive immunity for HIV prevention.

3.2 Introduction

Broadly neutralizing antibodies (bNAbs) block infection of diverse HIV strains by targeting relatively conserved epitopes on the HIV envelope trimer (1–3). An HIV vaccine will likely need to elicit these types of antibodies, but this has not yet been achieved. However, some HIV-infected individuals naturally generate bNAb responses, enabling the isolation of monoclonal antibodies with high potency and exceptional breadth and creating the possibility of passive immunization for HIV prevention (4, 5). Animal studies support this approach, with bNAbs such as CAP256-VRC26.25, PGT121, 3BNC117, 10-1074, PGDM1400, VRC01, and VRC07 shown to prevent simian-human immunodeficiency virus infection in nonhuman primate models (6–9). In addition, passively infused bNAbs 3BNC117, 10-1074, and VRC01 substantially reduce viral levels in viremic individuals, confirming their neutralization capacity in vivo (10–15). This has led to the antibody-mediated prevention (AMP) trial, a proof-of-concept study in Africa, Switzerland, and the Americas that is underway to assess whether the bNAb VRC01 can prevent HIV infection in humans (<https://ampstudy.org.za/>).

The promise of passive immunization opens up possibilities for using bNAbs in other formats. Small antibody fragments such as single-chain variable fragments (scFv) may offer advantages over IgG (16–18). scFv consist of the antibody variable heavy and variable light chains and are substantially smaller than IgG molecules (30 kDa versus approximately 147 kDa) (19). This may enhance some of their pharmacokinetic properties and improve their distribution throughout the body and absorption into various tissues. In the context of HIV, the smaller size may enhance diffusion into genital tract mucosal tissues, where most HIV infections occur (5, 16, 20–22). Moreover, as scFv do not have an Fc region, they are less likely to display nonspecific cytotoxicity through cytokine release, although this reduces their half-life significantly and removes their ability to perform Fc-mediated effector functions (16, 22). Antibody scFv have been successfully used in clinical settings particularly for cancer therapy, where their enhanced tissue penetration aids in tumour targeting, and are also being developed for neurological diseases, such as Parkinson's disease, although some anti-drug antibody responses were noted (16, 23, 24).

Antibody scFv targeting the HIV envelope have previously been isolated through phage display, but their neutralization capacity was limited (25, 26). Initial attempts to engineer scFv, immunoadhesins, or other antibody fragments from bNAbs VRC01, PG9, and PG16 showed

poor expression and loss of function compared to the IgG (18). The availability of a larger number of broad and potent bNAbs has revived interest in this area. We, therefore, made scFvs of bNAbs that target 4 distinct epitopes on the HIV envelope trimer, focusing on those undergoing human clinical testing. These included CAP256-VRC26.25 (which binds the V2 apex and is referred to here as CAP256.25) (28), PGT121 (V3-glycan supersite) (29), 3BNC117 (CD4 binding site [CD4bs]) (30), and 10E8v4 (MPER) (31). We found that a reduction in neutralization breadth and/or potency of the scFv compared to the IgG was due to differences in dependence on specific residues in the epitopes. However, overall, scFv largely retained breadth and potency against diverse global HIV isolates and may prove to be useful for future clinical development to prevent HIV acquisition.

3.3 Methods

3.3.1 Construction of scFv

scFv containing the variable regions of various bNAbs were cloned by overlapping PCR or ordered from GenScript (NJ, USA) (Table 1). For CAP256.25 and PGT121, 3 different glycine-serine linkers, namely, a 15-amino-acid linker [linker 1, (GGGGS)³], a 20-amino-acid linker [linker2, (GGGGS)⁴], and an 18-amino-acid linker (linker 3, GGSSRSSSSGGGSGGGG) were tested in the VH-VL orientation. L3 was also tested for the VL-VH orientation. We used an 8-histidine tag for purification purposes preceded by an HRV-3C site in case removal of the histidine tag was needed for future experiments. Table 1 shows the scFv constructs in VH-VL orientation with L3 for CAP256.25. All other scFv (3BNC117 and 10E8v4) were expressed in the VH-VL conformation with linker 1 as specified.

Table 3.1 Amino acid sequences of four scFv constructs derived from HIV bNAbs.

The variable heavy chain (light blue) and variable light chain (yellow) are indicated, as well as the glycine-serine linker (purple) and the 8x histidine tag (red), which is preceded by an HRV3C site (italicized) and is separated from the light chain by a small GG spacer.

scFv construct	Amino acid sequences
CAP256.25 L3 VH-VL	QVQLVESGGGVVQPGTSLRLSCAASQFRFDGYGMHWVRQAPGKGLEWVASISHDGIIKKY HAEKVGWGRFTISRDNKNTLYLQMNSLRPEDTALYYCAKDLREDECEEWSDYYDFGKQ LPCAKSRGGLVGIADNWGQGTMTVSS GGSSRSSSSGGGSGGGG QSVLTQPPSVSAAPGQKVTISCSGNTSNIGNNFVSWYQQRPGRAPQLLIYETDKRPSGI PDRFSASKSGTSGTLAITGLQTGDEADYYCATWAASLSSARVFGTGTKVIVL GGLEVLFGGPHHHHHHHH
PGT121 L1 VH-VL	QMQLQESGPGLVKPSSETLSLTCSVSGASISDSYWSWIRRSPPGKGLEWIGYVHKSGDTNY SPSLKSRVNLSDTSTKNQVLSLSVAATAADSGKYYCARTLHGRRIYGIVAFNEWFTYFY MDVWNGNTQVTVSS GGGSGGGSGGGSGGS DISVAPGETARISCGEKSLSRAVQWYQHRAGQAPSLIIYNNQDRPSGIPERFSGSPDS PFGTTATLTITSVEAGDEADYYCHIWDNRVPTKWVFGGGTTLTVL GGLEVLFGGPHHHHHHHH
3BNC117 L1 VH-VL	QVQLLQSGAAVTKPGASVRVSCASGYNIRDYFIHWWVRQAPGQGLQWVGWINPKTGQPN NPRQFQGRVSLTRHASWDFDTFSFYMDLKALRSDDTAVYFCARQSDYWDFDVWGSQTQ VTVSS GGGSGGGSGGGSGGS DIQMTQSPSSLSASVGDVTITTCQANGYLNWYQRRGKAPKLLIYDGSKLERGVPSRFS GRRWGQEYNLTINNLPEDIATYFCQVYEFVVPGTRLDLK GGLEVLFGGPHHHHHHHH
10E8v4 L1 VH-VL	EVRLVESGGGLVKPGGSLRLSCSASGFDNWMWVRQPPGKGLEWVGRITGPGEWGS VDYAESVKGRFTISRDNKNTLYLEMNNVRTEDTGYFCARTGKYYDFWSGYPPGEEYF QDWGQGTLLIVSS GGGSGGGSGGGSGGS ASELTQDPAVSVALKQTVTITCRGDSLRSHYASWYQKKPGQAPVLLFYGKNNRPSGIPD RFGSGASGNRASLTITGAQAEDEADYYCSSRDKSGSRLSVFGGGTKLTVL GGLEVLFGGPHHHHHHHH

3.3.2 Protein expression

All constructs were cloned into the pCMVR vector (AIDS Reagent Program, Division of AIDS, NIAID, NIH). For the lambda chain of PGT121, the pBR322-based IG-lambda expression vector was used (AIDS Reagent Program, Division of AIDS, NIAID, NIH). The constructs were grown in JM109 bacterial cells and extracted using a plasmid Maxiprep kit (Qiagen, Hilden Germany). Sequences were confirmed using the Applied Biosystems 3500xL Genetic Analyzer. Constructs were expressed as previously described. In short, HEK293F suspension cells at 1.5×10^6 to 2×10^6 cells/ml were cotransfected with linear polyethyleneimine hydrochloride (molecular weight, 40,000) at a 3:1 ratio with 1 µg of plasmid per 1 mL of culture. Supernatants were harvested after 6 days. Purification of scFv. scFv were purified using Ni-Sepharose beads (GE Healthcare, Massachusetts USA). Proteins were washed using a 30mM imidazole–phosphate-buffered saline (PBS) solution and eluted using 400mM imidazole in PBS. Glycerol was added to the elution at a final concentration of 5% to limit aggregation. scFv were applied to HiLoad Superdex 75 or Superdex 200 columns (GE Healthcare) equilibrated with PBS at pH 6.5 (5% glycerol with 0.02% sodium azide). The fractions corresponding to the size of the scFv were collected, pooled, and concentrated using Vivaspin concentrators or Vivapore static concentrators (GE Healthcare). The samples were dialyzed overnight at room temperature to remove sodium azide. Concentrations were measured on a NanoDrop device (ThermoFisher, MA, USA), with extinction coefficients at 1% calculated using ExPASy ProtParam and characterized by SDS-PAGE. Molar weight was determined by using ExPASy ProtParam (Figure 3.1A). scFv proteins were stored at -75°C.

3.3.3 IgG production

IgG constructs were expressed in HEK293F cells as described previously. Supernatants were harvested after 6 days and purified using a protein A affinity column. Proteins were eluted using a 0.15 M glycine buffer at pH 2.5 in 1 M Tris, pH 8, and were concentrated and dialyzed into PBS, pH 6.5, containing 5% glycerol. Concentrations were measured on a NanoDrop device using an extinction coefficient of 13.7 and a 1% solution. The molecular weight of the IgG was calculated using ExPASy ProtParam (69) for CAP256.25 (150.71 kDa), 10E8v4 (147.29 kDa), 3BNC117 (146.24 kDa), and PGT121 (146.2 kDa). IgG and proteins were stored at -75°C.

3.3.4 Neutralization assay

Single-round infection pseudoviruses were grown and titres determined as described previously (70–72). Briefly, plasmids were transfected with the pseudovirus backbone pSG3^{Δenv} into 293T cells at ratios of 1:3 PEI Max. Viruses were harvested after 48h, filtered, frozen, and titrated. A panel of 43 viruses plus BG505 N332 and CAP256_SU (CAP256.3 mo.9C), representing HIV-1 clades A, B, and C, was used to compare neutralization titres of IgG and scFv. Neutralization assays were performed in TZM-bl cells as described previously. Proteins were tested at 200μg/mL for the IgG (~146 kDa) and 50μg/mL for the scFv (28 to 32 kDa). scFv neutralization titres were recalculated by compensating for the fold difference in molecular weight (values for each scFv are shown in Figure 3.1A). (The IC₅₀ data for scFv and IgG molar concentrations are available on request). All assays were repeated at least twice. Geometric mean potency was calculated for both IgG and scFv using sensitive viruses only. When comparing viruses mutated by site-directed mutagenesis, a fold change of more than 3 compared to the parent virus was considered significant.

3.3.5 Site-directed mutagenesis

Mutations in the envelope gene of viruses were made using the QuikChange Lightning site-directed mutagenesis kit (Agilent). Sequences were confirmed using the Applied Biosystems 3500xL Genetic Analyzer. Pseudoviruses were grown as described above.

3.3.6 Statistics and sequence analysis

Wilcoxon matched-pair signed-rank test was used to assess differences between IgG and scFv within a subtype. Sequence alignments were made using the Analyze Align tool from the HIV LanL website

(https://www.hiv.lanl.gov/content/sequence/ANALYZEALIGN/analyze_align.html) .

3.3.7 Structural modelling

We used the PyMOL Molecular Graphics System (version 2.1.1; Schrödinger, LLC) to model a BG505 SOSIP.664 trimer (PDB entry 5V8L). Residues corresponding to the sequences in the CATNAP LANL for CAP256_SU and AC10.0.29 were modelled and coloured based on charge. For 3BNC117, we used Fab structures, either in complex with a clade A gp120 from 93TH057 with G429 (4JPV) or in complex with the clade A BG505 SOSIP.664 trimer with R429 (PDB entry

5V8L). We used PyMOL to visualize the 429 residue and used the built-in software to predict polar bonds.

3.4 Results

3.4.1 Construction and optimization of scFv derived from HIV bNAbs

We designed scFv of bNAbs that target four major epitopes on the HIV-1 envelope (CAP256.25, PGT121, 3BNC117, and 10E8v4) to determine if they retain neutralization breadth and potency. These scFv consisted of the variable heavy chain and the variable light chain of the parent IgG connected by a glycine-serine linker with a histidine tag for purification (amino acid sequences of each construct used to generate scFv are shown in Table 1). As linker length can be a major determinant of paratope formation, we tested linkers of three different lengths. Heavy (VH)- and light (VL)-chain orientation was also tested for one of the linkers. CAP256.25 and PGT121 were used to first assess expression and function of these different constructs (Figure 3.1A). For CAP256.25 scFv, the 18-aminoacid linker (termed L3) in both VH-VL and VL-VH orientations had the highest expression. The 15-amino-acid (GGGGS)³ linker (L1) showed lower expression levels, and the 20-amino-acid (GGGGS)⁴ linker (L2) was poorly expressed. For PGT121, the highest expression was observed for L1 in the VH-VL orientation. We next tested whether the different linkers affected neutralization breadth and potency using panels of sensitive viruses tailored to each bNAb. Since scFv are roughly 5 times smaller than IgG, the 50% inhibitory concentration (IC₅₀) values were multiplied by the molecular weight fold difference between each scFv and its matched IgG (values shown in Figure 3.1A and explained in Materials and Methods).

For CAP256.25, all three linkers in the VH-VL orientation showed similar neutralizing activity against a panel of 36 viruses, while the VL-VH L3 (purple) had a 2-fold lower geometric mean potency (Figure 3.1B). We therefore used CAP256.25 scFv VH-VL L3, which showed the highest expression levels, to conduct further experiments. Similarly, no significant differences were observed for the different PGT121 scFv constructs against an 18-virus panel (Figure 3.1A), and PGT121 VH-VL L1, which showed the highest expression levels, was used. Based on this, 3BNC117 and 10E8v4 scFv were expressed in the VH-VL conformation with the L1 linker. Both expressed relatively well and showed good neutralizing activity (Figure 3.1A).

A

	Construct		Molecular Weight	Molecular weight fold difference for IgG vs. scFv	Expression	Geometric Mean IC ₅₀ in µg/mL *	% viruses neutralized +
CAP256.25 <i>V2-Apex</i>	L1	V _H -V _L	30.96 kDa	4.86	1-2 mg/L	1.9	67%
	L2	V _H -V _L	31.27 kDa	4.82	<1 mg/L	1.4	67%
	L3	V _H -V _L	31.35 kDa	4.81	>2 mg/L	1.9	67%
	L3	V _L -V _H	31.35 kDa	4.81	>2 mg/L	3.8	53%
PGT121 <i>V3 Glycan</i>	L1	V _H -V _L	28.89 kDa	5.06	>2 mg/L	11.6	56%
	L2	V _H -V _L	29.20 kDa	5.00	< 1 mg/L	18.0	50%
	L3	V _H -V _L	29.28 kDa	4.99	1-2 mg/L	18.0	50%
	L3	V _L -V _H	29.28 kDa	4.99	1-2 mg/L	11.5	56%
3BNC117 <i>CD4bs</i>	L1	V _H -V _L	28.47 kDa	5.14	1-2 mg/L	2.3	82%
10E8v4 <i>MPER</i>	L1	V _H -V _L	29.30 kDa	5.03	> 2 mg/L	1.2	100%

* Geometric mean adjusted for molecular weight difference with IgG
+Smaller panel of viruses tested for CAP256.25 and PGT121

B

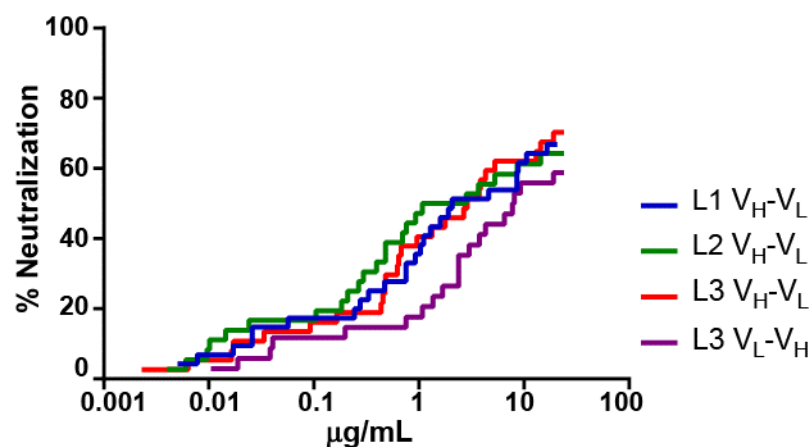


Figure 3.1 Design, expression, and testing of scFv constructs of HIV bNAbs.

(A) A total of 10 scFv constructs were made, with different glycine-serine linkers for CAP256.25 and PGT121 and L1 (GGGGGS)³, L2 (GGGGGS)⁴, and L3 (GGSSRSSSSGGGGSGGGG) for V_H-V_L and L3 for V_L-V_H. For 3BNC117 and 10E8v4, only L1 V_H-V_L was made. The molecular weights and protein expression levels for each scFv are shown together with the adjusted geometric mean neutralization potency. A selected panel of viruses was used to assess CAP256.25 scFv (34 viruses) and PGT121 scFv (18 viruses), whereas the full 45-virus panel is shown for 3BNC117 and 10E8v4. (B) Breadth-potency curves of CAP256.25 scFv made with 3 different linkers in the V_H-V_L orientation and for L3 in the V_L-V_H orientation. (scFv is corrected for molecular weight size difference by multiplying the IC₅₀ by the fold difference in molecular weight between the IgG and the scFv, a value of ~5x).

3.4.2 HIV bNAb scFv retain good neutralization breadth and potency

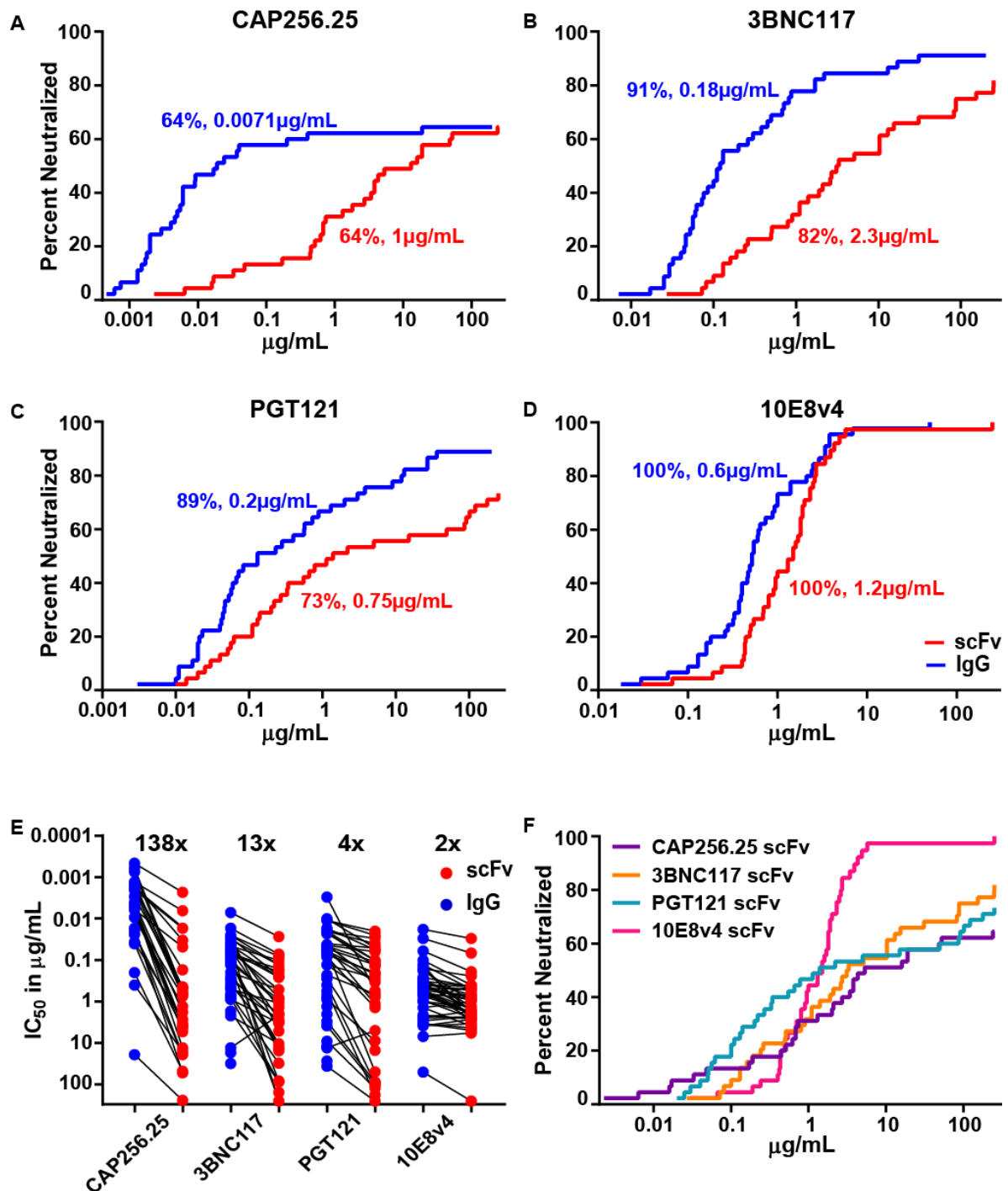


Figure 3.2 Neutralization breadth and potency of scFv compared to those of IgG for 4 HIV bNAbs. (A to D) Breadth-potency curves of scFv (red) and IgG (blue) of CAP256.25 (A), 3BNC117 (B), PGT121 (C), and 10E8v4 (D) using data from a 45-virus multisubtype panel tested in the TZM-bl neutralization assay. The IC₅₀ of viruses against the percentage of viruses neutralized is given. Percent neutralization and geometric mean IC₅₀ potency, in micrograms per milliliter (corrected for molecular weight size difference by multiplying their IC₅₀ by the fold difference in molecular weight between IgG and scFv, ~5x), are shown in each plot. (E) Potency losses of scFv (red) versus IgG (blue) against the same panel. Viruses resistant to both IgG and scFv were excluded. (F) Comparison of breadth-potency curves for all 4 scFv bNAbs against the 45-virus panel.

All four scFv were tested for neutralizing activity against a larger multiclade panel of 45 viruses for breadth and potency. Compared to their parental IgG proteins, all scFv retained substantial neutralizing activity, although they showed some reduction in breadth and/or potency. CAP256.25 scFv maintained breadth of 64% equivalent to the full IgG, but there was a significant ($P<0.0001$) 138-fold decrease in geometric mean potency compared to that of IgG (from 0.0071 to 1 µg/mL) (Figure 3.2A). While all viruses in the panel were less potently neutralized by CAP256.25 scFv, some viruses were more severely affected, with >1,000-fold loss of potency for Q23.17, Q842.D12, and AC10.0.29 (Figure 3.3).

The scFv of 3BNC117 and PGT121 showed an overall loss in both potency and breadth of neutralization. For 3BNC117, the scFv was 13-fold less potent than the IgG ($P<0.0001$) and had slightly lower breadth of 82% compared to 91% for the IgG (Figure 3.2B). PGT121 scFv had 4-fold reduced potency ($P<0.0001$) and breadth of 73% compared to 89% for the IgG (Figure 3.2C). This was a result of the scFv losing activity against viruses such as REJO.67 and QH0692.42 (Figure 3.3). However, even in these cases, the scFv showed low levels of maximum neutralization (>20% but <50%) at the highest concentration tested, indicating that the scFv still bound to resistant viruses (highlighted in Figure 3.3 with an asterisk). Despite the significant potency losses for some viruses, there were a number of viruses that were neutralized equally well by the scFv and by the IgG of PGT121 and 3BNC117, a phenomenon that was not seen with CAP256.25 scFv (Figure 3.3).

The MPER antibody 10E8v4, when expressed as an scFv, showed no loss of function. It maintained 100% breadth of the panel with a minor 2-fold reduction in geometric mean potency compared to the IgG version (Figure 3.2D). A significant reduction in potency was noted for only one virus, TRO.11, although it was still neutralized (Figure 3.3).

Comparison of the potency of all four scFv relative to their IgG clearly demonstrates the significant loss of function for CAP256.25 scFv (Figure 3.2E). Nonetheless, the breadth-potency curve of CAP256.25 scFv still overlapped that of the other scFv (Figure 3.2F), a consequence of the exceptional potency of the parental CAP256.25 IgG. The superior neutralization breadth of the 10E8v4 scFv was also evident in these breadth-potency curves. Overall, the median IC_{50} for all 4 scFv were in the range of 1 to 2 µg/mL, which is close to the therapeutic range for VRC01 that is currently undergoing human efficacy testing.

		IC ₅₀ of IgG and scFv in µg/mL											
		CAP256.25			3BNC117			PGT121			10E8v4		
		IgG	scFv	Fold	IgG	scFv	Fold	IgG	scFv	Fold	IgG	scFv	Fold
Subtype A	Q23.17	0.0017	2.1	1210	0.046	0.10	2	0.011	0.020	2	2.5	2.3	1
	Q168.A2	0.00074	0.62	844	0.076	0.19	2	36	250	7	0.93	2.5	3
	Q259.D2.17	0.0046	3.4	735	0.046	250	5482	13	5	0.4	6.9	5.7	1
	Q461.E2	0.024	4.3	183	0.044	0.26	6	>200	>250	~	2.1	2.6	1
	Q769.D22	>200	>250	~	0.017	0.072	4	>200	>250	~	3.8	2.7	1
	Q842.D12	0.019	19	1007	0.0071	0.027	4	0.083	1.39	17	4.1	2.8	1
	BG505.W6M 332N	0.0013	0.68	522	0.025	0.081	3	0.044	0.062	1	0.40	0.95	2
Subtype B	CAAN5342.A2	>200	>250	~	0.88	3.3	4	0.042	0.34	8	3.4	4.9	1
	AC10.0.29	0.017	52	3003	17	>250	>14.7	0.072	0.20	3	0.39	0.54	1
	RHPA4259.7	>200	>250	~	0.025	0.13	5	0.046	0.11	2	0.60	1.9	3
	TRO.11	>200	>250	~	0.029	0.24	8	0.067	0.33	5	0.16	1.8	12
	PVO.4	0.037	2.7	71	0.072	0.16	2	0.23	0.66	3	3.8	3.4	1
	SC422661.8	>200	>250	~	0.062	0.51	8	0.28	15	56	0.49	0.49	1
	REJO.67	>200	>250	~	0.04	1.4	33	1.3	>250*	>132	0.61	0.90	1
	TRJO4551.18	>200	>250	~	0.11	13	121	9.0	121	13	2.9	2.3	1
	WITO.33	>200	>250	~	0.032	0.13	4	0.73	>250*	>342	0.32	0.43	1
	THRO4156.18	19	240	13	2.2	10.3	5	>200	>250	~	0.46	0.80	2
	QH0692.42	>200	>250	~	0.2	0.5	2	0.56	>250	>444	0.52	1.9	4
	6535.3	>200	>250	~	0.71	30.8	44	0.020	0.014	1	0.18	0.95	5
Subtype C	Du151.2	0.0020	0.17	83	>200*	154	1	0.011	0.025	2	0.37	1.6	4
	Du156.12	0.0040	0.190	48	0.056	2.7	48	0.021	0.14	7	0.03	0.067	2
	Du172.17	>200	>250	~	0.68	>250	>367	0.057	49.4	865	0.10	0.4	4
	Du422.1	0.0092	1.3	145	>200	>250*	~	0.047	1.13	24	0.47	0.8	2
	ZM53M.PB12	0.00046	0.0023	5	0.38	10.3	27	0.0030	0.55	183	3.4	4.3	1
	ZM109F.PB4	0.0060	5.3	880	0.13	247	1962	12	>250*	>20	0.13	0.5	4
	ZM135M.PL10A	>200	>250	~	0.06	0.26	4	2.0	>250*	>125	0.13	0.4	3
	ZM197M.PB7	0.0060	0.048	8	0.45	2.6	6	>200	>250	~	0.06	0.19	3
	ZM214M.PL15	0.0060	3.8	640	0.12	0.9	7	0.06	0.22	4	0.54	1.5	3
	ZM233M.PB6	0.0020	0.017	9	0.30	1.9	6	3.0	86	29	0.16	0.24	2
	ZM249M.PL1	0.0050	2	442	0.10	1.1	11	0.88	2.2	2	0.37	1.5	4
	CAP8.6F	0.20	48	240	31	>250*	>8	0.017	0.056	3	0.88	0.42	0.5
	CAP45.2.00.G3	0.00060	0.51	851	1.7	>250	>147	0.57	177	310	0.33	0.69	2
	CAP61.4.22.F10A	0.040	19	480	0.11	15.4	144	0.053	0.13	2	2.4	1.7	1
	CAP63.A9	0.0090	0.74	82	0.26	3.1	12	0.13	0.78	6	0.52	2.7	5
	CAP84.32	0.40	16	40	0.085	82	966	0.010	0.010	1	1.0	1.3	1
	CAP85.9	>200	>250	~	1.7	87	51	0.13	0.27	2	0.40	0.43	1
	CAP88.B5	>200	>250	~	0.82	87	107	0.40	91	228	0.018	0.030	2
	CAP206.8	0.0020	0.68	340	>200	>250*	~	3.8	101	27	0.26	0.44	2
	CAP210.2.00.E8	0.0018	0.016	9	13	2.1	0.2	27	>250*	>9	1.0	1.8	2
	CAP228.2.00.51J	0.0055	3.8	686	0.056	>250*	>4400	27	>250	>9	50	250	5
	CAP239.2.00.G3J	0.0029	0.44	149	>200*	>250*	~	0.023	0.11	5	0.54	0.7	1
	CAP244.D3	>200	>250	~	0.13	10.3	78	>200	>250	~	0.28	1.3	5
	CAP255.2.00.16J	>200	>250	~	0.029	5.1	179	0.040	0.051	1	0.73	1.8	3
	CAP256.SU	0.0013	0.0064	5	0.50	1.1	2	0.020	0.040	2	1.4	3.9	3
	ConC	0.0015	0.033	22	0.052	6	121	0.020	0.030	2	1.4	1.0	1
Geometric mean (µg/mL)		0.0071	1.0	138	0.18	2.3	13	0.20	0.75	3.7	0.62	1.2	2
Breadth		64.4%	64.4%		91.1%	82.2%		88.9%	73.3%		100.0%	100.0%	

Figure 3.3 Neutralization data for IgG and scFv of HIV bNAbs tested against the 45-virus panel.

The IC₅₀ values for both IgG and scFv against 7 subtype A, 12 subtype B, and 26 subtype C viruses are shown in micrograms per milliliter. IgG was tested at a starting concentration of 50µg/mL, whereas scFv started at 250µg/mL. Fold changes between IgG and scFv are shown. The colors indicate the potency at which the viruses were neutralized, with the warmer colors indicating higher potency. Resistant viruses are colored in light blue. Maximum levels of neutralization for scFv and IgG of <50% but >20% are indicated with an asterisk. Geometric mean titers, fold difference from IgG, and breadth against the 45-virus panel are shown at the bottom of the table. (scFv titers were corrected for molecular weight size difference by multiplying their IC₅₀ by the fold difference in molecular weight between the IgG and the scFv, as noted in Figure 3.1A)

3.4.3 Loss of potency in CAP256.25 scFv linked to epitope charge

In order to probe the loss of potency for CAP256.25 scFv, we tested the effect of point mutations in the V2 epitope of the autologous virus CAP256_SU. Mutations L165V and K171N

had no effect on either the scFv or IgG (Figure 3.4A). Similarly, a conservative K169R mutation did not impact the neutralizing activity of either protein. The T162I mutation (which removes the conserved glycan at position 160) and the K170Q mutations had a small effect on the scFv (3- and 4-fold, respectively) but not on the IgG (below 3-fold). However, the scFv was more affected by the K169I substitution (11-fold for the scFv versus 3-fold for the IgG), the K169Q (20- versus 6-fold), the K169T (35- versus 12-fold), and the R166K (75- versus 4-fold) mutations. For both scFv and IgG, a K169E mutation resulted in complete neutralization resistance. Therefore, while neutralization by the scFv and IgG were overall similarly influenced by mutations in the epitope, the consequence of individual mutations was more pronounced for the scFv.

To further explore this, we partitioned CAP256.25-sensitive viruses into those with a greater than 10-fold potency loss for the scFv compared to that of the IgG (24 viruses) versus those with less than a 10-fold change (5 viruses) (Figure 3.4B; glycans, N(X)T/S, are indicated by a pink O in the logograms). Differences at residue 160 or 166 between these two groups did not explain the general loss of potency by the scFv. Some variation was noted at position 169, with 169M (2/24), 169N (1/24), 169V (1/24), and 169R (3/24) found in viruses where the scFv lost potency. However, in 21/24 viruses with a large potency loss, an uncharged glutamine (Q) was present at position 170 rather than a charged lysine (K) or arginine (R). We therefore introduced Q170K into three viruses (AC10.0.29, Q23.17, and Du156.12) along with 169K in AC10.0.29 (M169K) and Q23.17 (R169K). Individual 169K and 170K mutations significantly improved neutralization by the scFv in both Du156.12 and Q23.17 (but not AC10.0.29) but had no significant effect on the IgG (Figure 3.4C). However, the combination of these mutations significantly improved scFv neutralization in both Q23.17 (70-fold) and AC10.0.29 (86-fold) compared to a minimal effect (2- and 3-fold, respectively) for the IgG. These data suggested that CAP256.25 scFv neutralization was more dependent on the charge of the epitope than the matched IgG.

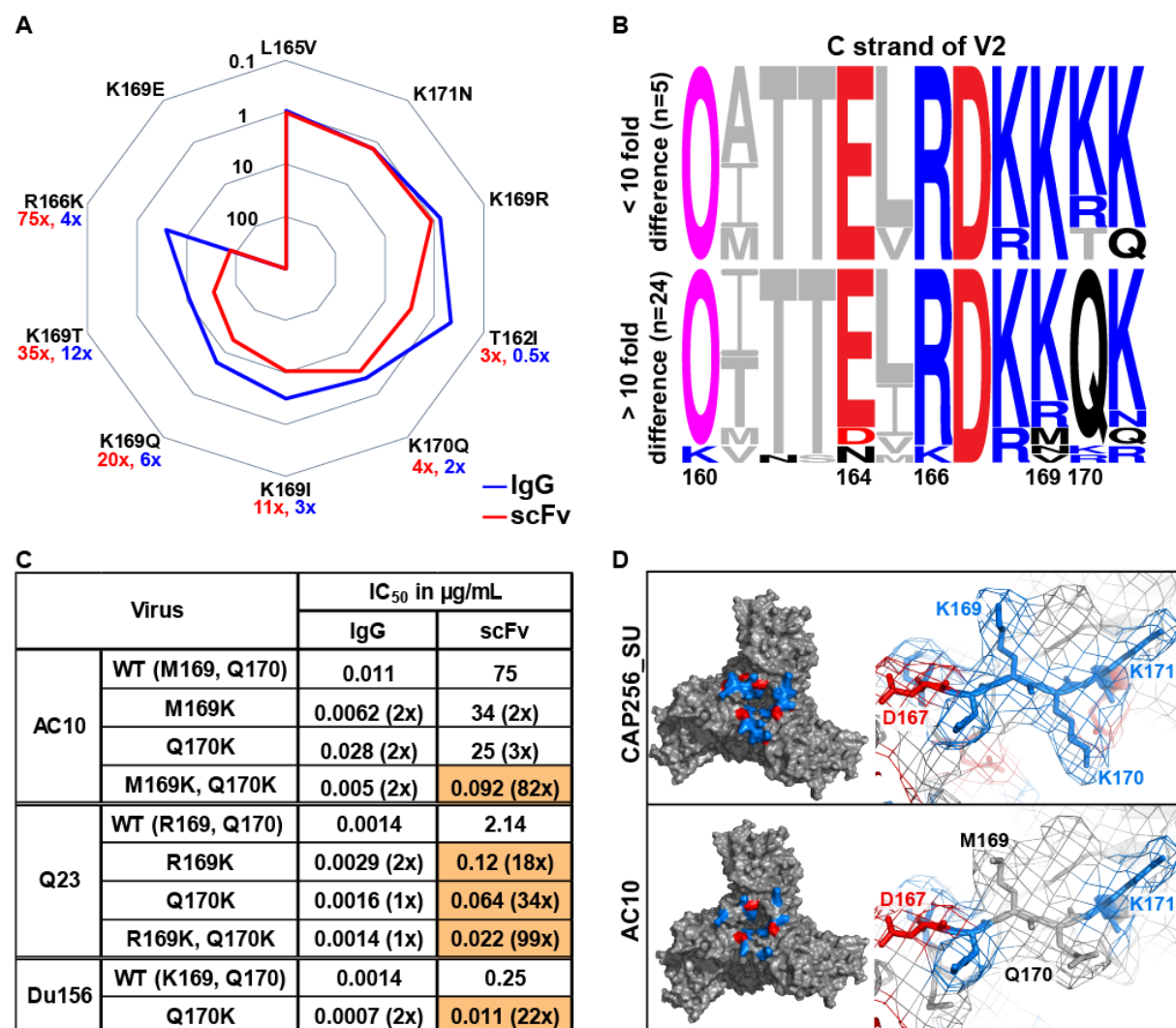


Figure 3.4 Charged amino acids in V2 reduced CAP256.25 scFv neutralization.

(A) Spiderplot showing fold change in neutralization of V2 mutants relative to CAP256_SU wild type (WT) by the CAP256.25 IgG (blue) and the scFv (red). A fold change of 1 indicates no difference from the WT virus. Values closer to the center indicate resistance, whereas those on the outer bounds indicate a sensitivity. (B) Amino acid sequence alignment of viruses neutralized by scFv and IgG with similar potency (top) or more than 10-fold decrease in potency (bottom). The N160 glycan is denoted by an O and is colored magenta. Negatively and positively charged residues are indicated by red and blue, respectively. (C) IgG and scFv neutralization (IC₅₀) of three viruses with point mutations at positions 169 and 170. Fold changes relative to the wild type are indicated in parentheses, and those greater than 5-fold are colored orange. scFv is corrected for the molecular weight size difference (multiplied ~5x). (D) Structural representation of the V2 apex of the HIV envelope trimer of a CAP256.25 scFv-sensitive virus (CAP256_SU) and a less sensitive virus (AC10.0.29). The apex of the trimer is shown on the left, and a close-up of positions 167 to 171 is shown on the right. Positive charges are colored blue, whereas negative charges are colored red. (Model is based on 5V8L structure of BG505 SOSIP.664 [78] and was modified using PyMOL software.)

To visualize the effect of charge in the V2 apex of different envelopes, models of the HIV Env of CAP256_SU and AC10.0.29 were generated (Figure 3.4D). CAP256_SU, which is similarly neutralized by both scFv and IgG, has charged lysine residues at positions 169 and 170 (Figure 3.4D). These 169K and 170K residues formed a cluster of positive charges, at the apex of the trimer. In contrast, AC10 displayed a reduced charge in the epitope, with neutral amino acids occupying these positions (M169 and Q170), possibly explaining the observed virus-specific loss of potency.

3.4.4 PGT121 scFv neutralization affected by both the N332 glycan and V1 length

The glycan at position N332 is a key part of the epitope of V3-directed bNAbs, such as PGT121 (29, 32). To determine the dependence of the scFv on the N332 glycan, we deleted this glycan in three viruses (Q23.17, Du156.12, and TRO.11) through an N332A mutation. There was a significant loss of scFv potency for all 3 viruses, while for the IgG, this effect was seen in 2 of the 3 viruses (Figure 3.5A, left). We also introduced this glycan into resistant viruses Q168.A2, REJO.67, CAP45.2.00.G3, and ZM197M.PB7, which conferred neutralization sensitivity to both the scFv and the IgG of PGT121 (Figure 3.5A, right), although not always to the same level as the IgG.

We next analysed the sequences of the 45 viruses tested, dividing them into 3 groups: viruses equally sensitive to the IgG and scFv (Figure 3.5B, upper, 21 viruses), viruses with a 10-fold-reduced sensitivity to the scFv and/or IC_{50} of $>200\mu\text{g/mL}$ for the scFv (Figure 3.5B, middle, 19 viruses), and viruses resistant to both the IgG and the scFv (Figure 3.5B, lower, 5 viruses). There was no difference in the GDIR motif between the groups. As expected, 21/21 viruses sensitive to PGT121 had a glycan at position N332 (indicated by a pink O in the logograms). In contrast, viruses resistant to both scFv and IgG lacked the N332 glycan, with 2 viruses showing a glycan shift to N334 and 3 lacking either glycan. In viruses where the scFv lost potency (Figure 3.5B, middle), only 7 out of 19 had either a glycan shift to N334 or lacked a glycan at N332/N334. This suggested that while both the PGT121 IgG and scFv were dependent on the N332 glycan, the reduced potency and breadth of the scFv was only partly due to an increased dependence on this glycan.

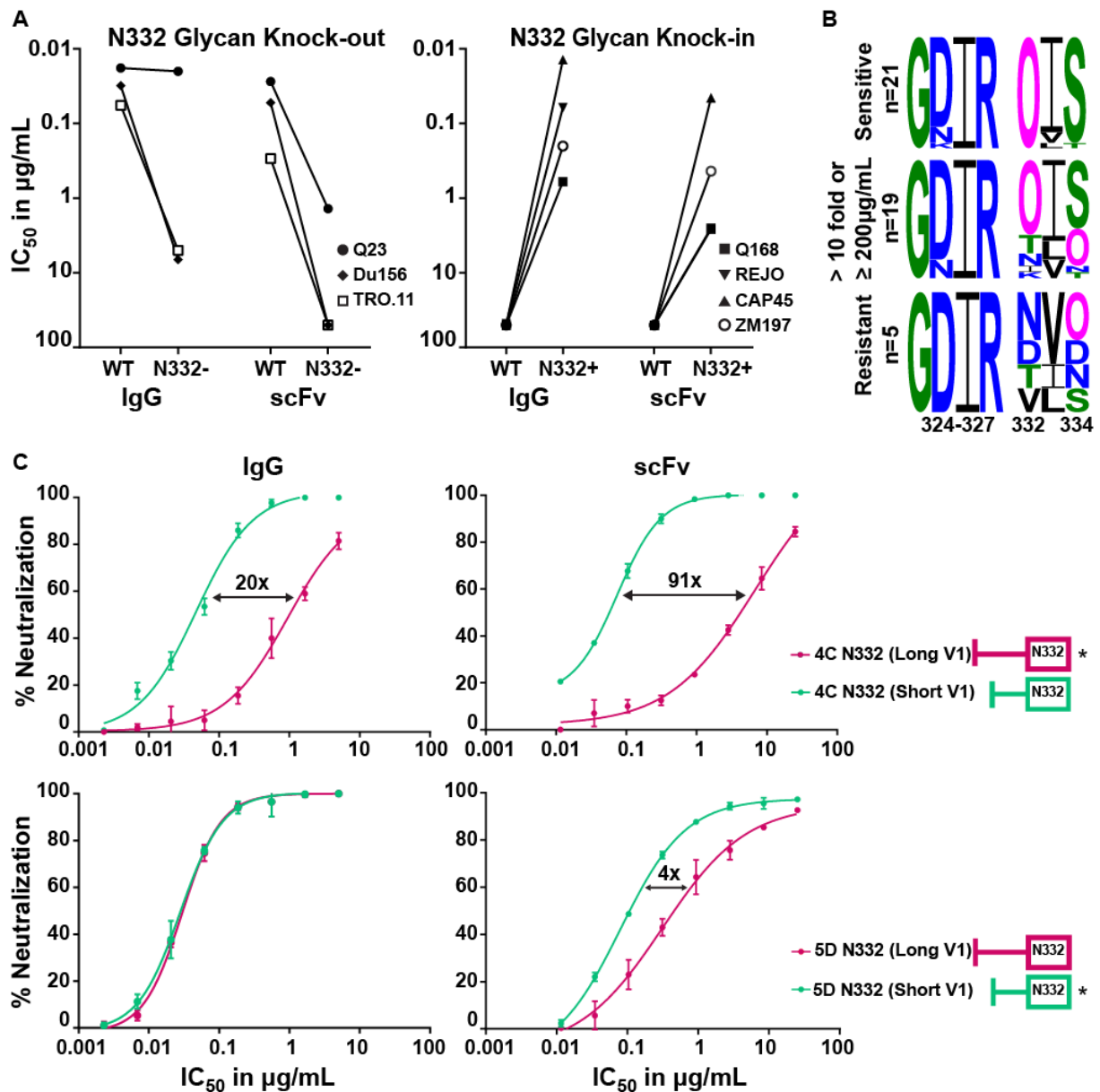


Figure 3.5 PGT121 scFv neutralization was dependent on the N332 glycan and the V1 loop length. (A) IC_{50} of IgG and scFv against 3 sensitive viruses (WT) with their N332 glycan knockouts (N332-) (left) and 4 resistant viruses (WT) with matched N332 glycan knockins (N332+) (right). (B) Noncontiguous amino acid sequences of the V3 region (positions 324 to 327 and 332 to 334, encompassing the GDIR motif and the N332/4 glycans). Viruses are divided into three groups: viruses neutralized equally well by IgG and scFv of PGT121 (top), those with >10 -fold difference and/or $>200\mu\text{g/mL}$ for scFv (middle), and viruses resistant to both scFv and IgG (bottom). Glycans [N(X)T/S] are shown as a pink O. (C) Neutralization curves of IgG and scFv (in micrograms per milliliter) against two CAP177 clones, 4C, which has a long V1 (dark pink, star), and 5D, with a short V1 (green, star), and their V1 mutant swaps (no stars). Fold differences between the wild-type and mutant swaps are indicated. scFv is corrected for the molecular weight size difference (multiplied ~ 5 times).

V3-directed bNABs are also affected by the length of the V1 region (33, 34), so we assessed whether this contributed to the loss of scFv neutralization. We used two related viruses isolated from the same individual, donor CAP177, that showed differential sensitivity to

PGT121. The CAP177-4C virus contains a long V1 loop (dark pink) (Figure 3.5C, upper), while CAP177-5D has a short V1 loop (green) (Figure 3.5C, lower). V1 swaps between these 2 clones were made and tested for sensitivity to both scFv and IgG. Inserting a short V1 in CAP177-4C (Figure 3.5C, upper) increased the potency of PGT121 IgG by 20-fold (from 0.94 μ g/mL to 0.047 μ g/mL) but had a substantially greater 91-fold effect on the scFv (from 6.7 μ g/mL to 0.074 μ g/mL). Similarly, there was a 4-fold loss in potency for the scFv when a long V1 was introduced into CAP177-5D (from 0.080 μ g/mL to 0.31 μ g/mL), with no effect on the IgG (Figure 3.5C, lower). The length of V1, therefore, had a greater effect on the neutralization potency of the scFv than the IgG of PGT121.

3.4.5 Loss of potency for the 3BNC117 scFv more pronounced for subtype C viruses

The N276 glycan is highly conserved and a major contact residue for CD4 binding site antibodies, including 3BNC117 (30, 35). To interrogate the loss of potency for the 3BNC117 scFv, we introduced this glycan into a resistant virus or removed it from three sensitive viruses (Figure 3.6A). Insertion of the N276 glycan into CAP239.2.00.G3J (through an L278T mutation) rendered this resistant virus highly sensitive to IgG, although the effect on the scFv was marginal. However, removal of the N276 glycan in three viruses had a more significant effect on the scFv than the IgG, with the N276A mutation causing a 10-fold increase in resistance or a knockout effect (Figure 3.6A). This indicated that the scFv was more dependent on the 276 glycan for neutralization than the IgG.

CD4bs antibodies have been shown to have some subtype preference, often exhibiting lower efficacy against subtype C viruses (36). To explore whether this also impacted the scFv, we compared the loss of potency, relative to that of IgG, for subtype A, B, and C viruses. There was a significant 9-fold difference in the IC₅₀ between the scFv and IgG for subtype B viruses (Figure 3.6B). However, there was a 28-fold difference for subtype C viruses, suggesting that the subtype-specific genetic differences have more of an impact on the scFv than the IgG. For subtype A, only one virus, Q259.D2.17, which lacked the N276 glycan (marked in green), showed more than a 10-fold difference (Figure 3.6B). This glycan was also missing in the subtype C virus, CAP228.51, which was significantly less well neutralized by the scFv.

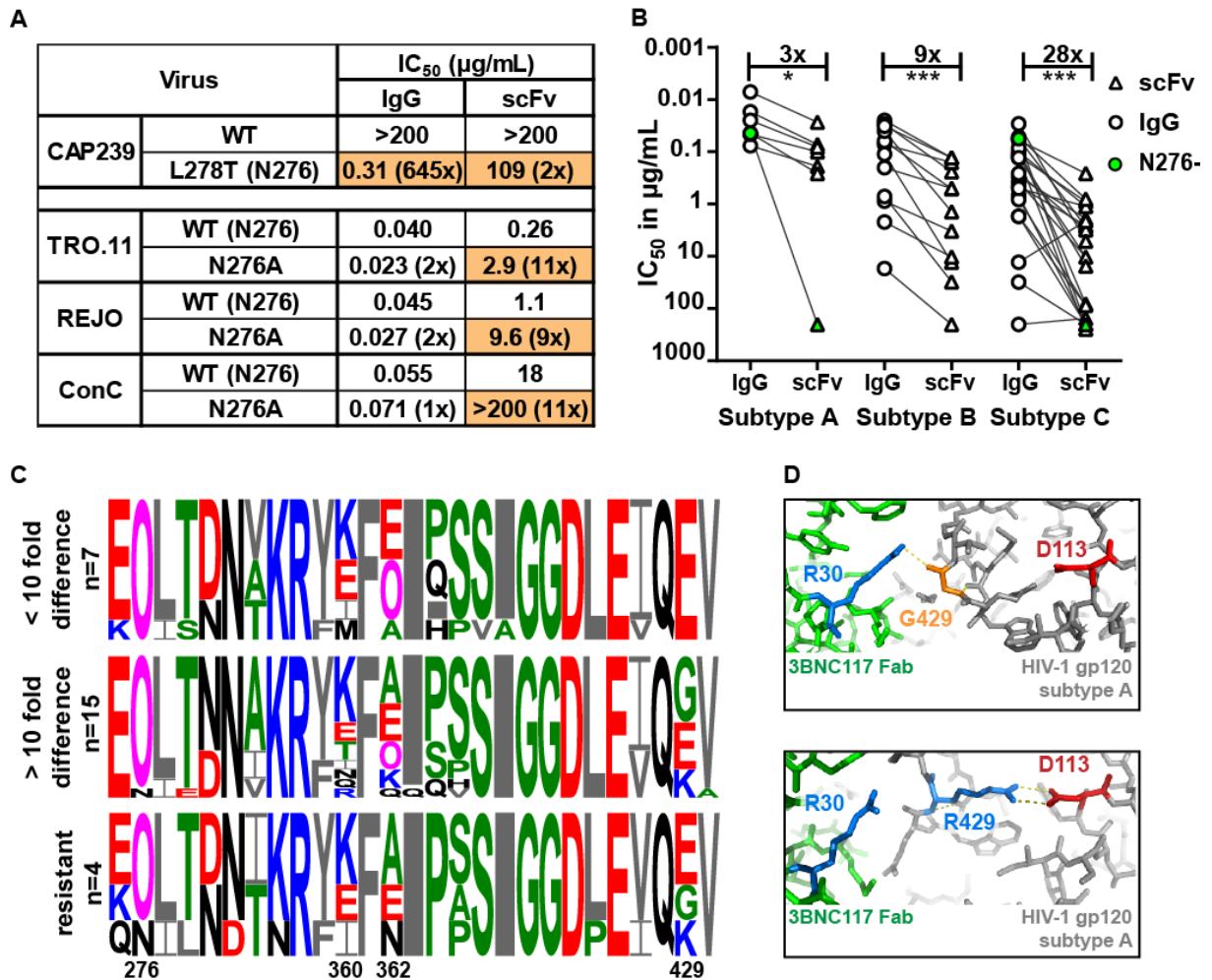


Figure 3.6 Loss of potency for the 3BNC117 scFv was more pronounced for subtype C viruses.

(A) IC₅₀ of IgG and scFv neutralization of one virus that lacked the N276 glycan with a glycan knockin (CAP239) and 3 viruses in which the N276 glycan has been deleted (scFv corrected for molecular weight difference). Fold changes compared to the wild type are shown in parentheses, with changes of >5-fold colored in orange. (B) IC₅₀ titers in micrograms per milliliter of IgG (circle) and scFv (triangle) against HIV-1 subtypes A, B, and C. Viruses lacking an N276 glycan are colored green. IgG- and scFv-resistant viruses are excluded. Fold differences are indicated above the graph and excluded the 2 viruses that lacked an N276 glycan. (C) Logogram of amino acid sequences of the CD4 binding site of HIV-1 subtype C viruses neutralized at the same level by scFv and IgG (<10-fold), those less well neutralized by scFv (>10-fold difference), or those resistant to both IgG and scFv. The N276 glycan and sites 360, 362, and 429, which exhibit the most variability, are indicated. Negatively and positively charged residues are colored red and blue, respectively. (D) Structural representation of the interaction of 3BNC117 Fab (green) and HIV Env (gray) containing an arginine (blue) or glycine (orange) at position 429. The aspartic acid at residue 113 in the HIV trimer is colored red, whereas the arginine at position 30 in the Fab is colored blue. Polar bonds are indicated by dotted lines (PDB entries 4JPV and 5V8L) (78, 79).

We next analysed the viral amino acid sequences in the CD4 binding site of subtype C viruses to identify other residues that might contribute to the potency difference between IgG and

scFv (Figure 3.6C). Viruses were separated into 3 groups based on their relative sensitivity to IgG and scFv. There was significant viral variation at positions 360, 362, and 429, all of which have been previously implicated in the binding of CD4bs bNAbs (36, 37), although position 429 has not specifically been shown to impact 3BNC117.

To further explore the possibility that residue 429 impacted the scFv, we compared crystal structures of the 3BNC117 Fab in complex with gp120 (PDB entries 4JPV and 5V8L). We found that a neutral or negative charge at residue 429 interacted with a positively charged residue 30 (arginine) on the 3BNC117 Fab (Figure 3.6D). In contrast, a positive charge at position 429 in the HIV-1 trimer could prevent 3BNC117 binding to this site by folding back and binding to an aspartic acid at position 113 in gp120, occluding this residue.

Therefore, the 3BNC117 scFv binding may in part be more reliant on the neutral or negative charge at position 429 than its IgG counterpart. Thus, virus-specific loss of potency for the 3BNC117 scFv was likely impacted by viral subtype, the 276 glycan, and charged residues within the epitope.

3.5 Discussion

We have designed and expressed scFv of HIV bNAbs that are undergoing human clinical testing to assess whether they maintained neutralization capacity in this format. These bNAbs targeted one of four well-defined epitopes on the viral envelope, namely, the V2 apex (CAP256.25), the V3 glycan (PGT121), the CD4bs (3BNC117), and the MPER (10E8v4). Remarkably, these much smaller scFv, engineered from the variable regions of the IgG, retained substantial breadth and potency, and 10E8v4 was equivalent to the full IgG molecule. While CAP256.25, 3BNC117, and PGT121 scFv lost potency, they were nonetheless in the therapeutic range of VRC01, a CD4bs antibody that is currently in clinical efficacy testing.

Newly designed scFv showed good levels of expression with different linker lengths of 15, 18, or 20 amino acids having minimal to no impact on neutralizing activity. This suggested that the loss of activity relative to IgG was due to the inherent nature rather than the design of the scFv, which at 30 kDa are 5 times smaller than IgG. Antibody scFv were more likely to lose neutralization potency rather than breadth, although for 3BNC117 and PGT121 the loss of potency often resulted in reduced breadth. Potency loss was most severe for CAP256.25 and was evident against all viruses tested, while for 3BNC117 and PGT121 this occurred in a virus-specific manner. Epitope mapping studies on selected viruses showed that scFv bound to their epitope in ways subtly different from those of the matched IgG. Thus, charged residues at positions 169 and 170 in V2 affected CAP256.25 scFv neutralization more than the IgG. For PGT121, interactions with the N332 glycan and the length of V1 impacted the scFv to a greater degree than the IgG, and for 3BNC117, loss of binding to the N276 glycan was more detrimental to the scFv than the IgG. Thus, while scFv retained the ability to interact with known epitopes, they were more sensitive to variations at single residues within these epitopes. Whether these potency losses are unique to each scFv or are dependent on the epitopes being targeted would need to be determined by designing and testing a larger number of scFv versions of bNAbs.

scFv have a single binding site compared to the 2 binding sites of IgG, which likely reduces their affinity for the antigen. It is known that smaller proteins tend to have higher off rates due to lower binding affinity (38–40). This was previously demonstrated for scFv-based fragments of VRC01 that had a 4-fold lower affinity than the IgG version (18). Affinity is influenced by noncovalent intermolecular interactions between proteins, such as electrostatic

charge and hydrogen bonding. Our data suggest that a charge change in the epitope had a greater effect than the loss of a glycan, although both likely impacted the binding affinity of the scFv, accounting for the potency losses. Loss of affinity could also be due to the inability of scFv with a single binding site to effectively cross-link protomers on the trimer. Even though trimers are present at low density on the HIV virion, the polarization of trimers at the cell contact site would allow the IgG but not the scFv to cross-link (41–45).

The constant region affects the angle at which the antibody paratope engages the viral epitope and also helps to stabilize the heavy-light chain interactions (46). As a result, scFv may have a somewhat less rigid paratope than IgG. Removal of the Fc region could, therefore, alter these interactions, contributing to the scFv being more dependent on key residues in the epitope. Structural studies in combination with affinity studies of scFv are needed to further inform the molecular interactions with the HIV trimer and provide insights into the stability of scFv paratopes. Others have shown that the lack of an Fc region also significantly reduces the half-life of scFv and removes their ability to mediate effector functions, which compromises their clinical development (16, 22). However, scFv may have lower immunogenicity and off-target effects than IgG as a result of not having an antibody Fc region to engage host Fc receptors (18, 47).

scFv may have better access to their epitopes than IgG due to their smaller size, as has been reported using other antibody constructs. For example, antigen-binding fragments (Fab) of antibodies targeting the CD4-induced (CD4i) epitope showed significant improvement in potency over the IgG versions (48–50). Moreover, scFv isolated from phage display libraries demonstrated good breadth and potency (25, 51). A number of antibody epitopes on the HIV trimer are not readily accessible by IgG molecules due to steric hindrance, including the MPER on gp41 (53–55). Remarkably, the scFv of 10E8v4, an MPER antibody that targets an epitope close to the viral of the 10E8v4 scFv to this recessed site could offset the loss of affinity that results from having only a single paratope. Alternatively, scFv targeting the MPER may be partially buried in the lipid membrane, which could stabilize the interaction with the epitope (54–56). scFv may also be important in preventing cell-cell transmission, where spatial restrictions within the virological synapse limit antibody access (57).

scFv may be useful in the context of passive immunization; however, the caveats associated with this format will need to be addressed, in particular their reduced half-life. Adeno-associated virus (AAV) vectors, which are being developed for continuous, delivery of antibody-based products as vectored immunoprophylaxis against HIV infection (58–61), may provide a solution. scFv, with their smaller gene inserts, may be more suitable for expression in vectors, such as AAV-8, that have been previously deployed for some of the IgG bNAbs described here (61). This approach could be particularly important in the context of bNAb combinations needed to optimize the coverage of global HIV strains (62–64). Since multiple scFv could be expressed from the same vector, their use in combination treatments may be a distinct advantage (47, 58, 62, 65). scFv derived from other HIV bNAbs will need to be tested to determine which combinations show optimal complementarity in their neutralization profiles given that scFv neutralization cannot be predicted.

Overall, our data indicate that scFv retain considerable breadth and potency and could perform better than IgG equivalents as combination therapeutics. In particular, 10E8v4 should be considered a leading candidate for human clinical testing, given that it retains all its functional activity as an scFv. Although there are some challenges with this format, there are also unique advantages of scFv over IgG. These smaller antibody proteins, therefore, have considerable potential in passive immunization strategies to prevent HIV infection.

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3.7 References

1. Bhiman JN, Lynch RM. 2017. Broadly neutralizing antibodies as treatment: effects on virus and immune system. *Curr HIV/AIDS Rep* 14:54–62. <https://doi.org/10.1007/s11904-017-0352-1>.
2. Burton DR, Hangartner L. 2016. Broadly neutralizing antibodies to HIV and their role in vaccine design. *Annu Rev Immunol* 34:635– 659. <https://doi.org/10.1146/annurev-immunol-041015-055515>.
3. Julg B, Barouch DH. 2019. Neutralizing antibodies for HIV-1 prevention. *Curr Opin HIV AIDS* 14:318–324. <https://doi.org/10.1097/COH.0000000000000556>.
4. Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE, Beary H, Hayes D, Frankel SS, Birx DL, Lewis MG. 2000. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat Med* 6:207–210. <https://doi.org/10.1038/72318>.
5. Cheeseman HM, Olejniczak NJ, Rogers PM, Evans AB, King DFL, Ziprin P, Liao H-X, Haynes BF, Shattock RJ. 2017. Broadly neutralizing antibodies display potential for prevention of HIV-1 infection of mucosal tissue superior to that of nonneutralizing antibodies. *J Virol* 91:e01762-16. <https://doi.org/10.1128/JVI.01762-16>.
6. Julg B, Liu P-T, Wagh K, Fischer WM, Abbink P, Mercado NB, Whitney JB, Nkolola JP, McMahan K, Tartaglia LJ, Borducchi EN, Khatiwada S, Kamath M, LeSuer JA, Seaman MS, Schmidt SD, Mascola JR, Burton DR, Korber BT, Barouch DH. 2017. Protection against a mixed SHIV challenge by a broadly neutralizing antibody cocktail. *Sci Transl Med* 9:eaao4235. <https://doi.org/10.1126/scitranslmed.aao4235>.
7. Julg B, Tartaglia LJ, Keele BF, Wagh K, Pegu A, Sok D, Abbink P, Schmidt SD, Wang K, Chen X, Joyce MG, Georgiev IS, Choe M, Kwong PD, Doria-Rose NA, Le K, Louder MK, Bailer RT, Moore PL, Korber B, Seaman MS, Abdool Karim SS, Morris L, Koup RA, Mascola JR, Burton DR, Barouch DH. 2017. Broadly neutralizing antibodies targeting the HIV-1

- envelope V2 apex confer protection against a clade C SHIV challenge. *Sci Transl Med* 9:eaal1321. <https://doi.org/10.1126/scitranslmed.aal1321>.
8. Gautam R, Nishimura Y, Pegu A, Nason MC, Klein F, Gazumyan A, Golijanin J, Buckler-White A, Sadjadpour R, Wang K, Mankoff Z, Schmidt SD, Lifson JD, Mascola JR, Nussenzweig MC, Martin MA. 2016. A single injection of anti-HIV-1 antibodies protects against repeated SHIV challenges. *Nature* 533:105–109. <https://doi.org/10.1038/nature17677>.
 9. Pegu A, Hessel AJ, Mascola JR, Haigwood NL. 2017. Use of broadly neutralizing antibodies for HIV-1 prevention. *Immunol Rev* 275:296 –312. <https://doi.org/10.1111/imr.12511>.
 10. Caskey M, Klein F, Lorenzi JCC, Seaman MS, West AP, Jr, Buckley N, Kremer G, Nogueira L, Braunschweig M, Scheid JF, Horwitz JA, Shimeliovich I, Ben-Avraham S, Witmer-Pack M, Platten M, Lehmann C, Burke LA, Hawthorne T, Gorelick RJ, Walker BD, Keler T, Gulick RM, Fätkenheuer G, Schlesinger SJ, Nussenzweig MC, West AP, Fätkenheuer G. 2015. Viraemia suppressed in HIV-1-infected humans by broadly neutralizing antibody 3BNC117. *Nature* 522:487–491. <https://doi.org/10.1038/nature14411>.
 11. Caskey M, Schoofs T, Gruell H, Settler A, Karagounis T, Kreider EF, Murrell B, Pfeifer N, Nogueira L, Oliveira TY, Learn GH, Cohen YZ, Lehmann C, Gillor D, Shimeliovich I, Unson-O'Brien C, Weiland D, Robles A, Kümmerle T, Wyen C, Levin R, Witmer-Pack M, Eren K, Ignacio C, Kiss S, West AP, Mouquet H, Zingman BS, Gulick RM, Keler T, Bjorkman PJ, Seaman MS, Hahn BH, Fätkenheuer G, Schlesinger SJ, Nussenzweig MC, Klein F. 2017. Antibody 10-1074 suppresses viremia in HIV-1-infected individuals. *Nat Med* 23:185–191. <https://doi.org/10.1038/nm.4268>.
 12. Lynch RM, Boritz E, Coates EE, DeZure A, Madden P, Costner P, Enama ME, Plummer S, Holman L, Hendel CS, Gordon I, Casazza J, Conan-Cibotti M, Migueles SA, Tressler R, Bailer RT, McDermott A, Narpala S, O'Dell S, Wolf G, Lifson JD, Freemire BA, Gorelick RJ, Pandey JP, Mohan S, Chomont N, Fromentin R, Chun T-W, Fauci AS, Schwartz RM, Koup RA, Douek DC, Hu Z, Capparelli E, Graham BS, Mascola JR, Ledgerwood JE. 2015. Virologic effects of broadly neutralizing antibody VRC01 administration during chronic HIV-1 infection. *Sci Transl Med* 7:319ra206. <https://doi.org/10.1126/scitranslmed.aad5752>.
 13. Mendoza P, Gruell H, Nogueira L, Pai JA, Butler AL, Millard K, Lehmann C, Suárez I, Oliveira TY, Lorenzi J, Cohen YZ, Wyen C, Kümmerle T, Karagounis T, Lu C-L, Handl L,

- Unson O'Brien C, Patel R, Ruping C, Schlotz M, Witmer-Pack M, Shimeliovich I, Kremer G, Thomas E, Seaton KE, Horowitz J, West AP, Bjorkman PJ, Tomaras GD, Gulick RM, Pfeifer N, Fätkenheuer G, Seaman MS, Klein F, Caskey M, Nussenzweig MC. 2018. Combination therapy with anti-HIV-1 antibodies maintains viral suppression. *Nature* 561:479–484. <https://doi.org/10.1038/s41586-018-0531-2>.
14. Bar KJ, Sneller MC, Harrison LJ, Justement JS, Overton ET, Petrone ME, Salantes DB, Seamon CA, Scheinfeld B, Kwan RW, Learn GH, Proschan MA, Kreider EF, Blazkova J, Bardsley M, Refsland EW, Messer M, Clarridge KE, Tustin NB, Madden PJ, Oden K, O'Dell SJ, Jarocki B, Shiakolas AR, Tressler RL, Doria-Rose NA, Bailer RT, Ledgerwood JE, Capparelli EV, Lynch RM, Graham BS, Moir S, Koup RA, Mascola JR, Hoxie JA, Fauci AS, Tebas P, Chun T-W. 2016. Effect of HIV antibody VRC01 on viral rebound after treatment interruption. *N Engl J Med* 375:2037–2050. <https://doi.org/10.1056/NEJMoa1608243>.
15. Scheid JF, Horwitz JA, Bar-On Y, Kreider EF, Lu C-L, Lorenzi JCC, Feldmann A, Braunschweig M, Nogueira L, Oliveira TY, Shimeliovich I, Patel R, Burke LA, Cohen YZ, Hadrigan S, Settler A, Witmer-Pack M, West AP, Jr, Juelg B, Keler T, Hawthorne T, Zingman BS, Gulick RM, Pfeifer N, Learn GH, Seaman MS, Bjorkman PJ, Klein F, Schlesinger SJ, Walker BD, Hahn BH, Nussenzweig MC, Caskey M. 2016. HIV-1 antibody 3BNC117 suppresses viral rebound in humans during treatment interruption. *Nature* 535:556–560. <https://doi.org/10.1038/nature18929>.
16. Ahmad ZA, Yeap SK, Ali AM, Ho WY, Alitheen NBM, Hamid M. 2012. scFv antibody: principles and clinical application. *Clin Dev Immunol* 2012: 980250–980215. <https://doi.org/10.1155/2012/980250>.
17. Chen W, Dimitrov DS. 2009. Human monoclonal antibodies and engineered antibody domains as HIV-1 entry inhibitors. *Curr Opin HIV AIDS* 4:112–117. <https://doi.org/10.1097/COH.0b013e328322f95e>.
18. West AP, Galimidi RP, Gnanapragasam PNP, Bjorkman PJ. 2012. Singlechain Fv-based anti-HIV proteins: potential and limitations. *J Virol* 86:195–202. <https://doi.org/10.1128/JVI.05848-11>.
19. Wang S, Zheng C, Liu Y, Zheng H, Wang Z. 2008. Construction of multiform scFv antibodies using linker peptide. *J Genet Genomics* 35:313–316. [https://doi.org/10.1016/S1673-8527\(08\)60045-4](https://doi.org/10.1016/S1673-8527(08)60045-4).

20. Colcher D, Pavlinkova G, Beresford G, Booth BJ, Choudhury A, Batra SK. 1998. Pharmacokinetics and biodistribution of genetically-engineered antibodies. *Q J Nucl Med* 42:225–241.
21. Yokota T, Milenic DE, Whitlow M, Schlom J. 1992. Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res* 52:3402–3408.
22. Unverdorben F, Richter F, Hutt M, Seifert O, Malinge P, Fischer N, Kontermann RE. 2016. Pharmacokinetic properties of IgG and various Fc fusion proteins in mice. *MAbs* 8:120 – 128. <https://doi.org/10.1080/19420862.2015.1113360>.
23. Holliger P, Hudson PJ. 2005. Engineered antibody fragments and the rise of single domains. *Nat Biotechnol* 23:1126 –1136. <https://doi.org/10.1038/nbt1142>.
24. Bivi N, Moore T, Rodgers G, Denning H, Shockley T, Swearingen CA, Gelfanova V, Calderon B, Peterson DA, Hodsdon ME, Siegel RW, Higgs RE, Konrad RJ. 2019. Investigation of pre-existing reactivity to biotherapeutics can uncover potential immunogenic epitopes and predict immunogenicity risk. *MAbs* 11:861– 869. <https://doi.org/10.1080/19420862.2019.1612699>.
25. Khan L, Kumar R, Thiruvengadam R, Parray HA, Makhdoomi MA, Kumar S, Aggarwal H, Mohata M, Hussain AW, Das R, Varadarajan R, Bhattacharya J, Vajpayee M, Murugavel KG, Solomon S, Sinha S, Luthra K. 2017. Cross-neutralizing anti-HIV-1 human single chain variable fragments (scFvs) against CD4 binding site and N332 glycan identified from a recombinant phage library. *Sci Rep* 7:45163. <https://doi.org/10.1038/srep45163>.
26. . Burton DR, Barbas CF, Persson MA, Koenig S, Chanock RM, Lerner RA. 1991. A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. *Proc Natl Acad Sci U S A* 88:10134 –10137. <https://doi.org/10.1073/pnas.88.22.10134>.
27. Reference deleted.
28. Doria-Rose NA, Bhiman JN, Roark RS, Schramm CA, Gorman J, Chuang G-Y, Pancera M, Cale EM, Ernandes MJ, Louder MK, Asokan M, Bailer RT, Druz A, Fraschilla IR, Garrett NJ, Jarosinski M, Lynch RM, McKee K, O'Dell S, Pegu A, Schmidt SD, Staupe RP, Sutton MS, Wang K, Wibmer CK, Haynes BF, Abdool-Karim S, Shapiro L, Kwong PD, Moore PL, Morris L, Mascola JR. 2016. New member of the V1V2-directed CAP256-VRC26 lineage that shows increased breadth and exceptional potency. *J Virol* 90:76 –91. <https://doi.org/10.1128/JVI.01791-15>.

29. Walker LM, Huber M, Doores KJ, Falkowska E, Pejchal R, Julien J-P, Wang S-KS-K, Ramos A, Chan-Hui P-Y, Moyle M, Mitcham JL, Hammond PW, Olsen OA, Phung P, Fling S, Wong C-H, Phogat SK, Wrin T, Simek MD, Principal Investigators PG, Koff WC, Wilson IA, Burton DR, Poignard P, Walker LM, Huber M, Doores KJ, Falkowska E, Pejchal R, Chan-Hui P-Y, Moyle M, Mitcham JL, Hammond PW, Olsen OA, Phung P, Principal Investigators PG, Koff WC, Wilson IA, Burton DR, Poignard P, Protocol G Principal Investigators. 2011. Broad neutralization coverage of HIV by multiple highly potent antibodies. *Nature* 477:466–470. <https://doi.org/10.1038/nature10373>.
30. Scheid JF, Mouquet H, Ueberheide B, Diskin R, Klein F, Oliveira TYK, Pietzsch J, Fenyo D, Abadir A, Velinzon K, Hurley A, Myung S, Boulad F, Poignard P, Burton DR, Pereyra F, Ho DD, Walker BD, Seaman MS, Bjorkman PJ, Chait BT, Nussenzweig MC. 2011. Sequence and structural convergence of broad and potent HIV antibodies that mimic CD4 binding. *Science* 333:1633–1637. <https://doi.org/10.1126/science.1207227>.
31. Kwon YD, Georgiev IS, Ofek G, Zhang B, Asokan M, Bailer RT, Bao A, Caruso W, Chen X, Choe M, Druz A, Ko S-Y, Louder MK, McKee K, O'Dell S, Pegu A, Rudicell RS, Shi W, Wang K, Yang Y, Alger M, Bender MF, Carlton K, Cooper JW, Blinn J, Eudailey J, Lloyd K, Parks R, Alam SM, Haynes BF, Padte NN, Yu J, Ho DD, Huang J, Connors M, Schwartz RM, Mascola JR, Kwong PD. 2016. Optimization of the solubility of HIV-1-neutralizing antibody 10E8 through somatic variation and structure-based design. *J Virol* 90:5899 – 5914. <https://doi.org/10.1128/JVI.03246-15>.
32. Julien J-P, Sok D, Khayat R, Lee JH, Doores KJ, Walker LM, Ramos A, Diwanji DC, Pejchal R, Cupo A, Katpally U, Depetris RS, Stanfield RL, McBride R, Marozsan AJ, Paulson JC, Sanders RW, Moore JP, Burton DR, Poignard P, Ward AB, Wilson IA. 2013. Broadly neutralizing antibody PGT121 allosterically modulates CD4 binding via recognition of the HIV-1 gp120 V3 base and multiple surrounding glycans. *PLoS Pathog* 9:e1003342. <https://doi.org/10.1371/journal.ppat.1003342>.
33. Anthony C, York T, Bekker V, Matten D, Selhorst P, Ferreria R-C, Garrett NJ, Karim SSA, Morris L, Wood NT, Moore PL, Williamson C. 2017. Cooperation between strain-specific and broadly neutralizing responses limited viral escape and prolonged the exposure of the broadly neutralizing epitope. *J Virol* 91:e00828-17. <https://doi.org/10.1128/JVI.00828-17>.

34. Deshpande S, Patil S, Kumar R, Hermanus T, Murugavel KG, Srikrishnan AK, Solomon S, Morris L, Bhattacharya J. 2016. HIV-1 clade C escapes broadly neutralizing autologous antibodies with N332 glycan specificity by distinct mechanisms. *Retrovirology* 13:60. <https://doi.org/10.1186/s12977-016-0297-2>.
35. McGuire AT, Hoot S, Dreyer AM, Lippy A, Stuart A, Cohen KW, Jardine J, Menis S, Scheid JF, West AP, Schief WR, Stamatatos L. 2013. Engineering HIV envelope protein to activate germline B cell receptors of broadly neutralizing anti-CD4 binding site antibodies. *J Exp Med* 210:655– 663. <https://doi.org/10.1084/jem.20122824>.
36. Bricault CA, Yusim K, Seaman MS, Yoon H, Theiler J, Giorgi EE, Wagh K, Theiler M, Hraber P, Macke JP, Kreider EF, Learn GH, Hahn BH, Scheid JF, Kovacs JM, Shields JL, Lavine CL, Ghantous F, Rist M, Bayne MG, Neubauer GH, McMahan K, Peng H, Chéneau C, Jones JJ, Zeng J, Ochsenbauer C, Nkolola JP, Stephenson KE, Chen B, Gnanakaran S, Bonsignori M, Williams LD, Haynes BF, Doria-Rose N, Mascola JR, Montefiori DC, Barouch DH, Korber B. 2019. HIV-1 neutralizing antibody signatures and application to epitope-targeted vaccine design. *Cell Host Microbe* 25: 59–72. <https://doi.org/10.1016/j.chom.2018.12.001>.
37. Liu Q, Lai YT, Zhang P, Louder MK, Pegu A, Rawi R, Asokan M, Chen X, Shen CH, Chuang GY, Yang ES, Miao H, Wang Y, Fauci AS, Kwong PD, Mascola JR, Lusso P. 2019. Improvement of antibody functionality by structure-guided paratope engraftment. *Nat Commun* 10:721. <https://doi.org/10.1038/s41467-019-08658-4>.
38. Schreiber G, Haran G, Zhou H-X. 2009. Fundamental aspects of protein-protein association kinetics. *Chem Rev* 109:839 – 860. <https://doi.org/10.1021/cr800373w>.
39. Pan AC, Borhani DW, Dror RO, Shaw DE. 2013. Molecular determinants of drug-receptor binding kinetics. *Drug Discov Today* 18:667– 673. <https://doi.org/10.1016/j.drudis.2013.02.007>.
40. Miller DC, Lunn G, Jones P, Sabnis Y, Davies NL, Driscoll P. 2012. Investigation of the effect of molecular properties on the binding kinetics of a ligand to its biological target. *Med Chem Commun* 3:449. <https://doi.org/10.1039/c2md00270a>.
41. Klasse PJ. 2007. Modeling how many envelope glycoprotein trimers per virion participate in human immunodeficiency virus infectivity and its neutralization by antibody. *Virology* 369:245–262. <https://doi.org/10.1016/j.virol.2007.06.044>.

42. Chien MP, Lin CH, Chang DK. 2009. Recruitment of HIV-1 envelope occurs subsequent to lipid mixing: a fluorescence microscopic evidence. *Retrovirology* 6:20. <https://doi.org/10.1186/1742-4690-6-20>.
43. Cohen FS, Melikyan GB. 2004. The energetics of membrane fusion from binding, through hemifusion, pore formation, and pore enlargement. *J Membr Biol* 199:1–14. <https://doi.org/10.1007/s00232-004-0669-8>.
44. Louder MK, Sambor A, Chertova E, Hunte T, Barrett S, Ojong F, Sanders- Buell E, Zolla-Pazner S, McCutchan FE, Roser JD, Gabuzda D, Lifson JD, Mascola JR. 2005. HIV-1 envelope pseudotyped viral vectors and infectious molecular clones expressing the same envelope glycoprotein have a similar neutralization phenotype, but culture in peripheral blood mononuclear cells is associated with decreased neutralization sensitivity. *Virology* 339:226 –238. <https://doi.org/10.1016/j.virol.2005.06.003>.
45. Cohen YZ, Lorenzi JCC, Seaman MS, Nogueira L, Schoofs T, Krassnig L, Butler A, Millard K, Fitzsimons T, Daniell X, Dizon JP, Shimeliovich I, Montefiori DC, Caskey M, Nussenzweig MC. 2017. Neutralizing activity of broadly neutralizing anti-HIV-1 antibodies against clade B clinical isolates produced in peripheral blood mononuclear cells. *J Virol* 92:e01883-17. <https://doi.org/10.1128/JVI.01883-17>.
46. Janda A, Bowen A, Greenspan NS, Casadevall A. 2016. Ig constant region effects on variable region structure and function. *Front Microbiol* 7:22. <https://doi.org/10.3389/fmicb.2016.00022>.
47. Monnier P, Vigouroux R, Tassew N. 2013. In vivo applications of single chain Fv (variable domain) (scFv) fragments. *Antibodies* 2:193–208. <https://doi.org/10.3390/antib2020193>.
48. Labrijn AF, Poignard P, Raja A, Zwick MB, Delgado K, Franti M, Binley JM, Vivona V, Grundner C, Huang C-C, Venturi M, Petropoulos C, Wrin T, Dimitrov DS, Robinson JE, Kwong PD, Wyatt RT, Sodroski JG, Burton DR. 2003. Access of antibody molecules to the conserved coreceptor binding site on glycoprotein gp120 is sterically restricted on primary human immunodeficiency virus type 1. *J Virol* 77:10557–10565. <https://doi.org/10.1128/jvi.77.19.10557-10565.2003>.
49. Zhang MY, Shu Y, Sidorov I, Dimitrov DS. 2004. Identification of a novel CD4i human monoclonal antibody Fab that neutralizes HIV-1 primary isolates from different clades. *Antiviral Res* 61:161–164. <https://doi.org/10.1016/j.antiviral.2003.09.009>.

50. Tanaka K, Kuwata T, Alam M, Kaplan G, Takahama S, Valdez KPR, Roitburd-Berman A, Gershoni JM, Matsushita S. 2017. Unique binding modes for the broad neutralizing activity of single-chain variable fragments (scFv) targeting CD4-induced epitopes. *Retrovirology* 14:44. <https://doi.org/10.1186/s12977-017-0369-y>.
51. Kumar S, Kumar R, Khan L, Makhdoomi MA, Thiruvengadam R, Mohata M, Agarwal M, Lodha R, Kabra SK, Sinha S, Luthra K. 2017. CD4-binding site directed cross-neutralizing scFv monoclonals from HIV-1 subtype C infected Indian children. *Front Immunol* 8:1568. <https://doi.org/10.3389/fimmu.2017.01568>.
52. Reference deleted
53. Zwick MB, Labrijn AF, Wang M, Spenlehauer C, Saphire EO, Binley JM, Moore JP, Stiegler G, Katinger H, Burton DR, Parren P. 2001. Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. *J Virol* 75:10892–10905. <https://doi.org/10.1128/JVI.75.22.10892-10905.2001>.
54. Irimia A, Sarkar A, Stanfield RL, Wilson IA. 2016. Crystallographic identification of lipid as an integral component of the epitope of HIV broadly neutralizing antibody 4E10. *Immunity* 44:21–31. <https://doi.org/10.1016/j.immuni.2015.12.001>.
55. Irimia A, Serra AM, Sarkar A, Jacak R, Kalyuzhniy O, Sok D, Saye-Francisco KL, Schiffner T, Tingle R, Kubitz M, Adachi Y, Stanfield RL, Deller MC, Burton DR, Schief WR, Wilson IA. 2017. Lipid interactions and angle of approach to the HIV-1 viral membrane of broadly neutralizing antibody 10E8: insights for vaccine and therapeutic design. *PLoS Pathog* 13: e1006212. <https://doi.org/10.1371/journal.ppat.1006212>.
56. Lee JH, Ozorowski G, Ward AB. 2016. Cryo-EM structure of a native, fully glycosylated, cleaved HIV-1 envelope trimer. *Science* 351:1043–1048. <https://doi.org/10.1126/science.aad2450>.
57. Zhang MY, Borges AR, Ptak RG, Wang Y, Dimitrov AS, Alam SM, Wieczorek L, Bouma P, Fouts T, Jiang S, Polonis VR, Haynes BF, Quinnan GV, Montefiori DC, Dimitrov DS. 2010. Potent and broad neutralizing activity of a single chain antibody fragment against cell-free and cell-associated HIV-1. *MAbs* 2:266–274. <https://doi.org/10.4161/mabs.2.3.11416>.

58. Naso MF, Tomkowicz B, Perry WL, Strohl WR. 2017. Adeno-associated virus (AAV) as a vector for gene therapy. *BioDrugs* 31:317–334. <https://doi.org/10.1007/s40259-017-0234-5>.
59. Balazs AB, Chen J, Hong CM, Rao DS, Yang L, Baltimore D. 2011. Antibody-based protection against HIV infection by vectored immunoprophylaxis. *Nature* 481:81– 86. <https://doi.org/10.1038/nature10660>.
60. Lin A, Balazs AB. 2018. Adeno-associated virus gene delivery of broadly neutralizing antibodies as prevention and therapy against HIV-1. *Retrovirology* 15:66. <https://doi.org/10.1186/s12977-018-0449-7>.
61. van den Berg FT, Makoah NA, Ali SA, Scott TA, Mapengo RE, Mutsvunguma LZ, Mkhize NN, Lambson BE, Kgagudi PD, Crowther C, Abdool Karim SS, Balazs AB, Weinberg MS, Ely A, Arbuthnot PB, Morris L. 2019. AAV-mediated expression of broadly neutralizing and vaccine-like antibodies targeting the HIV-1 envelope V2 region. *Mol Ther Methods Clin Dev* 14:100 –112. <https://doi.org/10.1016/j.omtm.2019.06.002>.
62. Wagh K, Bhattacharya T, Williamson C, Robles A, Bayne MG, Garrity J, Rist M, Rademeyer C, Yoon H, Lapedes A, Gao H, Greene K, Louder MK, Kong R, Karim SA, Burton DR, Barouch DH, Nussenzweig MC, Mascola JR, Morris L, Montefiori DC, Korber B, Seaman MS. 2016. Optimal combinations of broadly neutralizing antibodies for prevention and treatment of HIV-1 clade C infection. *PLoS Pathog* 12:e1005520. <https://doi.org/10.1371/journal.ppat.1005520>.
63. Doria-Rose NA, Louder MK, Yang Z, O'Dell S, Nason M, Schmidt SD, McKee K, Seaman MS, Bailer RT, Mascola JR. 2012. HIV-1 neutralization coverage is improved by combining monoclonal antibodies that target independent epitopes. *J Virol* 86:3393–3397. <https://doi.org/10.1128/JVI.06745-11>.
64. Kong R, Louder MK, Wagh K, Bailer RT, DeCamp A, Greene KM, Gao H, Taft JD, Gazumyan A, Liu C, Nussenzweig MC, Korber BT, Montefiori DC, Mascola JR. 2015. Improving neutralization potency and breadth by combining broadly reactive HIV-1 antibodies targeting major neutralization epitopes. *J Virol* 89:2659 –2671. <https://doi.org/10.1128/JVI.03136-14>.
65. Liu Z, Chen O, Wall JBJ, Zheng M, Zhou Y, Wang L, Ruth Vaseghi H, Qian L, Liu J. 2017. Systematic comparison of 2A peptides for cloning multigenes in a polycistronic vector. *Sci Rep* 7:2193. <https://doi.org/10.1038/s41598-017-02460-2>.

66. Portolano N, Watson PJ, Fairall L, Millard CJ, Milano CP, Song Y, Cowley SM, Schwabe JW. 2014. Recombinant protein expression for structural biology in HEK 293F suspension cells: a novel and accessible approach. *J Vis Exp* 92:e51897. <https://doi.org/10.3791/51897>.
67. Reference deleted.
68. Reference deleted.
69. Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A. 2009. Protein identification and analysis tools on the ExPASy server, p 571–607. In Walker JM (ed), *The proteomics protocols handbook*. Humana Press, Totowa, NJ.
70. Mascola JR, D'Souza P, Gilbert P, Hahn BH, Haigwood NL, Morris L, Petropoulos CJ, Polonis VR, Sarzotti M, Montefiori DC. 2005. Recommendations for the design and use of standard virus panels to assess neutralizing antibody responses elicited by candidate human immunodeficiency virus type 1 vaccines. *J Virol* 79:10103–10107. <https://doi.org/10.1128/JVI.79.16.10103-10107.2005>.
71. Seaman MS, Janes H, Hawkins N, Grandpre LE, Devoy C, Giri A, Coffey RT, Harris L, Wood B, Daniels MG, Bhattacharya T, Lapedes A, Polonis VR, McCutchan FE, Gilbert P, Self SG, Korber BT, Montefiori DC, Mascola JR. 2010. Tiered categorization of a diverse panel of HIV-1 Env pseudoviruses for assessment of neutralizing antibodies. *J Virol* 84:1439 – 1452. <https://doi.org/10.1128/JVI.02108-09>.
72. Todd CA, Greene KM, Yu X, Ozaki DA, Gao H, Huang Y, Wang M, Li G, Brown R, Wood B, D'Souza MP, Gilbert P, Montefiori DC, Sarzotti-Kelsoe M. 2012. Development and implementation of an international proficiency testing program for a neutralizing antibody assay for HIV-1 in TZM-bl cells. *J Immunol Methods* 375:57– 67. <https://doi.org/10.1016/j.jim.2011.09.007>.
73. Gray ES, Madiga MC, Hermanus T, Moore PL, Wibmer CK, Tumba NL, Werner L, Mlisana K, Sibeko S, Williamson C, Abdool Karim SS, Morris L. 2011. The neutralization breadth of HIV-1 develops incrementally over four years and is associated with CD4 T cell decline and high viral load during acute infection. *J Virol* 85:4828–4840. <https://doi.org/10.1128/JVI.00198-11>.
74. Moore PL, Sheward DJ, Nonyane M, Ranchobe N, Hermanus T, Gray ES, Abdool Karim SS, Williamson C, Morris L. 2013. Multiple pathways of escape from HIV broadly cross-

- p neutralizing V2-dependent antibodies. J Virol 87:4882– 4894.
-
- <https://doi.org/10.1128/JVI.03424-12>
- .
75. Doria-Rose NA, Schramm CA, Gorman J, Moore PL, Bhiman JN, DeKosky BJ, Ernandes MJ, Georgiev IS, Kim HJ, Pancera M, Staupe RP, Altae-Tran HR, Bailer RT, Crooks ET, Cupo A, Druz A, Garrett NJ, Hoi KH, Kong R, Louder MK, Longo NS, McKee K, Nonyane M, O'Dell S, Roark RS, Rudicell RS, Schmidt SD, Sheward DJ, Soto C, Wibmer CK, Yang Y, Zhang Z, Mullikin JC, Binley JM, Sanders RW, Wilson IA, Moore JP, Ward AB, Georgiou G, Williamson C, Abdool Karim SS, Morris L, Kwong PD, Shapiro L, Mascola JR. 2014. Developmental pathway for potent V1V2-directed HIV-neutralizing antibodies. *Nature* 509:55– 62. <https://doi.org/10.1038/nature13036>.
 76. Montefiori DC. 2009. Measuring HIV neutralization in a luciferase reporter gene assay, p 395–405. In Prasad VR, Kalpana GV (ed), *HIV protocols. Methods in molecular biology*. Humana Press, Totowa, NJ.
 77. Sarzotti-Kelsoe M, Bailer RT, Turk E, Lin C, Bilska M, Greene KM, Gao H, Todd CA, Ozaki DA, Seaman MS, Mascola JR, Montefiori DC. 2014. Optimization and validation of the TZM-bl assay for standardized assessments of neutralizing antibodies against HIV-1. *J Immunol Methods* 409:131–146. <https://doi.org/10.1016/j.jim.2013.11.022>.
 78. Lee JH, Andrabi R, Su C-Y, Yasmeen A, Julien J-P, Kong L, Wu NC, McBride R, Sok D, Pauthner M, Cottrell CA, Nieusma T, Blattner C, Paulson JC, Klasse PJ, Wilson IA, Burton DR, Ward AB. 2017. A broadly neutralizing antibody targets the dynamic HIV envelope trimer apex via a long, rigidified, and anionic hairpin structure. *Immunity* 46:690 –702. <https://doi.org/10.1016/j.immuni.2017.03.017>.
 79. Klein F, Diskin R, Scheid JF, Gaebler C, Mouquet H, Georgiev IS, Pancera M, Zhou T, Incesu RB, Fu BZ, Gnanapragasam PNP, Oliveira TY, Seaman MS, Kwong PD, Bjorkman PJ, Nussenzweig MC. 2013. Somatic mutations of the immunoglobulin framework are generally required for broad and potent HIV-1 neutralization. *Cell* 153:126 –138. <https://doi.org/10.1016/j.cell.2013.03.018>.

4 Combinations of scFv made from HIV broadly neutralizing antibodies demonstrate high potency and breadth against a multiclade panel of viruses.

4.1 Introduction

Broadly neutralizing antibodies (bNAbs), isolated from a subset of HIV-1 positive individuals, are capable of neutralizing a wide range of HIV viruses. Crucially, bNAbs have been shown to provide protection in non-human primate studies and it is thought that an effective HIV vaccine will need to elicit these kinds of antibodies (Parren *et al.*, 2001; Saunders *et al.*, 2015; Julg, Liu, *et al.*, 2017; Martinez-Navio *et al.*, 2019). However, to date, no candidate HIV vaccines have been able to elicit bNAbs in humans (Rerks-Ngarm *et al.*, 2009; Karasavvas *et al.*, 2012; Klein *et al.*, 2013; Bekker *et al.*, 2018). This has led the field to actively explore the possibility of using bNAbs as biological drugs for passive immunization against HIV (Marcotte *et al.*, 2015; Burton *et al.*, 2016; Bhiman *et al.*, 2017; Lin *et al.*, 2018; Crowell *et al.*, 2019).

The first efficacy trials of an antibody for HIV prevention are currently underway in Africa and the Americas. These two Antibody-Mediated Prevention (AMP) trials are testing the ability of a single antibody, VRC01, to prevent HIV infection in high-risk men and women (www.ampstudy.org.za). The results of these proof-of-principle trials are expected in December 2020 or early 2021. However, to target the extensive envelope diversity, minimize escape, and provide sufficient potency it is widely accepted that a combination of multiple antibodies will be needed. Several studies have investigated the potential of antibody combinations and observed, as expected, an increase in breadth and potency (Kong *et al.*, 2015; Wagh *et al.*, 2016). These studies demonstrate that the complementary neutralization profiles of single mAbs can improve the overall breadth and provide higher coverage of multiclade panels of viruses at much lower antibody concentrations (Wagh *et al.*, 2016; Wagh, Seaman, *et al.*, 2018). Several clinical trials are currently assessing the safety of antibody combinations. For example, HVTN 130 is assessing the safety of dual and triple antibody combinations of the bNAbs PGT121, PGDM1400, 10-1074, and VRC07-523LS in a Phase I trial that started in March 2019 (HVTN, 2019).

A study focusing on clade C viruses, the predominant clade in southern Africa, demonstrated that a combination of three or more antibodies provided superior coverage compared to using single or dual combinations of antibodies (Wagh *et al.*, 2016). Having multiple antibodies covering the same viruses also improved the maximum neutralization of those viruses, allowing for complete neutralization (Wagh *et al.*, 2016). By using those antibodies that specifically target the HIV subtypes predominant in a specific area, a geographically relevant set of antibodies may be selected to provide optimal coverage and potency (Wagh, Seaman, *et al.*, 2018). CAP256.25, which preferentially targets clade C, is currently being assessed in combination with PGT121 and VRC07-523LS in dual and triple combination in the South African-led CAPRISA 012B trial (CAPRISA, 2020; Mahomed *et al.*, 2020).

The ability to predict the breadth and potency of antibody combinations without the need for experimental validation enables the rapid identification of optimal combinations. Two models have been used to predict the IC_{50} and IC_{80} of antibody combinations based on single antibody titres, the Loewe additive model, and the Bliss-Hill additive model. Both models assume that there is no interaction between the different antibodies and that neutralization in combination will be additive. By comparison with experimental results, these models can also be used to determine whether synergy or antagonism occurs. When using the Loewe additive model three profiles for the potency of antibody combinations were found. Most combinations of two antibodies demonstrated additive potency where the experimental potency was close to the predicted IC_{50} . However, some synergistic effects were found, especially in combinations with the CD4 binding site, MPER, and V1/V2 antibodies (Kong *et al.*, 2015). Synergistic effects also depended on the viral sensitivity to the antibody combination resulting in an overall additive effect although individual viruses could demonstrate antagonism or synergy. This was mainly found for combinations containing V3 antibodies which were usually the most potent antibodies tested at the time (Kong *et al.*, 2015). Competition between adjacent epitopes also occurs where for example, the V2 antibody PG9 competes with the V3 antibodies PGT121-PGT123 or between CD4bs antibodies and the interface antibody 8ANC195 (Derking *et al.*, 2015).

Synergistic effects have been noted on rare occasions for bispecific antibodies that target the virus only. A bispecific antibody that simultaneously engaged the V2 and V3 epitopes showed moderate levels of synergy (Davis-Gardner *et al.*, 2020). Another bispecific employing a

CAP256.25 scFv and the antibody binding fragment (Fab) of 10-1074, showed moderate levels of improved potency against a few viruses (Davis-Gardner *et al.*, 2020). In this case, neutralization was compared to single scFv-Fc or IgG rather than to the predicted combination titres or experimental combinations of the two arms, which may have overestimated the level of synergy (West *et al.*, 2012; Huang *et al.*, 2016; Song *et al.*, 2016; Khan *et al.*, 2018; Wagh, Seaman, *et al.*, 2018; Davis-Gardner *et al.*, 2020). More convincing evidence of synergy was observed when antibodies targeting a host cell protein and the viral Env protein were combined particularly as part of bispecific or trispecific antibody constructs. This effect is due to the localization of the anti-HIV antibody close to the host cell membrane through CCR5 or CD4 binding, for example, the 10E8-iMab, which targets the MPER region on the HIV virion and the CD4 receptor on the HIV target cell. This bispecific antibody has a geometric mean IC₅₀ potency of 0.002µg/mL compared to 0.4µg/mL for 10E8 and 0.05µg/mL for the iMab indicating a 25-fold improvement over the expected activity (Huang *et al.*, 2016; Moshoeite *et al.*, 2019).

Single chain variable fragments (scFv) are small molecules, which contain the variable heavy and light chain of antibodies connected through a glycine linker. These molecules have some enhanced pharmacokinetic properties such as improved distribution and absorption into mucosal tissues despite a loss of half-life due to lacking an Fc region (Yokota *et al.*, 1992; Colcher *et al.*, 1998; Unverdorben *et al.*, 2012, 2016). They may also display less steric hindrance when used in combination with other molecules and other bNAbs or scFv (Kumar *et al.*, 2017). This may be especially true for epitopes in close proximity such as the V2 and V3, or V3 and CD4bs where competition between IgG occurs. A recent study demonstrated that scFv targeting the V3 and CD4bs could display synergy when combined with fusion inhibitors (Alam *et al.*, 2019). We, therefore, tested combinations of scFv that target major epitopes on the HIV trimer, namely CAP256.25 (V2 apex), PGT121 (N332-supersite), 3BNC117 (CD4bs), 8ANC195 (gp120-gp41), and 10E8v4 (MPER).

In the previous chapter, we demonstrated that these scFv of bNAbs retain significant breadth and potency. In particular, 10E8v4 maintained the same breadth and most of its potency as an scFv. In this chapter, we demonstrate that combinations of scFv generally follow a model of additive potency and complementary breadth. Some combinations displayed low levels of synergy and we found little to no evidence of antagonism. Overall combinations of three scFv

antibodies reached considerable breadth and potency indicating that scFv in combinations may be suitable for passive immunity purposes.

4.2 Methods

For ease of reference, a brief summary of the methods described previously (in Chapter 2) is provided below (4.2.1-4.2.3). The methods and analyses that are unique to this chapter are described in more detail below.

4.2.1 *scFv expression and purification*

Genes of scFv of five HIV directed bNAbs targeting key trimer epitopes (CAP256.25, PGT121, 3BNC117, 8ANC195, and 10E8v4) were cloned into a CMV/R expression plasmid. Proteins were expressed in 293F cells cultured in HEK293 Freestyle media at 37°C at 150rpm. After 6 days, the supernatants were harvested by centrifugation at 1500g at 4°C and filtered through a 0.22µm filter. The supernatants were then run over a Ni-NTA gravity column washed with 30mM imidazole in 1X PBS, pH7.5 and eluted using 400mM imidazole in 1X PBS, pH7.5. The procedure was repeated to capture all protein in the supernatant. This was followed by size exclusion through a Superdex HiLoad 16/600 or 26/600 (200 pg) gel filtration column on an ÅKTA purifier. Thereafter, the elutant was buffer exchanged with 150mM NaCl, 3mM HEPES, and 2.5-5% glycerol and concentrated using Vivapore table-top concentrators to approximately 1mg/mL (see Chapter 2).

4.2.2 *Pseudovirus production for TZM-bl assay*

Plasmids containing HIV-1 envelope (gp160) genes cloned in the pcDNATM3.1D/V5-His-TOPO[®] vector were co-transfected with pSG3^{Δenv} into HEK293T cells and cultured for 48-72hrs at 37°C. Supernatants were harvested and filtered through a 45µm filter and frozen at -80°C. Virus stocks were titrated on TZM-bl cells using a luciferase assay to determine a dilution yielding RLU at least 10-fold above the “cell only” background (40,000-100,000 RLU).

4.2.3 *Neutralization assay*

Neutralization was measured using pseudoviruses in a validated single-cycle assay in TZM-bl cells. scFv proteins or IgG versions were added to growth media at a starting concentration of 50µg/mL or ~1400nM and serially diluted 3-fold in duplicate before being added to a 96 well plate (25µL). Pseudovirus was added in an equal volume (25µL) to the plate containing the diluted scFv and incubated for 1hr at 37°C. TZM-bl cells (20µL containing 0.5*10⁶/mL cells) in growth media (GM) containing 70µg/mL of DEAE were added and the mixture incubated for 24hrs. 100µL of GM was added and the cells incubated for another 24-48hrs, then half the

media (100µL) was removed and Bright-Glo firefly luciferase substrate mixed with assay buffer was added according to manufacturer specifications. After a 2min incubation at Room Temperature (RT), the media and luciferase mixture was mixed and transferred to a black 96 well plate. The plate was then read on the Victor-X luminometer.

4.2.4 Combinations of scFv

Combinations were tested by adding equimolar amounts of two or three scFv proteins in a neutralization assay as described above. Pre-dilutions containing scFv at 2µM and 10µM each were used to facilitate the assay set-up. As a control, the single scFv were diluted to 10µM and 2µM as well and run alongside the combinations as a comparison. The highest concentration tested for the combinations was 30µg/mL or 1000nM.

The IC₅₀ and IC₈₀ values from experimental combinations were compared to those of the single scFv and to the predicted IC₅₀ and IC₈₀ based on the **Loewe Additive model**. This model uses IC₅₀ and/or IC₈₀ data and predicts the IC₅₀ or IC₈₀ of an antibody combination. It assumes non-interaction between the antibodies and therefore that the values are additive.

The following formula is used to calculate Loewe Additivity.

$$Predicted\ IC_{50} = 1 / \left(\frac{1}{IC_{50}(A)} + \frac{1}{IC_{50}(B)} + \dots \frac{1}{IC_{50}(N)} \right)$$

Post analysis values were recalculated from nM into µg/mL based on the following formula.

$$IC_{50}\ in\ \mu g/mL = \frac{(Mw(A) \times IC_{50}) + (Mw(B) \times IC_{50}) + \dots (Mw(N) \times IC_{50})}{n \times 1000}$$

Where (n) is the number of antibodies, Mw the molecular weight of the antibodies in the combination, and IC₅₀ the experimental IC₅₀ or theoretical IC₅₀ obtained. The same formula is used for IC₈₀ values replacing the IC₅₀ with IC₈₀ in the formula above.

4.2.5 Synergy based on the Loewe model

Synergy was predicted based on whether the experimental IC₅₀ and IC₈₀ were improved compared to the Loewe Additive model. The formula below was used to characterize this effect.

$$LogFold = Log10\left(\frac{Loewe\ IC_{50}}{Experimental\ IC_{50}}\right)$$

For a combination of antibodies, the mean and 95% Confidence intervals were calculated, where a positive value indicated synergy and a negative value antagonism and either did not include 0. We used a cut-off of log fold <-0.35 or >0.35 which equates to roughly 2.5x difference to determine which individual test points show a potential difference.

4.2.6 Statistics

All statistics were done using the GraphPad Prism 8 software. Fold differences of the experimental combinations were calculated with the most potent scFv in the mixture for both IC_{50} and IC_{80} . The experimental and model-predicted data were correlated using Pearson's correlation coefficient with a two-tailed p-value. A nonlinear fit model was used to predict the slope between the experimental and predicted data, using a least-squares regression. This created a log-log line between x and y. Moreover, weighing $(1/Y^2)$ was used to minimize the effect of outliers, as variation in the extreme IC_{50} values is likely to have a larger impact on the curve. To test if experimental values differed significantly from the predicted values for the individual combinations, a Wilcoxon paired t-test was done. A log-fold with a 95% confidence interval was used to determine if the fold difference is significantly different from 0, indicating either synergy or antagonism.

Breadth-potency curves were drawn using a survival model in GraphPad. Significance was tested using the log-rank Mantel-Cox significance test.

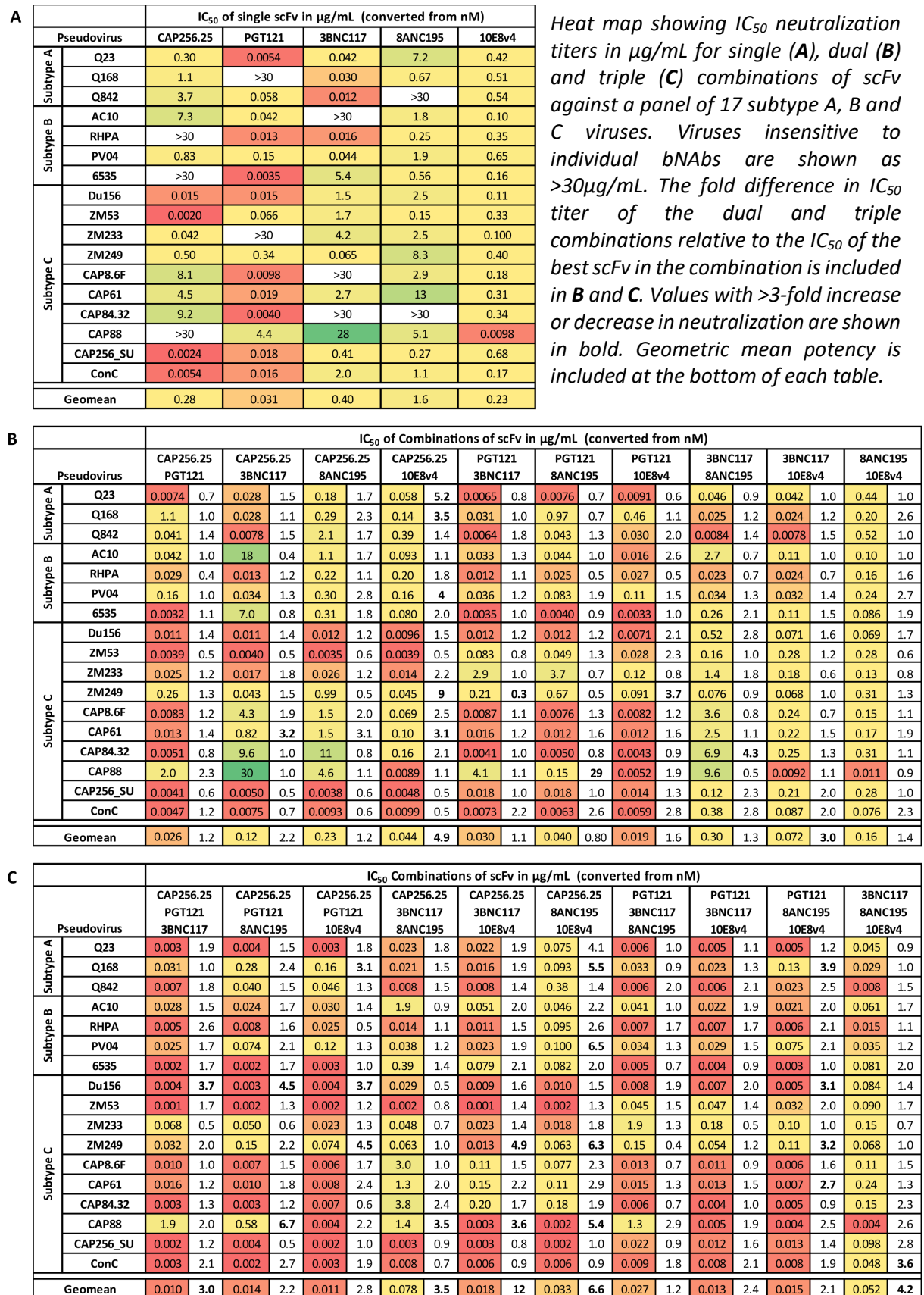
4.3 Results

4.3.1 Dual and Triple Combinations of scFv of HIV bNAbs

To assess whether scFv of HIV bNAbs show increased breadth and potency when used in combination, we tested five different scFv as part of dual and triple combinations. The scFv included those targeting the V2 (CAP256.25), the N332 glycan (PGT121), the CD4bs (3BNC117), and the MPER region (10E8v4), and all were previously shown in Chapter 3 to retain significant activity. For this study, we added the antibody 8ANC195 which targets the gp120-gp41 interface so that all five major epitopes on the HIV trimer were covered. All scFv were expressed and purified and size and purity were confirmed by running size exclusion columns and SDS-PAGE gels. Combinations of scFv were tested against a panel of 17 pseudoviruses from subtypes A, B, and C. These were selected based on their sensitivity to at least three of the five scFv under investigation. Equimolar amounts (based on their molecular weights shown in Chapter 2), of each scFv in combinations of two or three were tested, giving a total of 20 different sets of antibodies, which were compared to single scFv neutralization titres. To standardize the output data, the concentration of the single scFv and the scFv combinations in $\mu\text{g/mL}$ were calculated from the nM titres and the molecular weight of the scFvs (see Figure 4.1A).

As expected, combinations of two scFv improved the coverage, with active scFv making up for the inactive scFv. For some dual and triple combinations, breadth reached 100% at IC_{50} against this 17 virus panel. An improvement in potency was also observed for both the dual and triple combinations due to scFv with complementary potency profiles. The neutralization of individual viruses by two or three scFv, was usually similar to the IC_{50} of the most potent scFv in the combination (fold differences are shown in the columns next to the $\text{IC}_{50\text{s}}$) (Figure 4.1A). Except for a few cases, the virus neutralization titer of the single scFv and dual or triple combinations fell within 3-fold of the titer of the most potent single scFv in that combination. There was little to no antagonism for the entire panel for all 20 scFv combinations tested (a total of 340 single test titres). However, a few instances of potential synergy were noted with most of these linked to specific scFv combinations (fold differences with bolded values in Figure 4.1A).

Figure 4.1 Neutralization titers of single, dual and triple combinations of scFv.



The combination of CAP256.25+10E8v4 scFv stood out, demonstrating an overall 4.9-fold improvement in geometric mean titer (Figure 4.1B). This improvement was driven by five viruses of which two were subtype A, one was subtype B and two were subtype C indicating this was not subtype-specific. Five other dual scFv combinations showed improved potency over single scFv for specific viruses but showed less than 3-fold improvement in geometric mean potency of all viruses. The 3BNC117+10E8v4 combination also showed an overall 3-fold improvement in the geometric mean titer although this was not seen for individual viruses indicating complementarity of neutralization potency of the scFv rather than synergy.

For the triple combinations, five of the ten combinations showed improved geometric means compared to the most potent single scFv with an up to 12-fold improvement for the CAP256.25+3BNC117+10E8v4 combination (Figure 4.1C). This was higher than for the dual combinations where only two out of ten showed an improvement in geometric mean potency and was driven by improvement in potency against single viruses (16 of the 170 virus-antibody combinations). This was due to the different scFv such as is the case with CAP256.25 and 3BNC117 scFv, in the combinations being potent against different viruses allowing for complementarity in neutralization potency and coverage. Due to having more antibodies, this was to a higher degree observed in triple combinations compared to dual combinations leading to improved geometric means.

Some viruses appeared to be more sensitive to the effects of combined scFv. For example, a slight synergy was observed against the CAP61 and ZM249 viruses for the dual combinations, although for the latter loss of potency was also noted (fold difference <0.33 see Figure 4.1B). Some enhancement in potency was also seen with these viruses plus Du156 and CAP88 for the triple combinations (Figure 4.1C). Overall, we found either improvement or similar titres for combinations compared to single scFv particularly for triple combinations indicating that combinations of scFv improve the coverage of a panel of viruses and the potency at which viruses are neutralized.

4.3.2 scFv follow an additive model of interaction with low levels of synergy

To determine whether the combinations of scFv follow an additive model of interaction, we compared the experimental results with predictions based on the single scFv titres using the Loewe Additive model. For this, we also calculated the IC₈₀ of the dual and triple combinations

as this has a better predictive value for efficacy in HIV prevention (Webb *et al.*, 2015). This model assumes that two drugs or antibodies that do not interact will have an additive effect on their IC₅₀ and IC₈₀ values meaning that if equal amounts of antibody are present there will be no decrease or increase in potency compared to single antibodies. We calculated the IC₅₀ and IC₈₀ based on this model (the formula is given in the Methods section) and based on the geometric mean IC₅₀ and IC₈₀ of the repeats of single scFv obtained in the experiment. The predicted data was compared to the experimental IC₅₀ and IC₈₀ of the combinations. We compared the titres of dual combinations and triple combinations against viruses where both scFv are active (Figure 4.2A, C, E, G) and against all the viruses tested (Figure 4.2B, D, F, H).

There was a strong significant ($p < 0.0001$) correlation between the experimental IC₅₀ and IC₈₀ and the predicted IC₅₀ and IC₈₀ of the dual combinations with $r = 0.82$ and $r = 0.94$ respectively. This correlation was slightly weaker when combinations with inactive scFv were included, with a stronger effect seen in the IC₈₀ values. Similarly, for the triple combinations, there was a strong correlation between the predicted values and the experimental values $r = 0.96$ and $r = 0.83$ respectively for the IC₅₀ and IC₈₀ and a slightly weaker correlation when the pairs with inactive scFv were included in the IC₅₀ comparison. Although a few antibody combinations, showed some improved potency (red dots), or decreased potency (blue dots) to individual viruses compared to the Loewe model, overall, there was less than a 1-2 fold difference between the predicted values and the experimental data. This indicated that the IC₅₀ and IC₈₀ of the combinations could be predicted using the single scFv data in the Loewe model, although some variability existed.

To quantify the difference between the experimental data and the predicted data, the log-fold difference was calculated. Only viruses which were neutralized by all scFv in the specific combination were included in this analysis (see the formula in Methods). Comparing the experimental data to the expected titres allowed us to determine whether there is potential synergy (better than the model), as indicated by a log-fold difference > 0.35 and/or antagonism (worse than the model) as indicated by a log-fold difference < -0.35 (Figure 4.2I-L). Most dual combinations had a mean log-fold difference close to 0 (log-fold $-0.35 < CI < 0.35$) indicating that there was neither antagonism nor synergy.

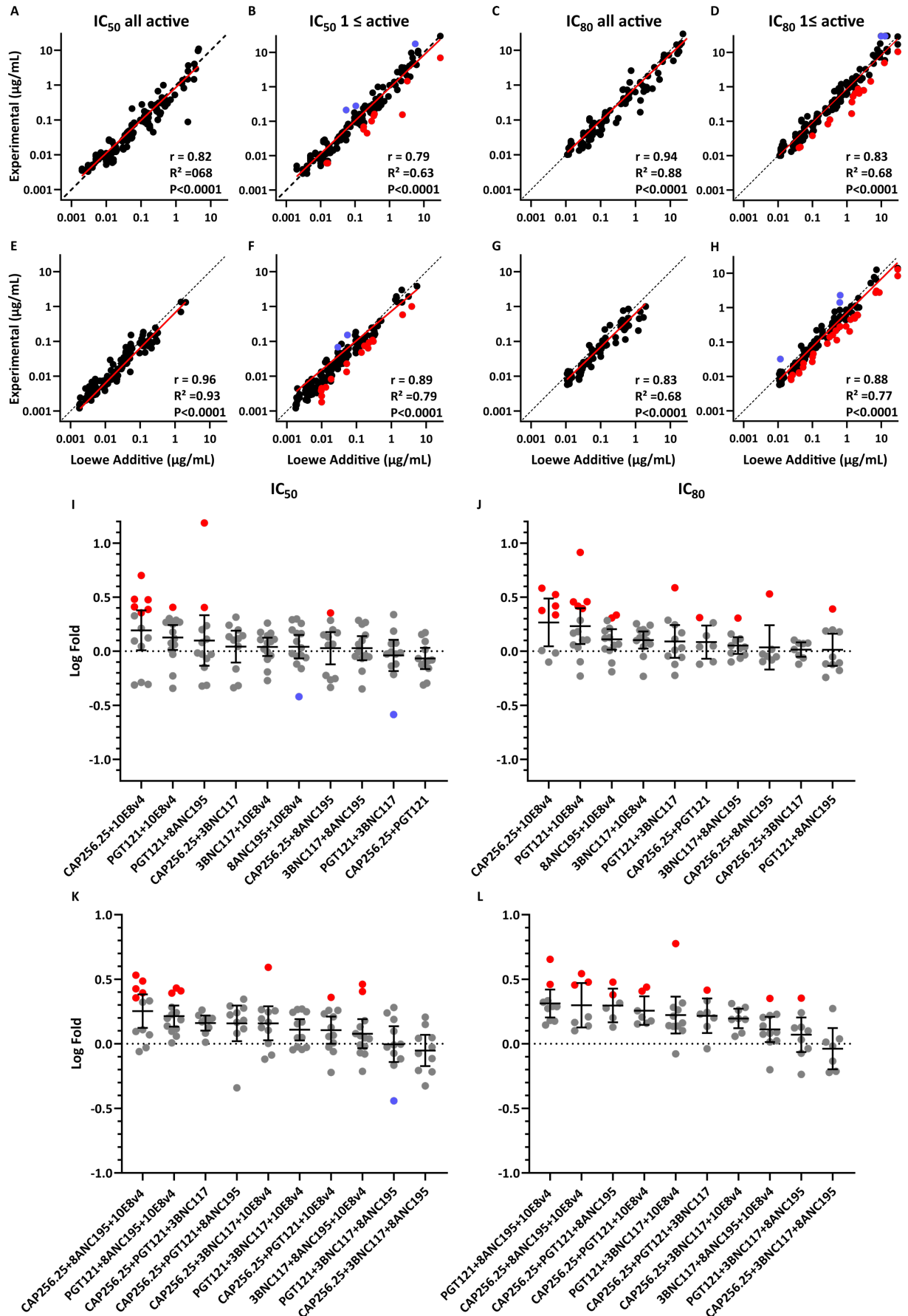


Figure 4.2 Comparison of experimental and predicted combinations of dual and triple scFv antibodies.

(A-D) IC_{50} and IC_{80} titers of dual antibody combinations plotted against the predicted IC_{50} and IC_{80} titers according to the Loewe Additive Model. Combinations where all scFv neutralized the virus (A, C) or where at least one scFv neutralized the virus (B, D). (Continued on next page)

Figure 4.2 continued. (E-H) Predicted IC_{50} and IC_{80} titers of triple combinations versus the experimental IC_{50} and IC_{80} titers where all scFv are active (**E, G**) and where at least one scFv is active (**F, H**). Red circles indicate improved potency (log-fold difference >0.35) of the combinations (synergy) whereas the blue circles represent reduced potency (antagonism, log-fold difference <-0.35). A nonlinear regression log-log line (red) and an equity line (black dotted) as well as r , R^2 and P values are shown. (**I-L**) Log-fold differences of double and triple IC_{50} (**I, K**) and IC_{80} (**J, L**) experimental combinations to the Loewe Additive model prediction. Mean and the 95% confidence interval are shown with a dotted line indicating no fold change. Red and blue are used as above. Only combinations where all single scFv are active and have a titer are included in the analysis for (**I-L**).

Some combinations had a mean improvement of potency compared to model predictions. For example, 10E8v4+CAP256.25 showed some potency improvement compared to the Loewe model in both the IC_{50} (CI: 0.0072-0.38) and IC_{80} (CI: 0.045-0.49) titres, albeit at low levels. Similarly, 10E8v4+PGT121 also showed a low level of synergy compared to the model (IC_{50} CI: 0.01-0.24, IC_{80} CI: 0.067-0.40). In general, more combinations showed a mean log-fold improvement (CI not including 0) in the IC_{80} compared to the IC_{50} for the dual combinations (four versus two combinations) (see first two dual combinations in IC_{80} and first four in IC_{80} Figure 4.2 I and J). Most of the dual combination neutralization titres against individual viruses for IC_{50} (119/131 virus-antibody combination titres) and IC_{80} (90/102) also ranged between a log-fold difference of -0.35 to 0.35 showing neither improvement nor decrease in potency compared to the model.

For the triple combinations, many of the combinations containing 10E8v4 showed a low level of synergy in either IC_{50} or IC_{80} or both (Figure 4.2K, L). The CAP256.25+PGT121+3BNC117 combination also showed an improvement compared to the model in both IC_{50} and IC_{80} titres. Indeed, only three combinations did not show an improvement compared to the model namely all triple combinations with 8ANC195+3BNC117 and either CAP256.25, PGT121, or 10E8v4. No loss of potency was observed (where CI did not include 0) for any of the dual or triple combinations in mean log-fold IC_{50} or IC_{80} . Individual triple combination neutralization titres against individual viruses for IC_{50} (100/113) and IC_{80} (70/83) mostly did not show less than a -0.35 or more than a 0.35 log-fold difference. Overall only a few cases of antagonism (log-fold difference <-0.35) were observed in the individual IC_{50} values of both dual and triple combinations, however, multiple instances of low-level synergy with improved potency compared to the model occurred for both IC_{50} and IC_{80} . This indicated that scFv in combinations show low levels of synergy and potency improvements both in individual titres

and on average compared to the prediction model. This also indicated that the model could accurately predict IC₅₀ and IC₈₀ titres, even though the model underestimated the potency of scFv combinations compared to the experimental results.

4.3.3 Neutralization curves of dual and triple combinations compared to single scFv

We overlaid the neutralization curves of the combinations with the single scFv used in the combination, to determine if there were distinct patterns associated with additive, synergistic, or antagonistic effects. We selected dual and triple combinations that showed synergy or antagonism using the Loewe model or potency improvements compared to the most potent scFv in the mix.

For most combinations, an additive effect was seen where the combination curve overlapped with the best scFv in the combination. This is demonstrated in Figure 4.3A for three different viruses and two scFv dual combinations. For Q842 and ZM233 the combination curve tracked with the 10E8v4 scFv, which was the most potent scFv in the combinations for these two viruses. In the case of ZM53, which is highly sensitive to CAP256.25, the combination curve overlapped with the CAP256.25 scFv.

A few dual combinations showed synergy with a left shift of the curve compared to the single scFv for individual viruses (10/170 viruses-antibody combinations at IC₅₀ and 14/170 viruses-antibody combinations at IC₈₀). Three examples are shown in Figure 4.3B that all include 10E8v4 with either PGT121 or CAP256.25. For all three viruses, the combination is more potent than either scFv alone. Besides this left shift, we also observed that the slope of the neutralization curve was improved or similar to the most potent scFv. This was seen for the CAP256.25+10E8v4 combination against ZM249 resulting in improved IC₅₀ and IC₈₀. Moreover, we also observed that the maximum neutralization could also be improved for example in the PGT121+8ANC195 combination against ZM249.

The levels of antagonism were generally very low with only one dual combination of PGT121+3BNC117 against the subtype C virus ZM249 showing less potency than either scFv alone. This could be partially mitigated by adding a third antibody (e.g. 8ANC195) in the mix. Otherwise, ZM249 was sensitive to the scFv ZM249 with three dual combinations showing

synergy (Figure 4.3C). Similarly, combinations against ZM53 and Q842 showed both additive effects and synergistic effects (Figure 4.3A, B).

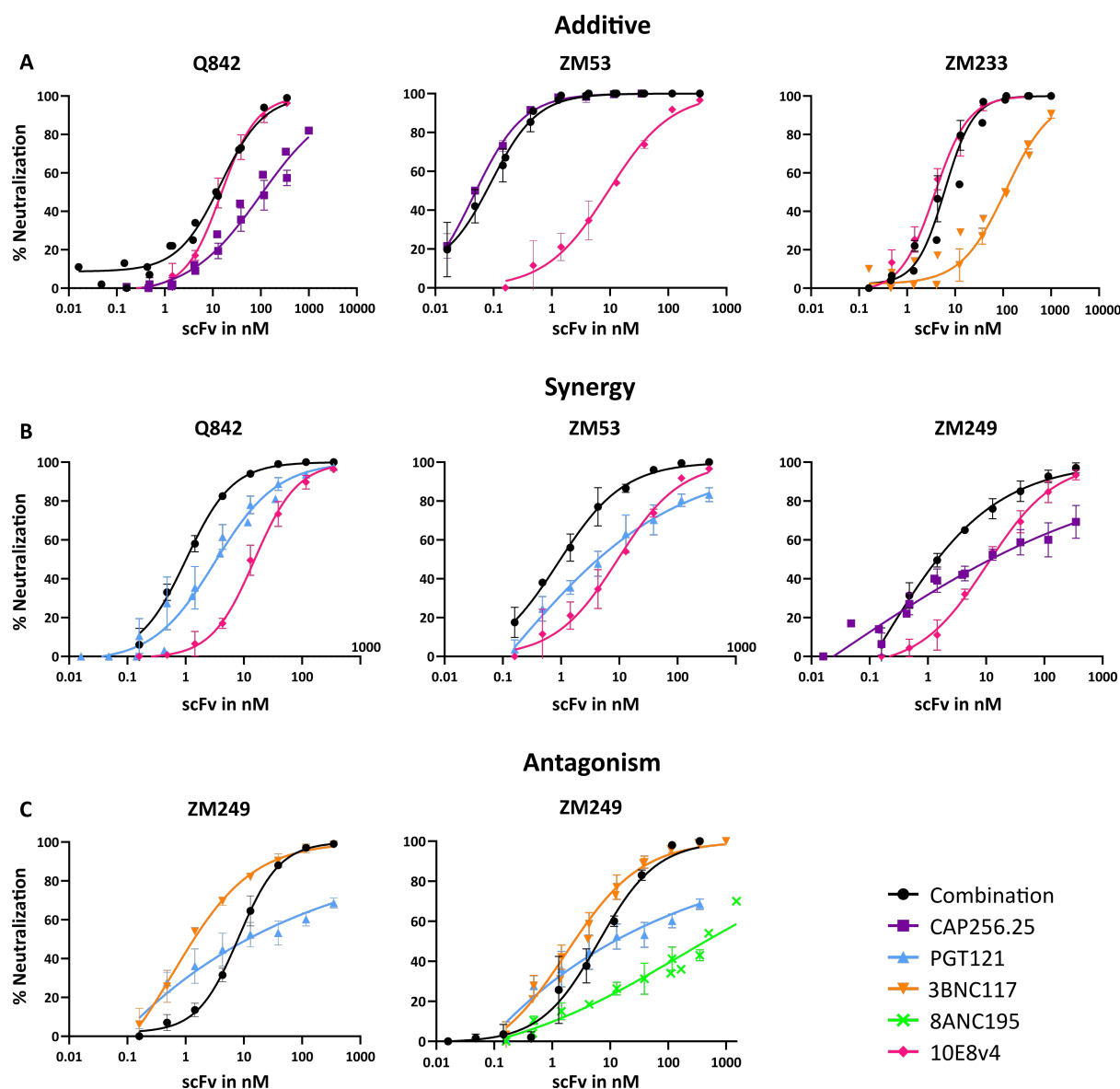


Figure 4.3 Neutralization curves showing additive, synergistic and antagonistic potency effects of combinations of antibodies.

Combinations of scFv compared to single scFv neutralization curves with CAP256.25 in purple, PGT121 in light blue, 3BNC117 in orange, 8ANC195 in green and 10E8v4 in pink. The dual or triple combinations are represented in black. Geometric mean values are used for each data point with error bars representing repeat experiments. (A) Examples of combinations where neither synergy nor antagonism is observed for three viruses tested against dual combinations of scFv. Additive potency is represented by the combination curve (black) overlapping with the best scFv in the mixture. (B) Synergy as observed in dual combinations of scFv against three different virus strains. Synergy was represented by a left shift of the combination curve (black) relative to the most potent scFv in the mixture, or by steeper neutralization curves resulting in improved IC_{80} . (C) Antagonism as represented by a right shift of the combination curve relative to the most potent scFv in the mixture, observed in combinations of two or three scFv tested against ZM249, which was sensitive to all scFv in the combinations.

We next looked at combinations of three scFv compared to single scFv neutralization curves to determine why more synergistic effects were observed in the triple combinations. Improvement observed in the triple combinations was often due to small improvements in potency observed in the dual combinations. For example, combinations of CAP256.25+10E8v4 or PGT121+10E8v4 showed potency improvement against CAP61, and similar effects were observed in triple combinations containing these scFv (Figure 4.4A). The addition of a third (weak) antibody did not affect the left shift of the combination of neutralization curves.

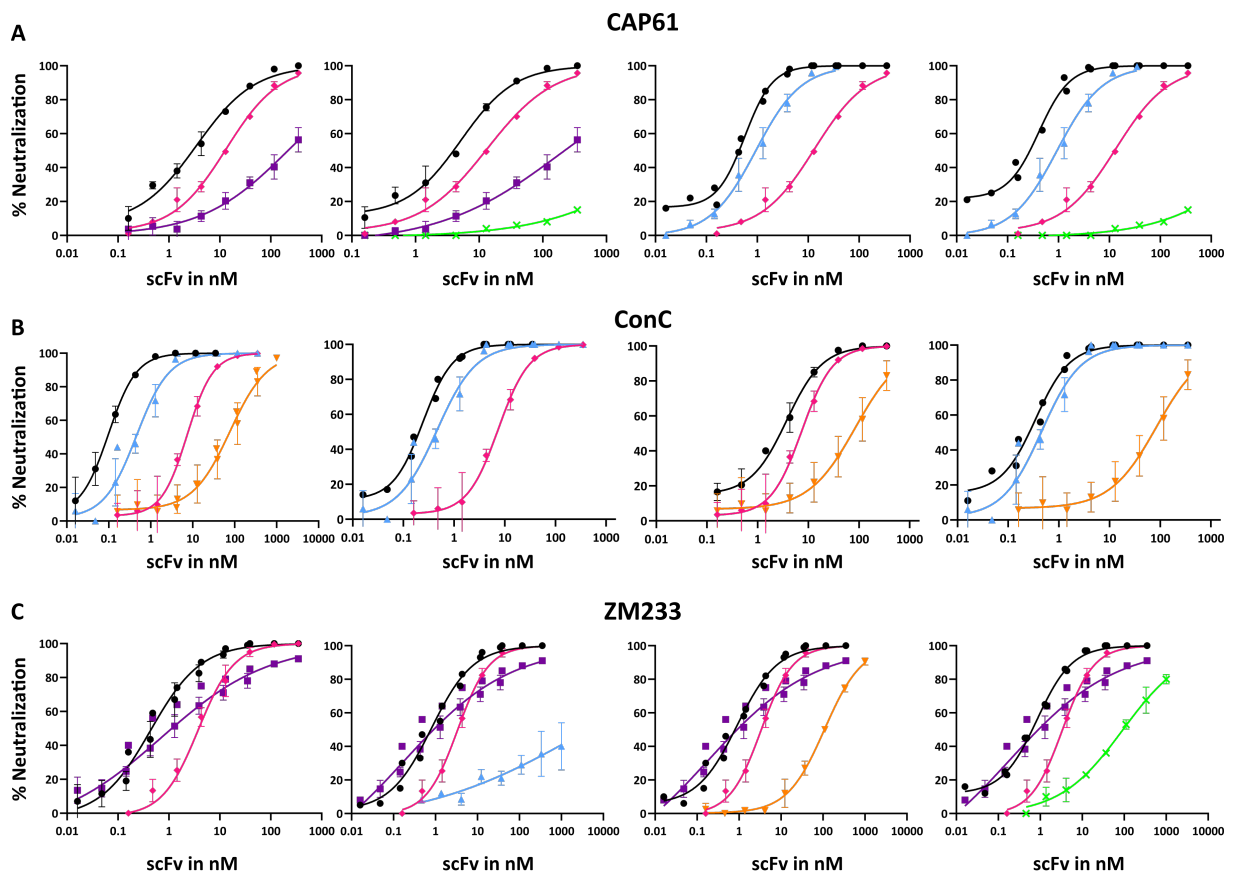


Figure 4.4 Combinations of three scFv show improved neutralization, maximum neutralization and improved IC_{80} .

(A) Neutralization of CAP61 showed synergistic effects when tested against combinations of 10E8v4 (pink) plus CAP256.25 (purple) or PGT121 (blue). Addition of 8ANC195 did not result in additional potency nor reduce the potency of the combination. (B) Neutralization of ConC demonstrated improved neutralization for some of the triple combinations, which was increased compared to dual combinations. (C) Neutralization for ZM233 was not improved at IC_{50} however, the IC_{80} did improve compared to the scFv, allowing for 100% neutralization at a lower concentration compared to the single scFv. Addition of PGT121, 3BNC117 or 8ANC195 did not improve neutralization above the combination of 10E8v4 and CAP256.25.

In some of the triple combinations, for example against ConC, we observed potency improvements which could be attributed to mild synergistic effects seen in the dual combinations where the effect was too low to be significant (Figure 4.4B). The synergistic effect of 10E8v4+CAP256.25 allowed for improved IC_{80} across multiple triple combinations tested against ZM233 as well mainly due to steeper slopes of the neutralization curve which was generally improved in the combinations. Here we also observed that the addition of a third antibody such as PGT121, 3BNC117, or 8ANC195 did not affect the combination neutralization curve (Figure 4.4C). Although neutralization of specific viruses such as CAP88, CAP61, ZM249, ZM233, and Du156, seems to have benefitted from combining antibodies, no clear pattern could be distinguished as IC_{50} or IC_{80} titres were often similar or showed only small improvements. Combinations containing CAP256.25+10E8v4 consistently showed the most enhancements in potency compared to the single scFv. Moreover, three antibodies in combination generally resulted in improved neutralization profiles, with improved potency at both IC_{50} and IC_{80} compared to dual combinations. Triple combinations, therefore, outperformed dual combinations overcoming antagonistic effects as well.

4.3.4 Using a model of Loewe Additivity to predict IC_{50} and IC_{80} titres of scFv combinations against a large virus panel

As demonstrated, IC_{50} and IC_{80} of scFv combinations could be accurately predicted using the Loewe additivity model, albeit with a slight underestimation. Therefore, we extrapolated the IC_{50} and IC_{80} data of single scFv for combinations of two and three scFv to the larger 45-virus panel described in Chapter 2. These theoretical scFv combinations were ranked based on their geometric mean potency using both IC_{50} and IC_{80} data for dual and triple combinations. Combinations with the expected highest breadth and potency were plotted in a breadth potency plot (Figure 4.5).

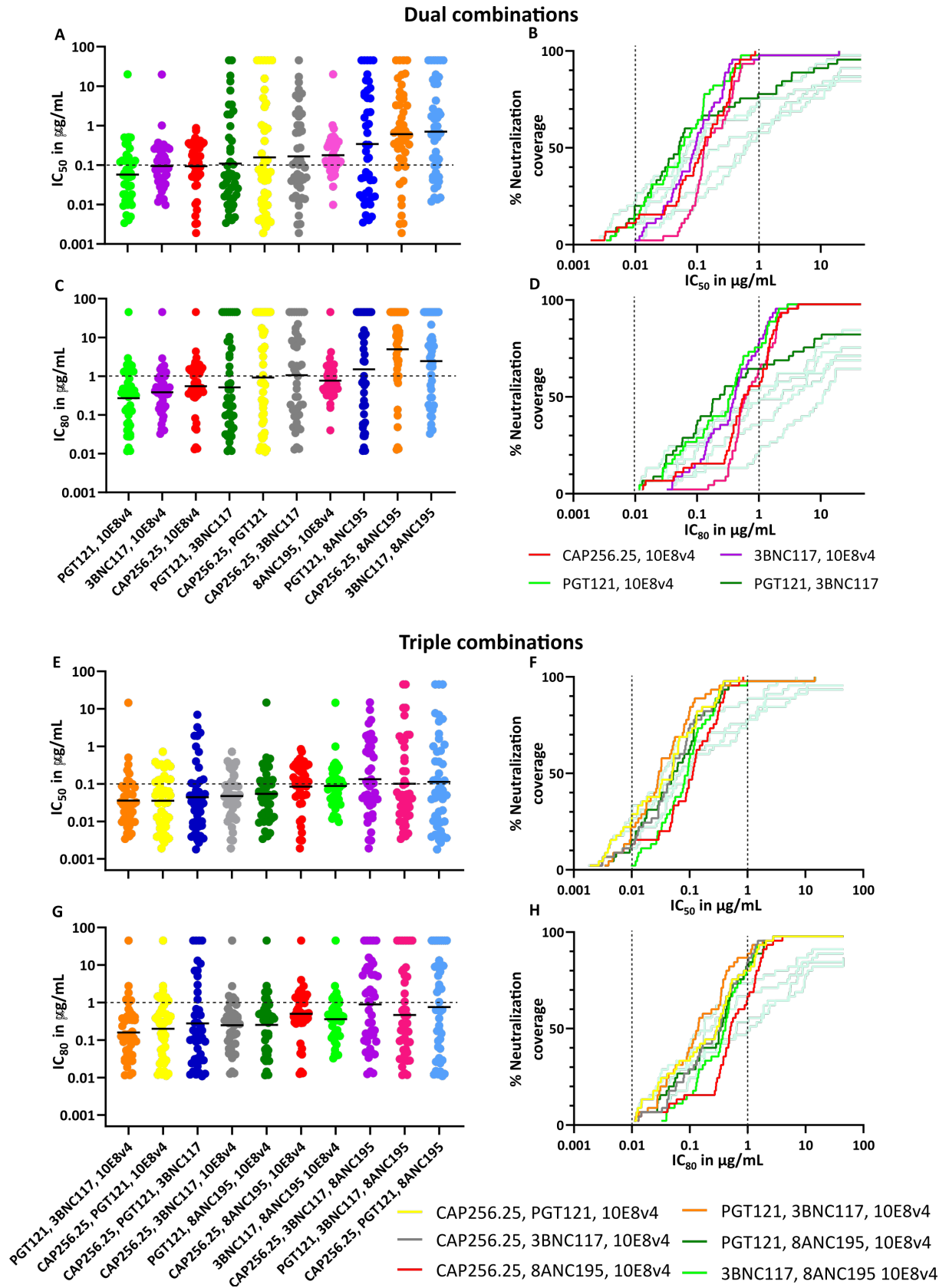


Figure 4.5 Breadth and potency of scFv of HIV bNAbs combinations.

(A) The titers of dual combinations were determined based on the single scFv data for a panel of 45 viruses. The IC_{50} data is plotted in $\mu g/mL$ recalculated from nM based on the formula given in the methods section. (B) The most potent and broad dual combinations were plotted for the IC_{50} , with the others in light blue (C) Predicted IC_{80} titers of dual combinations based on the Loewe additive model. (D) Breadth-potency curves from IC_{80} data for the most potent and broad dual combinations with the others shown in light blue. (Continued on next page).

Figure 4.5 continued. (E) IC_{50} titers for triple combinations based on the Loewe Additive model ranked based on geometric mean. **(F)** Breadth potency curves for the IC_{50} of the most potent and broad triple combinations are plotted with the other combinations shown in light blue. **(G)** Predicted IC_{80} titers for the triple combinations. **(H)** The breadth-potency curves based on IC_{80} titers of the most potent and broad combinations. The other combinations are shown in light blue. The maximum neutralization for the IC_{50} and IC_{80} data is set at $45\mu\text{g/mL}$ for all plots. Geometric mean is indicated by a black line in A, C, E and G. Dotted lines are shown at $0.1\mu\text{g/mL}$ for the IC_{50} (A, E) and at $1\mu\text{g/mL}$ for the IC_{80} values (C, G) and at $0.01\mu\text{g/mL}$ and $1\mu\text{g/mL}$ for breadth-potency plots (B, D, F, H). Ranking was based on geometric mean IC_{50} first then geometric mean IC_{80} . Second, number of viruses neutralized below $1\mu\text{g/mL}$ at IC_{50} and below $10\mu\text{g/mL}$ for IC_{80} was taken into account for the ranking.

The best dual combinations, namely PGT121+10E8v4 (light green), 3BNC117+10E8v4 (purple), and CAP256.25+10E8v4 (red) were able to neutralize most viruses below $1\mu\text{g/mL}$ at the IC_{50} and below $10\mu\text{g/mL}$ at the IC_{80} (Figure 4.5A, C). The breadth-potency curves of these three 10E8v4-containing combinations reached 100% neutralization at the IC_{50} and 98% neutralization at the IC_{80} (coloured breadth-potency plots versus pale blue, Figure 4.5B, D) with most viruses neutralized between 0.01 and $1\mu\text{g/mL}$ at IC_{50} . The fourth combination of 10E8v4 with 8ANC195 was less effective due to the low potency of both antibodies and the limited breadth of 8ANC195. None of the other dual combinations showed complete neutralization of the panel (Figure 4.5D). Furthermore, these combinations had larger numbers of resistant viruses (set at $>45\mu\text{g/mL}$), as demonstrated by the data points at the top of the potency plots (Figure 4.5A, C). While PGT121+3BNC117 had a geometric mean below $0.1\mu\text{g/mL}$, there was a larger spread of neutralization titres (Figure 4.5A, C, dark green). Moreover, neutralization breadth was lost at IC_{80} as indicated by the downward shift of the breadth potency curve compared to the IC_{50} curve (Figure 4.5B, D, green).

The triple combinations showed higher levels of potency and complete neutralization with 8/10 showing complete neutralization at IC_{50} and 6/10 showing near-complete neutralization at IC_{80} (Figure 4.5E-H). Moreover, 7/10 triple combinations had a geometric mean potency below $0.1\mu\text{g/mL}$ at IC_{50} and of these, three combinations neutralized all viruses below $1\mu\text{g/mL}$ (Figure 4.5E, yellow, grey, red). At IC_{80} these three combinations all had a geometric mean potency below $1\mu\text{g/mL}$ (Figure 4.5E, G). This indicated that triple combinations are able to reach nearly 100% neutralization at lower concentrations for both IC_{50} and IC_{80} .

The breadth of each theoretical combination was next determined at four concentrations: 0.1, 1, 10, and $45\mu\text{g/mL}$ in addition to the geometric mean for the combinations tested (with a cut off at $45\mu\text{g/mL}$) (see Figure 4.6). Combining these scFv, improved the coverage at all

concentrations for both IC₅₀ and IC₈₀. Breadth for dual combinations ranged from 87% to 100% at IC₅₀ and 64% to 98% at IC₈₀. In dual combinations, 100% breadth at IC₅₀ was reached for one combination at 1µg/mL and for three combinations at 45µg/mL. For the IC₈₀, 98% breadth was obtained at 10µg/mL for the same combinations. Most combinations of three scFv had 100% breadth for the IC₅₀ at the highest concentrations tested, except for PGT121+3BNC117+8ANC195 and CAP256.25+PGT121+8ANC195, which had a breadth of 96% and 93.3% respectively (Figure 4.6). Breadth was especially improved for lower concentrations at IC₈₀. For example, breadth for single scFv ranged from 0 to 44% for IC₅₀ and 0 to 24% for IC₈₀ compared to the lowest concentration, whereas dual combinations ranged from 22 to 62% for the IC₅₀ and 2 to 29% for IC₈₀, and combinations of 3 antibodies ranged from 47% to 82% for IC₅₀ and 16 to 40% for IC₈₀. Geometric mean potency for the panel also improved from 0.12-2.94 µg/mL for single scFv to 0.058-0.4µg/mL for dual combinations and 0.036-0.13µg/mL for triple combinations. This indicated that combinations of scFv were able to increase the coverage at lower concentrations, which will be vital in clinical settings.

Since PGT121, 3BNC117, and CAP256.25 all had good potencies as scFv, using these together with 10E8v4 resulted in improved potency of the combination. Moreover, scFv such as CAP256.25 and PGT121 and/or 3BNC117 have complementary profiles, with 3BNC117, for example, being especially potent against subtype B viruses and CAP256.25 being potent against subtype C viruses (also see Chapter 3). 10E8v4 is the broadest scFv and that resulted in combinations containing 10E8v4, having the highest breadth.

As a result, four of the five triple combinations with the best geometric mean titres included 10E8v4. On the contrary, introducing less potent or less broad antibodies such as 8ANC195 resulted in less favourable antibody combinations, where 5 out of 6 combinations with the lowest potency and breadth contained 8ANC195, consistent with the relatively lower potency and breadth of this antibody. Of the top five, three combinations, CAP256.25+PGT121+10E8v4 and CAP256.25+3BNC117+10E8v4 and CAP256.25+8ANC195+10E8v4 neutralized all viruses below 1µg/mL and 98% of viruses at IC₈₀ below 10µg/mL (see Table 4.1). The best ranked PGT121+3BNC117+10E8v4 combination neutralized 82% of viruses below 0.1µg/mL and 98% of viruses below 1µg/mL at the IC₅₀ value and 89% of viruses below 1µg/mL and 98% of viruses below 10µg/mL at the IC₈₀ value. This analysis indicated a significant gain of potency and neutralization at lower concentrations than single scFv at both IC₅₀ and IC₈₀. Moreover,

significant gains of breadth were observed at the IC₈₀ value for 6/10 triple combinations. These data demonstrate that the benefits of combinations of IgG targeting different HIV epitopes are transferable to the smaller and more versatile scFv fragments.

		IC ₅₀ in µg/mL					IC ₈₀ in µg/mL				
		0.1	1	10	45	Geometric mean	0.1	1	10	45	Geometric mean
1 scFv	CAP256.25	20%	49%	60%	62%	0.21	11%	16%	29%	47%	1.55
	3BNC117	29%	47%	69%	78%	0.39	13%	31%	62%	67%	0.86
	PGT121	44%	56%	62%	71%	0.12	24%	44%	51%	56%	0.20
	10E8v4	24%	96%	98%	100%	0.21	2%	49%	98%	98%	0.82
	8ANC195	0%	20%	51%	69%	2.94	0%	7%	24%	40%	4.95
2 scFv	PGT121 + 10E8v4	62%	98%	98%	100%	0.058	29%	76%	98%	98%	0.24
	PGT121 + 3BNC117	62%	78%	91%	96%	0.083	36%	64%	80%	82%	0.20
	3BNC117 + 10E8v4	56%	96%	98%	100%	0.0946	16%	78%	98%	98%	0.34
	CAP256.25 + PGT121	53%	73%	82%	84%	0.059	31%	53%	62%	71%	0.22
	CAP256.25 + 3BNC117	47%	71%	93%	98%	0.15	22%	47%	76%	84%	0.53
	PGT121 + 8ANC195	44%	60%	80%	87%	0.17	24%	47%	58%	69%	0.35
	CAP256.25 + 10E8v4	42%	100%	100%	100%	0.09	16%	56%	98%	98%	0.50
	3BNC117 + 8ANC195	29%	58%	76%	87%	0.37	13%	36%	69%	73%	0.86
	8ANC195 + 10E8v4	27%	96%	98%	100%	0.18	2%	64%	98%	98%	0.70
	CAP256.25 + 8ANC195	22%	58%	82%	91%	0.40	11%	20%	47%	64%	1.60
3 scFv	PGT121 + 3BNC117 + 10E8v4	82%	98%	98%	100%	0.036	40%	89%	98%	98%	0.14
	CAP256.25 + 3BNC117 + 10E8v4	73%	100%	100%	100%	0.047	29%	84%	98%	98%	0.22
	CAP256.25 + PGT121 + 3BNC117	73%	89%	100%	100%	0.044	42%	73%	87%	91%	0.17
	CAP256.25 + PGT121 + 10E8v4	71%	100%	100%	100%	0.035	33%	80%	98%	98%	0.18
	PGT121 + 8ANC195 + 10E8v4	62%	98%	98%	100%	0.054	29%	82%	98%	98%	0.23
	PGT121 + 3BNC117 + 8ANC195	62%	78%	91%	96%	0.076	36%	67%	84%	84%	0.20
	3BNC117 + 8ANC195 + 10E8v4	58%	98%	98%	100%	0.088	16%	82%	98%	98%	0.32
	CAP256.25 + PGT121 + 8ANC195	53%	78%	93%	93.3%	0.074	31%	53%	78%	82%	0.31
	CAP256.25 + 3BNC117 + 8ANC195	51%	78%	98%	100%	0.13	24%	49%	82%	89%	0.54
	CAP256.25 + 8ANC195 + 10E8v4	47%	100%	100%	100%	0.085	16%	67%	98%	98%	0.45

Figure 4.6 Predicted maximum percentage inhibition for single scFv and combinations of scFv against a 45-virus panel.

Percentage neutralization was calculated at 0.1, 1, 10 and 45µg/mL from single scFv titers and from predicted scFv dual and triple combination titers for the IC₅₀ and IC₈₀. Colors indicate percentage neutralization with 1-19% in green, 20-49% yellow, 50-79% yellow-orange, 80-89% orange, 90-99% red and 100% dark red. Geometric mean at 45µg/mL is also given.

4.4 Discussion

Combinations of scFv were able to enhance the breadth and potency of HIV-1 neutralization. Specifically, several triple combinations reached 100% coverage at IC₅₀ and 98% coverage at IC₈₀ and showed a dramatic improvement in potency due to complementary neutralization profiles. These findings are similar to earlier studies on IgG where the enhanced breadth and geometric mean potency were also observed using antibody combinations. These levels were slightly higher in the triple combinations compared to the dual combinations and suggested the possibility of additional benefits of using these small antibody molecules.

Generally, two-scFv combinations followed an additive model of potency. This finding is similar to combinations of IgG and is expected as these epitopes represent distinct areas on the trimer. Moreover, there was little to no antagonism observed in any of the combinations tested when compared to single scFv titres. This was also true when we compared the experimental combination titres to the predicted titres using the Loewe model. With IgG, some antagonism has been noted when using this model, however, head-to-head comparisons of IgG and scFv against a larger virus panel would be necessary to determine the extent of this difference (Kong *et al.*, 2015). Steric hindrance may impact scFv less than IgG although we observed some interference in the case of 3BNC117 and PGT121. Both these scFv have a similar potency against the ZM249 virus but some antagonism was noted compared to the single scFv titres. This is probably because the targets of these two antibodies are relatively closely positioned on the envelope trimer (also see Figure 1.7, Introduction). The addition of a third antibody (in a triple combination with e.g. 10E8v4) was, however, able to negate the negative effects of this combination against this virus. The few other cases of antagonism noted for scFv combinations may be due to conformational changes rather than steric hindrance, which would occur after antibody binding. To explore this, scFv-trimer complex crystallization studies would have to be done.

Using the Loewe additive model, low levels of synergy were observed for combinations containing the MPER antibody 10E8v4 and the V2 antibody CAP256.25. This may be due to overlapping neutralization profiles where both of the scFv potentially neutralize the same virus. Similar findings were noted in a previous study, where a combination of MPER (10E8) and V2 (PG9) IgG antibodies showed some synergistic effects in a subset of viruses using the Loewe model (Kong *et al.*, 2015). Overlapping neutralization may be beneficial as scFv can bind

trimers in different states, with CAP256.25 binding early, whereas 10E8v4 is likely to bind trimers that are in an intermediate state allowing for complementarity in virus neutralization (Doria-Rose *et al.*, 2015; Kong *et al.*, 2015; Zhou *et al.*, 2017; Pinto *et al.*, 2019). Potency improvements in dual combinations were often amplified in the triple combinations with, for example, 10E8v4 and CAP256.25 present in about half of the triple combinations that showed low-level synergy in both IC_{50} and IC_{80} . To determine if this low-level synergy is an epitope-dependent effect, different scFv targeting the same epitope would need to be tested (Kong *et al.*, 2015; Wagh *et al.*, 2016). Timed attachment assays and binding assays in combination with structural studies could also provide more clarity into this phenomenon. We are also exploring the use of a more recent model, the Bliss-Hill model to predict combined neutralization titres to determine if this synergy is a true improvement or is a feature of the Loewe Additive model. The Bliss-Hill model takes into account the slope of the neutralization curve and both IC_{50} and IC_{80} , and therefore may improve predictions of combined titres compared to the Loewe Additive model used here (Wagh *et al.*, 2016).

From a clinical perspective, the use of antibody combinations may limit the impact of suboptimal antibody levels. Overlapping neutralization can enhance potency against single viruses, which ensures that complete viral neutralization is achieved at lower concentrations of antibody. Neutralization slopes were also improved by combinations of antibodies, especially IC_{80} titres and neutralization plateaus (less than 100% neutralization of a virus) were also less common with antibody combinations. The selection of antibody combinations with high potency (IC_{80}) can more effectively counter declining antibody levels associated with passive administration. Although combinations of two scFv neutralized 100% of viruses at the highest concentration tested, the coverage was much reduced at lower concentrations, whereas triple antibody combinations demonstrated much higher coverage at lower concentrations by combining broad antibodies with very potent antibodies. For example, at an IC_{50} of 1 μ g/mL, 100% of viruses were neutralized and at an IC_{80} of 1 μ g/mL, 89% of viruses were neutralized. This demonstrates that complementary neutralization of the same virus is extremely advantageous, which was previously demonstrated for combinations of IgG (Wagh, Seaman, *et al.*, 2018). Titres are likely to improve if newer scFv are developed which retain better potency compared to the IgG. Moreover, the selection of antibodies with optimal potency to cover geographically relevant viruses will ensure ongoing efficacy in the region.

For long-term antibody-mediated protection, adherence, and maintaining sufficient plasma levels in a wider population is critically important. At lower concentrations, scFv and IgG lose coverage especially in the ability to fully neutralize the virus (IC₈₀ values). This is also observed for IgG which, means that all the antibodies in the combination must be kept at optimal levels. For IgG, one way to achieve this has been to introduce mutations in the Fc portion of the antibody to increase the half-life by up to 6 months (Liu *et al.*, 2019; Rosenberg *et al.*, 2019). A second option is to introduce them into the body via a stable expression system through a vectored immunoprophylaxis (VIP) approach. However, due to the space constraints on vectors, constructs with multi-specific modalities would prove difficult. Bispecific products, which would partially overcome this, are also limited by space constraints. scFv in comparison with their much smaller size may be more readily expressed on AAV, allowing for the expression of multiple scFv continuously. scFv have a genetic size approximately 2.6X smaller than IgG allowing for the expression of minimally three scFv per AAV. (Dong *et al.*, 2008; Z. Wu *et al.*, 2010; West *et al.*, 2012; Monnier *et al.*, 2013; Gruenert *et al.*, 2016; Liu *et al.*, 2017; Hay *et al.*, 2018; Lin *et al.*, 2018).

The scFv studied here demonstrated high breadth and potency when used in combinations. Although only a limited number of scFv were tested on a small panel of viruses we demonstrated their potential against a larger panel of viruses through modelling. By selecting bNAbs that target all five epitopes we also showed the potential for complementary and synergistic effects. These scFv showed little to no antagonism something which was previously observed with IgG which are five times larger. As more potent scFv are engineered, combinations with even better efficacy may be found. In addition, as scFv are more readily expressed on vectors, this could provide an alternative avenue for the use of bNAbs in passive immunization. The results of the AMP trials are expected to fast-track the need to consider long-term delivery approaches for those antibodies with clinical benefit.

Addendum

Subsequent to this work, we initiated a collaboration with Dr. Kshitij Wagh from the Los Alamos National Laboratory, who developed the Bliss-Hill model. This model incorporates both IC_{50} and IC_{80} data to predict combination neutralization curves. In general, it is slightly more accurate in predicting combination titres and shows lower levels of improved potency compared to the Loewe model. This additional data analysis will be included in the planned manuscript describing the breadth and potency of scFv combinations.

5 Prevention of cell-associated transmission of HIV by scFv of bNAbs.

5.1 Introduction

Transmission of HIV-1 can occur via either infection of cells by free virions or through cell-to-cell routes. Transmission of viral particles through cell-mediated contact is reported to be 18,000-fold more efficient than transmission of cell-free particles (Chen *et al.*, 2007; Martin *et al.*, 2010). This enhanced efficiency is thought to be due to simultaneous co-transmission of multiple viral particles, which also allows for multiple integration events (Chen *et al.*, 2007; Martin *et al.*, 2010). There is *in vivo* evidence for transmission of cell-associated HIV in both humans and macaques (Pais-Correia *et al.*, 2010; Sallé *et al.*, 2010; Ndirangu *et al.*, 2012; Bernard-Stoecklin *et al.*, 2013, 2014; Kolodkin-Gal *et al.*, 2013). Although the relative contribution of this mode of transmission, compared to that of cell-free transmission, is not entirely clear, HIV infected cells have been identified in breastmilk and genital secretions suggesting a potential role for cell-cell transmission in primary HIV infection (Sallé *et al.*, 2010; Anderson *et al.*, 2014; Milligan *et al.*, 2014; Sagar, 2014). New cell culture models have suggested that cell-cell transmission occurs in synergy with cell-free transmission and that depending on diverse factors, either mode of transmission may be favoured (Lopez *et al.*, 2019; Ahmed *et al.*, 2020). Therefore, HIV prevention strategies should ideally aim to block both cell-free and cell-cell transmission.

There are several mechanisms that may account for cell-cell transmission of HIV. One important mechanism is the virological synapse, which is very similar to the immunological synapse formed between immune cells (Bracq *et al.*, 2018). The formation of virological synapses has been demonstrated *in vivo* for murine leukaemia virus by intravital imaging, and for HIV in humanized mouse models and non-human primate models (Murooka *et al.*, 2012; Bernard-Stoecklin *et al.*, 2013, 2014; Law *et al.*, 2016). These studies showed that infected T-cells make contact with other T-cells, enabling cell-cell transfer of HIV to occur. During the formation of virological synapses, HIV production in the donor cell becomes polarized to the site of contact, resulting in the formation of a bridge between the cells, in a process mediated by CD4 and the co-receptors on the target cell and Envelope protein on the infected cell. Nef protein also induces increased filopodia formation in the infected T-cells, which in turn

increases contact with other T-cells, resulting in enhanced viral transfer (Nobile *et al.*, 2010; Aggarwal *et al.*, 2012). A second form of cell-cell transmission occurs between two different cell types, such as transmission between a Dendritic Cell (DC) and a T-cell. In this mode of transmission, HIV virions are captured by the macrophage or DC and concentrated in virion-containing vesicles (VCC), and then presented to T-cells through immunological synapses (Mothes *et al.*, 2010; Evans *et al.*, 2013; Manches *et al.*, 2014; Bracq *et al.*, 2018).

The use of virological synapses and existing T-cell contacts enables cell-cell transfer to facilitate the more rapid spread of HIV through T-cell networks than cell-free transmission. Moreover, cell-cell transmission is linked to the rapid depletion of T-cells through pyroptosis (Galloway *et al.*, 2015). Cell-cell transmission also allows for direct infection of memory T-cells and induces cell senescence after cell-cell transfer, significantly contributing to the establishment of a viral reservoir in memory T-cells (Agosto *et al.*, 2018; Rezaei *et al.*, 2018). Lastly, cell-cell transmission may provide a mechanism for continued replication and spread of HIV despite ART (Sigal *et al.*, 2011; Galloway *et al.*, 2015; Martinez-Picado *et al.*, 2016; Agosto *et al.*, 2018; Pedro *et al.*, 2019). These data further support the need for biomedical interventions able to interrupt cell-cell transmission.

Broadly neutralizing antibodies (bNAbs) that can neutralize large numbers of global HIV strains are a major focus for HIV prevention and treatment (Sok *et al.*, 2018). These bNAbs, isolated as monoclonal antibodies from HIV-infected donors can prevent cell-free HIV infection. For example, SHIV infection of macaques can be prevented by passively infused bNAbs (Parren *et al.*, 2001; Pegu *et al.*, 2014; Gautam *et al.*, 2016; Julg, Liu, *et al.*, 2017; Xu *et al.*, 2017). Such studies are the basis of the current clinical assessment of the bNAb VRC01 in the HVTN Antibody-Mediated Protection (AMP) trial (www.amp.org). However, the ability of bNAbs to prevent cell-cell transmission is less clear. Partly, this knowledge gap reflects technical challenges in accurately measuring cell-cell transmission and distinguishing this from fusion or cell-free transmission (Sigal *et al.*, 2011; Durham *et al.*, 2012; Malbec *et al.*, 2013). Newly developed assays, used in this study, have greatly facilitated accurate measurement of cell-cell transmission (Reh *et al.*, 2015). There is some evidence that some bNAbs show reduced activity against cell-cell transmission (Rosenberg *et al.*, 2016; Xu *et al.*, 2017; Julg *et al.*, 2019). First-generation bNAbs, such as b12, 4E10, and 2F5 showed variable capacity to neutralize cell-associated virus (Martin *et al.*, 2010; Durham *et al.*, 2012). More recently isolated bNAbs with greater breadth and potency, such as the CAP256-VRC26 lineage, PGT121 and 3BNC117

also show significant loss of potency in the context of cell-cell transmission (Abela *et al.*, 2012; McCoy *et al.*, 2012; Malbec *et al.*, 2013; Gombos *et al.*, 2015; Reh *et al.*, 2015). In addition to reduced potency, a decrease in maximum neutralization was also observed compared to free virus neutralization (McCoy *et al.*, 2014; Li *et al.*, 2017). Moreover, in macaque models, higher concentrations of bNAbs were required to prevent cell-cell transmission compared to cell-free transmission (Malbec *et al.*, 2013; Li *et al.*, 2017; Parsons *et al.*, 2017). Lastly, mathematical models suggested a higher likelihood of escape mutations occurring in cell-cell transmission compared to cell-free transmission (Reh *et al.*, 2015). This reduced activity of bNAbs in preventing cell-cell transmission may have implications for both HIV prevention and treatment, where sub-optimal neutralization may result in rapid viral escape.

The mechanism for the reduced potency of bNAbs in cell-cell transmission is unclear but appears to be independent of cell-free neutralization potency (Malbec *et al.*, 2013; Reh *et al.*, 2015). Access of bNAbs to the VCC in DC and macrophages and to virological synapses may be limited, providing a general mechanism for their reduced potency (Chu 2012). However, reduced activity in the cell-cell assay is also, in part, epitope-specific. Antibodies which bind to the HIV envelope before CD4 engagement, tend to lose potency in cell-cell transmission, whereas those that bind after CD4 engagement generally retain their potency (Abela *et al.*, 2012; Reh *et al.*, 2015). Thus, many CD4 binding site (CD4bs) antibodies such as VRC01, 3BNC117, NIH45-46 show significantly reduced potency in cell-cell transmission assays (Abela *et al.*, 2012; Malbec *et al.*, 2013). In contrast, V3 bNAbs (10-1074 and PGT121) retain considerable potency in preventing cell-cell transmission (Reh *et al.*, 2015).

In some studies, smaller antibody-derived molecules performed better at mediating cell-cell neutralization than full antibodies (Martin *et al.*, 2010; Malbec *et al.*, 2013). A llama-derived CD4bs-directed single domain antibody, J3 (15kDa), was highly potent in cell-cell transmission (McCoy *et al.*, 2014). Similarly, the Fab of an MPER-directed bNAbs, 10E8, retained potency in DC-T cell transmission assays compared to cell-free transmission, whereas the full-sized IgG lost significant potency (Duncan *et al.*, 2014). These data suggest that steric hindrance may play a role in the reduced ability of bNAbs to prevent cell-cell transmission and that smaller molecules derived from bNAbs may be a viable option for inhibition of cell-cell transmission.

In cell-free neutralization assays, single-chain variable fragments (scFv) of bNAbs, smaller antibody fragments that incorporate the full paratope, retained considerable breadth and

potency compared to their IgG counterparts (van Dorsten *et al.*, 2020). Furthermore, combinations of scFv of N332 bNAbs inhibited cell-associated virus transmission, unlike matched IgG (Alam *et al.*, 2019). As scFv are about five times smaller than IgG they could potentially be more effective at preventing cell-cell transmission. We, therefore, tested four matched scFv and IgG, derived from bNAbs CAP256.25, PGT121, 3BNC117, and 10E8v4 in a cell-cell transmission assay and a cell-free transmission assay. The scFv but showed reduced potency in the cell-free virus transmission assay, as we have previously reported (van Dorsten *et al.*, 2020). However, unlike the parental IgG which lost significant activity in the cell-cell neutralization assays, the scFv were able to neutralize cell-associated virus with comparable activity to the IgG. The ability of these smaller antibody fragments to maintain almost complete activity against cell-associated virus supports the use of scFv derived from HIV-1 directed bNAbs for HIV prevention and treatment.

5.2 Methods

For ease of reference, a brief summary of the methods described previously (in Chapter 2) is provided below (5.2.1-5.2.3). Methods and analyses that are unique to this chapter are described in more detail below.

5.2.1 *scFv expression and purification*

scFv based on five HIV-directed bNAbs targeting key trimer epitopes (CAP256.25, PGT121, 3BNC117, 8ANC195, and 10E8v4) were cloned into a CMV/R expression plasmid. Proteins were expressed in 293F cells cultured in HEK293 Freestyle media at 37°C at 150rpm. After 6 days, the supernatants were harvested by centrifugation at 1500g at 4°C and filtered through a 0.22µm filter. The supernatants were then run over a Ni-NTA gravity column, washed with 30mM imidazole in 1X PBS, pH7.5 and eluted using 400mM imidazole in 1X PBS, pH7.5. The procedure was repeated to capture all protein in the supernatant. This was followed by size exclusion through a Superdex HiLoad 16/600 or 26/600 (200 µg) gel filtration column on an ÅKTA purifier. Thereafter, the elutant was buffer exchanged with 150mM NaCl, 3mM HEPES, and 2.5-5% glycerol and concentrated using Vivapore table-top concentrators to approximately 1mg/mL (see Chapter 2). IgG expression and purification.

5.2.2 *IgG protein expression*

IgG constructs were expressed in HEK293F cells as described previously. Supernatants were harvested after 6 days and purified using a protein A affinity column. Proteins were eluted using a 0.15 M glycine buffer at pH 2.5 in 1 M Tris, pH 8, and were concentrated and dialyzed into PBS, pH 6.5, containing 5% glycerol. Concentrations were measured on a NanoDrop device using an extinction coefficient of 13.7 and a 1% solution. The molecular weight of the IgG was calculated using ExPASy ProtParam (69) for CAP256.25 (150.71 kDa), 10E8v4 (147.29 kDa), 3BNC117 (146.24 kDa), and PGT121 (146.2 kDa). IgG and proteins were stored at -75°C (see Chapter 2 for more detail).

5.2.3 *Pseudovirus production for TZM-bl assay*

Plasmids containing HIV-1 envelope (gp160) genes cloned in the pcDNATM3.1D/V5-His-TOPO[®] vector were co-transfected with pSG3^{Δenv} into HEK293T cells and cultured for 48-72hrs at 37°C. Supernatants were harvested, filtered through a 45µm filter, and frozen at -80°C. Virus stocks were titrated on TZM-bl cells using a luciferase assay to determine a dilution yielding RLU at least 10-fold above the “cell only” background (40,000-100,000 RLU).

5.2.4 Neutralization assay

Neutralization was measured using pseudoviruses in a validated single-cycle assay in TZM-bl cells. scFv or IgG proteins were added to growth media at a starting concentration of 50µg/mL and serially diluted 3-fold in duplicate before being added to a 96 well plate (25µL). Pseudovirus was added in an equal volume (25µL) to the plate containing the diluted scFv and incubated for 1hr at 37°C. TZM-bl cells (20µL containing 5×10^5 cells/mL cells) in growth media (GM) containing 70µg/ml of DEAE were added and the mixture incubated for 24hrs. 100µL of GM was added and the cells incubated for another 24-48hrs, then half the media (100µL) was removed and Bright-Glo firefly luciferase substrate mixed with assay buffer was added according to manufacturer specifications. After a 2min incubation at RT, the media and luciferase mixture was mixed and transferred to a black 96 well plate. The plate was then read on the Victor-X luminometer.

5.2.5 The cell-cell transmission assay

The cell-cell transmission assay employs two cell lines, the 293T cell line and the A3.01 cell line which is derived from T-cells (Reh *et al.*, 2015). This model for cell-cell transmission uses the 293T cell line as a donor cell, expressing pseudovirus on the cell surface. The A3.01 cells express CD4 receptors and co-receptors on the cell surface, support HIV infection, and replication, and can form virological synapses (Folks *et al.*, 1985; Gombos *et al.*, 2015). This makes them useful as a model for cell-cell transmission and in this assay, they are used as target cells for the cell-associated virus (Abela *et al.*, 2012). During the assay, a synapse is formed between the 293T cells and the A3.01 cells allowing for direct virus transfer between the two cells.

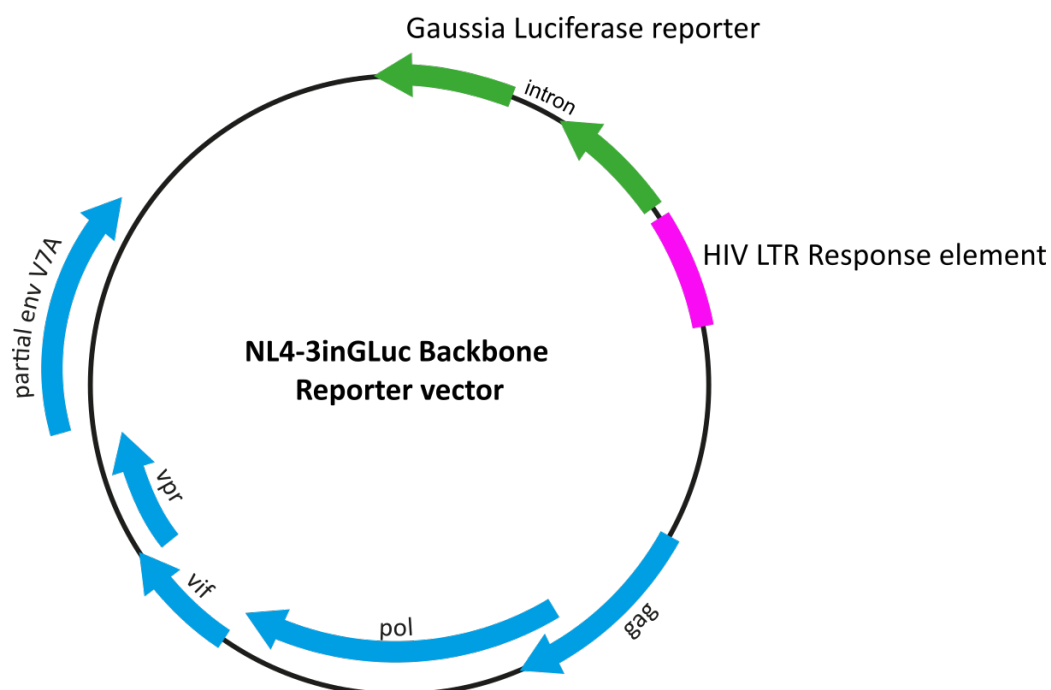


Figure 5.1 Representation of the NL4-3inGLuc vector

The NL4-3inGLuc reporter vector containing a reversed Gaussia Luciferase reporter gene regulated under an HIV response element. The pseudotyped HIV vector contains a truncation in env gene and no vpu gene. Co-transfection with an envelope expression vector results in pseudovirus with a reversed luciferase reporter. Co-culture with target cells (A3.01) results luciferase expression allowing for measurement of cell-cell transmission. Map adapted from (Janaka *et al.*, 2013; Reh *et al.*, 2015).

To ensure accurate measurement of cell-cell transmission, the assay uses a reversed luciferase reporter vector (NL4-3inGLuc) to generate pseudovirus in the 293T cells. The NL4-3inGLuc vector (backbone) is an HIV NL4-3 full-genome derived vector with a truncation in the Env gene and a reversed Gaussia Luciferase reporter gene (containing an intron) that is regulated by an HIV LTR response element (Figure 5.1) (Mazurov *et al.*, 2010; Janaka *et al.*, 2013; Zhong *et al.*, 2013). Due to the presence of the intron and the reversed luciferase gene, luciferase expression can only occur if there is correct packaging and splicing into HIV pseudoviruses followed by infection and expression in the target A3.01 cells. This backbone and an HIV-1 envelope-expressing plasmid were co-transfected into 293T cells, to generate pseudoviruses, but do not induce luciferase activity in the 293T cells. This allows the differentiation of viral fusion events and accurate measurement of the cell-to-cell transmission (Abela *et al.*, 2012; DeSantis *et al.*, 2016). The protocol is described in detail below.

293T cells were grown overnight to 50-80% confluence in DMEM containing 10% FCS, 0.05mg/mL gentamicin (Figure 5.2). A mixture of 120µL Polyethylenimine (PEI) (linear, MW

25,000Da) at 1mg/mL combined with 15µg of plasmid containing HIV-1 gp160 envelope genes and 45µg of NL4-3inGLuc backbone was added to the 293T cells and incubated for 6hrs at 37°C at 5% CO₂, to enable pseudovirus production but not budding from the 293T cells. After 6hrs the cells were physically detached, pelleted at 300g for 3min, and resuspended at 10⁵cells/mL. The transfected cells were plated at 5,000 cells/well (50µL) and incubated with duplicate serial dilutions of IgG or scFv for 1hr at 37°C (50µL). A3.01 cells were added (1.5 x 10⁴ cells per well in 100µL) and the co-culture was incubated for 60hrs at 37°C at 5% CO₂, to allow for infection and luciferase expression. DEAE was omitted from the culture media to eliminate free virus transmission. For the readout, the Renilla Luciferase kit from Promega (Wisconsin, USA) was used. 50µL of lysis buffer (diluted 5x with H₂O) was mixed with 50µL of cell supernatant containing the A3.01 suspension cells, but not the adherent 293T cells. After 10min of incubation, 20µL of the substrate (diluted 100x in assay buffer) was combined with 20µL of the supernatant lysate and read using the luminescence protocol on a Dynex luminometer (MTX, Florida, USA). Neutralization curves and IC₅₀ and or IC₈₀ values were extrapolated using Microsoft Office Excel and/or Graphpad Prism.

5.2.6 Cell-free assay using A3.01 cells

The cell-free assay was performed similarly to the neutralization assay described in Chapter 2 but using A3.01 suspension cells instead of TZM-bl cells to enable accurate comparison of cell-cell and cell-free infection. Pseudoviruses were generated as described above, using the pNL4-lucAM reporter vector containing HIV genes and a firefly luciferase reporter which is expressed in the target cells (A3.01) after infection (Rusert *et al.*, 2011). A mixture of 120µL linear PEI (MW 25,000Da) at 1mg/mL combined with 15µg env plasmid and 45µg pNL4-lucAM backbone was transfected into 293T cells and after 6hrs the media was refreshed to eliminate PEI-plasmid complexes. Virus-containing supernatants were harvested and viruses were titrated as described above and frozen at -80°C. For the neutralization assay, IgG or scFv were serially diluted in duplicate and added in equal volume to virus (50µL) to a 96-well plate and incubated for 1hr. 100µL of A3.01 cells were added at a concentration of 5 x 10⁵cells/mL. Similar to the TZM-bl assay, DEAE (70µg/mL) was added to this assay to enhance infectivity. After 48-72hrs, the A3.01 suspension cells were pelleted and the supernatant removed. Afterwards, 50µL of lysis buffer was added and incubated for 5-10 minutes. After mixing 50µL was transferred to a readout plate and 50µL of the luciferase substrate (diluted 1/10) was added and the signal was detected on a Dynex luminometer (MTX, Florida, USA).

Neutralization curves and IC₅₀ and or IC₈₀ values were extrapolated using Microsoft Office Excel and/or Graphpad Prism.

5.3 Results

5.3.1 IgG and scFv neutralize cell-free pseudovirus equivalently in a TZM-bl assay and an A3.01 assay

We tested four scFv, derived from bNAbs CAP256.25, PGT121, 3BNC117, and 10E8v4 in a cell-free transmission assay using A3.01 and TZM-bl cells, to ensure that both cell types produce similar titres for cell-free neutralization. A panel of seven viruses from subtype A (BG505), subtype B (PV04, RHPA, AC10) and subtype C (ZM53, ZM214, CAP256_SU) was selected based on their sensitivity to both IgG and scFv in the TZM-bl neutralization assay (van Dorsten *et al.*, 2020) and on their ability to infect the A3.01 cells (Reh *et al.*, 2015). Both IgG and scFv neutralized the same viruses in both assays, though some variation in titer by cell line was observed. For example, CAP256.25 (particularly as an IgG) showed slightly increased potency in the TZM-bl cells, whereas 10E8v4 showed the opposite trend. Overall, however, there was no significant difference in geometric mean neutralization titres in these two assays for either IgG or scFv (Figure 5.2) (paired Wilcoxon t-test). For the scFv, the IC_{50} values were multiplied by the molecular size difference compared with the IgG (as in Chapter 3) to compensate for the approximately 5-fold difference in protein size (Figure 5.2).

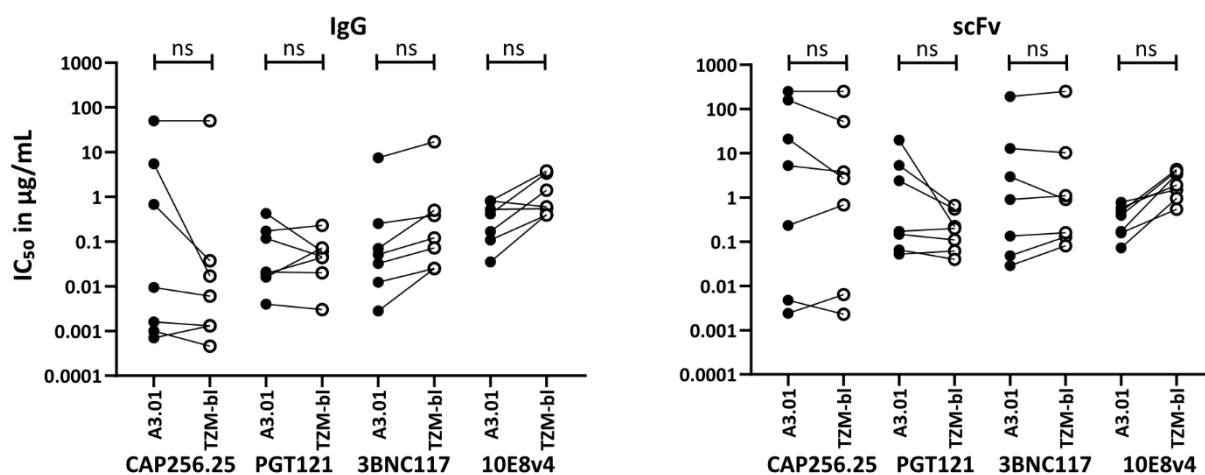


Figure 5.2 Comparison of cell-free neutralization titers in the A3.01 and TZM-bl cells for scFv and IgG
Cell-free neutralization in the A3.01 based assay compared the TZM-bl assay for 4 IgG (left) and 4 scFv (right), each tested against seven viruses. The IC_{50} of the scFv are adjusted to compensate for their 5-fold smaller size compared to the IgG. A Wilcoxon paired t-test was used to assess differences in geometric mean neutralization titers between the two assays. “ns” indicates “not significant”

5.3.2 scFv lose potency in cell-free neutralization but, unlike IgG, maintain potency against cell-associated virus.

We next compared the neutralization (as IC_{50}) of IgG to matched scFv in the cell-free and the cell-cell transmission assays using the same set of seven viruses (Figure 5.3).

As with the cell-free neutralization tested earlier, both the IgG and scFv were able to neutralize cell-associated virus in the A3.01 assay (see Figure 5.3). Viruses neutralized in the free virus assay were also neutralized in the cell-cell assay, and RHPA, which was not neutralized by CAP256.25 in the cell-free assay was also resistant to both scFv and IgG in the cell-cell assay. Geometric mean titres for cell-free neutralization and cell-cell neutralization were generally within the same order of magnitude. However, some variation in geometric mean titres, both between scFv and IgG and between cell-free and cell-cell neutralization assays were observed, leading us to further probe these relationships, below (Figure 5.4).

Antibody			BG505 N332+	PV04	RHPA	AC10	ZM53	ZM214	CAP256_SU	Geomean
CAP256.25	IgG	Free virus	0.0016	0.68	>250	5.5	0.0010	0.0094	0.00073	0.019
		Cell-Cell	0.022	2.4	>250	0.77	0.013	0.035	0.0034	0.063
	scFv	Free virus	0.23	21	>250	159	0.0047	5.2	0.0024	0.60
		Cell-Cell	0.50	18	>250	35	0.049	0.69	0.024	0.79
PGT121	IgG	Free virus	0.019	0.17	0.12	0.016	0.0037	0.42	0.021	0.041
		Cell-Cell	0.56	2.5	0.38	0.32	0.077	18.5	0.72	0.78
	scFv	Free virus	0.052	5.3	0.15	0.17	2.39	19.7	0.066	0.58
		Cell-Cell	0.38	6.3	0.48	0.76	0.45	56	0.31	1.3
3BNC117	IgG	Free virus	0.0028	0.03	0.012	7.5	0.25	0.051	0.071	0.069
		Cell-Cell	0.25	0.25	0.12	15.2	4.21	3.2	0.76	1.0
	scFv	Free virus	0.029	0.13	0.048	193	12.8	3.0	0.90	1.0
		Cell-Cell	0.43	0.19	0.087	257	13.6	8.2	1.5	2.3
10E8v4	IgG	Free virus	0.035	0.81	0.81	0.11	0.41	0.52	0.17	0.26
		Cell-Cell	0.59	2.0	4.4	2.5	0.60	2.7	0.29	1.3
	scFv	Free virus	0.072	0.17	0.59	0.16	0.48	0.78	0.40	0.29
		Cell-Cell	0.26	0.51	1.7	0.43	0.77	2.2	0.29	0.65

IC_{50} in $\mu g/ml$	<0.01	0.01-0.1	0.1-1	1-10	10-50	>50
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Figure 5.3 Potency of IgG and scFv neutralization of free virus and cell-associated virus in A3.01 cells. IC_{50} neutralization ($\mu g/mL$) of IgG and scFv are shown for cell-free virus (A3.01) and cell-cell transmission against seven viruses. The IC_{50} of the scFv are adjusted to compensate for their 5-fold smaller size compared to the IgG. The geometric mean IC_{50} is shown on the left of the table. The color gradient represents the potency of the antibodies with red being the most potent and green the least potent. ">250" denotes lack of neutralization at 250 $\mu g/mL$.

We represented the IC_{50} data of the IgG and scFv against these 7 viruses in a log-log plot and a potency plot (Figure 5.4). There was a significant correlation between the potency of the

parental IgG and that of the scFv for both assays ($r=0.987$, $p<0.0001$ for the cell-free assay and $r=0.806$, $p<0.0001$ for the cell-cell assay) (Figure 5.4). However, despite the overall correlation, there was a pronounced reduction in potency for the scFv in the cell-free assay where we observed an 8.7-fold geometric mean reduction in potency compared to the IgG. This was indicated by the leftward shift for the data points away from the identity line, which represents a one to one linear relationship between the IgG and the scFv, in Figure 5.4. This is consistent with our previous findings of reduced neutralization potency of scFv compared to IgG in the TZM-bl free virus neutralization assay (van Dorsten *et al.*, 2020).

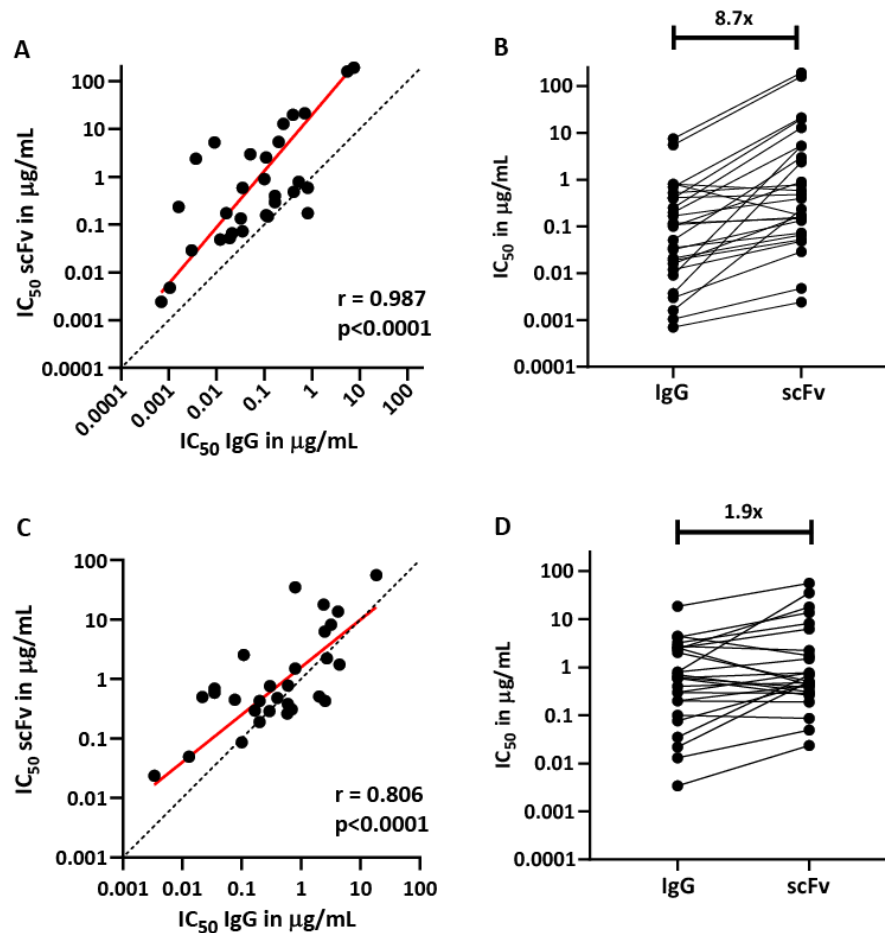


Figure 5.4 Comparison of the neutralization potency of IgG and scFv in the cell-free (top panels) and cell-cell assays (bottom panels).

(A) IC_{50} values for four scFv versus four IgG for the cell-free neutralization assay for 7 viruses as per figure 5.3. The dotted line represents an identity line. A log-log line is plotted in red to indicate the trend between IgG and scFv (B) IC_{50} of IgG and scFv are plotted. Geometric mean fold difference between IgG and scFv is indicated above the graph. (C) The IC_{50} values for scFv are plotted against the IgG for the cell-cell transmission assay. (D) IC_{50} of IgG and scFv in the cell-cell transmission assay. The geometric mean fold difference between IgG and scFv is indicated above the graph. For A-D the IC_{50} of the scFv are corrected for their molecular weight difference.

For the cell-cell neutralization assay, the loss of potency of the scFv compared to the IgG was less pronounced (1.9-fold, compared to 8.7-fold in the cell-free assay) (Figure 5.4C, D). Thus, although in both systems the scFv show reduced potency compared to IgG, this loss of potency was significantly less pronounced in cell-cell transmission.

This reduced potency of scFv compared to the IgG varied by antibody in both assays. For example, CAP256.25 scFv lost approximately 32-fold potency as an scFv compared to the IgG in the cell-free neutralization assay (Figure 5.5A), whereas 3BNC117 and PGT121 lost less potency (15- and 14-fold respectively). In contrast, 10E8v4 was able to maintain almost complete potency in the A3.01 based cell-free assay, as was previously observed for the TZM-bl assay (van Dorsten *et al.*, 2020) (Figure 5.5A).

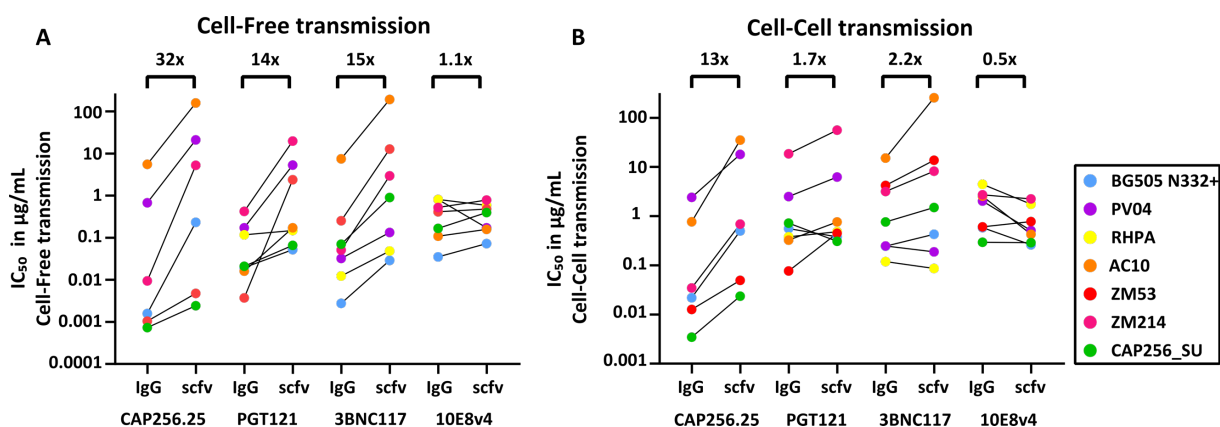


Figure 5.5 Comparison of the neutralization potency of four IgG to their scFv versions in cell-free transmission and cell-cell transmission.

(A) IC_{50} of IgG and scFv in the cell-free A3.01 transmission assay for 4 antibody pairs. (B) IC_{50} values of IgG and scFv in a cell-cell transmission assay for four antibody pairs. For both A and B the geometric mean potency difference between the IgG and scFv is given above the graph. For A and B colors correspond to different viruses as per legend

Similarly, in the cell-cell neutralization assay, CAP256.25 scFv demonstrated a potency loss of 13-fold compared to the IgG, with three of seven viruses showing a 10-20 fold potency difference between the IgG and the scFv (Figure 5.5B). In contrast, the geometric mean fold difference for PGT121 was 1.7-fold, with the scFv showing loss of potency against one of the seven viruses (ZM53). For 3BNC117, an overall change of 2.2-fold was observed with 5/7 viruses showing similar sensitivity to the scFv, but the remaining two viruses (AC10, ZM53) showing reduced sensitivity. Interestingly, for 10E8v4 there was an overall improvement in potency for the scFv (conferred by >2-fold enhanced neutralization for four of the seven-panel

viruses) compared to the IgG potency with an average 2-fold improvement in the geometric mean (Figure 5.5B, 5.4). Thus, unlike the cell-free model, most scFv and their parental IgG neutralized cell-associated virus with comparable potencies.

5.3.3 scFv neutralize cell-associated virus similarly to cell-free virus, whereas IgG lose potency against cell-associated virus transmission.

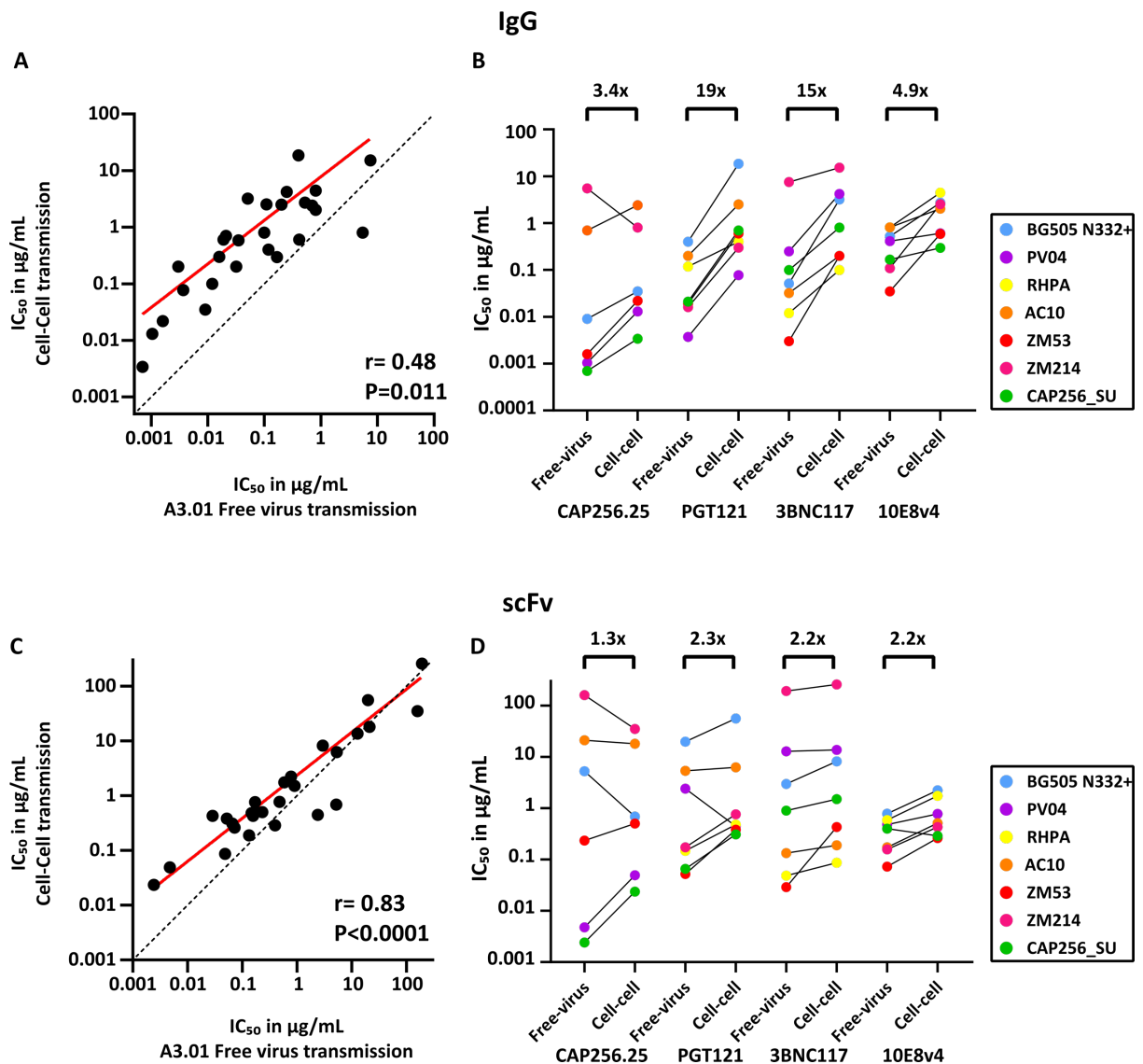


Figure 5.6 Comparison of cell-associated and cell-free virus neutralization for IgG and scFv

(A) Correlation between cell-free and cell-cell neutralization for IgG of four bNAbs. (B) IC₅₀ of four IgG of free-virus compared to cell-associated virus neutralization. (C) Correlation between cell-free neutralization and cell-associated neutralization for scFv of bNAbs. (D) IC₅₀ of 4 scFv of free-virus compared to cell-associated neutralization. A log-log trend line is drawn for A and C as well as an identity line. Pearson's correlation and p value is given in the graphs for both A and C. Geometric mean fold difference between free virus and cell-associated virus neutralization is given above the respective plots B and D. The colors in B and D correspond to viruses tested as per legend.

We hypothesized that scFv maintain equivalent activity in both assays, whereas IgG specifically lose potency against cell-associated virus. For IgG, we observed a weak correlation between the potency of cell-free neutralization and cell-associated neutralization ($r=0.48$ $p=0.011$, Figure 5.6A), with reduced potency in the cell-cell assay. Examination of each IgG confirmed significant loss of activity in the cell-cell assay for all antibody-virus combinations but one. These geometric mean potency losses in the cell-cell transmission assay were 3.4x for CAP256.25, 4.9-fold for 10E8v4, 15-fold for 3BNC117, and 19-fold for PGT121 (Figure 5.6B).

In contrast to the IgG, for the scFv, we observed a much stronger correlation between the potency of cell-free neutralization and cell-associated neutralization ($r=0.83$ $p<0.0001$) (Figure 5.6C). A comparison of the change in potency for each scFv confirmed this observation, with all scFv demonstrating geometric mean potency loss in cell-cell neutralization of <3-fold (Figure 5.6D). For CAP256.25, only two viruses, ZM53 and CAP256_SU, showed >10-fold loss in potency in the cell-cell model. In contrast, increased potency against cell-associated virus was observed for two viruses, AC10 and ZM214. Similarly, the PGT121 scFv showed slightly reduced potency against four viruses, but gained potency against one virus in the cell-cell assay (ZM53). Both the 3BNC117 and 10E8v4 scFvs showed only a marginal loss of activity in the cell-cell assay (approximately 2-fold in geometric mean potency) for all viruses tested.

The selective loss of potency for the IgG compared to the scFv were further illustrated by assessing the log-fold difference between neutralization of cell-associated and cell-free virus of IgG compared to the scFv (Figure 5.7A). For all IgG, the mean log-fold difference was greater than zero, and in many cases closer to one, suggesting a 10-fold loss of activity in the cell-cell neutralization assay. In contrast, all scFv had mean log-fold differences of close to zero, indicating no significant difference between the cell-free and cell-associated virus neutralization. Comparison of IgG and scFv log-fold differences confirmed that in three cases (PGT121, 3BNC117, and 10E8v4), the potency loss for the IgG was significantly higher than that of the scFv (Figure 5.7). Overall, these data highlight the fact that, unlike the IgG, the scFv of these four bNAbs maintain similar activity for both cell-free and cell-cell neutralization.

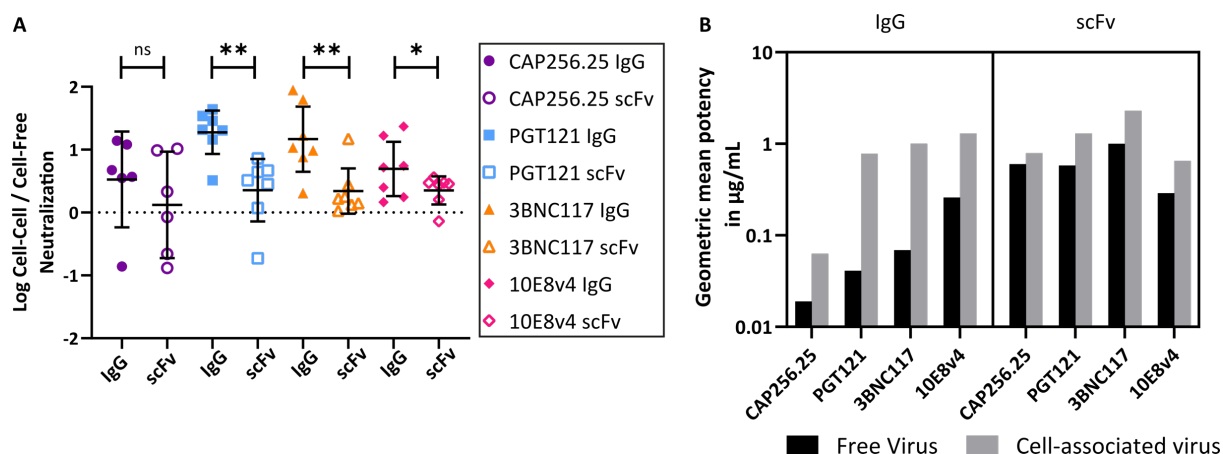


Figure 5.7 Fold differences in neutralization of cell-free and cell-cell transmission of IgG compared to the scFv illustrate selective loss of potency for the IgG in cell-cell transmission.

(A) Log-fold potency difference between the IC_{50} for cell-cell and cell-free neutralization. Closed symbols represent the IgG whereas the open symbols present the scFv. CAP256.25 is shown in purple, PGT121 in blue, 3BNC117 in orange and 10E8v4 in pink. The mean is given with the 95% confidence interval and a paired t-test was performed between the IgG and scFv with statistical significance given above the graphs. "ns" denotes not significant. (B) Geometric mean potency of four IgG (left) and four scFv (right) against free-virus transmission (black) and cell-cell transmission (grey).

5.5 Discussion

HIV-directed scFv hold substantial promise for HIV prevention and treatment, as these smaller antibody fragments retain activity in cell-free virus neutralization (van Dorsten *et al.*, 2020). However, the ability of scFv to block cell-cell transmission is unknown and has implications for their use as preventative and therapeutic modalities. The smaller size of these molecules suggests that these fragments may efficiently neutralize cell-associated virus, compared to the full-sized antibodies, a hypothesis this is supported by some, though not all studies in HIV (Zhang *et al.*, 2004, 2010; McCoy *et al.*, 2014; Alam *et al.*, 2019). We assessed the scFvs of four HIV-directed bNAbs for their ability to neutralize cell-cell transmission. As we have previously shown in a different cell line, scFv exhibited reduced neutralization of cell-free virus compared to IgG. However, unlike IgG, which lose substantial activity against cell-cell transmission, scFv generally showed equivalent neutralization of cell-cell and cell-free virus. The preserved potency of scFv in both modes of transmission makes these appealing candidates for HIV treatment and prevention.

Although overall, scFv maintain equivalent activity in cell-free and cell-cell assays, some scFv-specific effects were observed. For example, the scFv of CAP256.25 displayed considerably less activity against cell-cell transmission than the IgG for all seven viruses tested. This effect may be due to the differential sensitivity of the scFv to naturally occurring variations in the viral epitope. In the TZM-bl assay CAP256.25 scFv was strongly affected by the charge of the epitope in the C strand of the V2 loop which may also have affect neutralization of cell-associated virus leading to reduced potency of scFv compared to IgG in these viruses (van Dorsten *et al.*, 2020) (also see Chapter 3). This increased sensitivity of the scFv to viral variation may also strongly impact neutralization of cell-cell transmission and could explain the lower potency for CAP256.25 scFv in this assay. In contrast, the 10E8v4 scFv was slightly more potent than the IgG against cell-cell transmission. The 10E8v4 scFv retains almost complete activity in cell-free transmission (van Dorsten *et al.*, 2020). In comparison, the IgG showed reduced potency against the cell-cell transmission assay, giving the scFv an advantage. Similar effects have been reported previously for the 10E8 Fab which also retained potency in cell-cell assays (Duncan *et al.*, 2014). This may suggest that some MPER directed scFv and antibody fragments show particular promise in preventing both cell-cell and cell-free transmission.

Although some previous studies have suggested an enhanced capability of small fragments to prevent cell-cell transmission (Schiffner *et al.*, 2013; Duncan *et al.*, 2014; McCoy *et al.*, 2014), we did not find this in our study. Several factors make comparison across different studies difficult. Not all antibody fragments were compared to their IgG counterparts making comparison difficult (Duncan *et al.*, 2013; McCoy *et al.*, 2014). In addition, diverse assays were used in the different studies, with for example flow cytometry or luciferase-based readouts, which also accounts for variable results found across these studies (Durham *et al.*, 2012; Li *et al.*, 2017). Thirdly, different cell lines were used to compare cell-free and cell-associated neutralization, which may result in variable results. In this study, the same cell line was used to test neutralization of both modes of transmission (free-virus and cell-associated virus). Future standardization of the cell-cell transmission assays will enable a better understanding of the neutralization capacity of bNAbs and antibody fragments in a cell-to-cell setting (Reh *et al.*, 2015).

The biological contribution of cell-cell transmission, including its role in primary infection, is not clear. There are indications that cell-associated transmission may play a role in mother-to-child transmission (MTCT), both *in utero* and during breastfeeding (Rousseau *et al.*, 2004; Bulterys *et al.*, 2010; Milligan *et al.*, 2014; Showa *et al.*, 2019). It is not known if antibodies can provide protection against HIV in MTCT settings or if passively administered antibodies can transfer to breastmilk (Glass *et al.*, 1983; Harris *et al.*, 2006; Ndirangu *et al.*, 2012; Anderson *et al.*, 2014; Rogier *et al.*, 2014; Marcotte *et al.*, 2015). As bNAbs are further employed in passive immunization strategies, especially in paediatric populations, it will become increasingly important to determine if they can effectively protect from infection through the different transmission routes (Hessell *et al.*, 2016).

Cell-associated virus also plays a role in disseminating and maintaining HIV infection in the body after infection (Evans *et al.*, 2013; Galloway *et al.*, 2015; Smith *et al.*, 2017). In treatment settings, cell-associated virus may more easily escape bNAb neutralization, due to the higher potency needed to neutralize this virus and by “hiding” in intracellular spaces avoiding the antibody contact altogether. Therefore cell-associated virus could cause continued replication despite sufficient antibody levels (Horwitz *et al.*, 2013; Kolodkin-Gal *et al.*, 2013; Shingai *et al.*, 2013; Gombos *et al.*, 2015; Reh *et al.*, 2015; Caskey *et al.*, 2017; Bar-On *et al.*, 2018). Thus, cell-associated virus may hinder efforts at eradicating the HIV reservoir. In clinical settings,

additional studies may be required to select for bNAbs able to target both cell-free and cell-associated virus effectively.

Our data suggests potential for scFv in HIV prevention as they can neutralize cell-free and cell-associated virus. Although the lack of an Fc region will prevent scFv from eliminating infected cells (Ahamadi-Fesharaki et al., 2019; Su et al., 2019), the smaller size of these molecules provides other advantages. scFv could exhibit improved tissue penetration especially into mucosal tissues and lymph nodes where cell-associated transmission occurs (Balazs *et al.*, 2014; Naso *et al.*, 2017; Lin *et al.*, 2018; van den Berg *et al.*, 2019). The much smaller genetic size of constructs encoding scFv also allows for easier incorporation into vectors such as Adeno Associated Vectors (AAV) vectors. This may be vital due to the short half-life of scFv (Balazs *et al.*, 2014; Naso *et al.*, 2017; Lin *et al.*, 2018; van den Berg *et al.*, 2019). AAV would allow for continuous expression of scFv, which may especially be useful for long-term applications such as passive immunization.

Our study has limitations as we tested relatively few viruses, and measured neutralization using only one method of cell-associated transmission. Moreover, as we tested only one scFv and IgG for each epitope, we could not draw any conclusions regarding epitope-specific effects. However, in general, these four scFv retained substantial potency against cell-cell transmission compared to free-virus transmission, a feature that distinguishes them from their parental IgG. Along with the potential additional benefits of smaller molecules for passive immunization, these data support continued studies of HIV bNAb-derived scFv for clinical development in HIV prevention and treatment approaches.

6 Conclusion

6.1.1 *Historic use of plasma and antibodies*

Antibodies in the form of polyclonal plasma have long been applied to treat infectious diseases and also patients suffering from envenomation due to snakebites (C. H. Lee *et al.*, 2016; Mohan *et al.*, 2019). Plasma therapy was first developed in the late 19th- early 20th-century using anti-diphtheria plasma extracted from immunized horses to successfully treat diphtheria patients. Similarly, plasma therapy was applied during the 1918 influenza pandemic and was later used to treat diseases such as polio and measles. Following the discovery of antibiotics, serum therapy for bacterial diseases such as *Haemophilus influenza* and meningococcus declined, however, viral diseases and bacterial toxin derived diseases were still treated with polyclonal plasma. This approach is still in use today with polyclonal antibodies (derived from human plasma) used as post-exposure prophylaxis, for Varicella-zoster, hepatitis B, and rabies (Weber *et al.*, 2015; Albrecht, 2018; Brown *et al.*, 2019). Plasma therapy is also being tested in the current COVID-19 pandemic, showing modest results, with some studies showing decreasing duration and severity of disease and lower mortality, although the latter did not reach statistical significance and some studies showing no effect (Casadevall *et al.*, 2020; Duan *et al.*, 2020; L. Chen *et al.*, 2020; Simonovich *et al.*, 2020).

The advent of monoclonal antibody technology and the ability to identify antigen-specific B cells has revolutionized the field of antibody therapy. Much of the progress in using monoclonal antibodies for the treatment of infectious disease stems from the extensive and decades-long work in non-infectious diseases such as cancer, autoimmune diseases, and other chronic diseases. To date, approximately 96 monoclonal antibodies are approved in Europe and the USA for clinical use and 18 more are under consideration (Kaplon *et al.*, 2020; Reichert *et al.*, 2020). In cancer, tumour-specific antibodies targeting both solid and systemic cancers such as leukaemia, have significantly improved the quality and duration of life. Due to their superior tumour targeting, these antibodies have much fewer off-target effects when used for treatment. They are also being developed for diagnostics such as radiolabelling for PET-CT imaging and to improve drug targeting of current chemotherapeutics by drug antibody complexing. The use of engineered antibody fragments such as scFv are attractive for reasons discussed in this thesis but are still in the early research phase with 16 scFv based products in early clinical trials (Ahamadi-Fesharaki *et al.*, 2019).

6.1.2 The role of antibodies for infectious diseases in the current clinical climate

Despite the successful historical use of plasma therapy, only 4 monoclonal antibodies have been licensed for infectious diseases (Ebola, HIV, and RSV) with another 6 in active clinical development against diseases such as cytomegalovirus (CMV), rabies, and Ebola. (Salazar *et al.*, 2017; Hooft van Huijsduijnen *et al.*, 2020; Kaplon *et al.*, 2020; Reichert *et al.*, 2020). Many other antibodies are still in early testing stages such as antibodies targeting bacterial infections (Zurawski *et al.*, 2020). The slow uptake of monoclonal antibodies for infectious diseases is largely due to the existence of alternatives such as vaccines and other treatments, which are cheaper and have a proven track record. However, antibodies may offer distinct advantages especially for newly emerging infections given the slow rate and uncertainty of vaccine development. Such was the case for the recent pandemics of Zika virus, and Ebola, where antibody therapies were tested and showed efficacy in treatment (Xie *et al.*, 2017; Keeffe *et al.*, 2018; Branche *et al.*, 2019; Levine, 2019; Iversen *et al.*, 2020; van Rompay *et al.*, 2020). More recently monoclonal antibodies were rapidly developed for treatment and prevention purposes in the current SARS-CoV-2 pandemic outbreak. Two companies licensed antibody cocktails under the emergency use protocol of the FDA for the treatment of COVID-19 namely, casirivimab and imdevimab (REGN10933 and REGN10987 respectively) and bamlanivimab (LY-CoV555) (Baum *et al.*, 2020; Hansen *et al.*, 2020; Lilly, 2020; P. Chen *et al.*, 2020; Regeneron, 2020). Furthermore, the re-emergence of many vaccine-preventable diseases as a result of declining vaccine coverage may benefit from passive immunity approaches, able to provide instant protection in an outbreak situation. For some diseases such as malaria, vaccines show only a limited efficacy of 30% after four doses, and cannot fully prevent the disease. Adjunct prophylactic treatment with antibodies could, therefore, be applied in vulnerable populations to prevent malaria infection (Cockburn *et al.*, 2018; Draper *et al.*, 2018). There are also rare cases where vaccines cannot be used because they induce harmful immune responses. For example, vaccines against the mosquito-borne flavivirus that causes dengue fever failed, as they resulted in antibody-dependent enhancement (ADE) of infection. This type of enhancement is also observed in natural infection of individuals previously infected with dengue fever or with other flaviviruses such as yellow fever and Zika suggesting that an alternate approach is needed. Monoclonal antibodies can provide instant and specific immunity to vulnerable population groups such as young infants, the elderly, and immune-

compromised individuals. Therefore, their use against a known pathogen target may be especially useful in cases where vaccines are unavailable, dangerous, or ineffective.

The modern era of using antibodies as biological drugs for infectious diseases started with the monoclonal antibody paviluzimab targeting respiratory syncytial virus (RSV). This antibody is preferred over the polyclonal immunoglobulin, which was initially used, as there is higher production capacity and more consistent and safer production with no batch-to-batch variation and no coinfections from blood-borne pathogens. Moreover, monoclonal antibodies show reduced side effects due to their increased specificity compared to polyclonal preparations. There are also several antibodies in early research stages targeting diseases such as rabies, hepatitis A and C, measles, rubella, CMV, and tetanus (Hooft van Huijsduijnen *et al.*, 2020; Kaplon *et al.*, 2020; Reichert *et al.*, 2020).

6.1.3 The potential role of antibodies in HIV prevention

In HIV, antibody usage may be especially useful as no vaccine or cure is currently available. Broadly neutralizing antibodies are relatively rare antibodies but can target the multi-subtype diversity that exists in HIV. These antibodies were shown to protect against SHIV or SIV infection in simian models and this provided the rationale for testing them in humans (Parren *et al.*, 2001; Hessel *et al.*, 2009; Gautam *et al.*, 2016; Julg, Liu, *et al.*, 2017). The results of the two antibody-mediated protection (AMP) trials that are currently ongoing in Africa and the Americas are expected at the end of 2020 or early 2021 (www.ampstudy.org.za). These trials using VRC01, a CD4bs antibody, are a proof of concept trial and will demonstrate whether bNAbs can protect from HIV infection. Efficacy in the AMP trials will provide the much-needed evidence that bNAbs are a correlate of protection for vaccines. Moreover, it will also indicate that antibodies may successfully be employed for HIV prevention. In this context, a combination of antibodies will provide superior protection against HIV infection by increasing the coverage and potency to target the multi-clade viral diversity (Kong *et al.*, 2015; Wagh *et al.*, 2016; Wagh, Seaman, *et al.*, 2018; Schommers *et al.*, 2020). It is for this reason that we generated scFv of the broadly neutralizing antibodies targeting the major epitopes on the HIV trimer that are currently undergoing clinical testing in this study. The MPER antibody 10E8 or its re-engineered version 10E8v4 is currently being tested in combination with an anti-CD4 antibody as part of a multi-specific iMab (Huang *et al.*, 2016; Wagh, Seaman, *et al.*, 2018; ClinicalTrials.gov, 2020a). Similarly, the CD4 antibody 3BNC117 was tested in multiple treatment trials either alone or in combination with other antibodies such as the V3 antibody

10-1074 (Scheid *et al.*, 2016; Mendoza *et al.*, 2018). The V2 antibody CAP256.25, isolated from a South African individual in the CAPRISA cohort is being tested in a combination with PGT121 and /or the CD4 antibody VRC07 (Mahomed *et al.*, 2020).

It is widely anticipated that the AMP trials will demonstrate efficacy and will stimulate the rapid advancement of the antibody pipeline for HIV prevention (Corey *et al.*, 2021). It will also provide a benchmark for HIV vaccines by providing a titer required for prevention efficacy. Lack of efficacy in the AMP trial will imply that a successful vaccine will need a more potent or broader immune response that includes the elimination of infected cells via cellular immunity. It could also indicate that alternative transmission routes such as cell-cell transmission play a critical role during acquisition. Previous studies have shown that not all bNAbs are equally capable of preventing cell-cell transmission (Malbec *et al.*, 2013; Reh *et al.*, 2015; Li *et al.*, 2017; Smith *et al.*, 2017). Low efficacy may imply that a higher level of antibody is needed which may be achieved with more potent antibodies or with antibody combinations. Moreover, it may indicate that a vaccine will need to elicit multiple types of antibodies that function in complementarity (Morris *et al.*, 2017; Gruell *et al.*, 2018; Julg *et al.*, 2019).

6.1.4 The use of bNAbs for the treatment of HIV

Control of SHIV infection in non-human primate models demonstrated that bNAbs also hold potential for the treatment of HIV (Shingai *et al.*, 2013; Hessel *et al.*, 2016; Gruell *et al.*, 2018; Wu *et al.*, 2019; Shapiro *et al.*, 2020). In the last 5 years, several clinical trials have shown that bNAbs could achieve viral suppression. Antibodies targeting the CD4bs (3BNC117) and the N332 supersite (10-1074, PGT121) were administered singly or in combination to patients undergoing treatment interruption (Malbec *et al.*, 2013; Caskey *et al.*, 2017; Mendoza *et al.*, 2018; Niessl *et al.*, 2020; Shapiro *et al.*, 2020). These antibodies resulted in reductions of viral load, however, rebound from single antibodies occurred rapidly and was associated with the development of viral escape mutations, a limitation which could be overcome by combining antibodies. Antibody combinations applied in a therapeutic setting resulted in an increase in potency and breadth due to the complementary nature of these antibodies and a much lower frequency of escape mutations. A phase 2 trial using 3BNC117+10-1074 demonstrated a mean reduction of 2 logs of viral load on average for 3 months. Escape was associated with the infecting viruses being resistant to one of the antibodies in the mixture. Moreover, full viral load suppression could be achieved in patients with controlled disease (up to 30 weeks) after cessation of ART (Mendoza *et al.*, 2018). Viral suppression waned as the antibody

concentrations declined over time. This could be counteracted using antibodies engineered to have a longer half-life or by the introduction of antibodies into a vector for long-term expression. Despite the promise of antibody therapy, these products are still in the early stages of development compared to anti-retroviral therapy (ART). It is highly likely that they will need to be combined or used in conjunction with ART. In addition to having direct anti-viral effects, the highly specific binding properties of antibodies can be exploited for treatment optimization. For example, scFv have been used to make drug-scFv complexes to improve the targeting of ART to HIV-infected cells and virus particles (Berger, 2011; Safdari *et al.*, 2016). This would significantly reduce off-target effects and improve the quality of life of patients on lifelong ART. This approach has been successfully used in cancer settings linking scFv to cytotoxic drugs, greatly reducing the side effects. Early antibody-drug complexes for cancer are in late-stage trials and are likely to be applied clinically in the next few years (Safdari *et al.*, 2016; Woitok *et al.*, 2016; Deonarain, 2018).

6.1.5 The use of bNAbs in HIV cure approaches

As we move from chronic treatment to a possible cure, bNAbs and their fragments could play a large role, as they are uniquely suited to target latently infected cells. bNAbs facilitate the clearance of infected cells through immune activation mediated by the Fc receptors and therefore could target cells expressing HIV proteins on their surface (Bournazos *et al.*, 2014; Lu *et al.*, 2016; Schoofs *et al.*, 2016). However, they may need to be combined with latency reversal agents, as latent cells do not express high levels of HIV antigens on their surface. bNAbs will also need to be able to target cell-cell transmission which contributes to the maintenance and establishment of the HIV reservoir (Rasmussen *et al.*, 2016; Zerbato *et al.*, 2016; Clayton *et al.*, 2017; Kumar *et al.*, 2018; Rezaei *et al.*, 2018; Liu *et al.*, 2020). scFv of bNAbs do not have Fc functions eliminating their cytotoxic effects but they may diffuse more easily into reservoir sites and contribute by preventing reseeding of the viral reservoir. As shown in this thesis, scFv were equally capable of targeting cell-cell transmission compared to inhibiting free-virus transmission. Interrupting the re-establishment and maintenance of HIV reservoirs will likely need a multifaceted approach with the potential use for bNAbs in combination with latency reversal agents, drug-antibody approaches, and other strategies (Grobbs *et al.*, 2019).

6.1.6 Multi-specific antibodies and antibody fragments in HIV

As multiple antibody specificities are needed in both treatment and prevention and possibly cure, bispecific or trispecific antibodies would provide a substantial benefit over single paratope antibodies (Asokan *et al.*, 2015; Steinhardt *et al.*, 2018; Davis-Gardner *et al.*, 2020). Multi-specific antibodies with two or three modalities are in early phase clinical trials to assess for safety. These antibodies combine broad and potent bNAbs using Fab or scFv based proteins. For example, a trispecific antibody targeting the MPER (10E8v4), the CD4bs (VRC01), and the V2 apex (PGDM1400) is in a phase 1 trial (Xu *et al.*, 2017). Moreover, the 10E8-iMab, which targets both MPER and CD4 (targeting the T-cell) is an extremely potent and broad antibody and is also in a phase 1 clinical trials (Huang *et al.*, 2016; ClinicalTrials.gov, 2020a). The small protein size of antibody fragments can be particularly useful for administration via mucosal or nasal routes. For example, a heavy chain only small antibody fragment, targeting the Receptor Binding Domain was very potent against SARS-CoV-2 and could be nebulized for direct application in the lungs (Wrapp *et al.*, 2020). Due to the small size, of such antibody fragments, they would have a limited half-life, which can partially be overcome by PEGylating the nanobodies. Some of them may also be immunogenic as they do not naturally occur in humans.

6.1.7 The cost of antibody therapy

Current antibody therapy carries a considerable cost and the upscaling of production can be limited by manufacturing costs, expression levels, and the expense of material, reagents, and cell-lines. This cost can be partially mitigated by optimizing the expression levels of the antibodies and antibody products. For example, the 10E8v4 scFv expressed very high levels per litre of antibody whereas other antibodies such as 8ANC195 scFv needed extensive optimization. Antibody therapy for cancer costs around \$96,731 in the USA per patient per year (between 1.5-2 million Rands). The high cost of current antibody therapies results in economic inequality and potential under-use in regions where patients would highly benefit from these therapies. Should antibody therapy become a reality for HIV treatment and/or prevention, dealing with this issue would become imperative. One infectious disease where antibodies were successfully used for prophylaxis is RSV, where palivizumab is injected monthly into susceptible infants. However, this remains an expensive treatment for developing nations where it would be most needed. Newer antibodies with 100x the potency are in various stages of testing and clinical trials would dramatically improve this to one

injection per season and significantly decrease cost (Fujita-Yamaguchi, 2014; Park *et al.*, 2015; Wu *et al.*, 2017; Hooft van Huijsduijnen *et al.*, 2020). In addition, there have been significant improvements by optimizing half-life, and by using novel application methods. The manufacturing cost of antibodies can thus be significantly decreased with some ranging between \$20-80/gram of antibody, which may make them a viable option for continued development for use in infectious diseases.

6.1.8 Vectors for the expression of antibodies

Another way to significantly reduce the cost of antibody therapeutics is to make use of vectors and the host cells' machinery for continuous endogenous expression. There have been significant advances in improving vectored-mediated immunoprophylaxis (VIP) with the development of viral vectors and RNA based delivery systems (Van Hoecke *et al.*, 2019). For example, mRNA-based delivery of VRC01 protected humanized mice from HIV infection (Pardi *et al.*, 2017). This strategy makes use of the patients' cells, which produce the antibodies after transfection (Amanat *et al.*, 2020). Moreover, vectors such as Adeno-Associated Virus vectors (AAV), allow for administration and continued expression of these antibodies from muscle cells (Lin *et al.*, 2018). This technique has already been tested in several clinical trials of non-infectious diseases where AAV-based scFv expression was applied for Parkinson's disease targeting protein expression directly to the brain (Huang *et al.*, 2013; Qu *et al.*, 2019). Similarly, it has been tested for protein and/or enzymatic deficiencies to treat Haemophilia (Naso *et al.*, 2017). Many of the therapies using AAV are currently in Phase I and Phase II clinical trials, with one phase III trial using AAV-9 (NCT03505099) targeting spinal muscular atrophy (Qu *et al.*, 2019). Using AAV has the advantage that, in theory, long-term expression of antibodies or proteins can be achieved. However, there are several challenges as anti-AAV and anti-antibody form in patients, limiting the antibody half-life (Bivi *et al.*, 2019). This can partially be circumvented by using other non-immunogenic serotypes, which are not commonly present in human populations. These serotypes of AAV also have specific cell preferences, which can correspond with specific organ and or location preferences allowing for targeted antibody expression, limiting side effects, and maximizing the effectiveness of the treatment (Büning *et al.*, 2003; Flotte, 2006; Lin *et al.*, 2018; Martinez-Navio *et al.*, 2019; Ronzitti *et al.*, 2020).

AAV has been used to express the HIV bNAb PG9, which targets the V2 Apex. Unfortunately, production of anti-AAV and anti-PG9 antibodies resulted in low levels of PG9 expression (Priddy *et al.*, 2019). More recently CAP256.25, which is a much more potent V2 Apex-directed

antibody has also been expressed using AAV and tested in mice models (van den Berg *et al.*, 2019). Similarly, VRC07 expressed in AAV-8 has been tested in humans (NCT03374202). Preliminary results presented earlier this year showed that VRC07 was expressed at higher and at sustained levels over 36 weeks. This trial also demonstrated lower levels of anti-VRC07 antibodies, compared to previous trials with PG9 administered through AAV-1 (Casazza *et al.*, 2020). These trials indicate that not all bNAbs may be suited to the AAV platform and that much optimization in humans to prevent AAV or antibody-targeted immune response is required before this becomes a reliable prevention method.

In addition to this challenge, AAV vectors have limited space to package genetic material, restricting the number of antibodies that can be expressed from one vector. Recently some developments have allowed an increase in total genetic size from 5kb to 8.9 kb (AAV-5) (Dong *et al.*, 2008; Z. Wu *et al.*, 2010), however, vectors larger than 5kB resulted in truncated and heterogeneous expression of the vectors. In addition, the expression levels of AAV vectors decrease with larger genetic inserts, and self-complementary vectors, which generally have improved expression levels, are more constrained for size (McCarty, 2008; Fuchs *et al.*, 2016). Therefore, small antibody fragments are ideally suited for AAV expression, as their genetic size is smaller compared to IgG. With some engineering multiple scFv can be expressed from one vector, increasing the viral epitope coverage (Liu *et al.*, 2017). As scFv and other small fragments generally have shorter half-lives, AAV will also ensure more long-term expression.

6.1.9 Contribution of the thesis to the scientific field

In this thesis, we have explored the neutralization potency of scFv to determine whether they can be employed for passive immunization purposes. Only some scFv of HIV bNAbs have been studied (such as VRC01) but no in-depth analysis of the cause of the loss of potency had been done (West *et al.*, 2012). In chapter three of this thesis, we detailed the differential sensitivity of the scFv compared to the IgG. We showed that CAP256.25 scFv is more sensitive to charge differences in the C strand of the V2 loop. Moreover, both PGT121 and 3BNC117 scFv are sensitive to glycan differences in their respective epitopes resulting in a small loss of potency and some loss of neutralization coverage. We also found one scFv which did not show any potency loss compared to the IgG even when compensated for the size difference with the IgG, something which had not been demonstrated previously. This also indicated that each scFv of an HIV bNAb showed unique changes in neutralization potential which may be related

to their respective epitopes, requiring further investigation. Despite their potency losses, scFv still retained significant potency and breadth.

When scFv were tested in combinations, they followed an additive model of potency, similarly to IgG (Kong *et al.*, 2015; Wagh *et al.*, 2016; Wagh, Seaman, *et al.*, 2018). Using the Loewe model we demonstrated that these scFv show significant potency and breadth when combined in mixes of two or three antibodies. This is of particular importance as it is likely a combination of antibodies will be required to cover the wide variability of the HIV viral diversity. Moreover, high enough potency will also be required, and perhaps dual coverage of each viral strain to provide sufficient protection from HIV infection (Corey *et al.*, 2021). We show that these scFv can fulfil these conditions. As scFv may be more readily expressed on AAV compared to IgG, they could be applied for passive immunization purposes.

Lastly, we investigated the neutralization capacity of four IgG and scFv against cell-cell transmission. Although the exact role of cell-to-cell transmission in HIV remains unclear, there is evidence of its role in mother-to-child transmission (Milligan *et al.*, 2014). It is therefore imperative that bNAbs can protect from this mode of transmission but they have been shown to lose potency against cell-associated virus. Here we show that scFv although losing potency against the cell-free virus, can neutralize cell-associated virus with significant potency and in some cases better than IgG. More study of scFv against the cell-associated virus may be required using for example replicating virus and/or studying the different forms of cell-to-cell transmission. scFv, however, show potential against cell-associated virus and warrant further investigation in preventing this mode of HIV transmission

6.1.10 Final statement

The use of antibodies to prevent and treat infectious diseases will likely grow in the future as has been demonstrated with Ebola and now COVID-19. The imminent results from the VRC01 AMP trial are also expected to rapidly advance the testing of bNAbs for HIV prevention (Corey *et al.*, 2021). Although there is much to be discovered and understood about antibody fragments, this work provides important guidance for their future use. We have demonstrated that they not only retained much of their breadth and potency they also show potential against cell-cell transmission. Moreover, high levels of potency and breadth were reached in combinations of scFv, demonstrating promise for clinical applications such as passive

immunization through vectored immune prophylaxis. Further research to exploit the advantages afforded by scFv are therefore warranted.

7 References

- Abela, I. A. *et al.* (2012) 'Cell-cell transmission enables HIV-1 to evade inhibition by potent CD4bs directed antibodies.', *PLoS pathogens*, 8(4), p. e1002634. doi: 10.1371/journal.ppat.1002634.
- Ackerman, M. E. *et al.* (2016) 'Polyfunctional HIV-Specific Antibody Responses Are Associated with Spontaneous HIV Control', *PLoS Pathogens*, 12(1), pp. 1–14. doi: 10.1371/journal.ppat.1005315.
- Aggarwal, A. *et al.* (2012) 'Mobilization of HIV spread by diaphanous 2 dependent filopodia in infected dendritic cells', *PLoS Pathogens*, 8(6). doi: 10.1371/journal.ppat.1002762.
- Agosto, L. M. *et al.* (2007) 'HIV-1 integrates into resting CD4+ T cells even at low inoculums as demonstrated with an improved assay for HIV-1 integration', *Virology*, 368(1), pp. 60–72. doi: 10.1016/j.virol.2007.06.001.
- Agosto, L. M. *et al.* (2014) 'Highly Active Antiretroviral Therapies Are Effective against HIV-1 Cell-to-Cell Transmission', *PLoS Pathogens*. Edited by C. Aiken, 10(2), p. e1003982. doi: 10.1371/journal.ppat.1003982.
- Agosto, L. M. *et al.* (2018) 'HIV-1-Infected CD4+ T Cells Facilitate Latent Infection of Resting CD4+ T Cells through Cell-Cell Contact', *Cell Reports*, 24(8), pp. 2088–2100. doi: 10.1016/j.celrep.2018.07.079.
- Ahamadi-Fesharaki, R. *et al.* (2019) 'Single-Chain Variable Fragment-Based Bispecific Antibodies: Hitting Two Targets with One Sophisticated Arrow', *Molecular Therapy - Oncolytics*, 14, pp. 38–56. doi: 10.1016/j.omto.2019.02.004.
- Ahmed, S. S. *et al.* (2020) 'Environmental Restrictions: A New Concept Governing HIV-1 Spread Emerging from Integrated Experimental-Computational Analysis of Tissue-Like 3D Cultures', *Cells*, 9(5), p. 1112. doi: 10.3390/cells9051112.
- Akiyama, H. *et al.* (2015) 'CD169-Mediated Trafficking of HIV to Plasma Membrane Invaginations in Dendritic Cells Attenuates Efficacy of Anti-gp120 Broadly Neutralizing Antibodies', *PLoS Pathogens*. Edited by C. Aiken, 11(3), pp. 1–23. doi: 10.1371/journal.ppat.1004751.
- Alam, M. M. M. *et al.* (2019) 'Synergistic inhibition of cell-to-cell HIV-1 infection by combinations of single chain variable fragments and fusion inhibitors', *Biochemistry and Biophysics Reports*, 20(September), p. 100687. doi: 10.1016/j.bbrep.2019.100687.
- Albrecht, M. (2018) 'Post-exposure prophylaxis against varicella-zoster virus infection', in *UpToDate*, p. 1. Available at: https://www.uptodate.com/contents/post-exposure-prophylaxis-against-varicella-zoster-virus-infection?search=Varicella%2Fzosterimmunoglobulin&source=search_result&selectedTitle=1~27&usage_type=default&display_rank=1 (Accessed: 7 August 2020).
- Ali, Z. *et al.* (2009) 'Gammadelta T cell immune manipulation during chronic phase of simian-human immunodeficiency virus infection [corrected] confers immunological benefits', *J Immunol*, 183(8), pp. 5407–5417. doi: 10.4049/jimmunol.0901760.
- Almond, D. *et al.* (2010) 'Structural conservation predominates over sequence variability in

References

- the crown of HIV type 1's V3 loop', *AIDS Research and Human Retroviruses*, 26(6), pp. 717–723. doi: 10.1089/aid.2009.0254.
- Amanat, F. and Krammer, F. (2020) 'SARS-CoV-2 Vaccines: Status Report', *Immunity*. Cell Press, pp. 583–589. doi: 10.1016/j.immuni.2020.03.007.
- Ananthaswamy, N. *et al.* (2019) 'A sequestered fusion peptide in the structure of an HIV-1 transmitted founder envelope trimer', *Nature Communications*, 10(1), p. 873. doi: 10.1038/s41467-019-08825-7.
- Anderson, D. J. (2014) 'Modeling mucosal cell-Associated HIV type 1 transmission in vitro', *Journal of Infectious Diseases*, 210(Suppl 3), pp. S648–S653. doi: 10.1093/infdis/jiu537.
- Anderson, D. J. and Le Grand, R. (2014) 'Cell-Associated HIV mucosal transmission: The neglected pathway', *Journal of Infectious Diseases*, 210, pp. S606–S608. doi: 10.1093/infdis/jiu538.
- Andrabi, R. *et al.* (2015) 'Identification of Common Features in Prototype Broadly Neutralizing Antibodies to HIV Envelope V2 Apex to Facilitate Vaccine Design', *Immunity*, 43(5), pp. 959–973. doi: 10.1016/j.immuni.2015.10.014.
- Anthony, C. *et al.* (2017) 'Cooperation between Strain-Specific and Broadly Neutralizing Responses Limited Viral Escape and Prolonged the Exposure of the Broadly Neutralizing Epitope', *Journal of Virology*. Edited by G. Silvestri, 91(18), p. JVI.00828-17. doi: 10.1128/JVI.00828-17.
- Apellániz, B. *et al.* (2014) 'The three lives of viral fusion peptides', *Chemistry and Physics of Lipids*, 181, pp. 40–55. doi: 10.1016/j.chemphyslip.2014.03.003.
- Arts, E. J. and Hazuda, D. J. (2012) 'HIV-1 antiretroviral drug therapy', *Cold Spring Harbor Perspectives in Medicine*, 2(4). doi: 10.1101/cshperspect.a007161.
- Ashokkumar, M. *et al.* (2018) 'Unique Phenotypic Characteristics of Recently Transmitted HIV-1 Subtype C Envelope Glycoprotein gp120: Use of CXCR6 Coreceptor by Transmitted Founder Viruses', *Journal of Virology*, 92(9). doi: 10.1128/jvi.00063-18.
- Asokan, M. *et al.* (2015) 'Bispecific Antibodies Targeting Different Epitopes on the HIV-1 Envelope Exhibit Broad and Potent Neutralization', *Journal of Virology*. Edited by G. Silvestri, 89(24), pp. 12501–12512. doi: 10.1128/jvi.02097-15.
- Bailes, E. *et al.* (2003) 'Hybrid origin of SIV in chimpanzees', *Science*, 300(5626), p. 1713. doi: 10.1126/science.1080657.
- Balazs, A. B. *et al.* (2012) 'Antibody-based protection against HIV infection by vectored immunoprophylaxis', *Nature*, 481(7379), pp. 81–86. doi: 10.1038/nature10660.
- Balazs, A. B. *et al.* (2014) 'Vectored immunoprophylaxis protects humanized mice from mucosal HIV transmission', *Nature Medicine*, 20(3), pp. 296–300. doi: 10.1038/nm.3471.
- Bar-On, Y. *et al.* (2018) 'Safety and antiviral activity of combination HIV-1 broadly neutralizing antibodies in viremic individuals', *Nature Medicine*, 24(11), pp. 1701–1707. doi: 10.1038/s41591-018-0186-4.
- Bar, K. J. *et al.* (2012) 'Early low-titer neutralizing antibodies impede HIV-1 replication and select for virus escape', *PLoS Pathogens*, 8(5), p. e1002721. doi:

References

10.1371/journal.ppat.1002721.

Bar, K. J. *et al.* (2016) 'Effect of HIV Antibody VRC01 on Viral Rebound after Treatment Interruption', *New England Journal of Medicine*, 375(21), pp. 2037–2050. doi: 10.1056/NEJMoa1608243.

Barnabas, S. L. *et al.* (2018) 'Converging epidemics of sexually transmitted infections and bacterial vaginosis in southern African female adolescents at risk of HIV', *International Journal of STD & AIDS*, 29(6), pp. 531–539. doi: 10.1177/0956462417740487.

Barnes, C. O. *et al.* (2018) 'Structural characterization of a highly-potent V3-glycan broadly neutralizing antibody bound to natively-glycosylated HIV-1 envelope', *Nature Communications*, 9(1), p. 1251. doi: 10.1038/s41467-018-03632-y.

Barouch, D. H. *et al.* (2014) 'Therapeutic Efficacy of Potent Neutralizing HIV-1-Specific Monoclonal Antibodies in SHIV-Infected Rhesus Monkeys', *Nature*, 503(7475), pp. 224–228. doi: 10.1038/nature12744.

Barré-Sinoussi, F. *et al.* (2004) 'Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS)', *Revista de Investigacion Clinica*, 56(2), pp. 126–129. doi: 10.1126/science.6189183.

Bartesaghi, A. *et al.* (2013) 'Prefusion structure of trimeric HIV-1 envelope glycoprotein determined by cryo-electron microscopy', *Nature Structural and Molecular Biology*, 20(12), pp. 1352–1357. doi: 10.1038/nsmb.2711.

Baum, A. *et al.* (2020) 'REGN-COV2 antibodies prevent and treat SARS-CoV-2 infection in rhesus macaques and hamsters', *Science*, 370(6520), p. eabe2402. doi: 10.1126/science.abe2402.

Baxter, A. E. *et al.* (2014) 'Macrophage infection via selective capture of HIV-1-infected CD4 + T cells', *Cell Host and Microbe*, 16(6), pp. 711–721. doi: 10.1016/j.chom.2014.10.010.

Bbosa, N., Kaleebu, P. and Ssemwanga, D. (2019) 'HIV subtype diversity worldwide', *Current Opinion in HIV and AIDS*. Lippincott Williams and Wilkins, pp. 153–160. doi: 10.1097/COH.0000000000000534.

Behrens, A.-J. *et al.* (2016) 'Composition and Antigenic Effects of Individual Glycan Sites of a Trimeric HIV-1 Envelope Glycoprotein', *Cell Reports*, 14(11), pp. 2695–2706. doi: 10.1016/j.celrep.2016.02.058.

Bekker, L.-G. *et al.* (2018) 'Subtype C ALVAC-HIV and bivalent subtype C gp120/MF59 HIV-1 vaccine in low-risk, HIV-uninfected, South African adults: a phase 1/2 trial.', *The lancet. HIV*, 5(7), pp. e366–e378. doi: 10.1016/S2352-3018(18)30071-7.

van den Berg, F. T. *et al.* (2019) 'AAV-Mediated Expression of Broadly Neutralizing and Vaccine-like Antibodies Targeting the HIV-1 Envelope V2 Region', *Molecular Therapy - Methods & Clinical Development*, 14(September), pp. 100–112. doi: 10.1016/j.omtm.2019.06.002.

Berger, E. A. (2011) 'Targeted cytotoxic therapy: Adapting a rapidly progressing anticancer paradigm for depletion of persistent HIV-infected cell reservoirs', *Current Opinion in HIV and AIDS*, 6(1), pp. 80–85. doi: 10.1097/COH.0b013e3283412515.

Bernard-Stoecklin, S. *et al.* (2013) 'Semen CD4+ T Cells and Macrophages Are Productively Infected at All Stages of SIV infection in Macaques', *PLoS Pathogens*, 9(12), pp. 1–13. doi:

References

10.1371/journal.ppat.1003810.

Bernard-Stoecklin, S. *et al.* (2014) 'Nonhuman Primate Models for Cell-Associated Simian Immunodeficiency Virus Transmission: The Need to Better Understand the Complexity of HIV Mucosal Transmission', *Journal of Infectious Diseases*, 210(suppl 3), pp. S660–S666. doi: 10.1093/infdis/jiu536.

Bhiman, J. N. *et al.* (2015) 'Viral variants that initiate and drive maturation of V1V2-directed HIV-1 broadly neutralizing antibodies', *Nature Medicine*, 21(11), pp. 1332–1336. doi: 10.1038/nm.3963.

Bhiman, J. N. and Lynch, R. M. (2017) 'Broadly Neutralizing Antibodies as Treatment: Effects on Virus and Immune System', *Current HIV/AIDS Reports*, 14(2), pp. 54–62. doi: 10.1007/s11904-017-0352-1.

Bivi, N. *et al.* (2019) 'Investigation of pre-existing reactivity to biotherapeutics can uncover potential immunogenic epitopes and predict immunogenicity risk', *mAbs*, 11(5), pp. 861–869. doi: 10.1080/19420862.2019.1612699.

Blattner, C. *et al.* (2014) 'Structural delineation of a quaternary, cleavage-dependent epitope at the gp41-gp120 interface on intact HIV-1 env trimers', *Immunity*, 40(5), pp. 669–680. doi: 10.1016/j.immuni.2014.04.008.

Boeras, D. I. *et al.* (2011) 'Role of donor genital tract HIV-1 diversity in the transmission bottleneck', *Proceedings of the National Academy of Sciences of the United States of America*, 108(46), pp. E1156–63. doi: 10.1073/pnas.1103764108.

Bolton, D. L. *et al.* (2016) 'Human Immunodeficiency Virus Type 1 Monoclonal Antibodies Suppress Acute Simian-Human Immunodeficiency Virus Viremia and Limit Seeding of Cell-Associated Viral Reservoirs', *Journal of virology*, 90(3), pp. 1321–1332. doi: 10.1128/JVI.02454-15.Editor.

Bonsignori, M. *et al.* (2017) 'Antibody-virus co-evolution in HIV infection: paths for HIV vaccine development', *Immunological Reviews*, 275(1), pp. 145–160. doi: 10.1111/imr.12509.

Borman, S. A. (2009) *Building HIV's Curvaceous Coat*, C&EN. Available at: <https://cen.acs.org/articles/87/i25/Building-HIVs-Curvaceous-Coat.html>.

Bournazos, S. *et al.* (2014) 'Broadly neutralizing anti-HIV-1 antibodies require Fc effector functions for in vivo activity.', *Cell*, 158(6), pp. 1243–1253. doi: 10.1016/j.cell.2014.08.023.

Bournazos, S. *et al.* (2016) 'Bispecific Anti-HIV-1 Antibodies with Enhanced Breadth and Potency', *Cell*, 165(7), pp. 1609–1620. doi: 10.1016/j.cell.2016.04.050.

Bowers, P. M. *et al.* (2014) 'Nucleotide insertions and deletions complement point mutations to massively expand the diversity created by somatic hypermutation of antibodies', *Journal of Biological Chemistry*, 289(48), pp. 33557–33567. doi: 10.1074/jbc.M114.607176.

Bracq, L. *et al.* (2018) 'Mechanisms for Cell-to-Cell Transmission of HIV-1', *Frontiers in Immunology*, 9(FEB), p. 1. doi: 10.3389/fimmu.2018.00260.

Branche, E. *et al.* (2019) 'Human Polyclonal Antibodies Prevent Lethal Zika Virus Infection in Mice', *Scientific Reports*, 9(1), pp. 1–12. doi: 10.1038/s41598-019-46291-9.

Bricault, C. A. *et al.* (2019) 'HIV-1 Neutralizing Antibody Signatures and Application to Epitope-

References

- Targeted Vaccine Design', *Cell Host & Microbe*, 25(1), pp. 59-72.e8. doi: 10.1016/j.chom.2018.12.001.
- Brown, C. M. and DeMaria, A. (2019) 'Rabies immune globulin and vaccine', in *UpToDate*, pp. 1–16. Available at: <https://www.uptodate.com/contents/rabies-immune-globulin-and-vaccine> (Accessed: 7 August 2020).
- Buchbinder, S. P. *et al.* (2008) 'Efficacy assessment of a cell-mediated immunity HIV-1 vaccine (the Step Study): a double-blind, randomised, placebo-controlled, test-of-concept trial.', *Lancet (London, England)*, 372(9653), pp. 1881–1893. doi: 10.1016/S0140-6736(08)61591-3.
- Buffalo, C. Z. *et al.* (2019) 'How HIV Nef Proteins Hijack Membrane Traffic To Promote Infection'. doi: 10.1128/JVI.
- Bulterys, P. L., Dalai, S. C. and Katzenstein, D. A. (2010) 'Viral sequence analysis from HIV-infected mothers and infants: Molecular evolution, diversity, and risk factors for mother-to-child transmission', *Clinics in Perinatology*. NIH Public Access, pp. 739–750. doi: 10.1016/j.clp.2010.08.003.
- Büning, H. *et al.* (2003) 'Receptor targeting of adeno-associated virus vectors', *Gene Therapy*, 10(14), pp. 1142–1151. doi: 10.1038/sj.gt.3301976.
- Buonaguro, L., Tornesello, M. L. and Buonaguro, F. M. (2007) 'Human Immunodeficiency Virus Type 1 Subtype Distribution in the Worldwide Epidemic: Pathogenetic and Therapeutic Implications', *Journal of Virology*, 81(19), pp. 10209–10219. doi: 10.1128/JVI.00872-07.
- Burton, D. R. *et al.* (2009) 'Broad and potent neutralizing antibodies from an african donor reveal a new HIV-1 vaccine target', *Science*, 326(5950), pp. 285–289. doi: 10.1126/science.1178746.
- Burton, D. R. and Hangartner, L. (2016) 'Broadly Neutralizing Antibodies to HIV and Their Role in Vaccine Design', *Annual Review of Immunology*, 34(1), pp. 635–659. doi: 10.1146/annurev-immunol-041015-055515.
- Buzon, V. *et al.* (2010) 'Crystal structure of HIV-1 gp41 including both fusion peptide and membrane proximal external regions', *PLoS Pathogens*, 6(5), pp. 1–7. doi: 10.1371/journal.ppat.1000880.
- Caetano, D. G. *et al.* (2019) 'A case report of HIV-1 superinfection in an HIV controller leading to loss of viremia control: a retrospective of 10 years of follow-up', *BMC Infectious Diseases*, 19(1), p. 588. doi: 10.1186/s12879-019-4229-3.
- Calenda, G. *et al.* (2019) 'Delayed vaginal SHIV infection in VRC01 and anti- $\alpha 4\beta 7$ treated rhesus macaques', *PLoS Pathogens*. Edited by G. Silvestri, 15(5), p. e1007776. doi: 10.1371/journal.ppat.1007776.
- CAPRISA (2020) *CAPRISA Studies / CAPRISA*. Available at: <https://www.caprisa.org/Pages/CAPRISASTudies> (Accessed: 3 September 2020).
- Cardaci, S. *et al.* (2013) 'The V1/V2 loop of HIV-1 gp120 is necessary for Tat binding and consequent modulation of virus entry', *FEBS Letters*, 587(18), pp. 2943–2951. doi: 10.1016/j.febslet.2013.07.039.
- Carr, J. M. *et al.* (1999) 'Rapid and efficient cell-to-cell transmission of human immunodeficiency virus infection from monocyte-derived macrophages to peripheral blood

References

- lymphocytes', *Virology*, 265(2), pp. 319–329. doi: 10.1006/viro.1999.0047.
- Casadevall, A., Joyner, M. J. and Pirofski, L. A. (2020) 'A Randomized Trial of Convalescent Plasma for COVID-19 - Potentially Hopeful Signals', *JAMA - Journal of the American Medical Association*, 324(5), pp. 455–457. doi: 10.1001/jama.2020.10218.
- Casazza, J. P. *et al.* (2020) 'DURABLE HIV-1 ANTIBODY PRODUCTION IN HUMANS AFTER AAV8-MEDIATED GENE TRANSFER - CROI Conference', in *CROI*. Boston, Massachusetts. Available at: <https://www.croiconference.org/abstract/durable-hiv-1-antibody-production-in-humans-after-aav8-mediated-gene-transfer/> (Accessed: 23 June 2020).
- Caskey, M. *et al.* (2017) 'Antibody 10-1074 suppresses viremia in HIV-1-infected individuals', *Nature Medicine*, 23(2), pp. 185–191. doi: 10.1038/nm.4268.
- Centers for Disease Control (CDC) (1981) 'Kaposi's sarcoma and Pneumocystis pneumonia among homosexual men--New York City and California.', *MMWR. Morbidity and mortality weekly report*, 30(25), pp. 305–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/6789108> (Accessed: 21 June 2019).
- Chang, J. J. and Altfeld, M. (2010) 'Innate immune activation in primary HIV-1 infection', *J Infect Dis*, 202 Suppl, pp. S297-301. doi: 10.1086/655657.
- Chavez, L., Calvanese, V. and Verdin, E. (2015) 'HIV Latency Is Established Directly and Early in Both Resting and Activated Primary CD4 T Cells', *PLoS Pathogens*. Edited by M. Emerman, 11(6), p. e1004955. doi: 10.1371/journal.ppat.1004955.
- Checkley, M. A., Luttge, B. G. and Freed, E. O. (2011) 'HIV-1 Envelope Glycoprotein Biosynthesis, Trafficking, and Incorporation', *Journal of Molecular Biology*, 410(4), pp. 582–608. doi: 10.1016/j.jmb.2011.04.042.
- Cheeseman, H. M. *et al.* (2017) 'Broadly Neutralizing Antibodies Display Potential for Prevention of HIV-1 Infection of Mucosal Tissue Superior to That of Nonneutralizing Antibodies', *Journal of Virology*, 91(1), pp. e01762-16. doi: 10.1128/JVI.01762-16.
- Chen, B. (2019) 'Molecular Mechanism of HIV-1 Entry', *Trends in Microbiology*, 27(10), pp. 878–891. doi: 10.1016/j.tim.2019.06.002.
- Chen, L. *et al.* (2020) 'Convalescent plasma as a potential therapy for COVID-19', *The Lancet Infectious Diseases*, 20(4), pp. 398–400. doi: 10.1016/S1473-3099(20)30141-9.
- Chen, P. *et al.* (2007) 'Predominant Mode of Human Immunodeficiency Virus Transfer between T Cells Is Mediated by Sustained Env-Dependent Neutralization-Resistant Virological Synapses', *Journal of Virology*, 81(22), pp. 12582–12595. doi: 10.1128/JVI.00381-07.
- Chen, P. *et al.* (2020) 'SARS-CoV-2 Neutralizing Antibody LY-CoV555 in Outpatients with Covid-19', *New England Journal of Medicine*. doi: 10.1056/nejmoa2029849.
- Chien, M.-P., Jiang, S. and Chang, D.-K. (2007) 'The function of coreceptor as a basis for the kinetic dissection of HIV type 1 envelope protein-mediated cell fusion', *The FASEB Journal*, 22(4), pp. 1179–1192. doi: 10.1096/fj.07-9576com.
- Chien, M. P., Lin, C. H. and Chang, D. K. (2009) 'Recruitment of HIV-1 envelope occurs subsequent to lipid mixing: A fluorescence microscopic evidence', *Retrovirology*, 6, p. 20. doi: 10.1186/1742-4690-6-20.

References

- Chimukangara, B. *et al.* (2019) 'Trends in Pretreatment HIV-1 Drug Resistance in Antiretroviral Therapy-naïve Adults in South Africa, 2000–2016: A Pooled Sequence Analysis', *EClinicalMedicine*, 9(7451), pp. 26–34. doi: 10.1016/j.eclinm.2019.03.006.
- Chuang, G. Y. *et al.* (2013) 'Residue-Level Prediction of HIV-1 Antibody Epitopes Based on Neutralization of Diverse Viral Strains', *Journal of Virology*, 87(18), pp. 10047–10058. doi: 10.1128/JVI.00984-13.
- Churchill, M. J. *et al.* (2016) 'HIV reservoirs: what, where and how to target them', *Nature Reviews Microbiology*, 14(1), pp. 55–60. doi: 10.1038/nrmicro.2015.5.
- Cicala, C., Arthos, J. and Fauci, A. S. (2019) 'Role of T-cell trafficking in the pathogenesis of HIV disease', *Current Opinion in HIV and AIDS*, 14(2), pp. 115–120. doi: 10.1097/COH.0000000000000529.
- Clapham, P. R. and McKnight, Á. (2001) 'HIV-1 receptors and cell tropism', *Br Med Bull*, 58, pp. 43–59. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11714623>.
- Clayton, K. L. *et al.* (2017) 'HIV Infection of Macrophages: Implications for Pathogenesis and Cure', *Pathogens and Immunity*, 2(2), p. 179. doi: 10.20411/10.20411/pai.v2i2.204.
- clinicaltrials.gov (2018) *A Randomized, Double-blinded, Placebo-controlled, Dose-escalation Phase 1 Clinical Trial to Evaluate the Safety and Immunogenicity of Recombinant HIV Envelope Protein BG505 SOSIP.664 gp140 Vaccine, Adjuvanted, in Healthy, HIV-1 Uninfected Adults*. Available at: <https://clinicaltrials.gov/ct2/show/NCT03699241>.
- ClinicalTrials.gov (2020a) *10E8.4/iMab Bispecific Antibody in HIV-uninfected and HIV-infected Adults - NCT03875209*. Available at: <https://clinicaltrials.gov/ct2/show/NCT03875209> (Accessed: 16 September 2020).
- ClinicalTrials.gov (2020b) *Pharmacokinetics of SAR441236, October 15, 2018*. Available at: <https://clinicaltrials.gov/ct2/show/NCT03705169> (Accessed: 31 March 2020).
- Cockburn, I. A. and Seder, R. A. (2018) 'Malaria prevention: from immunological concepts to effective vaccines and protective antibodies', *Nature Immunology*, 19(11), pp. 1199–1211. doi: 10.1038/s41590-018-0228-6.
- Cohen, F. S. and Melikyan, G. B. (2004) 'The energetics of membrane fusion from binding, through hemifusion, pore formation, and pore enlargement.', *The Journal of membrane biology*, 199(1), pp. 1–14. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15366419> (Accessed: 19 July 2019).
- Colcher, D. *et al.* (1998) 'Pharmacokinetics and biodistribution of genetically-engineered antibodies', *Q J Nucl Med*, 42(4), pp. 225–41. Available at: <https://search.proquest.com/openview/aad96f811b8e44830fd2f5817ff5b54b/1?pq-origsite=gscholar&cbl=27900> (Accessed: 14 August 2018).
- Corey, L. *et al.* (2021) 'Two Randomized Trials of Neutralizing Antibodies to Prevent HIV-1 Acquisition', *New England Journal of Medicine*, 384(11), pp. 1003–1014. doi: 10.1056/nejmoa2031738.
- Corti, D. *et al.* (2010) 'Analysis of memory B cell responses and isolation of novel monoclonal antibodies with neutralizing breadth from HIV-1-infected individuals', *PLoS ONE*. Edited by D. Unutmaz, 5(1), p. e8805. doi: 10.1371/journal.pone.0008805.

References

- Costiniuk, C. T. and Jenabian, M.-A. (2014) 'Cell-to-cell transfer of HIV infection: implications for HIV viral persistence', *Journal of General Virology*, 95(Pt_11), pp. 2346–2355. doi: 10.1099/vir.0.069641-0.
- Crowell, T. A. *et al.* (2019) 'Safety and efficacy of VRC01 broadly neutralising antibodies in adults with acutely treated HIV (RV397): a phase 2, randomised, double-blind, placebo-controlled trial', *The Lancet HIV*, 6(5), pp. e297–e306. doi: 10.1016/S2352-3018(19)30053-0.
- Dale, B. M., Alvarez, R. A. and Chen, B. K. (2013) 'Mechanisms of enhanced HIV spread through T-cell virological synapses', *Immunological Reviews*, 251(1), pp. 113–124. doi: 10.1111/imr.12022.
- Davis-Gardner, M. E. *et al.* (2020) 'A bispecific antibody that simultaneously recognizes the V2-and V3-glycan epitopes of the hiv-1 envelope glycoprotein is broader and more potent than its parental antibodies', *mBio*, 11(1). doi: 10.1128/mBio.03080-19.
- Deeks, S. G., Tracy, R. and Douek, D. C. (2013) 'Systemic Effects of Inflammation on Health during Chronic HIV Infection', *Immunity*. NIH Public Access, pp. 633–645. doi: 10.1016/j.immuni.2013.10.001.
- Deleage, C. *et al.* (2019) 'Impact of early cART in the gut during acute HIV infection', *JCI Insight*, 1(10). doi: 10.1172/jci.insight.87065.
- Deonarain, M. P. (2018) 'Miniaturised 'antibody'-drug conjugates for solid tumours?', *Drug Discovery Today: Technologies*, 30(xx), pp. 47–53. doi: 10.1016/j.ddtec.2018.09.006.
- Derking, R. *et al.* (2015) 'Comprehensive Antigenic Map of a Cleaved Soluble HIV-1 Envelope Trimer', *PLoS Pathogens*, 11(3), pp. 1–22. doi: 10.1371/journal.ppat.1004767.
- DeSantis, M. C. *et al.* (2016) 'Quantitative correlation between infectivity and Gp120 Density on HIV-1 Virions revealed by optical trapping virometry', *Journal of Biological Chemistry*, 291(25), pp. 13088–13097. doi: 10.1074/jbc.M116.729210.
- Desmonde, S. *et al.* (2011) 'Severe morbidity and mortality in untreated HIV-infected children in a paediatric care programme in Abidjan, Côte d'Ivoire, 2004-2009', *BMC Infectious Diseases*, 11, p. 182. doi: 10.1186/1471-2334-11-182.
- Ding, S. *et al.* (2016) 'A highly conserved gp120 inner domain residue modulates Env conformation and trimer stability', *Journal of Virology*, 90(19), pp. 8395–8409. doi: 10.1128/JVI.01068-16.
- Dixon, S. (2002) 'The impact of HIV and AIDS on Africa's economic development', *BMJ*, 324(7331), pp. 232–234. doi: 10.1136/bmj.324.7331.232.
- Doitsh, G. and Greene, W. C. (2016) 'Dissecting How CD4 T Cells Are Lost during HIV Infection', *Cell Host and Microbe*. Cell Press, pp. 280–291. doi: 10.1016/j.chom.2016.02.012.
- Dong, J.-Y., Fan, P.-D. and Frizzell, R. A. (2008) 'Quantitative Analysis of the Packaging Capacity of Recombinant Adeno-Associated Virus', *Human Gene Therapy*, 7(17), pp. 2101–2112. doi: 10.1089/hum.1996.7.17-2101.
- Doores, K. J. (2015) 'The HIV glycan shield as a target for broadly neutralizing antibodies', *FEBS Journal*, 282(24), pp. 4679–4691. doi: 10.1111/febs.13530.
- Doria-Rose, N. A., Georgiev, I. S., *et al.* (2012) 'A Short Segment of the HIV-1 gp120 V1/V2

References

- Region Is a Major Determinant of Resistance to V1/V2 Neutralizing Antibodies', *Journal of Virology*, 86(15), pp. 8319–8323. doi: 10.1128/JVI.00696-12.
- Doria-Rose, N. A., Louder, M. K., *et al.* (2012) 'HIV-1 Neutralization Coverage Is Improved by Combining Monoclonal Antibodies That Target Independent Epitopes', *Journal of Virology*, 86(6), pp. 3393–3397. doi: 10.1128/JVI.06745-11.
- Doria-Rose, N. A. *et al.* (2014) 'Developmental pathway for potent V1V2-directed HIV-neutralizing antibodies', *Nature*, 509(7498), pp. 55–62. doi: 10.1038/nature13036.
- Doria-Rose, N. A. *et al.* (2015) 'New member of the V1V2-directed CAP256-VRC26 lineage that shows increased breadth and exceptional potency', *J Virol*, 90(1), pp. 76–91. doi: 10.1128/JVI.01791-15.
- van Dorsten, R. T. *et al.* (2020) 'Neutralization Breadth and Potency of Single-Chain Variable Fragments Derived from Broadly Neutralizing Antibodies Targeting Multiple Epitopes on the HIV-1 Envelope', *Journal of Virology*. Edited by G. Silvestri, 94(2). doi: 10.1128/JVI.01533-19.
- Draper, S. J. *et al.* (2018) 'Malaria Vaccines: Recent Advances and New Horizons', *Cell Host and Microbe*, 24(1), pp. 43–56. doi: 10.1016/j.chom.2018.06.008.
- Duan, H. *et al.* (2018) 'Glycan Masking Focuses Immune Responses to the HIV-1 CD4-Binding Site and Enhances Elicitation of VRC01-Class Precursor Antibodies', *Immunity*, 49(2), pp. 301-311.e5. doi: 10.1016/j.immuni.2018.07.005.
- Duan, K. *et al.* (2020) 'Effectiveness of convalescent plasma therapy in severe COVID-19 patients', *Proceedings of the National Academy of Sciences of the United States of America*, 117(17), pp. 9490–9496. doi: 10.1073/pnas.2004168117.
- Dubrovskaya, V. *et al.* (2019) 'Vaccination with Glycan-Modified HIV NFL Envelope Trimer-Liposomes Elicits Broadly Neutralizing Antibodies to Multiple Sites of Vulnerability', *Immunity*, 51(5), pp. 915-929.e7. doi: 10.1016/j.immuni.2019.10.008.
- Duerr, A. *et al.* (2012) 'Extended follow-up confirms early vaccine-enhanced risk of HIV acquisition and demonstrates waning effect over time among participants in a randomized trial of recombinant adenovirus HIV vaccine (Step Study).', *The Journal of infectious diseases*, 206(2), pp. 258–66. doi: 10.1093/infdis/jis342.
- Dufloo, J., Bruel, T. and Schwartz, O. (2018) 'HIV-1 cell-to-cell transmission and broadly neutralizing antibodies', *Retrovirology*, 15(1), p. 51. doi: 10.1186/s12977-018-0434-1.
- Duncan, C. J. A. *et al.* (2014) 'High-Multiplicity HIV-1 Infection and Neutralizing Antibody Evasion Mediated by the Macrophage-T Cell Virological Synapse', *Journal of Virology*, 88(4), pp. 2025–2034. doi: 10.1128/JVI.03245-13.
- Duncan, C. J. A., Russell, R. A. and Sattentau, Q. J. (2013) 'High multiplicity HIV-1 cell-to-cell transmission from macrophages to CD4+ T cells limits antiretroviral efficacy', *AIDS*, 27(14), pp. 2201–2206. doi: 10.1097/QAD.0b013e3283632ec4.
- Durham, N. D. *et al.* (2012) 'Neutralization Resistance of Virological Synapse-Mediated HIV-1 Infection Is Regulated by the gp41 Cytoplasmic Tail', *Journal of Virology*, 86(14), pp. 7484–7495. doi: 10.1128/jvi.00230-12.
- Dwyer-Lindgren, L. *et al.* (2019) 'Mapping HIV prevalence in sub-Saharan Africa between 2000 and 2017', *Nature*, 570(7760), pp. 189–193. doi: 10.1038/s41586-019-1200-9.

References

- Elliott, H., Hoyne, G. F. and Hoyne, G. (2015) 'The Role of the Nef Protein in MHC-I Downregulation and Viral Immune Evasion by HIV-1'. doi: 10.4172/2155-9899.1000375.
- Evans, V. A. *et al.* (2013) 'Myeloid Dendritic Cells Induce HIV-1 Latency in Non-proliferating CD4+ T Cells', *PLoS Pathogens*. Edited by S. R. Ross, 9(12), pp. 1–14. doi: 10.1371/journal.ppat.1003799.
- Falkowska, E. *et al.* (2014) 'Broadly neutralizing HIV antibodies define a glycan-dependent epitope on the prefusion conformation of gp41 on cleaved envelope trimers.', *Immunity*, 40(5), pp. 657–668. doi: 10.1016/j.immuni.2014.04.009.
- Faria, N. R. *et al.* (2014) 'The early spread and epidemic ignition of HIV-1 in human populations', *Science*, 346(6205), pp. 56–61. doi: 10.1126/science.1256739.
- Ferreira, R. C. *et al.* (2018) 'Structural Rearrangements Maintain the Glycan Shield of an HIV-1 Envelope Trimer After the Loss of a Glycan', *Scientific Reports*, 8(1). doi: 10.1038/s41598-018-33390-2.
- Ferry, T. *et al.* (2009) 'Uncontrolled viral replication as a risk factor for non-AIDS severe clinical events in HIV-infected patients on long-term antiretroviral therapy: APROCO/COPILOTE (ANRS CO8) cohort study', *J Acquir Immune Defic Syndr*, 51(4), pp. 407–415. doi: 10.1097/QAI.0b013e3181acb65f.
- Flotte, T. R. (2006) 'New AAV serotypes may broaden the therapeutic pipeline to human gene therapy', *Molecular Therapy*, 13(1), pp. 1–2. doi: 10.1016/j.ymthe.2005.11.004.
- Folks, T. *et al.* (1985) 'Characterization of a continuous T-cell line susceptible to the cytopathic effects of the acquired immunodeficiency syndrome (AIDS)-associated retrovirus', *Proceedings of the National Academy of Sciences of the United States of America*, 82(13), pp. 4539–4543. doi: 10.1073/pnas.82.13.4539.
- Fuchs, S. P. *et al.* (2015) 'AAV-Delivered Antibody Mediates Significant Protective Effects against SIVmac239 Challenge in the Absence of Neutralizing Activity', *PLoS Pathogens*. Edited by A. Trkola, 11(8), p. e1005090. doi: 10.1371/journal.ppat.1005090.
- Fuchs, S. P. and Desrosiers, R. C. (2016) 'Promise and problems associated with the use of recombinant AAV for the delivery of anti-HIV antibodies', *Molecular Therapy - Methods and Clinical Development*, 3(July), p. 16068. doi: 10.1038/mtm.2016.68.
- Fujita-Yamaguchi, Y. (2014) 'Production of Single-Chain Variable-Fragments against Carbohydrate Antigens', *Antibodies*, 3(1), pp. 155–168. doi: 10.3390/antib3010155.
- G. Williams, B., Lima, V. and Gouws, E. (2011) 'Modelling the Impact of Antiretroviral Therapy on the Epidemic of HIV', *Current HIV Research*, 9(6), pp. 367–382. doi: 10.2174/157016211798038533.
- Galloway, N. L. *et al.* (2015) 'Cell-to-Cell Transmission of HIV-1 Is Required to Trigger Pyroptotic Death of Lymphoid-Tissue-Derived CD4 T Cells.', *Cell reports*, 12(10), pp. 1555–1563. doi: 10.1016/j.celrep.2015.08.011.
- Garces, F. *et al.* (2015) 'Affinity Maturation of a Potent Family of HIV Antibodies Is Primarily Focused on Accommodating or Avoiding Glycans', *Immunity*, 43(6), pp. 1053–1063. doi: 10.1016/j.immuni.2015.11.007.
- Gautam, R. *et al.* (2016) 'A single injection of anti-HIV-1 antibodies protects against repeated

References

- SHIV challenges', *Nature*, 533(7601), pp. 105–109. doi: 10.1038/nature17677.
- Georgiev, I. S. *et al.* (2014) 'Antibodies VRC01 and 10E8 neutralize HIV-1 with high breadth and potency even with Ig-framework regions substantially reverted to germline', *J Immunol*, 192(3), pp. 1100–1106. doi: 10.4049/jimmunol.1302515.
- Ghosn, J. *et al.* (2018) 'HIV', *The Lancet*, 392(10148), pp. 685–697. doi: 10.1016/S0140-6736(18)31311-4.
- Gift, S. K. *et al.* (2017) 'Functional Stability of HIV-1 Envelope Trimer Affects Accessibility to Broadly Neutralizing Antibodies at Its Apex', *Journal of Virology*. Edited by V. Simon, 91(24), pp. 176–193. doi: 10.1128/JVI.01216-17.
- Glass, R. I. *et al.* (1983) 'Protection against Cholera in Breast-Fed Children by Antibodies in Breast Milk', *New England Journal of Medicine*, 308(23), pp. 1389–1392. doi: 10.1056/nejm198306093082304.
- Goetghebuer, T. *et al.* (2009) 'Effect of early antiretroviral therapy on the risk of AIDS/death in HIV-infected infants', *AIDS*, 23(5), pp. 597–604. doi: 10.1097/QAD.0b013e328326ca37.
- Goetz, M. B. *et al.* (2009) 'Relationship between HIV coreceptor tropism and disease progression in persons with untreated chronic HIV infection', *Journal of Acquired Immune Deficiency Syndromes*, 50(3), pp. 259–266. doi: 10.1097/QAI.0b013e3181989a8b.
- Gombos, R. B. *et al.* (2015) 'Inhibitory Effect of Individual or Combinations of Broadly Neutralizing Antibodies and Antiviral Reagents against Cell-Free and Cell-to-Cell HIV-1 Transmission', *Journal of Virology*, 89(15), pp. 7813–7828. doi: 10.1128/jvi.00783-15.
- Gousset, K. *et al.* (2008) 'Real-time visualization of HIV-1 GAG trafficking in infected macrophages', *PLoS Pathogens*. Edited by T. J. Hope, 4(3), p. e1000015. doi: 10.1371/journal.ppat.1000015.
- Gray, E. S. *et al.* (2007) 'Neutralizing Antibody Responses in Acute Human Immunodeficiency Virus Type 1 Subtype C Infection', *Journal of Virology*, 81(12), pp. 6187–6196. doi: 10.1128/jvi.00239-07.
- Gray, E. S. *et al.* (2011) 'The neutralization breadth of HIV-1 develops incrementally over four years and is associated with CD4+ T cell decline and high viral load during acute infection', *J Virol*. 2011/03/11, 85(10), pp. 4828–4840. doi: JVI.00198-11 [pii] 10.1128/JVI.00198-11.
- Grobben, M., Stuart, R. AL and van Gils, M. J. (2019) 'The potential of engineered antibodies for HIV-1 therapy and cure', *Current Opinion in Virology*, 38, pp. 70–80. doi: 10.1016/j.coviro.2019.07.007.
- Gruell, H. and Klein, F. (2018) 'Antibody-mediated prevention and treatment of HIV-1 infection', *Retrovirology*, 15(1), p. 73. doi: 10.1186/s12977-018-0455-9.
- Gruenert, A. K. *et al.* (2016) 'Self-complementary adeno-associated virus vectors improve transduction efficiency of corneal endothelial cells', *PLoS ONE*. Edited by G. Di Pasquale, 11(3), p. e0152589. doi: 10.1371/journal.pone.0152589.
- Guglielmi, L. and Martineau, P. (2009) 'Expression of single-chain Fv fragments in *E. coli* cytoplasm.', *Methods in molecular biology (Clifton, N.J.)*, 562, pp. 215–224. doi: 10.1007/978-1-60327-302-2_17.

References

- Gust, D. A. *et al.* (2009) 'HIV testing outside of the study among men who have sex with men participating in an HIV vaccine efficacy trial', *J Acquir Immune Defic Syndr*, 52(2), pp. 294–298. doi: 10.1097/QAI.0b013e3181ab6e93.
- Halper-Stromberg, A. *et al.* (2014) 'Broadly Neutralizing Antibodies and Viral Inducers Decrease Rebound from HIV-1 Latent Reservoirs in Humanized Mice', *Cell*, 158(5), pp. 989–999. doi: 10.1016/j.cell.2014.07.043.
- Han, Y. *et al.* (2007) 'Experimental approaches to the study of HIV-1 latency', *Nature Reviews Microbiology*, 5(2), pp. 95–106. doi: 10.1038/nrmicro1580.
- Hansen, J. *et al.* (2020) 'Studies in humanized mice and convalescent humans yield a SARS-CoV-2 antibody cocktail', *Science*, 369(6506), pp. 1010–1014. doi: 10.1126/science.abd0827.
- Harris, N. L. *et al.* (2006) 'Mechanisms of Neonatal Mucosal Antibody Protection', *The Journal of Immunology*, 177(9), pp. 6256–6262. doi: 10.4049/jimmunol.177.9.6256.
- Hay, C. E. *et al.* (2018) 'Development and testing of AAV-delivered single-chain variable fragments for the treatment of methamphetamine abuse', *PLoS ONE*. Edited by M. Taffe, 13(6), p. e0200060. doi: 10.1371/journal.pone.0200060.
- Haynes, B. F. *et al.* (2012) 'Immune-Correlates Analysis of an HIV-1 Vaccine Efficacy Trial', *New England Journal of Medicine*, 366(14), pp. 1275–1286. doi: 10.1056/nejmoa1113425.
- Hemelaar, J. *et al.* (2006) 'Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004', *AIDS*, 20(16), pp. W13–W23. doi: 10.1097/01.aids.0000247564.73009.bc.
- Henderson, L. J. *et al.* (2012) 'Human immunodeficiency virus type 1 (HIV-1) transactivator of transcription through its intact core and cysteine-rich domains inhibits wnt/ β -catenin signaling in astrocytes: Relevance to HIV neuropathogenesis', *Journal of Neuroscience*, 32(46), pp. 16306–16313. doi: 10.1523/JNEUROSCI.3145-12.2012.
- Hernandez, L. D. *et al.* (1996) 'Virus-Cell and Cell-Cell Fusion', *Annual Review of Cell and Developmental Biology*, 12(1), pp. 627–661. doi: 10.1146/annurev.cellbio.12.1.627.
- Hessell, A. J. *et al.* (2009) 'Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques', *Nature Medicine*, 15(8), pp. 951–954. doi: 10.1038/nm.1974.
- Hessell, A. J. *et al.* (2016) 'Early short-term treatment with neutralizing human monoclonal antibodies halts SHIV infection in infant macaques', *Nature Medicine*, 22(4), pp. 362–368. doi: 10.1038/nm.4063.
- Van Heuverswyn, F. *et al.* (2006) 'Human immunodeficiency viruses: SIV infection in wild gorillas', *Nature*, 444(7116), p. 164. doi: 10.1038/444164a.
- Hileman, C. O. and Funderburg, N. T. (2017) 'Inflammation, Immune Activation, and Antiretroviral Therapy in HIV', *Current HIV/AIDS Reports*, 14(3), pp. 93–100. doi: 10.1007/s11904-017-0356-x.
- Hill, M. K. *et al.* (2002) 'Proline Residues within Spacer Peptide p1 Are Important for Human Immunodeficiency Virus Type 1 Infectivity, Protein Processing, and Genomic RNA Dimer Stability', *Journal of Virology*, 76(22), pp. 11245–11253. doi: 10.1128/jvi.76.22.11245-11253.2002.

References

- Van Hoecke, L. and Roose, K. (2019) 'How mRNA therapeutics are entering the monoclonal antibody field', *Journal of Translational Medicine*, 17(1), p. 54. doi: 10.1186/s12967-019-1804-8.
- Hooft van Huijsduijnen, R. *et al.* (2020) 'Reassessing therapeutic antibodies for neglected and tropical diseases', *PLoS neglected tropical diseases*, 14(1), p. e0007860. doi: 10.1371/journal.pntd.0007860.
- Horwitz, J. A. *et al.* (2013) 'HIV-1 suppression and durable control by combining single broadly neutralizing antibodies and antiretroviral drugs in humanized mice.', *Proceedings of the National Academy of Sciences of the United States of America*, 110(41), pp. 16538–43. doi: 10.1073/pnas.1315295110.
- Huang, J. *et al.* (2012) 'Broad and potent neutralization of HIV-1 by a gp41-specific human antibody', *Nature*, 491(7424), pp. 406–412. doi: 10.1038/nature11544.
- Huang, J. *et al.* (2014) 'Broad and potent HIV-1 neutralization by a human antibody that binds the gp41-gp120 interface', *Nature*, 515(7525), pp. 138–142. doi: 10.1038/nature13601.
- Huang, L., Su, X. and Federoff, H. J. (2013) 'Single-chain fragment variable passive immunotherapies for neurodegenerative diseases', *International Journal of Molecular Sciences*, 14(9), pp. 19109–19127. doi: 10.3390/ijms140919109.
- Huang, Y. *et al.* (2016) 'Engineered Bispecific Antibodies with Exquisite HIV-1-Neutralizing Activity', *Cell*, 165(7), pp. 1621–1631. doi: 10.1016/j.cell.2016.05.024.
- Hunt, P. W. (2017) 'Very early ART and persistent inflammation in treated HIV', *Clinical Infectious Diseases*, 64(2), pp. 132–133. doi: 10.1093/cid/ciw697.
- HVTN (2019) *HVTN 130/HPTN 089, bNAb combinations*. Available at: https://www.hptn.org/research/studies/130_089 (Accessed: 2 September 2020).
- HVTN (2020) *Ongoing HVTN trials*, www.hvtn.org/en/science/HVTN-studies.html. Available at: <https://www.hvtn.org/en/science/HVTN-studies.html> (Accessed: 3 July 2019).
- Hymes, K. B. *et al.* (1981) 'Kaposi's sarcoma in homosexual men. A report of eight cases', *Lancet*, 318(8247), pp. 598–600. doi: 10.1016/S0140-6736(81)92740-9.
- INSIGHT START Study Group *et al.* (2015) 'Initiation of Antiretroviral Therapy in Early Asymptomatic HIV Infection.', *The New England journal of medicine*, 373(9), pp. 795–807. doi: 10.1056/NEJMoa1506816.
- International Union of Immunological Societies (2020) *Antibody Structure and Classes / Immunopaedia*. Available at: <https://www.immunopaedia.org.za/immunology/basics/7-antibody-structure-and-classes/> (Accessed: 9 April 2020).
- Irimia, A. *et al.* (2016) 'Crystallographic Identification of Lipid as an Integral Component of the Epitope of HIV Broadly Neutralizing Antibody 4E10', *Immunity*, 44(1), pp. 21–31. doi: 10.1016/j.immuni.2015.12.001.
- Irimia, A. *et al.* (2017) 'Lipid interactions and angle of approach to the HIV-1 viral membrane of broadly neutralizing antibody 10E8: Insights for vaccine and therapeutic design', *PLoS Pathogens*. Edited by A. Trkola, 13(2), p. e1006212. doi: 10.1371/journal.ppat.1006212.
- Iversen, P. L. *et al.* (2020) 'Recent successes in therapeutics for Ebola virus disease: no time

References

- for complacency', *The Lancet Infectious Diseases*, 20(9), pp. e231–e237. doi: 10.1016/S1473-3099(20)30282-6.
- Izquierdo-Useros, N. *et al.* (2014) 'HIV-1 Capture and Transmission by Dendritic Cells: The Role of Viral Glycolipids and the Cellular Receptor Siglec-1', *PLoS Pathogens*. Edited by T. C. Hobman, 10(7), p. e1004146. doi: 10.1371/journal.ppat.1004146.
- J.C., G. *et al.* (2012) 'Incomplete immune recovery in HIV infection: Mechanisms, relevance for clinical care, and possible solutions', *Clinical and Developmental Immunology*, 2012. Available at: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3312328/> (Accessed: 30 March 2020).
- Janaka, S. K., Gregory, D. A. and Johnson, M. C. (2013) 'Retrovirus Glycoprotein Functionality Requires Proper Alignment of the Ectodomain and the Membrane-Proximal Cytoplasmic Tail', *Journal of Virology*, 87(23), pp. 12805–12813. doi: 10.1128/jvi.01847-13.
- Janeway, C. J., Travers, P. and Walport, M. (2001) 'The structure of a typical antibody molecule', in *Immunobiology: The Immune System in Health and Disease. 5th edition. Chapter 3: Antigen Recognition by B-cell and T-cell Receptors*. New York: Garland Science. Available at: <http://www.ncbi.nlm.nih.gov/books/NBK27144/>.
- Jardine, J. G. *et al.* (2015) 'Priming a broadly neutralizing antibody response to HIV-1 using a germline-targeting immunogen', *Science*, 349(6244), pp. 156–161. doi: 10.1126/science.aac5894.
- Jardine, J. G. *et al.* (2016) 'Minimally Mutated HIV-1 Broadly Neutralizing Antibodies to Guide Reductionist Vaccine Design.', *PLoS pathogens*. Edited by A. Trkola, 12(8), p. e1005815. doi: 10.1371/journal.ppat.1005815.
- Jewkes, R. K. *et al.* (2010) 'Intimate partner violence, relationship power inequity, and incidence of HIV infection in young women in South Africa: A cohort study', *The Lancet*, 376(9734), pp. 41–48. doi: 10.1016/S0140-6736(10)60548-X.
- Johnson, P. R. *et al.* (2009) 'Vector-mediated gene transfer engenders long-lived neutralizing activity and protection against SIV infection in monkeys', *Nature Medicine*, 15(8), pp. 901–906. doi: 10.1038/nm.1967.
- Jolly, C. *et al.* (2004) 'HIV-1 cell to cell transfer across an Env-induced, actin-dependent synapse', *J Exp Med*, 199(2), pp. 283–293. doi: 10.1084/jem.20030648.
- Jolly, C. and Sattentau, Q. J. (2007) 'Human immunodeficiency virus type 1 assembly, budding, and cell-cell spread in T cells take place in tetraspanin-enriched plasma membrane domains', *J Virol*, 81(15), pp. 7873–7884. doi: 10.1128/JVI.01845-06.
- Joseph, S. B. *et al.* (2015) 'Bottlenecks in HIV-1 transmission: insights from the study of founder viruses', *Nature Reviews Microbiology*, 13(7), pp. 414–425. doi: 10.1038/nrmicro3471.
- Julg, B., Tartaglia, L. J., *et al.* (2017) 'Broadly neutralizing antibodies targeting the HIV-1 envelope V2 apex confer protection against a clade C SHIV challenge', *Science Translational Medicine*, 9(406), p. eaal1321. doi: 10.1126/scitranslmed.aal1321.
- Julg, B., Liu, P.-T., *et al.* (2017) 'Protection against a mixed SHIV challenge by a broadly neutralizing antibody cocktail', *Science Translational Medicine*, 9(408), p. eaao4235. doi: 10.1126/scitranslmed.aao4235.
- Julg, B. and Barouch, D. H. (2019) 'Neutralizing antibodies for HIV-1 prevention', *Current*

References

- opinion in HIV and AIDS*, 14(4), pp. 318–324. doi: 10.1097/COH.0000000000000556.
- Julien, J.-P., Lee, J. H., *et al.* (2013) 'Asymmetric recognition of the HIV-1 trimer by broadly neutralizing antibody PG9', *Proceedings of the National Academy of Sciences*, 110(11), pp. 4351–4356. doi: 10.1073/pnas.1217537110.
- Julien, J.-P., Cupo, A., *et al.* (2013) 'Crystal Structure of a Soluble Cleaved HIV-1 Envelope Trimer', *Science*, 342(6165), pp. 1477–1483. doi: 10.1126/science.1245625.
- Kaplon, H. *et al.* (2020) 'Antibodies to watch in 2020', *mAbs*, 12(1), p. 1703531. doi: 10.1080/19420862.2019.1703531.
- Karasavvas, N. *et al.* (2012) 'The Thai Phase III HIV Type 1 Vaccine Trial (RV144) Regimen Induces Antibodies That Target Conserved Regions Within the V2 Loop of gp120', *AIDS Research and Human Retroviruses*, 28(11), pp. 1444–1457. doi: 10.1089/aid.2012.0103.
- Keefe, J. R. *et al.* (2018) 'A Combination of Two Human Monoclonal Antibodies Prevents Zika Virus Escape Mutations in Non-human Primates', *Cell Reports*, 25(6), pp. 1385–1394.e7. doi: 10.1016/j.celrep.2018.10.031.
- van den Kerkhof, T. L. G. M. *et al.* (2016) 'HIV-1 escapes from N332-directed antibody neutralization in an elite neutralizer by envelope glycoprotein elongation and introduction of unusual disulfide bonds', *Retrovirology*, 13(1), p. 48. doi: 10.1186/s12977-016-0279-4.
- Khan, L. *et al.* (2017) 'Cross-neutralizing anti-HIV-1 human single chain variable fragments (scFvs) against CD4 binding site and N332 glycan identified from a recombinant phage library', *Scientific Reports*, 7(September 2016), p. 45163. doi: 10.1038/srep45163.
- Khan, S. N. *et al.* (2018) 'Targeting the HIV-1 Spike and Coreceptor with Bi- and Trispecific Antibodies for Single-Component Broad Inhibition of Entry', *Journal of Virology*, 92(18). doi: 10.1128/jvi.00384-18.
- Kharsany, A. B. M. M. *et al.* (2018) 'Community-based HIV prevalence in KwaZulu-Natal, South Africa: results of a cross-sectional household survey', *The Lancet HIV*, 5(8), pp. e427–e437. doi: 10.1016/S2352-3018(18)30104-8.
- Klasse, P. J. (2016) 'How to assess the binding strength of antibodies elicited by vaccination against HIV and other viruses', *Expert Review of Vaccines*, 15(3), pp. 295–311. doi: 10.1586/14760584.2016.1128831.
- Klein, C., Schaefer, W. and Regula, J. T. (2016) 'The use of CrossMAb technology for the generation of bi- and multispecific antibodies', *mAbs*, 8(6), pp. 1010–1020. doi: 10.1080/19420862.2016.1197457.
- Klein, F. *et al.* (2012) 'HIV therapy by a combination of broadly neutralizing antibodies in humanized mice', *Nature*, 492(7427), pp. 118–122. doi: 10.1038/nature11604.
- Klein, F. *et al.* (2013) 'Antibodies in HIV-1 vaccine development and therapy', *Science*, 341(6151), pp. 1199–1204. doi: 10.1126/science.1241144.
- Kolodkin-Gal, D. *et al.* (2013) 'Efficiency of Cell-Free and Cell-Associated Virus in Mucosal Transmission of Human Immunodeficiency Virus Type 1 and Simian Immunodeficiency Virus', *Journal of Virology*, 87(24), pp. 13589–13597. doi: 10.1128/jvi.03108-12.
- Kong, L. *et al.* (2013) 'Supersite of immune vulnerability on the glycosylated face of HIV-1

References

- envelope glycoprotein gp120', *Nature Structural and Molecular Biology*, 20(7), pp. 796–803. doi: 10.1038/nsmb.2594.
- Kong, R. *et al.* (2015) 'Improving neutralization potency and breadth by combining broadly reactive HIV-1 antibodies targeting major neutralization epitopes.', *Journal of virology*, 89(5), pp. 2659–71. doi: 10.1128/JVI.03136-14.
- Kong, R. *et al.* (2016) 'Fusion peptide of HIV-1 as a site of vulnerability to neutralizing antibody', *Science*, 352(6287), pp. 828–833. doi: 10.1126/science.aae0474.
- Kräusslich, H. G. *et al.* (1995) 'The spacer peptide between human immunodeficiency virus capsid and nucleocapsid proteins is essential for ordered assembly and viral infectivity.', *Journal of virology*, 69(6), pp. 3407–3419. doi: 10.1128/jvi.69.6.3407-3419.1995.
- Krumm, S. A. *et al.* (2016) 'Mechanisms of escape from the PGT128 family of anti-HIV broadly neutralizing antibodies', *Retrovirology*, 13(1), p. 8. doi: 10.1186/s12977-016-0241-5.
- Kubetzko, S. *et al.* (2006) 'PEGylation and multimerization of the anti-p185HER-2single chain Fv fragment 4D5: Effects on tumor targeting', *Journal of Biological Chemistry*, 281(46), pp. 35186–35201. doi: 10.1074/jbc.M604127200.
- Kumar, R. *et al.* (2018) 'Broadly neutralizing antibodies in HIV-1 treatment and prevention', *Therapeutic Advances in Vaccines and Immunotherapy*, 6(4), pp. 61–68. doi: 10.1177/2515135518800689.
- Kumar, S. *et al.* (2017) 'CD4-binding site directed cross-neutralizing scFv monoclonals from HIV-1 subtype C infected Indian children', *Frontiers in Immunology*, 8(NOV), p. 1568. doi: 10.3389/fimmu.2017.01568.
- Kwon, Y. Do *et al.* (2016) 'Optimization of the Solubility of HIV-1-Neutralizing Antibody 10E8 through Somatic Variation and Structure-Based Design', *J Virol*, 90(13), pp. 5899–5914. doi: 10.1128/JVI.03246-15.
- Kwong, P. D. *et al.* (1998) 'Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody', *Nature*, 393(6686), pp. 648–659. doi: 10.1038/31405.
- Landais, E. and Moore, P. L. (2018) 'Development of broadly neutralizing antibodies in HIV-1 infected elite neutralizers', *Retrovirology*, 15(1), p. 61. doi: 10.1186/s12977-018-0443-0.
- LANL (2015) *HIV Circulating Recombinant Forms (CRFs)*, *HIV sequence database*. Available at: <https://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html> (Accessed: 21 October 2019).
- Law, K. M. *et al.* (2016) 'In Vivo HIV-1 Cell-to-Cell Transmission Promotes Multicopy Micro-compartmentalized Infection', *Cell Reports*, 15(12), pp. 2771–2783. doi: 10.1016/j.celrep.2016.05.059.
- Lee, C. H. *et al.* (2016) 'Antibodies against venom of the snake *Deinagkistrodon acutus*', *Applied and Environmental Microbiology*, 82(1), pp. 71–80. doi: 10.1128/AEM.02608-15.
- Lee, J. H., Ozorowski, G. and Ward, A. B. (2016) 'Cryo-EM structure of a native, fully glycosylated, cleaved HIV-1 envelope trimer', *Science*, 351(6277), pp. 1043–1048. doi: 10.1126/science.aad2450.

References

- Len, A. C. L. *et al.* (2017) 'HIV-1 Activates T Cell Signaling Independently of Antigen to Drive Viral Spread', *Cell Reports*, 18(4), pp. 1062–1074. doi: 10.1016/j.celrep.2016.12.057.
- Levine, M. M. (2019) 'Monoclonal Antibody Therapy for Ebola Virus Disease', *New England Journal of Medicine*, 381(24), pp. 2365–2366. doi: 10.1056/nejme1915350.
- Li, B. *et al.* (2006) 'Evidence for Potent Autologous Neutralizing Antibody Titers and Compact Envelopes in Early Infection with Subtype C Human Immunodeficiency Virus Type 1', *Journal of Virology*, 80(11), pp. 5211–5218. doi: 10.1128/jvi.00201-06.
- Li, H. *et al.* (2017) 'Reduced Potency and Incomplete Neutralization of Broadly Neutralizing Antibodies against Cell-to-Cell Transmission of HIV-1 with Transmitted Founder Envs', *Journal of Virology*, 91(9), pp. e02425-16. doi: 10.1128/jvi.02425-16.
- Li, H. and Chen, B. K. (2019) 'Variable infectivity and conserved engagement in cell-to-cell viral transfer by HIV-1 Env from Clade B transmitted founder clones', *Virology*, 526, pp. 189–202. doi: 10.1016/j.virol.2018.10.016.
- Liao, H.-X. *et al.* (2013) 'Co-evolution of a broadly neutralizing HIV-1 antibody and founder virus', *Nature*, 496(7446), pp. 469–476. doi: 10.1038/nature12053.
- Lilly (2020) *Lilly's neutralizing antibody bamlanivimab (LY-CoV555) receives FDA emergency use authorization for the treatment of recently diagnosed COVID-19*. Available at: <https://investor.lilly.com/news-releases/news-release-details/lillys-neutralizing-antibody-bamlanivimab-ly-cov555-receives-fda> (Accessed: 30 November 2020).
- Lin, A. and Balazs, A. B. (2018) 'Adeno-associated virus gene delivery of broadly neutralizing antibodies as prevention and therapy against HIV-1', *11 Medical and Health Sciences 1103 Clinical Sciences 11 Medical and Health Sciences 1108 Medical Microbiology Marit van Gils, m.j.vangils@amc.*, *Retrovirology*, 15(1), p. 66. doi: 10.1186/s12977-018-0449-7.
- Lingappa, J. R. *et al.* (2014) 'How HIV-1 Gag assembles in cells: Putting together pieces of the puzzle', *Virus Research*, 193, pp. 89–107. doi: 10.1016/j.virusres.2014.07.001.
- Liu, J. *et al.* (2008) 'Molecular architecture of native HIV-1 gp120 trimers', *Nature*, 455(7209), pp. 109–113. doi: 10.1038/nature07159.
- Liu, Q. *et al.* (2019) 'Improvement of antibody functionality by structure-guided paratope engraftment', *Nature Communications*, 10(1). doi: 10.1038/s41467-019-08658-4.
- Liu, Y. *et al.* (2020) 'Broadly neutralizing antibodies for HIV-1: efficacies, challenges and opportunities', *Emerging Microbes and Infections*, 9(1), pp. 194–206. doi: 10.1080/22221751.2020.1713707.
- Liu, Z. *et al.* (2017) 'Systematic comparison of 2A peptides for cloning multi-genes in a polycistronic vector', *Scientific Reports*, 7(1), p. 2193. doi: 10.1038/s41598-017-02460-2.
- Lopez, P. *et al.* (2019) 'HIV Infection Stabilizes Macrophage-T Cell Interactions To Promote Cell-Cell HIV Spread', *Journal of Virology*. Edited by F. Kirchhoff, 93(18). doi: 10.1128/JVI.00805-19.
- Los Alamos National Laboratory (2017) *Landmarks of the HIV genome*, <https://www.hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html>. Available at: <https://www.hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html> (Accessed: 21 October 2019).

References

- Lu, C.-L. L. *et al.* (2016) 'Enhanced clearance of HIV-1-infected cells by broadly neutralizing antibodies against HIV-1 in vivo', *Science*, 352(6288), pp. 1001–1004. doi: 10.1126/science.aaf1279.
- Lynch, R. M. *et al.* (2012) 'The Development of CD4 Binding Site Antibodies during HIV-1 Infection', *Journal of Virology*, 86(14), pp. 7588–7595. doi: 10.1128/jvi.00734-12.
- Maartens, G., Celum, C. and Lewin, S. R. (2014) 'HIV infection: Epidemiology, pathogenesis, treatment, and prevention', *The Lancet*, 384(9939), pp. 258–271. doi: 10.1016/S0140-6736(14)60164-1.
- Mabvakure, B. M. *et al.* (2018) 'Positive Selection at Key Residues in the HIV Envelope Distinguishes Broad and Strain-Specific Plasma Neutralizing Antibodies', *Journal of Virology*, 93(6). doi: 10.1128/jvi.01685-18.
- Mabvakure, B. M. *et al.* (2019) 'Evidence for both Intermittent and Persistent Compartmentalization of HIV-1 in the Female Genital Tract', *Journal of Virology*, 93(10). doi: 10.1128/jvi.00311-19.
- Mahomed, S. *et al.* (2020) 'Assessing the safety and pharmacokinetics of the anti-HIV monoclonal antibody CAP256V2LS alone and in combination with VRC07-523LS and PGT121 in South African women: study protocol for the first-in-human CAPRISA 012B phase I clinical trial', *BMJ open*, 10(11), p. e042247. doi: 10.1136/bmjopen-2020-042247.
- Malbec, M. *et al.* (2013) 'Broadly neutralizing antibodies that inhibit HIV-1 cell to cell transmission', *The Journal of Experimental Medicine*, 210(13), pp. 2813–2821. doi: 10.1084/jem.20131244.
- Manches, O., Frleta, D. and Bhardwaj, N. (2014) 'Dendritic cells in progression and pathology of HIV infection', *Trends in Immunology*, 35(3), pp. 114–122. doi: 10.1016/j.it.2013.10.003.
- Marcobal, A. *et al.* (2016) 'Expression of Human Immunodeficiency Virus Type 1 Neutralizing Antibody Fragments Using Human Vaginal Lactobacillus', *AIDS Research and Human Retroviruses*, 32(10–11), pp. 964–971. doi: 10.1089/aid.2015.0378.
- Marcotte, H. and Hammarström, L. (2015) 'Passive Immunization', in *Mucosal Immunology*. Fourth Edi. Elsevier, pp. 1403–1434. doi: 10.1016/B978-0-12-415847-4.00071-9.
- Martin, N. *et al.* (2010) 'Virological Synapse-Mediated Spread of Human Immunodeficiency Virus Type 1 between T Cells Is Sensitive to Entry Inhibition', *Journal of Virology*, 84(7), pp. 3516–3527. doi: 10.1128/jvi.02651-09.
- Martinez-Navio, J. M. *et al.* (2019) 'Adeno-Associated Virus Delivery of Anti-HIV Monoclonal Antibodies Can Drive Long-Term Virologic Suppression', *Immunity*, 50(3), pp. 567–575.e5. doi: 10.1016/j.immuni.2019.02.005.
- Martinez-Picado, J. and Deeks, S. G. (2016) 'Persistent HIV-1 replication during antiretroviral therapy', *Current Opinion in HIV and AIDS*, 11(4), pp. 417–423. doi: 10.1097/COH.0000000000000287.
- Mascola, J. R. *et al.* (2000) 'Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies', *Nature Medicine*, 6(2), pp. 207–210. doi: 10.1038/72318.
- Masur, H. *et al.* (2010) 'An Outbreak of Community-Acquired *Pneumocystis carinii*

References

- Pneumonia', *New England Journal of Medicine*, 305(24), pp. 1431–1438. doi: 10.1056/nejm198112103052402.
- Maughan-Brown, B. *et al.* (2018) 'HIV Risk Among Adolescent Girls and Young Women in Age-Disparate Partnerships', *JAIDS Journal of Acquired Immune Deficiency Syndromes*, 78(2), pp. 155–162. doi: 10.1097/QAI.0000000000001656.
- Mazurov, D. *et al.* (2010) 'Quantitative comparison of HTLV-1 and HIV-1 cell-to-cell infection with new replication dependent vectors', *PLoS Pathogens*. Edited by T. J. Hope, 6(2), p. e1000788. doi: 10.1371/journal.ppat.1000788.
- McCarty, D. M. (2008) 'Self-complementary AAV Vectors; Advances and Applications', *Molecular Therapy*, 16(10), pp. 1648–1656. doi: 10.1038/mt.2008.171.
- McCoy, L. E. *et al.* (2012) 'Potent and broad neutralization of HIV-1 by a llama antibody elicited by immunization', *The Journal of Experimental Medicine*, 209(6), pp. 1091–1103. doi: 10.1084/jem.20112655.
- McCoy, L. E. *et al.* (2014) 'Neutralisation of HIV-1 cell-cell spread by human and llama antibodies', *Retrovirology*, 11(1), p. 83. doi: 10.1186/s12977-014-0083-y.
- McCoy, L. E. *et al.* (2016) 'Holes in the Glycan Shield of the Native HIV Envelope Are a Target of Trimer-Elicited Neutralizing Antibodies', *Cell Reports*, 16(9), pp. 2327–2338. doi: 10.1016/j.celrep.2016.07.074.
- McGuire, A. T. *et al.* (2013) 'Engineering HIV envelope protein to activate germline B cell receptors of broadly neutralizing anti-CD4 binding site antibodies', *Journal of Experimental Medicine*, 210(4), pp. 655–663. doi: 10.1084/jem.20122824.
- McLellan, J. S. *et al.* (2011) 'Structure of HIV-1 gp120 V1/V2 domain with broadly neutralizing antibody PG9', *Nature*, 480(7377), pp. 336–343. doi: 10.1038/nature10696.
- McPhee, E. *et al.* (2019) 'Short Communication: The Interaction of HIV Set Point Viral Load and Subtype on Disease Progression', *AIDS Research and Human Retroviruses*, 35(1), pp. 49–51. doi: 10.1089/aid.2018.0165.
- Mendoza, P. *et al.* (2018) 'Combination therapy with anti-HIV-1 antibodies maintains viral suppression', *Nature*, 561(7724), pp. 479–484. doi: 10.1038/s41586-018-0531-2.
- Mesin, L., Ersching, J. and Victora, G. D. (2016) 'Germinal Center B Cell Dynamics', *Immunity*, 45(3), pp. 471–482. doi: 10.1016/j.immuni.2016.09.001.
- Miller, C. M. *et al.* (2010) 'Why are antiretroviral treatment patients lost to follow-up? A qualitative study from South Africa.', *Tropical medicine & international health : TM & IH*, 15 Suppl 1(s1), pp. 48–54. doi: 10.1111/j.1365-3156.2010.02514.x.
- Milligan, C. and Overbaugh, J. (2014) 'The role of cell-Associated virus in mother-to-child HIV transmission', *Journal of Infectious Diseases*, 210(Suppl 3), pp. S631–S640. doi: 10.1093/infdis/jiu344.
- Mohan, G., Guduri, P. R. and Shastry, S. (2019) 'Role of therapeutic plasma exchange in snake bite associated thrombotic microangiopathy—A case report with review of literature', *Journal of Clinical Apheresis*, 34(4), pp. 507–509. doi: 10.1002/jca.21691.
- Monnier, P., Vigouroux, R. and Tassew, N. (2013) 'In Vivo Applications of Single Chain Fv

References

- (Variable Domain) (scFv) Fragments', *Antibodies*, 2(4), pp. 193–208. doi: 10.3390/antib2020193.
- Montefiori, D. C. *et al.* (2018) 'Neutralization tiers of HIV-1', *Current Opinion in HIV and AIDS*, 13(2), pp. 128–136. doi: 10.1097/COH.0000000000000442.
- Moore, P. L. *et al.* (2006) 'Nature of Nonfunctional Envelope Proteins on the Surface of Human Immunodeficiency Virus Type 1', *Journal of Virology*, 80(5), pp. 2515–2528. doi: 10.1128/jvi.80.5.2515-2528.2006.
- Moore, P. L. *et al.* (2008) 'The C3-V4 Region Is a Major Target of Autologous Neutralizing Antibodies in Human Immunodeficiency Virus Type 1 Subtype C Infection', *Journal of Virology*, 82(4), pp. 1860–1869. doi: 10.1128/jvi.02187-07.
- Moore, P. L. *et al.* (2009) 'Limited neutralizing antibody specificities drive neutralization escape in early HIV-1 subtype C infection', *PLoS Pathog*, 5(9), p. e1000598. doi: 10.1371/journal.ppat.1000598.
- Moore, P. L. *et al.* (2013) 'Multiple Pathways of Escape from HIV Broadly Cross-Neutralizing V2-Dependent Antibodies', *Journal of Virology*, 87(9), pp. 4882–4894. doi: 10.1128/JVI.03424-12.
- Moore, P. L. (2018) 'The Neutralizing Antibody Response to the HIV-1 Env Protein', *Current HIV Research*, 16(1), pp. 21–28. doi: 10.2174/1570162X15666171124122044.
- Morris, L. and Mkhize, N. N. (2017) 'Prospects for passive immunity to prevent HIV infection', *PLoS Medicine*, 14(11), p. e1002436. doi: 10.1371/journal.pmed.1002436.
- Moscato, C. G. *et al.* (2014) 'Trimeric HIV Env provides epitope occlusion mediated by hypervariable loops', *Scientific Reports*, 4. doi: 10.1038/srep07025.
- Moshoette, T. *et al.* (2019) 'Engineering and characterising a novel, highly potent bispecific antibody iMab-CAP256 that targets HIV-1', *Retrovirology*, 16(1), pp. 1–12. doi: 10.1186/s12977-019-0493-y.
- Mothes, W. *et al.* (2010) 'Virus cell-to-cell transmission', *J Virol*, 84(17), pp. 8360–8368. doi: 10.1128/JVI.00443-10.
- Murooka, T. T. *et al.* (2012) 'HIV-infected T cells are migratory vehicles for viral dissemination', *Nature*, 490(7419), pp. 283–289. doi: 10.1038/nature11398.
- Narasimhulu, V. G. S. *et al.* (2018) 'Distinct functions for the membrane-proximal ectodomain region (MPER) of HIV-1 gp41 in cell-free and cell-cell viral transmission and cell-cell fusion', *Journal of Biological Chemistry*, 293(16), pp. 6099–6120. doi: 10.1074/jbc.RA117.000537.
- Naso, M. F. *et al.* (2017) 'Adeno-Associated Virus (AAV) as a Vector for Gene Therapy', *BioDrugs*, 31(4), pp. 317–334. doi: 10.1007/s40259-017-0234-5.
- National Institute of Allergy and Infectious Diseases (2020) *Experimental HIV Vaccine Regimen Ineffective in Preventing HIV*. Available at: <https://www.niaid.nih.gov/news-events/experimental-hiv-vaccine-regimen-ineffective-preventing-hiv>.
- Ndirangu, J. *et al.* (2012) 'Cell-Free (RNA) and Cell-Associated (DNA) HIV-1 and Postnatal Transmission through Breastfeeding', *PLoS ONE*, 7(12). doi: 10.1371/journal.pone.0051493.
- Niessl, J. *et al.* (2020) 'Combination anti-HIV-1 antibody therapy is associated with increased

References

- virus-specific T cell immunity', *Nature Medicine*, 26(2), pp. 222–227. doi: 10.1038/s41591-019-0747-1.
- Nish, S. A. *et al.* (2017) 'CD4+ T cell effector commitment coupled to self-renewal by asymmetric cell divisions', *Journal of Experimental Medicine*, 214(1), pp. 39–47. doi: 10.1084/jem.20161046.
- Nixon, C. C. *et al.* (2017) 'In Vivo Models of Human Immunodeficiency Virus Persistence and Cure Strategies', *The Journal of Infectious Diseases*, 215(suppl_3), pp. S142–S151. doi: 10.1093/infdis/jiw637.
- Nobile, C. *et al.* (2010) 'HIV-1 Nef Inhibits Ruffles, Induces Filopodia, and Modulates Migration of Infected Lymphocytes', *Journal of Virology*, 84(5), pp. 2282–2293. doi: 10.1128/jvi.02230-09.
- Okoye, A. A. and Picker, L. J. (2013) 'CD4+ T-Cell Depletion In Hiv Infection: Mechanisms Of Immunological Failure', *Immunological Reviews*, 254(1), pp. 54–64. doi: 10.1111/imr.12066.
- Okulicz, J. F. and Lambotte, O. (2011) 'Epidemiology and clinical characteristics of elite controllers', *Current Opinion in HIV and AIDS*, 6(3), pp. 163–168. doi: 10.1097/COH.0b013e328344f35e.
- Pace, M. J. *et al.* (2011) 'HIV reservoirs and latency models', *Virology*, 411(2), pp. 344–354. doi: 10.1016/j.virol.2010.12.041.
- Pace, M. J. *et al.* (2012) 'Directly Infected Resting CD4+T Cells Can Produce HIV Gag without Spreading Infection in a Model of HIV Latency', *PLoS Pathogens*. Edited by G. Silvestri, 8(7), p. e1002818. doi: 10.1371/journal.ppat.1002818.
- Padte, N. N. *et al.* (2018) 'Engineering multi-specific antibodies against HIV-1', *Retrovirology*, 15(1), p. 60. doi: 10.1186/s12977-018-0439-9.
- Pais-Correia, A. M. *et al.* (2010) 'Biofilm-like extracellular viral assemblies mediate HTLV-1 cell-to-cell transmission at virological synapses', *Nature Medicine*, 16(1), pp. 83–89. doi: 10.1038/nm.2065.
- Pancera, M. *et al.* (2013) 'Structural basis for diverse N-glycan recognition by HIV-1-neutralizing V1-V2-directed antibody PG16', *Nature Structural and Molecular Biology*, 20(7), pp. 804–813. doi: 10.1038/nsmb.2600.
- Pancera, M. *et al.* (2014) 'Structure and immune recognition of trimeric pre-fusion HIV-1 Env', *Nature*, 514(7523), pp. 455–461. doi: 10.1038/nature13808.
- Panico, M. *et al.* (2016) 'Mapping the complete glycoproteome of virion-derived HIV-1 gp120 provides insights into broadly neutralizing antibody binding', *Scientific Reports*, 6(1), p. 32956. doi: 10.1038/srep32956.
- Pardi, N. *et al.* (2017) 'Administration of nucleoside-modified mRNA encoding broadly neutralizing antibody protects humanized mice from HIV-1 challenge', *Nature Communications*, 8. doi: 10.1038/ncomms14630.
- Park, T., Griggs, S. K. and Suh, D. C. (2015) 'Cost Effectiveness of Monoclonal Antibody Therapy for Rare Diseases: A Systematic Review', *BioDrugs*. Springer International Publishing, pp. 259–274. doi: 10.1007/s40259-015-0135-4.

References

- Parren, P. W. *et al.* (2001) 'Antibody Protects Macaques against Vaginal Challenge with a Pathogenic R5 Simian/Human Immunodeficiency Virus at Serum Levels Giving Complete Neutralization In Vitro', *Journal of Virology*, 75(17), pp. 8340–8347. doi: 10.1128/JVI.75.17.8340-8347.2001.
- Parsons, M. S. *et al.* (2017) 'Partial efficacy of a broadly neutralizing antibody against cell-associated SHIV infection', *Science Translational Medicine*, 9(402), pp. 1–13. doi: 10.1126/scitranslmed.aaf1483.
- Patel, M. B., Hoffman, N. G. and Swanstrom, R. (2008) 'Subtype-Specific Conformational Differences within the V3 Region of Subtype B and Subtype C Human Immunodeficiency Virus Type 1 Env Proteins', *Journal of Virology*, 82(2), pp. 903–916. doi: 10.1128/jvi.01444-07.
- Patel, P. *et al.* (2014) 'Estimating per-act HIV transmission risk', *AIDS*, 28(10), pp. 1509–1519. doi: 10.1097/QAD.0000000000000298.
- Pedro, K. D., Henderson, A. J. and Agosto, L. M. (2019) 'Mechanisms of HIV-1 cell-to-cell transmission and the establishment of the latent reservoir', *Virus Research*, 265, pp. 115–121. doi: 10.1016/j.virusres.2019.03.014.
- Pegu, A. *et al.* (2014) 'Neutralizing antibodies to HIV-1 envelope protect more effectively in vivo than those to the CD4 receptor', *Science Translational Medicine*, 6(243), pp. 243ra88–243ra88. doi: 10.1126/scitranslmed.3008992.
- Pejchal, R. *et al.* (2010) 'Structure and function of broadly reactive antibody PG16 reveal an H3 subdomain that mediates potent neutralization of HIV-1', *Proceedings of the National Academy of Sciences*, 107(25), pp. 11483–11488. doi: 10.1073/pnas.1004600107.
- Pinto, D. *et al.* (2019) 'Structural Basis for Broad HIV-1 Neutralization by the MPER-Specific Human Broadly Neutralizing Antibody LN01', *Cell Host and Microbe*, 26(5), pp. 623–637.e8. doi: 10.1016/j.chom.2019.09.016.
- Popovic, M. *et al.* (1984) 'Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS', *Science*, 224(4648), pp. 497–500. doi: 10.1126/science.6200935.
- Pornillos, O. *et al.* (2009) 'X-Ray Structures of the Hexameric Building Block of the HIV Capsid', *Cell*, 137(7), pp. 1282–1292. doi: 10.1016/j.cell.2009.04.063.
- Del Portillo, A. *et al.* (2011) 'Multiploid Inheritance of HIV-1 during Cell-to-Cell Infection', *Journal of Virology*, 85(14), pp. 7169–7176. doi: 10.1128/jvi.00231-11.
- Priddy, F. H. *et al.* (2019) 'Adeno-associated virus vectored immunoprophylaxis to prevent HIV in healthy adults: a phase 1 randomised controlled trial', *The Lancet HIV*, 6(4), pp. e230–e239. doi: 10.1016/S2352-3018(19)30003-7.
- Qu, Y. *et al.* (2019) 'Characteristics and advantages of adeno-Associated virus vector-mediated gene therapy for neurodegenerative diseases', *Neural Regeneration Research*, 14(6), pp. 931–938. doi: 10.4103/1673-5374.250570.
- Rasmussen, T. A., Tolstrup, M. and Søgaaard, O. S. (2016) 'Reversal of Latency as Part of a Cure for HIV-1', *Trends in Microbiology*, 24(2), pp. 90–97. doi: 10.1016/j.tim.2015.11.003.
- Ratner, L., Gallo, R. C. and Wong-Staal, F. (1985) 'HTLV-III, LAV, ARV are variants of same AIDS virus [8]', *Nature*. Nature Publishing Group, pp. 636–637. doi: 10.1038/313636c0.

References

- Regeneron (2020) *Regeneron's Casirivimab and Imdevimab Antibody Cocktail for COVID-19 is First Combination Therapy to Receive FDA Emergency Use Authorization | Regeneron Pharmaceuticals Inc.* Available at: <https://investor.regeneron.com/news-releases/news-release-details/regenerons-regen-cov2-first-antibody-cocktail-covid-19-receive> (Accessed: 30 November 2020).
- Reh, L. *et al.* (2015) 'Capacity of Broadly Neutralizing Antibodies to Inhibit HIV-1 Cell-Cell Transmission Is Strain- and Epitope-Dependent', *PLoS Pathogens*, 11(7), p. e1004966. doi: 10.1371/journal.ppat.1004966.
- Reh, L. *et al.* (2018) 'Phenotypic deficits in the HIV-1 envelope are associated with the maturation of a V2-directed broadly neutralizing antibody lineage', *PLoS Pathogens*, 14(1). doi: 10.1371/journal.ppat.1006825.
- Reichert, J. M. and The Antibody Society (2020) *Antibody therapeutics approved or in regulatory review in the EU or US*, www.antibodysociety.org. Available at: <https://www.antibodysociety.org/resources/approved-antibodies/> (Accessed: 5 August 2020).
- Reks-Ngarm, S. *et al.* (2009) 'Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand', *N Engl J Med*, 361(23), pp. 2209–2220. doi: 10.1056/NEJMoa0908492.
- Rezaei, S. D. *et al.* (2018) 'The Pathway To Establishing HIV Latency Is Critical to How Latency Is Maintained and Reversed', *Journal of Virology*. Edited by G. Silvestri, 92(13), pp. e02225-17. doi: 10.1128/jvi.02225-17.
- Ribeiro, R. M. *et al.* (2006) 'Naive and Memory Cell Turnover as Drivers of CCR5-to-CXCR4 Tropism Switch in Human Immunodeficiency Virus Type 1: Implications for Therapy', *Journal of Virology*, 80(2), pp. 802–809. doi: 10.1128/jvi.80.2.802-809.2006.
- Ringe, R. P. *et al.* (2018) 'Closing and Opening Holes in the Glycan Shield of HIV-1 Envelope Glycoprotein SOSIP Trimers Can Redirect the Neutralizing Antibody Response to the Newly Unmasked Epitopes', *Journal of Virology*, 93(4), pp. e01656-18. doi: 10.1128/jvi.01656-18.
- Robb, M. L., Eller, L. A., *et al.* (2016) 'Prospective study of acute HIV-1 infection in adults in East Africa and Thailand', *New England Journal of Medicine*, 374(22), pp. 2120–2130. doi: 10.1056/NEJMoa1508952.
- Robb, M. L. and Ananworanich, J. (2016) 'Lessons from acute HIV infection', *Current Opinion in HIV and AIDS*, 11(6), pp. 555–560. doi: 10.1097/COH.0000000000000316.
- Rodriguez-Plata, M. T. *et al.* (2013) 'The infectious synapse formed between mature dendritic cells and CD4+ T cells is independent of the presence of the HIV-1 envelope glycoprotein', *Retrovirology*, 10(1), p. 42. doi: 10.1186/1742-4690-10-42.
- Rogier, E. W. *et al.* (2014) 'Secretory antibodies in breast milk promote long-term intestinal homeostasis by regulating the gut microbiota and host gene expression', *Proceedings of the National Academy of Sciences of the United States of America*, 111(8), pp. 3074–3079. doi: 10.1073/pnas.1315792111.
- van Rompay, K. K. A. *et al.* (2020) 'A combination of two human monoclonal antibodies limits fetal damage by Zika virus in macaques', *Proceedings of the National Academy of Sciences of the United States of America*, 117(14), pp. 7981–7989. doi: 10.1073/pnas.2000414117.
- Ronzitti, G., Gross, D.-A. and Mingozzi, F. (2020) 'Human Immune Responses to Adeno-

References

- Associated Virus (AAV) Vectors', *Frontiers in Immunology*, 11, p. 670. doi: 10.3389/fimmu.2020.00670.
- Rosenberg, Y. J. *et al.* (2016) 'Protection against SHIV Challenge by Subcutaneous Administration of the Plant-Derived PGT121 Broadly Neutralizing Antibody in Macaques', *PLoS One*, 11(3), p. e0152760. doi: 10.1371/journal.pone.0152760.
- Rosenberg, Y. J. *et al.* (2019) 'Introduction of the YTE mutation into the non-immunogenic HIV bnAb PGT121 induces anti-drug antibodies in macaques', *PLoS ONE*, 14(2). doi: 10.1371/journal.pone.0212649.
- Rousseau, C. M. *et al.* (2004) 'Association of Levels of HIV-1–Infected Breast Milk Cells and Risk of Mother-to-Child Transmission', *The Journal of Infectious Diseases*, 190(10), pp. 1880–1888. doi: 10.1086/425076.
- Rubens, M. *et al.* (2015) 'HIV Vaccine: Recent Advances, Current Roadblocks, and Future Directions', *Journal of Immunology Research*, 2015, pp. 1–9. doi: 10.1155/2015/560347.
- Rudicell, R. S. *et al.* (2014) 'Enhanced Potency of a Broadly Neutralizing HIV-1 Antibody In Vitro Improves Protection against Lentiviral Infection In Vivo', *Journal of Virology*, 88(21), pp. 12669–12682. doi: 10.1128/JVI.02213-14.
- Rusert, P. *et al.* (2011) 'Interaction of the gp120 V1V2 loop with a neighboring gp120 unit shields the HIV envelope trimer against cross-neutralizing antibodies', *Journal of Experimental Medicine*, 208(7), pp. 1419–1433. doi: 10.1084/jem.20110196.
- Rusert, P. *et al.* (2016) 'Determinants of HIV-1 broadly neutralizing antibody induction', *Nature Medicine*, 22(11), pp. 1260–1267. doi: 10.1038/nm.4187.
- Sacks, D. *et al.* (2019) 'Somatic hypermutation to counter a globally rare viral immunotype drove off-track antibodies in the CAP256-VRC26 HIV-1 V2-directed bNAbs lineage', *PLoS Pathogens*. Edited by R. C. Desrosiers, 15(9), p. e1008005. doi: 10.1371/journal.ppat.1008005.
- Safdari, Y. *et al.* (2016) 'Use of Single-Chain Antibody Derivatives for Targeted Drug Delivery', *Molecular Medicine*, 22(1), pp. 258–270. doi: 10.2119/molmed.2016.00043.
- Sagar, M. (2014) 'Origin of the transmitted virus in HIV infection: Infected cells versus cell-free virus', *Journal of Infectious Diseases*, 210, pp. S667–S673. doi: 10.1093/infdis/jiu369.
- Salazar, G. *et al.* (2017) 'Antibody therapies for the prevention and treatment of viral infections', *npj Vaccines*, 2(1), pp. 1–12. doi: 10.1038/s41541-017-0019-3.
- Sallé, B. *et al.* (2010) 'Infection of Macaques after Vaginal Exposure to Cell-Associated Simian Immunodeficiency Virus', *The Journal of Infectious Diseases*, 202(3), pp. 337–344. doi: 10.1086/653619.
- Sanders, R. W. *et al.* (2002) 'Differential Transmission of Human Immunodeficiency Virus Type 1 by Distinct Subsets of Effector Dendritic Cells', *Journal of Virology*, 76(15), pp. 7812–7821. doi: 10.1128/jvi.76.15.7812-7821.2002.
- Sanders, R. W. *et al.* (2013) 'A Next-Generation Cleaved, Soluble HIV-1 Env Trimer, BG505 SOSIP.664 gp140, Expresses Multiple Epitopes for Broadly Neutralizing but Not Non-Neutralizing Antibodies', *PLoS Pathogens*. Edited by A. Trkola, 9(9), p. e1003618. doi: 10.1371/journal.ppat.1003618.

References

- Sanders, R. W. *et al.* (2015) 'HIV-1 neutralizing antibodies induced by native-like envelope trimers', *Science*, 349(6244), pp. aac4223–aac4223. doi: 10.1126/science.aac4223.
- Saunders, K. O. *et al.* (2015) 'Broadly Neutralizing Human Immunodeficiency Virus Type 1 Antibody Gene Transfer Protects Nonhuman Primates from Mucosal Simian-Human Immunodeficiency Virus Infection', *Journal of Virology*, 89(16), pp. 8334–8345. doi: 10.1128/jvi.00908-15.
- Schacker, T. W. *et al.* (2002) 'Collagen deposition in HIV-1 infected lymphatic tissues and T cell homeostasis', *Journal of Clinical Investigation*, 110(8), pp. 1133–1139. doi: 10.1172/jci16413.
- Scharf, L. *et al.* (2014) 'Antibody 8ANC195 reveals a site of broad vulnerability on the HIV-1 envelope spike', *Cell Rep*, 7(3), pp. 785–795. doi: 10.1016/j.celrep.2014.04.001.
- Scharf, L. *et al.* (2015) 'Broadly Neutralizing Antibody 8ANC195 Recognizes Closed and Open States of HIV-1 Env', *Cell*, 162(6), pp. 1379–1390. doi: 10.1016/j.cell.2015.08.035.
- Scheid, J. F. *et al.* (2011) 'Sequence and Structural Convergence of Broad and Potent HIV Antibodies That Mimic CD4 Binding', *Science*, 333(6049), pp. 1633–1637. doi: 10.1126/science.1207227.
- Scheid, J. F. *et al.* (2016) 'HIV-1 antibody 3BNC117 suppresses viral rebound in humans during treatment interruption', *Nature*, 535(7613), pp. 556–560. doi: 10.1038/nature18929.
- Schiffner, T., Sattentau, Q. J. and Duncan, C. J. A. (2013) 'Cell-to-cell spread of HIV-1 and evasion of neutralizing antibodies', *Vaccine*, 31(49), pp. 5789–5797. doi: 10.1016/j.vaccine.2013.10.020.
- Schommers, P. *et al.* (2020) 'Restriction of HIV-1 Escape by a Highly Broad and Potent Neutralizing Antibody', *Cell*, 180(3), pp. 471–489.e22. doi: 10.1016/j.cell.2020.01.010.
- Schoofs, T. *et al.* (2016) 'HIV-1 therapy with monoclonal antibody 3BNC117 elicits host immune responses against HIV-1', *Science*, 352(6288), pp. 997–1001. doi: 10.1126/science.aaf0972.
- Seaman, M. S. *et al.* (2010) 'Tiered Categorization of a Diverse Panel of HIV-1 Env Pseudoviruses for Assessment of Neutralizing Antibodies', *Journal of Virology*, 84(3), pp. 1439–1452. doi: 10.1128/JVI.02108-09.
- Seitz, R. (2016) 'Human Immunodeficiency Virus (HIV)', *Transfusion Medicine and Hemotherapy*, 43(3), pp. 203–222. doi: 10.1159/000445852.
- Shapiro, M. B. *et al.* (2020) 'Single-dose bNAb cocktail or abbreviated ART post-exposure regimens achieve tight SHIV control without adaptive immunity', *Nature Communications*, 11(1), pp. 1–13. doi: 10.1038/s41467-019-13972-y.
- Sharp, P. M. and Hahn, B. H. (2011) 'Origins of HIV and the AIDS Pandemic', *Cold Spring Harbor Perspectives in Medicine*, 1(1), pp. a006841–a006841. doi: 10.1101/cshperspect.a006841.
- Shingai, M. *et al.* (2013) 'Antibody-mediated immunotherapy of macaques chronically infected with SHIV suppresses viraemia', *Nature*, 503(7475), pp. 277–280. doi: 10.1038/nature12746.
- Showa, S. P., Nyabadza, F. and Hove-Musekwa, S. D. (2019) 'On the efficiency of HIV transmission: Insights through discrete time HIV models', *PLoS ONE*, 14(9), p. e0222574. doi:

References

10.1371/journal.pone.0222574.

Sigal, A. *et al.* (2011) 'Cell-to-cell spread of HIV permits ongoing replication despite antiretroviral therapy', *Nature*, 477(7362), pp. 95–98. doi: 10.1038/nature10347.

da Silva, E. S., Mulinge, M. and Bercoff, D. P. (2013) 'The frantic play of the concealed HIV envelope cytoplasmic tail', *Retrovirology*, 10(1), p. 54. doi: 10.1186/1742-4690-10-54.

Silver, Z. A. *et al.* (2020) 'Discovery of O-Linked Carbohydrate on HIV-1 Envelope and Its Role in Shielding against One Category of Broadly Neutralizing Antibodies', *Cell Reports*, 30(6), pp. 1862-1869.e4. doi: 10.1016/j.celrep.2020.01.056.

Simonovich, V. A. *et al.* (2020) 'A Randomized Trial of Convalescent Plasma in Covid-19 Severe Pneumonia', *New England Journal of Medicine*. doi: 10.1056/nejmoa2031304.

Sloan, D. D. *et al.* (2015) 'Targeting HIV Reservoir in Infected CD4 T Cells by Dual-Affinity Re-targeting Molecules (DARTs) that Bind HIV Envelope and Recruit Cytotoxic T Cells', *PLoS Pathogens*, 11(11), pp. 1–29. doi: 10.1371/journal.ppat.1005233.

Smith, S. A. and Derdeyn, C. A. (2017) 'New Connections: Cell-to-Cell HIV-1 Transmission, Resistance to Broadly Neutralizing Antibodies, and an Envelope Sorting Motif', *Journal of Virology*. Edited by G. Silvestri, 91(9), pp. e00149-17. doi: 10.1128/jvi.00149-17.

Sok, D. and Burton, D. R. (2018) 'Recent progress in broadly neutralizing antibodies to HIV', *Nature Immunology*, 19(11), pp. 1179–1188. doi: 10.1038/s41590-018-0235-7.

Song, R. *et al.* (2016) 'Distinct HIV-1 neutralization potency profiles of Ibalizumab-Based Bispecific antibodies', *Journal of Acquired Immune Deficiency Syndromes*, 73(4), pp. 365–373. doi: 10.1097/QAI.0000000000001119.

Soto, C. *et al.* (2016) 'Developmental Pathway of the MPER-Directed HIV-1-Neutralizing Antibody 10E8', *PLoS One*, 11(6), p. e0157409. doi: 10.1371/journal.pone.0157409.

Sowinski, S. *et al.* (2008) 'Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission', *Nat Cell Biol*, 10(2), pp. 211–219. doi: 10.1038/ncb1682.

Stamatatos, L., Pancera, M. and McGuire, A. T. (2017) 'Germline-targeting immunogens', *Immunological Reviews*, 275(1), pp. 203–216. doi: 10.1111/imr.12483.

Stanley P, Taniguchi N, A. M. (2017) 'N-glycans', in Varki A, Cummings RD, Esko JD, *et al.* (ed.) *Essentials of Glycobiology*. Cold Spring Harbor Laboratory Press. doi: 10.1101/glycobiology.3e.009.

Statistics South Africa (2018) 'Mid-year population estimates 2018 | Statistics South Africa', *Web Page*. Available at: www.statssa.gov.za/info@statssa.gov.za (Accessed: 21 October 2019).

Steichen, J. M. *et al.* (2016) 'HIV Vaccine Design to Target Germline Precursors of Glycan-Dependent Broadly Neutralizing Antibodies', *Immunity*, 45(3), pp. 483–496. doi: 10.1016/j.immuni.2016.08.016.

Steinhardt, J. J. *et al.* (2018) 'Rational design of a trispecific antibody targeting the HIV-1 Env with elevated anti-viral activity', *Nature Communications*, 9(1), p. 877. doi: 10.1038/s41467-018-03335-4.

Su, B. *et al.* (2019) 'Update on Fc-Mediated Antibody Functions Against HIV-1 Beyond

References

- Neutralization', *Frontiers in Immunology*, 10, p. 2968. doi: 10.3389/fimmu.2019.02968.
- Tabler, C. O. *et al.* (2014) 'CD4+ Memory Stem Cells Are Infected by HIV-1 in a Manner Regulated in Part by SAMHD1 Expression', *Journal of Virology*, 88(9), pp. 4976–4986. doi: 10.1128/JVI.00324-14.
- Tanaka, K. *et al.* (2017) 'Unique binding modes for the broad neutralizing activity of single-chain variable fragments (scFv) targeting CD4-induced epitopes', *Retrovirology*, 14(1), p. 44. doi: 10.1186/s12977-017-0369-y.
- Tanser, F. *et al.* (2018) 'Identifying "corridors of HIV transmission" in a severely affected rural South African population: A case for a shift toward targeted prevention strategies', *International Journal of Epidemiology*, 47(2), pp. 537–549. doi: 10.1093/IJE/DYX257.
- TEMPRANO ANRS 12136 Study Group (2015) 'A Trial of Early Antiretrovirals and Isoniazid Preventive Therapy in Africa', *New England Journal of Medicine*, 373(9), pp. 808–822. doi: 10.1056/NEJMoa1507198.
- The rgp120 HIV Vaccine Study Group (2005) 'Placebocontrolled Phase 3 Trial of a Recombinant Glycoprotein 120 Vaccine to Prevent HIV-1 Infection', *Journal of Infectious Diseases*, 191(5), pp. 654–665. doi: 10.1016/s0084-3873(08)70372-9.
- Tian, H., Lan, C. and Chen, Y. H. (2002) 'Sequence variation and consensus sequence of V3 loop on HIV-1 gp120', *Immunology Letters*, 83(3), pp. 231–233. doi: 10.1016/S0165-2478(02)00101-3.
- van Tienen, C. and van der Loeff, M. S. (2018) 'Epidemiology of HIV-2 Infection in West Africa', in *Encyclopedia of AIDS*. New York, NY: Springer New York, pp. 513–523. doi: 10.1007/978-1-4939-7101-5_34.
- Tincati, C., Douek, D. C. and Marchetti, G. (2016) 'Gut barrier structure, mucosal immunity and intestinal microbiota in the pathogenesis and treatment of HIV infection', *AIDS Research and Therapy*, 13(1), p. 19. doi: 10.1186/s12981-016-0103-1.
- Tomaras, G. D. *et al.* (2008) 'Initial B-Cell Responses to Transmitted Human Immunodeficiency Virus Type 1: Virion-Binding Immunoglobulin M (IgM) and IgG Antibodies Followed by Plasma Anti-gp41 Antibodies with Ineffective Control of Initial Viremia', *Journal of Virology*, 82(24), pp. 12449–12463. doi: 10.1128/jvi.01708-08.
- Tomaras, G. D. and Haynes, B. F. (2009) 'HIV-1-specific antibody responses during acute and chronic HIV-1 infection', *Current Opinion in HIV and AIDS*. NIH Public Access, pp. 373–379. doi: 10.1097/COH.0b013e32832f00c0.
- Turner, B. G. and Summers, M. F. (1999) 'Structural biology of HIV', *J Mol Biol*, 285(1), pp. 1–32. doi: 10.1006/jmbi.1998.2354.
- Umotoy, J. *et al.* (2019) 'Rapid and Focused Maturation of a VRC01-Class HIV Broadly Neutralizing Antibody Lineage Involves Both Binding and Accommodation of the N276-Glycan', *Immunity*. doi: 10.1016/j.immuni.2019.06.004.
- United Nations (2019) *World Population Prospects 2019: Highlights*, Department of Economic and Social Affairs. Available at: https://population.un.org/wpp/Publications/Files/WPP2019_Highlights.pdf (Accessed: 16 October 2019).

References

- Unverdorben, F. *et al.* (2012) 'Half-life extension of a single-chain diabody by fusion to domain B of staphylococcal protein A', *Protein Engineering, Design and Selection*, 25(2), pp. 81–88. doi: 10.1093/protein/gzr061.
- Unverdorben, F. *et al.* (2016) 'Pharmacokinetic properties of IgG and various Fc fusion proteins in mice', *mAbs*, 8(1), pp. 120–128. doi: 10.1080/19420862.2015.1113360.
- Vogt, V. (1997) *Retroviral Virions and Genomes, Retroviruses*. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21433348> (Accessed: 21 October 2019).
- Voss, J. E. *et al.* (2017) 'Elicitation of Neutralizing Antibodies Targeting the V2 Apex of the HIV Envelope Trimer in a Wild-Type Animal Model', *Cell Reports*, 21(1), pp. 222–235. doi: 10.1016/j.celrep.2017.09.024.
- Wagh, K. *et al.* (2016) 'Optimal Combinations of Broadly Neutralizing Antibodies for Prevention and Treatment of HIV-1 Clade C Infection', *PLOS Pathogens*. Edited by R. C. Desrosiers, 12(3), p. e1005520. doi: 10.1371/journal.ppat.1005520.
- Wagh, K., Kreider, E. F., *et al.* (2018) 'Completeness of HIV-1 Envelope Glycan Shield at Transmission Determines Neutralization Breadth', *Cell Reports*, 25(4), pp. 893–908.e7. doi: 10.1016/j.celrep.2018.09.087.
- Wagh, K., Seaman, M. S., *et al.* (2018) 'Potential of conventional & bispecific broadly neutralizing antibodies for prevention of HIV-1 subtype A, C & D infections', *PLoS Pathogens*. Edited by A. Trkola, 14(3), p. e1006860. doi: 10.1371/journal.ppat.1006860.
- Walker, L. M. *et al.* (2011) 'Broad neutralization coverage of HIV by multiple highly potent antibodies', *Nature*, 477(7365), pp. 466–470. doi: 10.1038/nature10373.
- Wang, H. *et al.* (2017) 'Asymmetric recognition of HIV-1 envelope trimer by V1V2 loop-targeting antibodies', *eLife*, 6. doi: 10.7554/eLife.27389.001.
- Wang, H. *et al.* (2018) 'Partially Open HIV-1 Envelope Structures Exhibit Conformational Changes Relevant for Coreceptor Binding and Fusion', *Cell Host and Microbe*, 24(4), pp. 579–592.e4. doi: 10.1016/j.chom.2018.09.003.
- Wang, L.-X. *et al.* (2015) 'Escherichia coli surface display of single-chain antibody VRC01 against HIV-1 infection', *Virology*, 475, pp. 179–186. doi: 10.1016/j.virol.2014.11.018.
- Wang, L. *et al.* (2019) 'Sequential trafficking of Env and Gag to HIV-1 T cell virological synapses revealed by live imaging', *Retrovirology*, 16(1), p. 2. doi: 10.1186/s12977-019-0464-3.
- Watts, J. M. *et al.* (2009) 'Architecture and secondary structure of an entire HIV-1 RNA genome', *Nature*, 460(7256), pp. 711–716. doi: 10.1038/nature08237.
- Webb, N. E., Montefiori, D. C. and Lee, B. (2015) 'Dose–response curve slope helps predict therapeutic potency and breadth of HIV broadly neutralizing antibodies', *Nature Communications*, 6(1), p. 8443. doi: 10.1038/ncomms9443.
- Weber, D. J., Rutala, W. A. and Bartlett, J. G. (2015) 'Prevention of hepatitis B virus and hepatitis C virus infection among healthcare providers', in *UpToDate*. Available at: https://www.uptodate.com/contents/prevention-of-hepatitis-b-virus-and-hepatitis-c-virus-infection-among-health-care-providers?search=hepatitis+b+immune+globulin&source=search_result&selectedTitle=1~110&usage_type=default&display_rank=1 (Accessed: 7 August 2020).

References

- Wei, X. *et al.* (2002) 'Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy', *Antimicrobial Agents and Chemotherapy*, 46(6), pp. 1896–1905. doi: 10.1128/AAC.46.6.1896-1905.2002.
- Wei, X. *et al.* (2003) 'Antibody neutralization and escape by HIV-1', *Nature*, 422(6929), pp. 307–312. doi: 10.1038/nature01470.
- Weiss and Verrips (2019) 'Nanobodies that Neutralize HIV', *Vaccines*, 7(3), p. 77. doi: 10.3390/vaccines7030077.
- West, A. P. *et al.* (2012) 'Single-Chain Fv-Based Anti-HIV Proteins: Potential and Limitations', *Journal of Virology*, 86(1), pp. 195–202. doi: 10.1128/JVI.05848-11.
- WHO (2019) *UPDATE OF RECOMMENDATIONS ON FIRST-AND SECOND-LINE ANTIRETROVIRAL REGIMENS*. Available at: <http://apps.who.int/bookorders>. (Accessed: 18 September 2019).
- Wibmer, C. K. *et al.* (2013) 'Viral Escape from HIV-1 Neutralizing Antibodies Drives Increased Plasma Neutralization Breadth through Sequential Recognition of Multiple Epitopes and Immunotypes', *PLoS Pathogens*. Edited by A. Trkola, 9(10), p. e1003738. doi: 10.1371/journal.ppat.1003738.
- Wibmer, C. K. *et al.* (2017) 'Structure and Recognition of a Novel HIV-1 gp120-gp41 Interface Antibody that Caused MPER Exposure through Viral Escape', *PLoS Pathogens*. Edited by R. C. Desrosiers, 13(1), p. e1006074. doi: 10.1371/journal.ppat.1006074.
- Wiehe, K. *et al.* (2018) 'Functional Relevance of Improbable Antibody Mutations for HIV Broadly Neutralizing Antibody Development', *Cell Host and Microbe*, 23(6), pp. 759–765.e6. doi: 10.1016/j.chom.2018.04.018.
- Wilens, C. B., Tilton, J. C. and Doms, R. W. (2012) 'HIV: Cell binding and entry', *Cold Spring Harbor Perspectives in Medicine*, 2(8). doi: 10.1101/cshperspect.a006866.
- Woitok, M. *et al.* (2016) 'The efficient elimination of solid tumor cells by EGFR-specific and HER2-specific scFv-SNAP fusion proteins conjugated to benzylguanine-modified auristatin F', *Cancer Letters*, 381(2), pp. 323–330. doi: 10.1016/j.canlet.2016.08.003.
- World Health Organization (2019) *HIV/AIDS*. Available at: <http://www.who.int/mediacentre/factsheets/fs360/en/>.
- Worobey, M. *et al.* (2008) 'Direct evidence of extensive diversity of HIV-1 in Kinshasa by 1960', *Nature*, 455(7213), pp. 661–664. doi: 10.1038/nature07390.
- Wrapp, D. *et al.* (2020) 'Structural Basis for Potent Neutralization of Betacoronaviruses by Single-domain Camelid Antibodies', *Cell*, p. 2020.03.26.010165. doi: 10.1016/j.cell.2020.04.031.
- Wu, X. *et al.* (2010) 'Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1', *Science*, 329(5993), pp. 856–861. doi: 10.1126/science.1187659.
- Wu, Y. *et al.* (2019) 'Rapid Elimination of Broadly Neutralizing Antibodies Correlates with Treatment Failure in the Acute Phase of Simian-Human Immunodeficiency Virus Infection', *Journal of Virology*, 93(20). doi: 10.1128/jvi.01077-19.
- Wu, Y., Jiang, S. and Ying, T. (2017) 'Single-Domain Antibodies As Therapeutics against Human

References

- Viral Diseases.', *Frontiers in immunology*, 8, p. 1802. doi: 10.3389/fimmu.2017.01802.
- Wu, Z., Yang, H. and Colosi, P. (2010) 'Effect of genome size on AAV vector packaging', *Molecular Therapy*, 18(1), pp. 80–86. doi: 10.1038/mt.2009.255.
- Wyss, S. *et al.* (2005) 'Regulation of Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Fusion by a Membrane-Interactive Domain in the gp41 Cytoplasmic Tail', *Journal of Virology*, 79(19), pp. 12231–12241. doi: 10.1128/jvi.79.19.12231-12241.2005.
- Xiang, Y. *et al.* (2014) 'The variable loop 3 in the envelope glycoprotein is critical for the atypical coreceptor usage of an HIV-1 strain', *PLoS ONE*. Edited by W. A. Paxton, 9(6), p. e98058. doi: 10.1371/journal.pone.0098058.
- Xie, X. *et al.* (2017) 'Small Molecules and Antibodies for Zika Therapy', *Journal of Infectious Diseases*, 216(suppl_10), pp. S945–S950. doi: 10.1093/infdis/jix406.
- Xu, K. *et al.* (2018) 'Epitope-based vaccine design yields fusion peptide-directed antibodies that neutralize diverse strains of HIV-1', *Nature Medicine*, 24(6), pp. 857–867. doi: 10.1038/s41591-018-0042-6.
- Xu, L. *et al.* (2017) 'Trispecific broadly neutralizing HIV antibodies mediate potent SHIV protection in macaques', *Science*, 356(6359), pp. 85–90. doi: 10.1126/science.aan8630.
- Yokota, T. *et al.* (1992) 'Rapid Tumor Penetration of a Single-Chain Fv and Comparison with Other Immunoglobulin Forms', *Cancer Research*, 52(12), pp. 3402–3408. doi: 10.1007/s00429-013-0597-4.
- Zerbato, J. M. *et al.* (2016) 'Establishment and Reversal of HIV-1 Latency in Naive and Central Memory CD4 + T Cells In Vitro', *Journal of Virology*, 90(18), pp. 8059–8073. doi: 10.1128/jvi.00553-16.
- Zhang, L. *et al.* (2019) 'An MPER antibody neutralizes HIV-1 using germline features shared among donors', *Nature Communications*, 10(1), pp. 1–16. doi: 10.1038/s41467-019-12973-1.
- Zhang, M. Y. *et al.* (2004) 'Identification of a novel CD4i human monoclonal antibody Fab that neutralizes HIV-1 primary isolates from different clades', *Antiviral Research*, 61(3), pp. 161–164. doi: 10.1016/j.antiviral.2003.09.009.
- Zhang, M. Y. *et al.* (2010) 'Potent and broad neutralizing activity of a single chain antibody fragment against cell-free and cell-associated HIV-1', *mAbs*, 2(3), pp. 266–274. doi: 10.4161/mabs.2.3.11416.
- Zhong, P. *et al.* (2013) 'Cell-to-Cell Transmission Can Overcome Multiple Donor and Target Cell Barriers Imposed on Cell-Free HIV', *PLoS ONE*. Edited by F. Mammano, 8(1), p. e53138. doi: 10.1371/journal.pone.0053138.
- Zhou, P. *et al.* (2019) 'Broadly resistant HIV-1 against CD4-binding site neutralizing antibodies', *PLoS Pathogens*. Edited by A. Trkola, 15(6), p. e1007819. doi: 10.1371/journal.ppat.1007819.
- Zhou, T. *et al.* (2010) 'Structural basis for broad and potent neutralization of HIV-1 by antibody VRC01', *Science*, 329(5993), pp. 811–817. doi: 10.1007/s00572-014-0566-7.
- Zhou, T. *et al.* (2017) 'Quantification of the Impact of the HIV-1-Glycan Shield on Antibody Elicitation', *Cell Reports*, 19(4), pp. 719–732. doi: 10.1016/j.celrep.2017.04.013.
- Zolla-Pazner, S. *et al.* (2016) 'Structure/Function Studies Involving the V3 Region of the HIV-1

References

Envelope Delineate Multiple Factors That Affect Neutralization Sensitivity Downloaded from', *Journal of Virology* on July, 90(2). doi: 10.1128/JVI.01645-15.

Zurawski, D. V. and McLendon, M. K. (2020) 'Monoclonal antibodies as an antibacterial approach against bacterial pathogens', *Antibiotics*, 9(4). doi: 10.3390/antibiotics9040155.

Zwahlen, M. and Egger, M. (2006) *Progression and mortality of untreated HIV-positive individuals living in resource-limited settings: Update of literature review and evidence synthesis UNAIDS Obligation HQ/05/422204*. Available at: <http://www.epidem.org> (Accessed: 30 March 2020).

8 Appendices

8.1 IgG and scFv sequences

IgG	Amino acid sequences
CAP256.25 Heavy chain	QVQLVESGGGVVQPGTSLRLSCAASQFRFDGYGMHWVRQAPGKGLEWVASISHDGIKK YHAEKVVWGRFTISRDN SKNTLYLQMNSLRPEDTALYYCAKDLREDECEEWWSDYYDFGK QLPCA KSRGGLVGIADNWGQGTMVTVSS
CAP256.25 lambda Chain	QSVLTQPPSVSAAPGQKVTISCSGNTSNIGNNFVSWYQQRPGRAPQLLIYETDKRPSGIP DRFSASKSGTSGTLAITGLQTGDEADYYCATWAASLSSARVFGTGTKVIVL
PGT121 Heavy Chain	QMQLQESGPGLVKPSETLSLTCSVSGASISDSYWSWIRrspgkglewigyvHKSGDTNYS PSLKSRVNLSDTSKNQVSLSLVAATAADSGKYCARTLHGRIYGIVAFNEWFTYFYMDV WGNGTQVTVSS
PGT121 lambda Chain	DISVAPGETARISCGEKSLSRAVQWYQHRAGQAPSLIYNNQDRPSGIPERFSGSPDSPF GTTATLTITSVEAGDEADYYCHIWD SRVP TKWVF GGGTTLTVL
3BNC117 Heavy Chain	QVQLLQSGAAVTKPGASVRVSCEASGYNIRDYFIHWWRQAPGQGLQWVGWINPKTGQ PNNPRQFQGRVSLTRHASWDFDTFSFYMDLKALRSDDTAVYFCARQRSDYWDFDVWG SGTQVTVSS
3BNC117 kappa Chain	DIQMTQSPSSLSASVGDVTITCQANGYLNWYQRRGKAPKLLIYDGSKL ERGVPSRFSG RRWGQEYNLTINNLPEDIATYFCQVYEFVVP GTRLDLK
10E8v4 Heavy Chain	EVRLVESGGGLVKPGGSLRLSCSASGFDFAWMWVRQPPGKGLEWVGRITGPGE WSVDYAESVKGRFTISRDN TKNTLYLEMNNV R T E D T G Y Y F C A R T G K Y Y D F W S G Y P P G E E YFQDWGQGTLVIVSS
10E8v4 lambda Chain	ASELTQDPAVSVALKQTVTITCRGDSLRSYASWYQKKPGQAPVLLFYGKNRPSGIPDR FSGSASGNRASLTITGAQAEDEADYYCSSRDKSGSRLSVFGGGTKLTVL

8ANC195 Heavy Chain	QIHLVQSGTEVKKPGSSVTVSCKAYGVNTFGLYAVNWVRQAPGQSLEYIGQIWRWKSSA SHHFRGRVLISAVDLTGSSPPISSLEIKNLTSDDTAVYFCTTTSTYDKWSGLHHDGVMFAFSS WGQGTLSVSA
8ANC195 kappa Chain	DIQMTQSPSTLSASIGDTRISCRASQITGNWVAWYQQRPGKAPRLIYRGAALLGGVP SRFSGSAAGTDFLTIGNLQAEDFGTFYCQQYDTYPGTFGQGTKVEVK
Heavy constant region	ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPTVSWNSGALTSGVHTFPAVLQSSG LYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCP APELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWYVDGVEVHNAKTK PREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAK GQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDS DGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGK
Lambda constant region	SQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQ SNNKYAASSYLSLTPEQWKSQRSYSCQVTHEGSTVEKTVAPTECS
Kappa constant region	RTVAAPSVFIFPPSDEQLKSGTASVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQD SKDSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

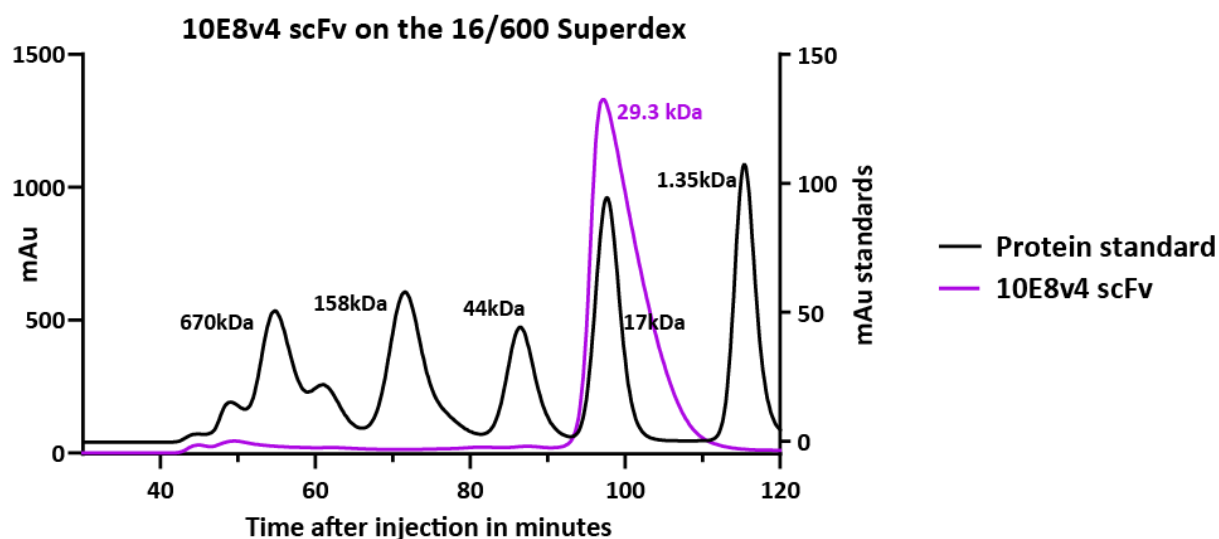
All IgG were cloned in the CMV/R plasmid

scFv construct	Amino acid sequences
CAP256.25 L3 V _H -V _L	QVQLVESGGGVVQPGTSLRLSCAASQFRFDGYGMHWVRQAPGKGLEWVASISHDGIKK YHAEKVWGRFTISRDNKNTLYLQMNSLRPEDTALYYCAKDLREDECEEWWSDYYDFGK QLPCAASRGGLVGIADNWGQGTMTVSS GGSSRSSSSGGGSGGGG QSVLTQPPSVSAAPGQKVTISCSGNTSNIGNNFVSWYQQRPGRAPQLLIYETDKRPSGIP DRFSASKSGTSGTLAITGLQTGDEADYYCATWAASLSSARVFGTGTKVIVL GGLEVLFGGPHHHHHHHH
PGT121 L1 V _H -V _L	QMQLQESGPGLVKPSSETLSLTCSVSGASISDSYWSWIRRS PGKGLEWIGYVHKSGDTNYS PSLKSRVNLSDTSKNQVSLSLVAATAADSGKYYCARTLHGRRYIGVAFNEWFTYFYMDV WNGTQVTVSS GGGSGGGSGGGSGGSS DISVAPGETARISCGEKSLSRAVQWYQHRAGQAPSLIYNNQDRPSGIPERFSGSPDSPF GTTATLTITSVEAGDEADYYCHIWDSDRVPTKWVFGGGTTLTVL GGLEVLFGGPHHHHHHHH
3BNC117 L1 V _H -V _L	QVQLQSGAAVTKPGASVRVSCEASGYNIRDYFIHWWRQAPGQGLQWVGWINPKTGQ PNNPRQFQGRVSLTRHASWDFDTFSFYMDLKALRSDDTAVYFCARQRSYWDVDFVWG SGTQVTVSS GGGSGGGSGGGSGGSS DIQMTQSPSSLSASVGDVTITCQANGYLNWYQRRGKAPKLLIYDGSKLERGVPSRFSG RRWGQEYNLTINNLPEDIATYFCQVYEFVVPGTRLDLK GGLEVLFGGPHHHHHHHH
10E8v4 L1 V _H -V _L	EVRLVESGGGLVKPGGSLRLSCSASGFDNNAWMTWVRQPPGKLEWVGRITGPGE WSVDYAESVKGRFTISRDNKNTLYLEMNNVRTEDTGYFCARTGKYDFWSGYPPGEE YFQDWGQGLVIVSS GGGSGGGSGGGSGGSS ASELTQDPAVSVALKQTVTITCRGDSLRSHYASWYQKKPGQAPVLLFYGKNNRPSGIPDR FSGSASGNRASLTITGAQAEDEADYYCSSRDKSGSRLSVFGGGTKLTVL GGLEVLFGGPHHHHHHHH
8ANC195 L1 V _H -V _L	QIHLVQSGTEVKKPGSSVTVSCKAYGVNTFGLYAVNWVRQAPGQSLEYIGQIWRWKSSA SHHFRGRVLISAVDLTGSSPPISSLEIKNLTSDDTAVYFCTTTSTYDKWSGLHHDGVMAFSS WGQGLISVSA GGGSGGGSGGGSGGSS DIQMTQSPSTLSASIGDTRISCRASQITGNWVAWYQQRPGKAPRLLIYRGAALLGGVP SRFSGSAAGTDFTLTIGNLQAEDFGTFYCCQYDTYPGTFGQGTKEVEK GGLEVLFGGPHHHHHHHH

All scFv were cloned into the CMV/R plasmid

8.2 scFv protein purification curves

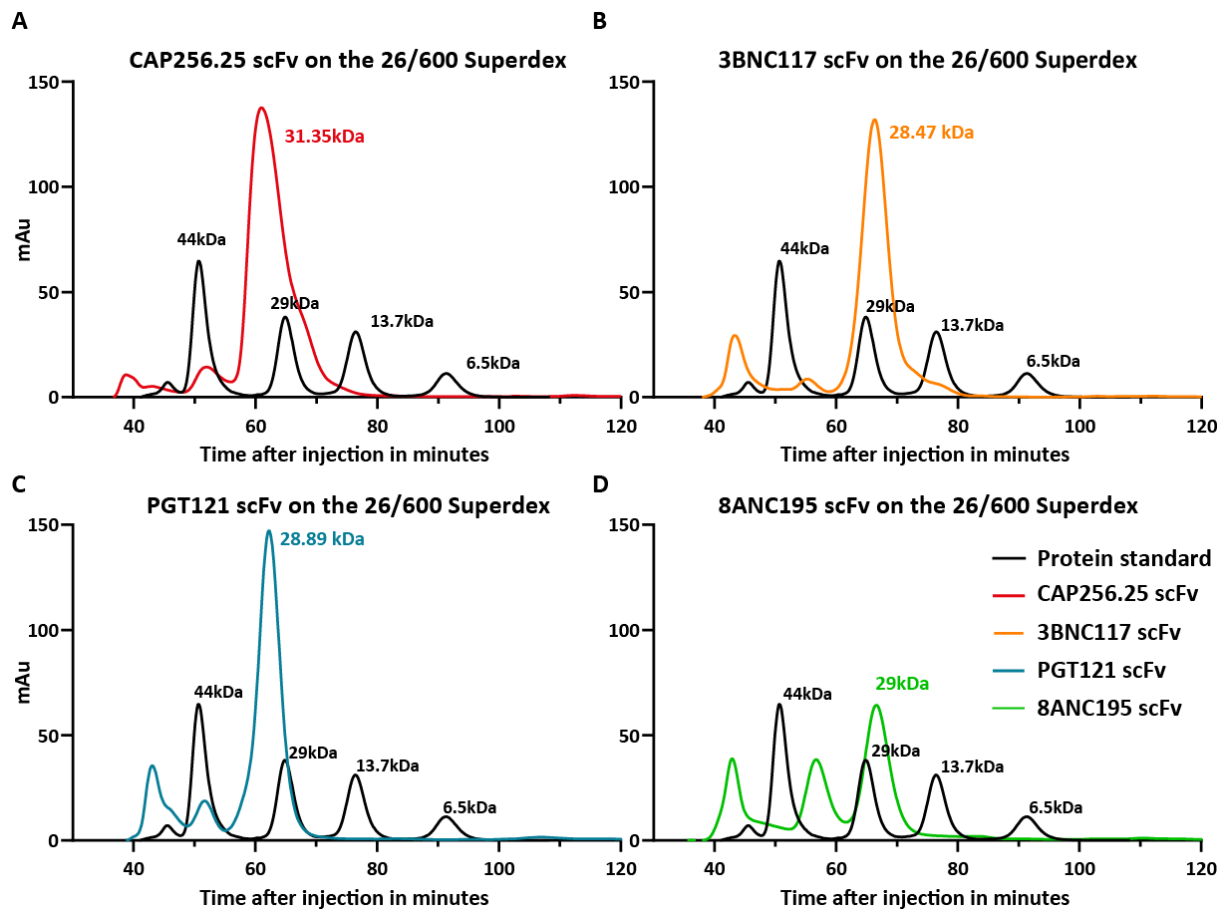
8.2.1 10E8v4 scFv on the 16/600 Superdex



Appendix 8.2.1: 10E8v4 scFv as measured and purified on the HiLoad 16/600 Superdex (200pg).

10E8v4 (purple) was run on the 16/600 HiLoad Superdex, which separates proteins up to 150kDa. The molecular weight marker consists of Thyroglobulin (670kDa), γ -globulin (158kDa), Ovalbumin (44kDa), Myoglobin (17kDa) and Vitamin B12 (1.35kDa). The fractions corresponding to 10E8v4 were collected and pooled.

8.2.2 scFv proteins on the HiLoad 26/600 Superdex



Appendix 8.2.2. scFv as measured and purified on the HiLoad 26/600 Superdex (200pg).

(A-D) ScFv of (A) CAP256.25 (pink), (B) 3BNC117 (orange), (C) PGT121 (blue) and (D) 8ANC195 (green) run on the 26/600 Hi Load Superdex size exclusion column. This column separates proteins up to 75kDa and is more accurate than the 16/600 for smaller proteins. A mixture of different sized proteins was used to determine the fraction profile of the size exclusion column (black) made of a mixture of Carbonic anhydrase (29kDa), Ribonuclease A (13.7kDa), Ovalbumin (43kDa) and Aprotinin (6.5kDa).

8.3 Primers used in this thesis

8.3.1 Primers for mutagenesis of viruses

R-CAP239 A236T	ACA TTA GTG CAT GGT CCC GTT CCA TTG AAT GTC TTA TTA TTA CAC TTT AGA
F-CAP239 A236T	TCT AAA GTG TAA TAA TAA GAC ATT CAA TGG AAC GGG ACC ATG CAC TAA TGT
R-CAP239 L278T	AGC CTA GCA GAA GAG GAT ATA ATA ATT AGA TCT CAA AAT ATA ACG GAC AAT ACC AAG ACA ATA ATA G
F-CAP239 L278T	CTA TTA TTG TCT TGG TAT TGT CCG TTA TAT TTT GAG ATC TAA TTA TTA TAT CCT CTT CTG CTA GGC T
F-Q842 I236T	TCT TGC ATG GCC CCG TTC CAT TGA ACT CCT CAT CCT TAC A
R-Q842 I236T	TGT AAG GAT GAG GAG TTC AAT GGA ACG GGG CCA TGC AAG A
R-WITO K236T	GAC ATT TCT ACA TGG TCC CGT TCC ATT GAA TGT CTT ATT ATT ACA CTT
F-WITO K236T	AAG TGT AAT AAT AAG ACA TTC AAT GGA ACG GGA CCA TGT AGA AAT GTC
R-ZM197 D332N	ATT ACT TTT ACT AAG GTT ACA ATG TGC CTG TCT TAT GTC TCC TAT T
F-ZM197 D332N	ATT ACT TTT ACT AAG GTT ACA ATG TGC CTG TCT TAT GTC TCC TAT T
R-Q168 T332N N334S	TAT TCC ATT CTG ATC CAC TGA CAT TAC AAT ATG CTT GTC TTA TAT CCC CTA T
F-Q168 T332N N334S	ATA GGG GAT ATA AGA CAA GCA TAT TGT AAT GTC AGT GGA TCA GAA TGG AAT A
R-CAP256 SU K170Q	AGT GCA TAT TCT TTC TGT TTC TTA TCT CTT AAT TCT GTG GTC GCA TT
F-CAP256SU K170Q	AAT GCG ACC ACA GAA TTA AGA GAT AAG AAA CAG AAA GAA TAT GCA CT
R-ZM53 K170Q	GCA ACC ACA GAA CTA AGA GAT AAG AAA CAG CAA GTG TAT GCA
F-ZM53 K170Q	TGC ATA CAC TTG CTG TTT CTT ATC TCT TAG TTC TGT GGT TGC
R-Du156 Q170K	AGT GCA TAC ACT TTC TTT TTC TTA TCT CTT AGT TCT GTG GTT GTA TT
F-Du156 Q170K	AAT ACA ACC ACA GAA CTA AGA GAT AAG AAA AAG AAA GTG TAT GCA CT
F-Q23 R169K Q170K	TTC AAT ATG ACC ACA GAA CTA AGG GAT AAG AAA AAG AAA GTA TAT TCA CTT TTT TAT AGA C
R-Q23 R169K Q170K	GTC TAT AAA AAA GTG AAT ATA CTT TCT TTT TCT TAT CCC TTA GTT CTG TGG TCA TAT TGA A
F-Q23 Q170K	TGACCACAGAATAAGGGATAAGAGAAAGAAAGTATATTTCACTTTT
R-Q23 Q170K	AAAAGTGAATATACTTTCTTTCTTTATCCCTTAGTTCTGTGGTCA
F-Q23 R169K	TATGACCACAGAATAAGGGATAAGAAACAGAAAGTATATTTCACTTT
R-Q23 R169K	AAAGTGAATATACTTTCTGTTTCTTTATCCCTTAGTTCTGTGGTCATA
F-AC10 Q170K	CACCACAAACATGAGAGATAAGATGAAGAAACAATATGCACTTTTTTATAA
R-AC10 Q170K	TTATAAAAAAGTGCATATTGTTTCTTCATCTTATCTCTCATGTTTGTGGTG
F-AC10 M169K	CACCACAAACATGAGAGATAAGAAGCAGAAACAATATGCAC
R-AC10 M169K	GTGCATATTGTTTCTGCTTCTTATCTCTCATGTTTGTGGTG
R-AC10 M169K Q170K	GTT TAT AAA AAA GTG CAT ATT GTT TCT TCT TCT TAT CTC TCA TGT TTG TGG TGA TAT TGA A
F-AC10 M169K Q170K	TTC AAT ATC ACC ACA AAC ATG AGA GAT AAG AAG AAG AAA CAA TAT GCA CTT TTT TAT AAA C

8.3.2 Primers for scFv cloning

CAP256.25 scFv cloning

F1 CAP256.25 scFv	GGTGGT ACCGGTGTACATTCC CAG GTG CAG TTG GTG GAG TCT GGG GGA
R1 CAP256.25 scFv	ATG GTG GGG TCC CTG GAA CAG CAC TTC CAG CCC TCC TAG GAC GAT GAC CTT GGT CCC AGT TCC GAA
R2 CAP256.25 scFv	ACC ACC GGA TCC TCA ATG GTG ATG GTG ATG GTG ATG GTG GGG TCC CTG GAA CAG CAC
Linker changes and VL-VH reverse for CAP256.25	
F-linker1 CAP256.25_2VH	GGC TCA GGA GGC GGA GGC AGT CAGGTGCAGTTGGTGGAGTCTGGGGGA
CAP256 scFv R1_2VH	ATG GTG GGG TCC CTG GAA CAG CAC TTC CAG CCC TCC TGA AGA GAC GGT GAC CAT TGT CCC TTG
CAP256.25 scFv F1_2VL	GGTGGTACCGGTGTACAT TCC CAG TCT GTG CTG ACG CAG CCG
R-linker1 CAP256.25_2VL	GCC TCC GCT GCC TCC GCC TCC TAG GAC GAT GAC CTT GGT CCC AGT TCC GAA

PGT121 scFv cloning and linker changes, and VL-VH reverse

F-PGT121 VL	TCC GAC ATC TCG GTG GCT CCG GGA GAA
R-PGT121 VL	CAA GAC AGT CAA GGT AGT TCC ACC TCC GAA
F-PGT121 VH	CAG ATG CAG CTC CAA GAG TCG GGG
R-PGT121 VH	CGA CGA CAC CGT GAC TTG CGT TCC ATT
PGT121 scFv F1 (VH)	GGT GGT ACC GGT GTA CAT TCC CAG ATG CAG CTC CAA GAG TCG GGG
PGT121 scFv R1 (VL)	ATG GTG GGG TCC CTG GAA CAG CAC TTC CAG CCC TCC CAA GAC AGT CAA GGT AGT TCC ACC TCC GAA
F-linker PGT121(VL)	GGC TCA GGA GGC GGA GGC AGT TCC GAC ATC TCG GTG GCT CCG GGA GAA
R linker PGT121(VH)	GCC TCC GCT GCC TCC GCC TCC CGA CGA CAC CGT GAC TTG CGT TCC
PGT121 scFv R1 2VH	ATG GTG GGG TCC CTG GAA CAG CAC TTC CAG CCC TCC CGA CGA CAC CGT GAC TTG CGT TCC ATT
PGT121 scFv F1 2VL	GGTGGTACCGGTGTACAT TCC TCC GAC ATC TCG GTG GCT CCG GGA GAA
R-linker1 PGT121_2VL	GCC TCC GCT GCC TCC GCC TCC CAA GAC AGT CAA GGT AGT TCC ACC TCC GAA

Linker changes

Linker 2 mutagenesis	
F linker1 ins 15nt_after_465	GGAGGCGGAGGCTCAGGAGGCGGAGGCTCCGGAGGCGG
R linker1 ins 15nt_after_465	CCGCCTCCGGAGCCTCCGCCTCCTGAGCCTCCGCCTCC

Linker 1 cloning	
R2-linker (GGGS)3	ACT GCC TCC GCC TCC TGA GCC TCC GCC TCC GCT GCC TCC GCC TCC
F2-linker (GGGS)3	GGA GGC GGA GGC AGC GGA GGC GGA GGC TCA GGA GGC GGA GGC AGT
F3 (GGGS)3	GGA GGC GGA GGC AGC GGA GGC
R3 (GGGS)3	ACT GCC TCC GCC TCC TGA GCC

VCR01 cloning: (low expression levels), replaced with 3BNC117

F-VRC01 VL	GAA ATT GTG TTG ACA CAG TCT CCA GGC ACC
R-VRC01 VL	ACG TTT AAT GTC GAC CTG GAC CTT GGT CCC
F-VRC01 VH	CAG GTG CAG CTG GTG CAG TCT GGA
R-VRC01 VH	TGA TGA GAC GAT GAC CGG GGT GCC
VRC01 scFv F1 (VH)	GGT GGC ACC GGT GTA CAT TCC CAG GTG CAG CTG GTG CAG TCT GGA
VRC01 scFv R1 (VL)	ATG GTG GGG TCC CTG GAA CAG CAC TTC CAG CCC TCC ACG TTT AAT GTC GAC CTG GAC CTT GGT CCC
F-linker1.2 VRC01 (VL)	GGC TCA GGA GGC GGA GGC AGT GAA ATT GTG TTG ACA CAG TCT CCA GGC ACC
R linker1.2 VRC01 (VH)	GCC TCC GCT GCC TCC GCC TCC TGA TGA GAC GAT GAC CGG GGT GCC
VRC01 scFv R1 2VH	ATG GTG GGG TCC CTG GAA CAG CAC TTC CAG CCC TCC TGA TGA GAC GAT GAC CGG GGT GCC
VRC01 scFv F1 2VL	GGTGGCACC GG GTG TACAT TCC GAA ATT GTG TTG ACA CAG TCT CCA GGC ACC
R linker1 VRC01_2VL	GCC TCC GCT GCC TCC GCC TCC ACG TTT AAT GTC GAC CTG GAC CTT GGT CCC

10E8 cloning (replaced later with 10E8v4)

F-10E8 VH	GAG GTG CAG CTG GTG GAG TCT GGG
R-10E8 VH	TGA GGA GAC GGT GAC CAG GGT GCC
F-10E8 VL	TCC TAT GAG CTG ACT CAG GAG ACT GGT
10E8 scFv F1 (VH)	GGT GGT ACC GGT GTA CAT TCC GAG GTG CAG CTG GTG GAG TCT GGG
10E8 scFv R1 (VL)	ATG GTG GGG TCC CTG GAA CAG CAC TTC CAG CCC TCC GAG GAC GGT CAG TTT GGT CCC
10E8 scFv R2 (VL)	ACC ACC AGA TCT TCA ATG GTG ATG GTG ATG GTG ATG GTG GGG TCC CTG GAA CAG CAC
F-linker1.2 10E8(VL)	GGC TCA GGA GGC GGA GGC AGT TCC TAT GAG CTG ACT CAG GAG ACT GGT
R linker1.2 10E8(VH)	GCC TCC GCT GCC TCC GCC TCC TGA GGA GAC GGT GAC CAG GGT GCC
F-linker1 10E8_2VH	GGC TCA GGA GGC GGA GGC AGT GAG GTG CAG CTG GTG GAG TCT GGG
10E8 scFv R1 2VH	ATG GTG GGG TCC CTG GAA CAG CAC TTC CAG CCC TCC TGA GGA GAC GGT GAC CAG GGT GCC
10E8 scFv F1 2VL	GGTGGTACC GG GTG TACAT TCC TCC TAT GAG CTG ACT CAG GAG ACT GGT

*3BNC117 L1 V_H-V_L, 8ANC195 L1 V_H-V_L and 10E8v4 L1 V_H-V_L scFv were ordered from Genscript.

8.4 Papers submitted



Journal of
Virology®

VACCINES AND ANTIVIRAL AGENTS



Neutralization Breadth and Potency of Single-Chain Variable Fragments Derived from Broadly Neutralizing Antibodies Targeting Multiple Epitopes on the HIV-1 Envelope

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ABSTRACT Passive administration of HIV-directed broadly neutralizing antibodies (bNAbs) can prevent infection in animal models, and human efficacy trials are under way. Single-chain variable fragments (scFv), comprised of only the variable regions of antibody heavy and light chains, are smaller molecules that may offer advantages over full-length IgG. We designed and expressed scFv of HIV bNAbs prioritized for clinical testing that target the V2-apex (CAP256-VRC26.25), V3-glycan supersite (PGT121), CD4 binding site (3BNC117), and MPER (10E8v4). The use of either a 15- or 18-amino-acid glycine-serine linker between the heavy- and light-chain fragments provided adequate levels of scFv expression. When tested against a 45-multisubtype virus panel, all four scFv retained good neutralizing activity, although there was variable loss of function compared to the parental IgG antibodies. For CAP256-VRC26.25, there was a significant 138-fold loss of potency that was in part related to differential interaction with charged amino acids at positions 169 and 170 in the V2 epitope. Potency was reduced for the 3BNC117 (13-fold) and PGT121 (4-fold) scFv among viruses lacking the N276 and N332 glycans, respectively, and in viruses with a longer V1 loop for PGT121. This suggested that scFv interacted with their epitopes in subtly different ways, with variation at key residues affecting scFv neutralization more than the matched IgGs. Remarkably, the scFv of 10E8v4 maintained breadth of 100% with only a minor reduction in potency. Overall, scFv of clinically relevant bNAbs had significant neutralizing activity, indicating that they are suitable for passive immunization to prevent HIV-1 infection.

IMPORTANCE Monoclonal antibodies have been isolated against conserved epitopes on the HIV trimer and are being investigated for passive immunization. Some of the challenges associated with full-sized antibody proteins may be overcome by using single-chain variable fragments (scFv). These smaller forms of antibodies can be produced more efficiently, may show fewer off-target effects with increased tissue penetration, and are more adaptable to vectored-mediated expression than IgG. Here, we demonstrate that scFv of four HIV-directed bNAbs (CAP256-VRC26.25, PGT121, 3BNC117, and 10E8v4) had significant neutralizing activity against diverse global strains of HIV. Loss of potency and/or breadth was shown to be due to increased dependence of the scFv on key residues within the epitope. These smaller antibody molecules with functional activity in the therapeutic range may be suitable for further development as passive immunity for HIV prevention.

Citation van Dorsten RT, Lambson BE, Wibmer CK, Weinberg MS, Moore PL, Morris L. 2020. Neutralization breadth and potency of single-chain variable fragments derived from broadly neutralizing antibodies targeting multiple epitopes on the HIV-1 envelope. *J Virol* 94:e01533-19 <https://doi.org/10.1128/JVI.01533-19>

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Broadly neutralizing antibodies (bNAbs) block infection of diverse HIV strains by targeting relatively conserved epitopes on the HIV envelope trimer (1–3). An HIV vaccine will likely need to elicit these types of antibodies, but this has not yet been achieved. However, some HIV-infected individuals naturally generate bNAb responses, enabling the isolation of monoclonal antibodies with high potency and exceptional breadth and creating the possibility of passive immunization for HIV prevention (4, 5). Animal studies support this approach, with bNAbs such as CAP256-VRC26.25, PGT121, 3BNC117, 10-1074, PGDM1400, VRC01, and VRC07 shown to prevent simian-human immunodeficiency virus infection in nonhuman primate models (6–9). In addition, passively infused bNAbs 3BNC117, 10-1074, and VRC01 substantially reduce viral levels in viremic individuals, confirming their neutralization capacity *in vivo* (10–15). This has led to the antibody-mediated prevention (AMP) trial, a proof-of-concept study in Africa, Switzerland, and the Americas that is under way to assess whether the bNAb VRC01 can prevent HIV infection in humans (<https://ampstudy.org.za/>).

The promise of passive immunization opens up possibilities for using bNAbs in other formats. Small antibody fragments such as single-chain variable fragments (scFv) may offer advantages over IgG (16–18). scFv consist of the antibody variable heavy and variable light chains and are substantially smaller than IgG molecules (30 kDa versus approximately 147 kDa) (19). This may enhance some of their pharmacokinetic properties and improve their distribution throughout the body and absorption into various tissues. In the context of HIV, the smaller size may enhance diffusion into genital tract mucosal tissues, where most HIV infections occur (5, 16, 20–22). Moreover, as scFv do not have an Fc region, they are less likely to display nonspecific cytotoxicity through cytokine release, although this reduces their half-life significantly and removes their ability to perform Fc-mediated effector functions (16, 22). Antibody scFv have been successfully used in clinical settings particularly for cancer therapy, where their enhanced tissue penetration aids in tumor targeting, and are also being developed for neurological diseases, such as Parkinson's disease, although some anti-drug antibody responses were noted (16, 23, 24).

Antibody scFv targeting the HIV envelope have previously been isolated through phage display, but their neutralization capacity was limited (25, 26). Initial attempts to engineer scFv, immunoadhesins, or other antibody fragments from bNAbs VRC01, PG9, and PG16 showed poor expression and loss of function compared to the IgG (18). The availability of a larger number of broad and potent bNAbs has revived interest in this area. We therefore made scFvs of bNAbs that target 4 distinct epitopes on the HIV envelope trimer, focusing on those undergoing human clinical testing. These included CAP256-VRC26.25 (which binds the V2 apex and is referred to here as CAP256.25) (28), PGT121 (V3-glycan supersite) (29), 3BNC117 (CD4 binding site [CD4bs]) (30), and 10E8v4 (MPER) (31). We found that a reduction in neutralization breadth and/or potency of the scFv compared to the IgG was due to differences in dependence on specific residues in the epitopes. However, overall, scFv largely retained breadth and potency against diverse global HIV isolates and may prove to be useful for future clinical development to prevent HIV acquisition.

RESULTS

Construction and optimization of scFv derived from HIV bNAbs. We designed scFv of bNAbs that target four major epitopes on the HIV-1 envelope (CAP256.25, PGT121, 3BNC117, and 10E8v4) to determine if they retain neutralization breadth and potency. These scFv consisted of the variable heavy chain and the variable light chain of the parent IgG connected by a glycine-serine linker with a histidine tag for purification (amino acid sequences of each construct used to generate scFv are shown in Table 1). As linker length can be a major determinant of paratope formation, we tested

TABLE 1 Amino acid sequences of four scFv constructs derived from HIV bNAbs ^a

scFv construct	Amino acid sequences
CAP256.25 L3 V _H -V _L	QVQLVESGGGVVQPGLTSLRLSCAASQFRFDGYGMHWVRQAPGKGLEWVASISHDGIKKY HAELVWGRFTISRDNKNTLYLQMNSLRPEDTALYYCAKDLREDECEEWSDYYDFGKQ LPCAASRGGLVGIADNWQGMVTVSS GGSSRSSSSGGGGSGGGG QSVLTQPPSVSAAPGQKVTISCSGNTSNIGNNFVSWYQQRPGRAPQLLIYETDKRPSGI PDRFSASKSGTSGTLAITGLQTGDEADYYCATWAASLSSARVFGTGKTVIVL GGLEVLFGQPHHHHHHHH
PGT121 L1 V _H -V _L	QMQLQESGPGVLKPSSETLSLTCSVSGASISDSYWSWIRRSPPGKGLEWIGYVHKSGDNTY SPSLKSRVNLSDTSKNQVSLSLVAATAADSGKYYCARTLHGRRIYGIVAFNEWFTYFY MDVWNGNTQVTVSS GGGGSGGGSGGGSGGGG DISVAPGETARISCGEKSLSRAVQWYQHRAGQAPSLIIYNNQDRPSGIPERFSGSPDS PFGTTATLTITSVEAGDEADYYCHIWDSDRVPTKWVFGGGTTLTVL GGLEVLFGQPHHHHHHHH
3BNC117 L1 V _H -V _L	QVQLLQSGAAVTKPGASVRVSCASGYNIRDYFIHWWVRQAPGQGLQWVGWINPKTGQPN NPRQFQGRVSLTRHASWDFDTFSFYMDLKLRSDDTAVYFCARQSDYWDFDVWGSSTQ VTVSS GGGGSGGGSGGGSGGGG DIQMTQSPSSLSASVGDVTITTCQANGYLNWYQRRGKAPKLLIYDGSKLERGVPSRFS GRRWQGEYNLTINNLPEDIAFYFCQVYEFVVPGLRLDLK GGLEVLFGQPHHHHHHHH
10E8v4 L1 V _H -V _L	EVRLVESGGGLVKGPSLRLSCSASGDFDFAWMWVRQPPGKGLEWVGRIITGPGEGWS VDYAESVKGRTISRDNKNTLYLEMNNVTEDTGYFCARTGKYDFWGSYPPGEEYF QDWGGTGLIVVSS GGGGSGGGSGGGSGGGG ASELTQDPAVSVALKQTVTITCRGDSLRSYASWYQKPKGQAPVLLFYGKNNRPSGIPD RFSGSASGNRASLTITGAQAEDEADYYCSSRDKSGSRLSVFGGGTKLTVL GGLEVLFGQPHHHHHHHH

^aThe variable heavy chain (light blue) and variable light chain (yellow) are indicated, as well as the glycine-serine linker (purple) and the 8× histidine tag (red), which is preceded by an HRV3C site (italicized) and is separated from the light chain by a small GG spacer.

linkers of three different lengths. Heavy (V_H)- and light (V_L)-chain orientation was also tested for one of the linkers. CAP256.25 and PGT121 were used to first assess expression and function of these different constructs (Fig. 1A). For CAP256.25 scFv, the 18-amino-acid linker (termed L3) in both V_H-V_L and V_L-V_H orientations had the highest expression. The 15-amino-acid (GGGG)₃ linker (L1) showed lower expression levels, and the 20-amino-acid (GGGG)₄ linker (L2) was poorly expressed. For PGT121, the highest expression was observed for L1 in the V_H-V_L orientation. We next tested whether the different linkers affected neutralization breadth and potency using panels of sensitive viruses tailored to each bNAb. Since scFv are roughly 5 times smaller than IgG, the 50% inhibitory concentration (IC₅₀) values were multiplied by the molecular weight fold difference between each scFv and its matched IgG (values shown in Fig. 1A and explained in Materials and Methods).

For CAP256.25, all three linkers in the V_H-V_L orientation showed similar neutralizing activity against a panel of 36 viruses, while the V_L-V_H L3 (purple) had a 2-fold lower geometric mean potency (Fig. 1B). We therefore used CAP256.25 scFv V_H-V_L L3, which showed the highest expression levels, to conduct further experiments. Similarly, no significant differences were observed for the different PGT121 scFv constructs against an 18-virus panel (Fig. 1A), and PGT121 V_H-V_L L1, which showed the highest expression levels, was used. Based on this, 3BNC117 and 10E8v4 scFv were expressed in the V_H-V_L conformation with the L1 linker. Both expressed relatively well and showed good neutralizing activity (Fig. 1A).

HIV bNAb scFv retain good neutralization breadth and potency. All four scFv were tested for neutralizing activity against a larger multiclade panel of 45 viruses for breadth and potency. Compared to their parental IgG proteins, all scFv retained substantial neutralizing activity, although they showed some reduction in breadth

A

	Construct		Molecular Weight	Molecular weight fold difference for IgG vs. scFv	Expression	Geometric Mean IC ₅₀ in µg/mL*	% viruses neutralized*
CAP256.25 V2-Apex	L1	V _H -V _L	30.96 kDa	4.86	1-2 mg/L	1.9	67%
	L2	V _H -V _L	31.27 kDa	4.82	<1 mg/L	1.4	67%
	L3	V _H -V _L	31.35 kDa	4.81	>2 mg/L	1.9	67%
	L3	V _L -V _H	31.35 kDa	4.81	>2 mg/L	3.8	53%
PGT121 V3 Glycan	L1	V _H -V _L	28.89 kDa	5.06	>2 mg/L	11.6	56%
	L2	V _H -V _L	29.20 kDa	5.00	< 1 mg/L	18.0	50%
	L3	V _H -V _L	29.28 kDa	4.99	1-2 mg/L	18.0	50%
	L3	V _L -V _H	29.28 kDa	4.99	1-2 mg/L	11.5	56%
3BNC117 CD4bs	L1	V _H -V _L	28.47 kDa	5.14	1-2 mg/L	2.3	82%
10E8v4 MPER	L1	V _H -V _L	29.30 kDa	5.03	> 2 mg/L	1.2	100%

*Geometric mean adjusted for molecular weight difference with IgG

*Smaller panel of viruses tested for CAP256.25 and PGT121

B

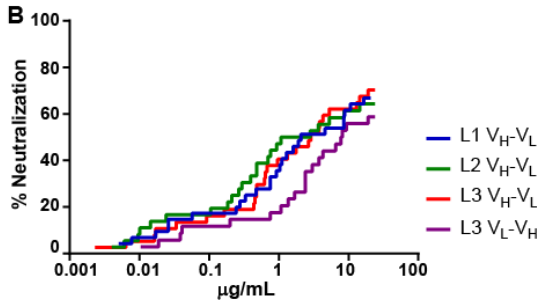


FIG 1 Design, expression, and testing of scFv constructs of HIV bNAbs. (A) A total of 10 scFv constructs were made, with different glycine-serine linkers for CAP256.25 and PGT121 and L1 (GGGGG)₃, L2 (GGGGG)₄, and L3 (GGSSRSSSSGGGGGGGG) for V_H-V_L and L3 for V_L-V_H. For 3BNC117 and 10E8v4, only L1 V_H-V_L was made. The molecular weights and protein expression levels for each scFv are shown together with the adjusted geometric mean neutralization potency. A selected panel of viruses was used to assess CAP256.25 scFv (34 viruses) and PGT121 scFv (18 viruses), whereas the full 45-virus panel is shown for 3BNC117 and 10E8v4. (B) Breadth-potency curves of CAP256.25 scFv made with 3 different linkers in the V_H-V_L orientation and for L3 in the V_L-V_H orientation. (scFv is corrected for molecular weight size difference by multiplying the IC₅₀ by the fold difference in molecular weight between the IgG and the scFv, a value of ~5×).

and/or potency. CAP256.25 scFv maintained breadth of 64% equivalent to the full IgG, but there was a significant ($P < 0.0001$) 138-fold decrease in geometric mean potency compared to that of IgG (from 0.0071 to 1 µg/ml) (Fig. 2A). While all viruses in the panel were less potently neutralized by CAP256.25 scFv, some viruses were more severely affected, with >1,000-fold loss of potency for Q23.17, Q842.D12, and AC10.0.29 (Fig. 3). The scFv of 3BNC117 and PGT121 showed an overall loss in both potency and breadth of neutralization. For 3BNC117, the scFv was 13-fold less potent than the IgG ($P < 0.0001$) and had slightly lower breadth of 82% compared to 91% for the IgG (Fig. 2B). PGT121 scFv had 4-fold reduced potency ($P < 0.0001$) and breadth of 73% compared to 89% for the IgG (Fig. 2C). This was a result of the scFv losing activity against viruses such as REJO.67 and QH0692.42 (Fig. 3). However, even in these cases, the scFv showed low levels of maximum neutralization (>20% but <50%) at the highest concentration tested, indicating that the scFv still bound to resistant viruses (highlighted in Fig. 3 with an asterisk). Despite the significant potency losses for some viruses, there were a number of viruses that were neutralized equally well by the scFv

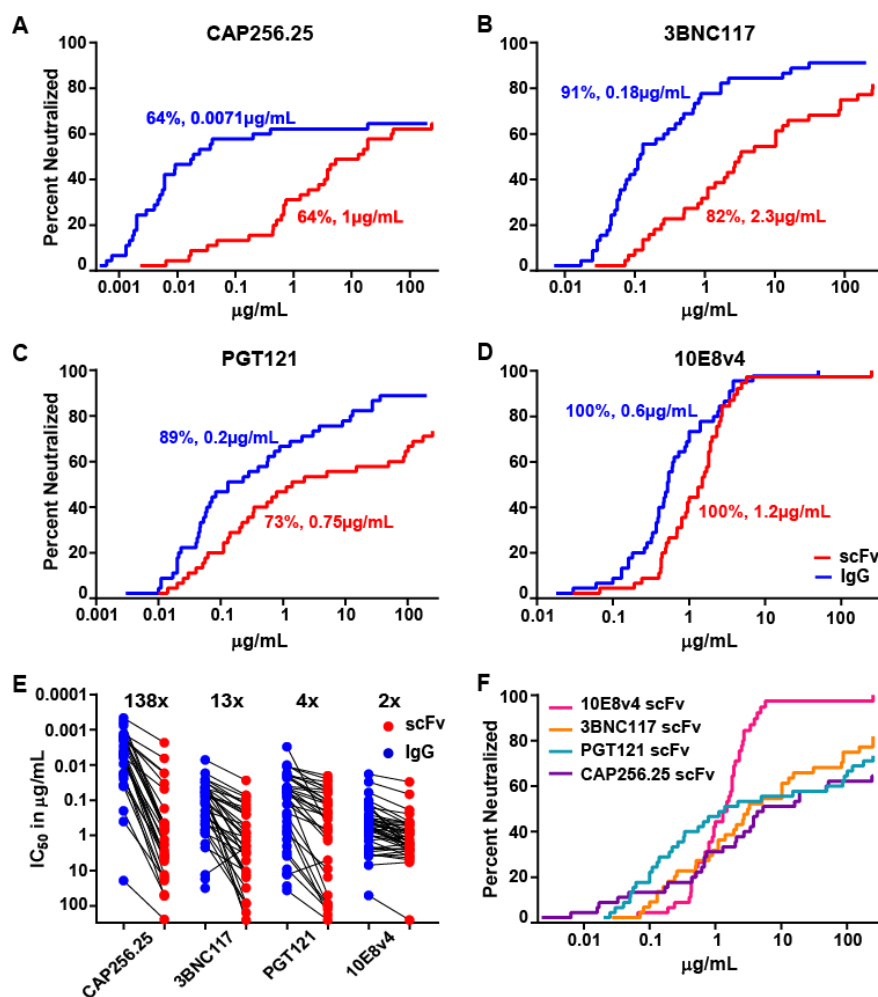


FIG 2 Neutralization breadth and potency of scFv compared to those of IgG for 4 HIV bNAbs. (A to D) Breadth-potency curves of scFv (red) and IgG (blue) of CAP256.25 (A), 3BNC117 (B), PGT121 (C), and 10E8v4 (D) using data from a 45-virus multisubtype panel tested in the TZM-bl neutralization assay. The IC_{50} of viruses against the percentage of viruses neutralized is given. Percent neutralization and geometric mean IC_{50} potency, in micrograms per milliliter (corrected for molecular weight size difference by multiplying their IC_{50} by the fold difference in molecular weight between IgG and scFv, $\sim 5\times$), are shown in each plot. (E) Potency losses of scFv (red) versus IgG (blue) against the same panel. Viruses resistant to both IgG and scFv were excluded. (F) Comparison of breadth-potency curves for all 4 scFv bNAbs against the 45-virus panel.

and by the IgG of PGT121 and 3BNC117, a phenomenon that was not seen with CAP256.25 scFv (Fig. 3).

The MPER antibody 10E8v4, when expressed as an scFv, showed no loss of function. It maintained 100% breadth of the panel with a minor 2-fold reduction in geometric mean potency compared to the IgG version (Fig. 2D). A significant reduction in potency was noted for only one virus, TRO.11, although it was still neutralized (Fig. 3).

Comparison of the potency of all four scFv relative to their IgG clearly demonstrates the significant loss of function for CAP256.25 scFv (Fig. 2E). Nonetheless, the breadth-potency curve of CAP256.25 scFv still overlapped that of the other scFv (Fig. 2F), a consequence of the exceptional potency of the parental CAP256.25 IgG. The superior neutralization breadth of the 10E8v4 scFv was also evident in these breadth-potency curves. Overall, the median IC_{50} for all 4 scFv were in the range of 1 to 2 $\mu\text{g/mL}$, which

IC ₅₀ of IgG and scFv in µg/mL													
		CAP256.25				3BNC117				PGT121			
		IgG	scFv	Fold		IgG	scFv	Fold		IgG	scFv	Fold	
Subtype A	Q23.17	0.0017	2.1	1210		0.046	0.10	2		0.011	0.020	2	2.5
	Q168.A2	0.00074	0.62	844		0.076	0.19	2		36	250	7	0.93
	Q259.D2.17	0.0046	3.4	735		0.046	250	5482		13	5	0.4	6.9
	Q461.E2	0.024	4.3	183		0.044	0.26	6		>200	>250	~	2.1
	Q769.D22	>200	>250	~		0.017	0.072	4		>200	>250	~	3.8
	Q842.D12	0.019	19	1007		0.0071	0.027	4		0.083	1.39	17	4.1
	BG505.W6M 332N	0.0013	0.68	522		0.025	0.081	3		0.044	0.062	1	0.40
Subtype B	CAAN5342.A2	>200	>250	~		0.88	3.3	4		0.042	0.34	8	3.4
	AC10.0.29	0.017	52	3003		17	>250	>14.7		0.072	0.20	3	0.39
	RHPA4259.7	>200	>250	~		0.025	0.13	5		0.046	0.11	2	0.60
	TRO.11	>200	>250	~		0.029	0.24	8		0.067	0.33	5	0.16
	PVO.4	0.037	2.7	71		0.072	0.16	2		0.23	0.66	3	3.8
	SC422661.8	>200	>250	~		0.062	0.51	8		0.28	15	56	0.49
	REJO.67	>200	>250	~		0.04	1.4	33		1.3	>250*	>132	0.61
	TRJO4551.18	>200	>250	~		0.11	13	121		9.0	121	13	2.9
	WITO.33	>200	>250	~		0.032	0.13	4		0.73	>250*	>342	0.32
	THRO4156.18	19	240	13		2.2	10.3	5		>200	>250	~	0.46
	QH0692.42	>200	>250	~		0.2	0.5	2		0.56	>250	>444	0.52
	6535.3	>200	>250	~		0.71	30.8	44		0.020	0.014	1	0.18
Subtype C	Du151.2	0.0020	0.17	83		>200*	154	1		0.011	0.025	2	0.37
	Du156.12	0.0040	0.190	48		0.056	2.7	48		0.021	0.14	7	0.03
	Du172.17	>200	>250	~		0.68	>250	>367		0.057	49.4	865	0.10
	Du422.1	0.0092	1.3	145		>200	>250*	~		0.047	1.13	24	0.47
	ZM53M.PB12	0.00046	0.0023	5		0.38	10.3	27		0.0030	0.55	183	3.4
	ZM109F.PB4	0.0060	5.3	880		0.13	247	1962		12	>250*	>20	0.13
	ZM135M.PL10A	>200	>250	~		0.06	0.26	4		2.0	>250*	>125	0.13
	ZM197M.PB7	0.0060	0.048	8		0.45	2.6	6		>200	>250	~	0.06
	ZM214M.PL15	0.0060	3.8	640		0.12	0.9	7		0.06	0.22	4	0.54
	ZM233M.PB6	0.0020	0.017	9		0.30	1.9	6		3.0	86	29	0.16
	ZM249M.PL1	0.0050	2	442		0.10	1.1	11		0.88	2.2	2	0.37
	CAP8.6F	0.20	48	240		31	>250*	>8		0.017	0.056	3	0.88
	CAP45.2.00.G3	0.00060	0.51	851		1.7	>250	>147		0.57	177	310	0.33
	CAP61.4.22.F10A	0.040	19	480		0.11	15.4	144		0.053	0.13	2	2.4
	CAP63.A9	0.0090	0.74	82		0.26	3.1	12		0.13	0.78	6	0.52
	CAP84.32	0.40	16	40		0.085	82	966		0.010	0.010	1	1.0
	CAP85.9	>200	>250	~		1.7	87	51		0.13	0.27	2	0.40
	CAP88.B5	>200	>250	~		0.82	87	107		0.40	91	228	0.018
	CAP206.8	0.0020	0.68	340		>200	>250*	~		3.8	101	27	0.26
	CAP210.2.00.E8	0.0018	0.016	9		13	2.1	0.2		27	>250*	>9	1.0
	CAP228.2.00.51J	0.0055	3.8	686		0.056	>250*	>4400		27	>250	>9	50
	CAP239.2.00.G3J	0.0029	0.44	149		>200*	>250*	~		0.023	0.11	5	0.54
	CAP244.D3	>200	>250	~		0.13	10.3	78		>200	>250	~	0.28
	CAP255.2.00.16J	>200	>250	~		0.029	5.1	179		0.040	0.051	1	0.73
	CAP256.SU	0.0013	0.0064	5		0.50	1.1	2		0.020	0.040	2	1.4
	ConC	0.0015	0.033	22		0.052	6	121		0.020	0.030	2	1.4
Geometric mean (µg/mL)		0.0071	1.0	138		0.18	2.3	13		0.20	0.75	3.7	0.62
Breadth		64.4%	64.4%			91.1%	82.2%			88.9%	73.3%		100.0%

FIG 3 Neutralization data for IgG and scFv of HIV bNABs tested against the 45-virus panel. The IC₅₀ values for both IgG and scFv against 7 subtype A, 12 subtype B, and 26 subtype C viruses are shown in micrograms per milliliter. IgG was tested at a starting concentration of 50 µg/ml, whereas scFv started at 250 µg/ml. Fold changes between IgG and scFv are shown. The colors indicate the potency at which the viruses were neutralized, with the warmer colors indicating higher potency. Resistant viruses are colored in light blue. Maximum levels of neutralization for scFv and IgG of <50% but >20% are indicated with an asterisk. Geometric mean titers, fold difference from IgG, and breadth against the 45-virus panel are shown at the bottom of the table. (scFv titers were corrected for molecular weight size difference by multiplying their IC₅₀ by the fold difference in molecular weight between the IgG and the scFv, as noted in Fig. 1A).

is close to the therapeutic range for VRC01 that is currently undergoing human efficacy testing.

Loss of potency in CAP256.25 scFv linked to epitope charge. In order to probe the loss of potency for CAP256.25 scFv, we tested the effect of point mutations in the V2 epitope of the autologous virus CAP256_SU. Mutations L165V and K171N had no effect on either the scFv or IgG (Fig. 4A). Similarly, a conservative K169R mutation did not impact the neutralizing activity of either protein. The T162I mutation (which removes the conserved glycan at position 160) and the K170Q mutations had a small effect on the scFv (3- and 4-fold, respectively) but not on the IgG (below 3-fold).

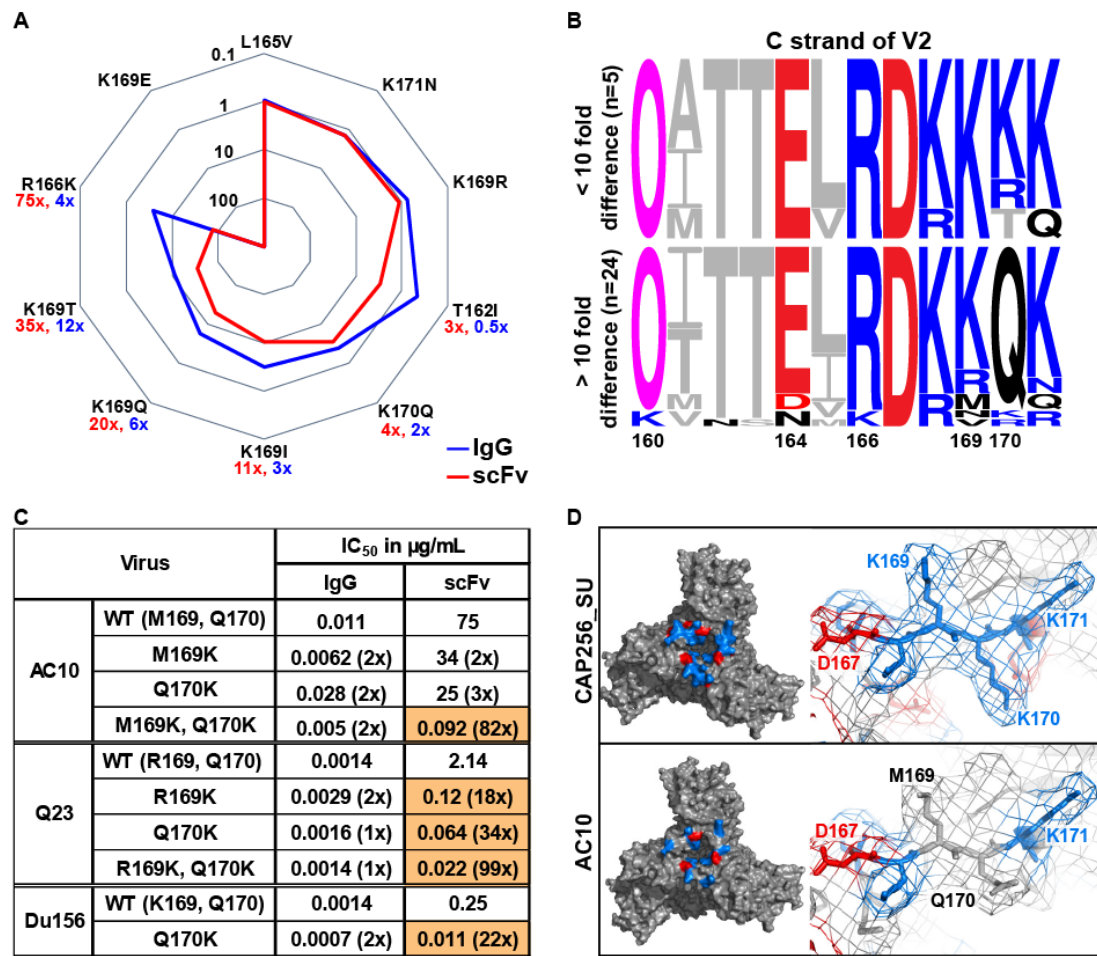


FIG 4 Charged amino acids in V2 reduced CAP256.25 scFv neutralization. (A) Spiderplot showing fold change in neutralization of V2 mutants relative to CAP256_SU wild type (WT) by the CAP256.25 IgG (blue) and the scFv (red). A fold change of 1 indicates no difference from the WT virus. Values closer to the center indicate resistance, whereas those on the outer bounds indicate a sensitivity. (B) Amino acid sequence alignment of viruses neutralized by scFv and IgG with similar potency (top) or more than 10-fold decrease in potency (bottom). The N160 glycan is denoted by an O and is colored magenta. Negatively and positively charged residues are indicated by red and blue, respectively. (C) IgG and scFv neutralization (IC₅₀) of three viruses with point mutations at positions 169 and 170. Fold changes relative to the wild type are indicated in parentheses, and those greater than 5-fold are colored orange. scFv is corrected for the molecular weight size difference (multiplied ~5×). (D) Structural representation of the V2 apex of the HIV envelope trimer of a CAP256.25 scFv-sensitive virus (CAP256_SU) and a less sensitive virus (AC10.0.29). The apex of the trimer is shown on the left, and a close-up of positions 167 to 171 is shown on the right. Positive charges are colored blue, whereas negative charges are colored red. (Model is based on 5V8L structure of BG505 SOSIP.664 [78] and was modified using PyMOL software.)

However, the scFv was more affected by the K169I substitution (11-fold for the scFv versus 3-fold for the IgG), the K169Q (20- versus 6-fold), the K169T (35- versus 12-fold), and the R166K (75- versus 4-fold) mutations. For both scFv and IgG, a K169E mutation resulted in complete neutralization resistance. Therefore, while neutralization by the scFv and IgG were overall similarly influenced by mutations in the epitope, the consequence of individual mutations was more pronounced for the scFv.

To further explore this, we partitioned CAP256.25-sensitive viruses into those with a greater than 10-fold potency loss for the scFv compared to that of the IgG (24 viruses) versus those with less than a 10-fold change (5 viruses) (Fig. 4B; glycans, N(X)T/S, are indicated by a pink O in the logograms). Differences at residue 160 or 166 between these two groups did not explain the general loss of potency by the scFv. Some variation was noted at position 169, with 169M (2/24), 169N (1/24), 169V (1/24), and

169R (3/24) found in viruses where the scFv lost potency. However, in 21/24 viruses with a large potency loss, an uncharged glutamine (Q) was present at position 170 rather than a charged lysine (K) or arginine (R). We therefore introduced Q170K into three viruses (AC10.0.29, Q23.17, and Du156.12) along with 169K in AC10.0.29 (M169K) and Q23.17 (R169K). Individual 169K and 170K mutations significantly improved neutralization by the scFv in both Du156.12 and Q23.17 (but not AC10.0.29) but had no significant effect on the IgG (Fig. 4C). However, the combination of these mutations significantly improved scFv neutralization in both Q23.17 (70-fold) and AC10.0.29 (86-fold) compared to a minimal effect (2- and 3-fold, respectively) for the IgG. These data suggested that CAP256.25 scFv neutralization was more dependent on the charge of the epitope than the matched IgG.

To visualize the effect of charge in the V2 apex of different envelopes, models of the HIV Env of CAP256_SU and AC10.0.29 were generated (Fig. 4D). CAP256_SU, which is similarly neutralized by both scFv and IgG, has charged lysine residues at positions 169 and 170 (Fig. 4D). These 169K and 170K residues formed a cluster of positive charges at the apex of the trimer. In contrast, AC10 displayed a reduced charge in the epitope, with neutral amino acids occupying these positions (M169 and Q170), possibly explaining the observed virus-specific loss of potency.

PGT121 scFv neutralization affected by both the N332 glycan and V1 length.

The glycan at position N332 is a key part of the epitope of V3-directed bNAbs, such as PGT121 (29, 32). To determine the dependence of the scFv on the N332 glycan, we deleted this glycan in three viruses (Q23.17, Du156.12, and TRO.11) through an N332A mutation. There was a significant loss of scFv potency for all 3 viruses, while for the IgG, this effect was seen in 2 of the 3 viruses (Fig. 5A, left). We also introduced this glycan into resistant viruses Q168.A2, REJO.67, CAP45.2.00.G3, and ZM197M.PB7, which conferred neutralization sensitivity to both the scFv and the IgG of PGT121 (Fig. 5A, right), although not always to the same level as the IgG.

We next analyzed the sequences of the 45 viruses tested, dividing them into 3 groups: viruses equally sensitive to the IgG and scFv (Fig. 5B, upper, 21 viruses), viruses with a 10-fold-reduced sensitivity to the scFv and/or IC_{50} of ≥ 200 μ g/ml for the scFv (Fig. 5B, middle, 19 viruses), and viruses resistant to both the IgG and the scFv (Fig. 5B, lower, 5 viruses). There was no difference in the GDIR motif between the groups. As expected, 21/21 viruses sensitive to PGT121 had a glycan at position N332 (indicated by a pink O in the logograms). In contrast, viruses resistant to both scFv and IgG lacked the N332 glycan, with 2 viruses showing a glycan shift to N334 and 3 lacking either glycan. In viruses where the scFv lost potency (Fig. 5B, middle), only 7 out of 19 had either a glycan shift to N334 or lacked a glycan at N332/N334. This suggested that while both the PGT121 IgG and scFv were dependent on the N332 glycan, the reduced potency and breadth of the scFv was only partly due to an increased dependence on this glycan.

V3-directed bNAbs are also affected by the length of the V1 region (33, 34), so we assessed whether this contributed to the loss of scFv neutralization. We used two related viruses isolated from the same individual, donor CAP177, that showed differential sensitivity to PGT121. The CAP177-4C virus contains a long V1 loop (dark pink) (Fig. 5C, upper), while CAP177-5D has a short V1 loop (green) (Fig. 5C, lower). V1 swaps between these 2 clones were made and tested for sensitivity to both scFv and IgG. Inserting a short V1 in CAP177-4C (Fig. 5C, upper) increased the potency of PGT121 IgG by 20-fold (from 0.94 μ g/ml to 0.047 μ g/ml) but had a substantially greater 91-fold effect on the scFv (from 6.7 μ g/ml to 0.074 μ g/ml). Similarly, there was a 4-fold loss in potency for the scFv when a long V1 was introduced into CAP177-5D (from 0.080 μ g/ml to 0.31 μ g/ml), with no effect on the IgG (Fig. 5C, lower). The length of V1, therefore, had a greater effect on the neutralization potency of the scFv than the IgG of PGT121.

Loss of potency for the 3BNC117 scFv more pronounced for subtype C viruses.

The N276 glycan is highly conserved and a major contact residue for CD4 binding site antibodies, including 3BNC117 (30, 35). To interrogate the loss of potency for the 3BNC117 scFv, we introduced this glycan into a resistant virus or removed it from three sensitive viruses (Fig. 6A). Insertion of the N276 glycan into CAP239.2.00.G3J (through

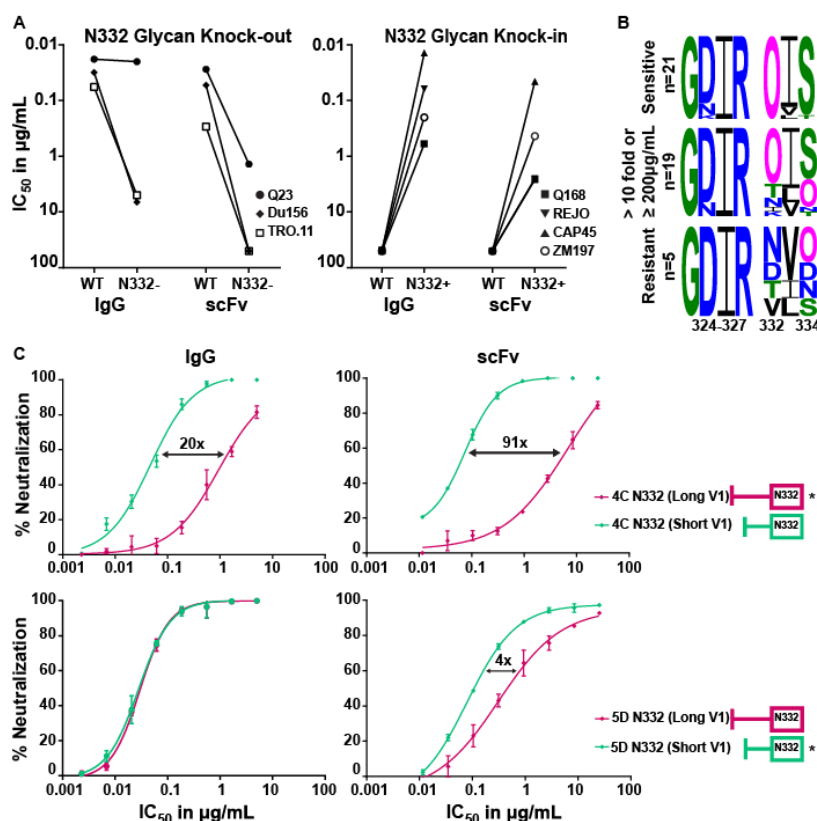


FIG 5 PGT121 scFv neutralization was dependent on the N332 glycan and the V1 loop length. (A) IC_{50} of IgG and scFv against 3 sensitive viruses (WT) with their N332 glycan knockouts (N332-) (left) and 4 resistant viruses (WT) with matched N332 glycan knockins (N332+) (right). (B) Noncontiguous amino acid sequences of the V3 region (positions 324 to 327 and 332 to 334, encompassing the GDIR motif and the N332/4 glycans). Viruses are divided into three groups: viruses neutralized equally well by IgG and scFv of PGT121 (top), those with >10-fold difference and/or $\geq 200 \mu\text{g/mL}$ for scFv (middle), and viruses resistant to both scFv and IgG (bottom). Glycans [N(X)T/S] are shown as a pink O. (C) Neutralization curves of IgG and scFv (in micrograms per milliliter) against two CAP177 clones, 4C, which has a long V1 (dark pink, star), and 5D, with a short V1 (green, star), and their V1 mutant swaps (no stars). Fold differences between the wild-type and mutant swaps are indicated. scFv is corrected for the molecular weight size difference (multiplied ~ 5 times).

an L278T mutation) rendered this resistant virus highly sensitive to IgG, although the effect on the scFv was marginal. However, removal of the N276 glycan in three viruses had a more significant effect on the scFv than the IgG, with the N276A mutation causing a 10-fold increase in resistance or a knockout effect (Fig. 6A). This indicated that the scFv was more dependent on the 276 glycan for neutralization than the IgG.

CD4bs antibodies have been shown to have some subtype preference, often exhibiting lower efficacy against subtype C viruses (36). To explore whether this also impacted the scFv, we compared the loss of potency, relative to that of IgG, for subtype A, B, and C viruses. There was a significant 9-fold difference in the IC_{50} between the scFv and IgG for subtype B viruses (Fig. 6B). However, there was a 28-fold difference for subtype C viruses, suggesting that the subtype-specific genetic differences have more of an impact on the scFv than the IgG. For subtype A, only one virus, Q259.D2.17, which lacked the N276 glycan (marked in green), showed more than a 10-fold difference (Fig. 6B). This glycan was also missing in the subtype C virus, CAP228.51, which was significantly less well neutralized by the scFv.

We next analyzed the viral amino acid sequences in the CD4 binding site of subtype C viruses to identify other residues that might contribute to the potency difference

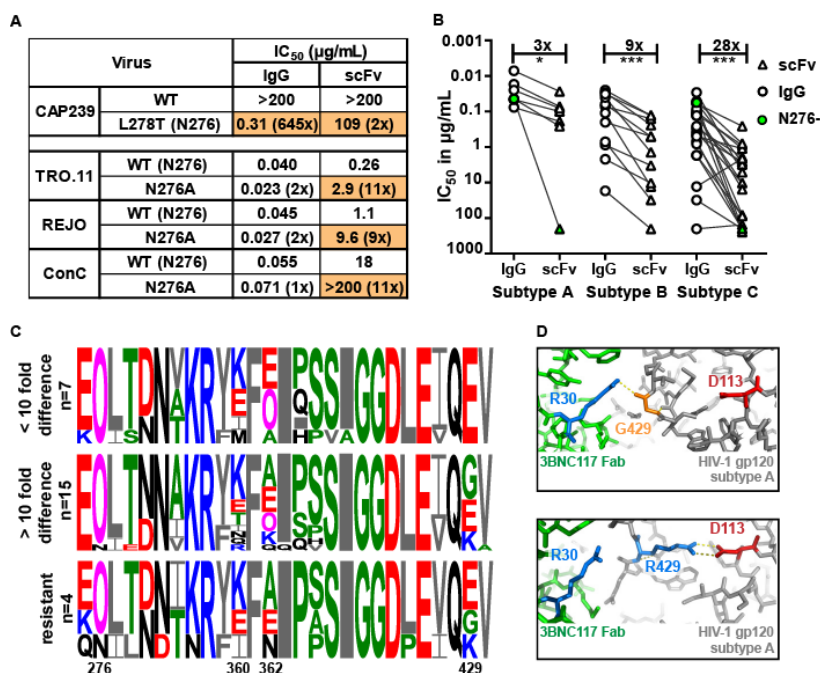


FIG 6 Loss of potency for the 3BNC117 scFv was more pronounced for subtype C viruses. (A) IC₅₀ of IgG and scFv neutralization of one virus that lacked the N276 glycan with a glycan knockin (CAP239) and 3 viruses in which the N276 glycan has been deleted (scFv corrected for molecular weight difference). Fold changes compared to the wild type are shown in parentheses, with changes of >5-fold colored in orange. (B) IC₅₀ titers in micrograms per milliliter of IgG (circle) and scFv (triangle) against HIV-1 subtypes A, B, and C. Viruses lacking an N276 glycan are colored green. IgG- and scFv-resistant viruses are excluded. Fold differences are indicated above the graph and excluded the 2 viruses that lacked an N276 glycan. (C) Logogram of amino acid sequences of the CD4 binding site of HIV-1 subtype C viruses neutralized at the same level by scFv and IgG (<10-fold), those less well neutralized by scFv (>10-fold difference), or those resistant to both IgG and scFv. The N276 glycan and sites 360, 362, and 429, which exhibit the most variability, are indicated. Negatively and positively charged residues are colored red and blue, respectively. (D) Structural representation of the interaction of 3BNC117 Fab (green) and HIV Env (gray) containing an arginine (blue) or glycine (orange) at position 429. The aspartic acid at residue 113 in the HIV trimer is colored red, whereas the arginine at position 30 in the Fab is colored blue. Polar bonds are indicated by dotted lines (PDB entries 4JPV and 5V8L) (78, 79).

between IgG and scFv (Fig. 6C). Viruses were separated into 3 groups based on their relative sensitivity to IgG and scFv. There was significant viral variation at positions 360, 362, and 429, all of which have been previously implicated in the binding of CD4bs bNAbs (36, 37), although position 429 has not specifically been shown to impact 3BNC117.

To further explore the possibility that residue 429 impacted the scFv, we compared crystal structures of the 3BNC117 Fab in complex with gp120 (PDB entries 4JPV and 5V8L). We found that a neutral or negative charge at residue 429 interacted with a positively charged residue 30 (arginine) on the 3BNC117 Fab (Fig. 6D). In contrast, a positive charge at position 429 in the HIV-1 trimer could prevent 3BNC117 binding to this site by folding back and binding to an aspartic acid at position 113 in gp120, occluding this residue. Therefore, the 3BNC117 scFv binding may in part be more reliant on the neutral or negative charge at position 429 than its IgG counterpart. Thus, virus-specific loss of potency for the 3BNC117 scFv was likely impacted by viral subtype, the 276 glycan, and charged residues within the epitope.

DISCUSSION

We have designed and expressed scFv of HIV bNAbs that are undergoing human clinical testing to assess whether they maintained neutralization capacity in this format.

These bNAbs targeted one of four well-defined epitopes on the viral envelope, namely, the V2 apex (CAP256.25), the V3 glycan (PGT121), the CD4bs (3BNC117), and the MPER (10E8v4). Remarkably, these much smaller scFv, engineered from the variable regions of the IgG, retained substantial breadth and potency, and 10E8v4 was equivalent to the full IgG molecule. While CAP256.25, 3BNC117, and PGT121 scFv lost potency, they were nonetheless in the therapeutic range of VRC01, a CD4bs antibody that is currently in clinical efficacy testing.

Newly designed scFv showed good levels of expression with different linker lengths of 15, 18, or 20 amino acids having minimal to no impact on neutralizing activity. This suggested that the loss of activity relative to IgG was due to the inherent nature rather than the design of the scFv, which at ~30 kDa are 5 times smaller than IgG. Antibody scFv were more likely to lose neutralization potency rather than breadth, although for 3BNC117 and PGT121 the loss of potency often resulted in reduced breadth. Potency loss was most severe for CAP256.25 and was evident against all viruses tested, while for 3BNC117 and PGT121 this occurred in a virus-specific manner. Epitope mapping studies on selected viruses showed that scFv bound to their epitope in ways subtly different from those of the matched IgG. Thus, charged residues at positions 169 and 170 in V2 affected CAP256.25 scFv neutralization more than the IgG. For PGT121, interactions with the N332 glycan and the length of V1 impacted the scFv to a greater degree than the IgG, and for 3BNC117, loss of binding to the N276 glycan was more detrimental to the scFv than the IgG. Thus, while scFv retained the ability to interact with known epitopes, they were more sensitive to variations at single residues within these epitopes. Whether these potency losses are unique to each scFv or are dependent on the epitopes being targeted would need to be determined by designing and testing a larger number of scFv versions of bNAbs.

scFv have a single binding site compared to the 2 binding sites of IgG, which likely reduces their affinity for the antigen. It is known that smaller proteins tend to have higher off rates due to lower binding affinity (38–40). This was previously demonstrated for scFv-based fragments of VRC01 that had a 4-fold lower affinity than the IgG version (18). Affinity is influenced by noncovalent intermolecular interactions between proteins, such as electrostatic charge and hydrogen bonding. Our data suggest that a charge change in the epitope had a greater effect than the loss of a glycan, although both likely impacted the binding affinity of the scFv, accounting for the potency losses. Loss of affinity could also be due to the inability of scFv with a single binding site to effectively cross-link protomers on the trimer. Even though trimers are present at low density on the HIV virion, polarization of trimers at the cell contact site would allow the IgG but not the scFv to cross-link (41–45).

The constant region affects the angle at which the antibody paratope engages the viral epitope and also helps to stabilize the heavy-light chain interactions (46). As a result, scFv may have a somewhat less rigid paratope than IgG. Removal of the Fc region could, therefore, alter these interactions, contributing to the scFv being more dependent on key residues in the epitope. Structural studies in combination with affinity studies of scFv are needed to further inform the molecular interactions with the HIV trimer and provide insights into the stability of scFv paratopes. Others have shown that the lack of an Fc region also significantly reduces the half-life of scFv and removes their ability to mediate effector functions, which compromises their clinical development (16, 22). However, scFv may have lower immunogenicity and off-target effects than IgG as a result of not having an antibody Fc region to engage host Fc receptors (18, 47).

scFv may have better access to their epitopes than IgG due to their smaller size, as has been reported using other antibody constructs. For example, antigen-binding fragments (Fab) of antibodies targeting the CD4-induced (CD4i) epitope showed significant improvement in potency over the IgG versions (48–50). Moreover, scFv isolated from phage display libraries demonstrated good breadth and potency (25, 51). A number of antibody epitopes on the HIV trimer are not readily accessible by IgG molecules due to steric hindrance, including the MPER on gp41 (53–55). Remarkably, the scFv of 10E8v4, an MPER antibody that targets an epitope close to the viral

membrane, largely maintained potency similar to that of the IgG. The improved access of the 10E8v4 scFv to this recessed site could offset the loss of affinity that results from having only a single paratope. Alternatively, scFv targeting the MPER may be partially buried in the lipid membrane, which could stabilize the interaction with the epitope (54–56). scFv may also be important in preventing cell-cell transmission, where spatial restrictions within the virological synapse limit antibody access (57).

scFv may be useful in the context of passive immunization; however, the caveats associated with this format will need to be addressed, in particular their reduced half-life. Adeno-associated virus (AAV) vectors, which are being developed for continuous delivery of antibody-based products as vectored immunoprophylaxis against HIV infection (58–61), may provide a solution. scFv, with their smaller gene inserts, may be more suitable for expression in vectors, such as AAV-8, that have been previously deployed for some of the IgG bNABs described here (61). This approach could be particularly important in the context of bNAB combinations needed to optimize the coverage of global HIV strains (62–64). Since multiple scFv could be expressed from the same vector, their use in combination treatments may be a distinct advantage (47, 58, 62, 65). scFv derived from other HIV bNABs will need to be tested to determine which combinations show optimal complementarity in their neutralization profiles given that scFv neutralization cannot be predicted.

Overall, our data indicate that scFv retain considerable breadth and potency and could perform better than IgG equivalents as combination therapeutics. In particular, 10E8v4 should be considered a leading candidate for human clinical testing, given that it retains all its functional activity as an scFv. Although there are some challenges with this format, there are also unique advantages of scFv over IgG. These smaller antibody proteins, therefore, have considerable potential in passive immunization strategies to prevent HIV infection.

MATERIALS AND METHODS

Construction of scFv. scFv containing the variable regions of various bNABs were cloned by overlapping PCR or ordered from GenScript (NJ, USA) (Table 1). For CAP256.25 and PGT121, 3 different glycine-serine linkers, namely, a 15-amino-acid linker [linker 1, (GGGGS)₃], a 20-amino-acid linker [linker 2, (GGGGS)₄], and an 18-amino-acid linker (linker 3, GGSSSSSSGGGGGGGG) were tested in the V_H-V_L orientation. L3 was also tested for the V_L-V_H orientation. We used an 8×-histidine tag for purification purposes preceded by an HRV-3C site in case removal of the histidine tag was needed for future experiments. Table 1 shows the scFv constructs in V_H-V_L orientation with L3 for CAP256.25. All other scFv (3BNC117 and 10E8v4) were expressed in the V_H-V_L conformation with linker 1 as specified.

Protein expression. All constructs were cloned into the pCMV vector (AIDS Reagent Program, Division of AIDS, NIAID, NIH). For the lambda chain of PGT121, the pBR322-based Ig-lambda expression vector was used (AIDS Reagent Program, Division of AIDS, NIAID, NIH). The constructs were grown in JM109 bacterial cells and extracted using a plasmid Maxiprep kit (Qiagen, Hilden Germany). Sequences were confirmed using the Applied Biosystems 3500xL Genetic Analyzer. Constructs were expressed as previously described (66). In short, HEK293F suspension cells at 1.5×10^6 to 2×10^6 cells/ml were cotransfected with linear polyethylenimine hydrochloride (molecular weight, 40,000) at a 3:1 ratio with 1 µg of plasmid per 1 ml of culture. Supernatants were harvested after 6 days.

Purification of scFv. scFv were purified using Ni-Sepharose beads (GE Healthcare, Massachusetts USA). Proteins were washed using a 30 mM imidazole-phosphate-buffered saline (PBS) solution and eluted using 400 mM imidazole in PBS. Glycerol was added to the elution at a final concentration of 5% to limit aggregation. scFv were applied to hiload Superdex 75 or Superdex 200 columns (GE Healthcare) equilibrated with PBS at pH 6.5 (5% glycerol with 0.02% sodium azide). The fractions corresponding to the size of the scFv were collected, pooled, and concentrated using Vivaspin concentrators or Vivapure static concentrators (GE Healthcare). The samples were dialyzed overnight at room temperature to remove sodium azide. Concentrations were measured on a NanoDrop device (ThermoFisher, MA, USA), with extinction coefficients at 1% calculated using ExPASy ProtParam (69) and characterized by SDS-PAGE. Molar weight was determined by using ExPASy ProtParam (Fig. 1A). scFv proteins were stored at –75°C.

IgG production. IgG constructs were expressed in HEK293F cells as described previously (66). Supernatants were harvested after 6 days and purified using a protein A affinity column. Proteins were eluted using a 0.15 M glycine buffer at pH 2.5 in 1 M Tris, pH 8, and were concentrated and dialyzed into PBS, pH 6.5, containing 5% glycerol. Concentrations were measured on a NanoDrop device using an extinction coefficient of 13.7 and a 1% solution. The molecular weight of the IgG was calculated using ExPASy ProtParam (69) for CAP256.25 (150.71 kDa), 10E8v4 (147.29 kDa), 3BNC117 (146.24 kDa), and PGT121 (146.2 kDa). IgG and proteins were stored at –75°C.

Neutralization assay. Single-round infection pseudoviruses were grown and titers determined as described previously (70–72). Briefly, plasmids were transfected with the pseudovirus backbone

pSG3Δ^{env} into 293T cells at ratios of 1:3 PEI Max. Viruses were harvested after 48 h, filtered, frozen, and titrated. A panel of 43 viruses (73) plus BG505 N332 and CAP256_SU (CAP256.3 mo.9C) (74, 75), representing HIV-1 clades A, B, and C, was used to compare neutralization titers of IgG and scFv. Neutralization assays were performed in TZM-bl cells as described previously (76, 77). Proteins were tested at 200 μg/ml for the IgG (~146 kDa) and 50 μg/ml for the scFv (28 to 32 kDa). scFv neutralization titers were recalculated by compensating for the fold difference in molecular weight (values for each scFv are shown in Fig. 1A). (The IC₅₀ data for scFv and IgG molar concentrations are available on request). All assays were repeated at least twice. Geometric mean potency was calculated for both IgG and scFv using sensitive viruses only. When comparing viruses mutated by site-directed mutagenesis, a fold change of more than 3 compared to the parent virus was considered significant.

Site-directed mutagenesis. Mutations in the envelope gene of viruses were made using the QuikChange Lightning site-directed mutagenesis kit (Agilent). Sequences were confirmed using the Applied Biosystems 3500xL Genetic Analyzer. Pseudoviruses were grown as described above.

Statistics and sequence analysis. Wilcoxon matched-pair signed-rank test was used to assess differences between IgG and scFv within a subtype.

Sequence alignments were made using the Analyze Align tool from the HIV LanL website (https://www.hiv.lanl.gov/content/sequence/ANALYZEALIGN/analyze_align.html).

Structural modeling. We used the PyMOL Molecular Graphics System (version 2.1.1; Schrödinger, LLC) to model a BG505 SOSIP.664 trimer (PDB entry 5V8L). Residues corresponding to the sequences in the CATNAP LANL for CAP256_SU and AC10.0.29 were modeled and colored based on charge.

For 3BNC117, we used Fab structures, either in complex with a clade A gp120 from 93TH057 with G429 (4JPV) or in complex with the clade A BG505 SOSIP.664 trimer with R429 (PDB entry 5V8L). We used PyMOL to visualize the 429 residue and used the built-in software to predict polar bonds.

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REFERENCES

- Bhiman JN, Lynch RM. 2017. Broadly neutralizing antibodies as treatment: effects on virus and immune system. *Curr HIV/AIDS Rep* 14:54–62. <https://doi.org/10.1007/s11904-017-0352-1>.
- Burton DR, Hangartner L. 2016. Broadly neutralizing antibodies to HIV and their role in vaccine design. *Annu Rev Immunol* 34:635–659. <https://doi.org/10.1146/annurev-immunol-041015-055515>.
- Julg B, Barouch DH. 2019. Neutralizing antibodies for HIV-1 prevention. *Curr Opin HIV AIDS* 14:318–324. <https://doi.org/10.1097/COH.0000000000000556>.
- Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE, Beary H, Hayes D, Frankel SS, Bix DL, Lewis MG. 2000. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat Med* 6:207–210. <https://doi.org/10.1038/72318>.
- Cheeseman HM, Olejniczak NJ, Rogers PM, Evans AB, King DFL, Ziprin P, Liao H-X, Haynes BF, Shattock RJ. 2017. Broadly neutralizing antibodies display potential for prevention of HIV-1 infection of mucosal tissue superior to that of nonneutralizing antibodies. *J Virol* 91:e01762-16. <https://doi.org/10.1128/JVI.01762-16>.
- Julg B, Liu P-T, Wagh K, Fischer WM, Abbink P, Mercado NB, Whitney JB, Nkolola JP, McMahan K, Tartaglia LJ, Borducchi EN, Khatiwada S, Kamath M, LeSuer JA, Seaman MS, Schmidt SD, Mascola JR, Burton DR, Korber BT, Barouch DH. 2017. Protection against a mixed SHIV challenge by a broadly neutralizing antibody cocktail. *Sci Transl Med* 9:eaao4235. <https://doi.org/10.1126/scitranslmed.aao4235>.
- Julg B, Tartaglia LJ, Keele BF, Wagh K, Pegu A, Sok D, Abbink P, Schmidt SD, Wang K, Chen X, Joyce MG, Georgiev IS, Choe M, Kwong PD, Doria-Rose NA, Le K, Louder MK, Bailer RT, Moore PL, Korber B, Seaman MS, Abdool Karim SS, Morris L, Koup RA, Mascola JR, Burton DR, Barouch DH. 2017. Broadly neutralizing antibodies targeting the HIV-1 envelope V2 apex confer protection against a clade C SHIV challenge. *Sci Transl Med* 9:eaal1321. <https://doi.org/10.1126/scitranslmed.aal1321>.
- Gautam R, Nishimura Y, Pegu A, Nason MC, Klein F, Gazumyan A, Golijanin J, Buckler-White A, Sadjadpour R, Wang K, Mankoff Z, Schmidt SD, Lifson JD, Mascola JR, Nussenzweig MC, Martin MA. 2016. A single injection of anti-HIV-1 antibodies protects against repeated SHIV challenges. *Nature* 533:105–109. <https://doi.org/10.1038/nature17677>.
- Pegu A, Hessel AJ, Mascola JR, Haigwood NL. 2017. Use of broadly

- neutralizing antibodies for HIV-1 prevention. *Immunol Rev* 275:296–312. <https://doi.org/10.1111/immr.12511>.
10. Caskey M, Klein F, Lorenzi JCC, Seaman MS, West AP, Jr, Buckley N, Kremer G, Nogueira L, Braunschweig M, Scheid JF, Horwitz JA, Shimeliovich I, Ben-Avraham S, Witmer-Pack M, Platten M, Lehmann C, Burke LA, Hawthorne T, Gorelick RJ, Walker BD, Keler T, Gulick RM, Fätkenheuer G, Schlesinger SJ, Nussenzweig MC, West AP, Fätkenheuer G. 2015. Viraemia suppressed in HIV-1-infected humans by broadly neutralizing antibody 3BNC117. *Nature* 522:487–491. <https://doi.org/10.1038/nature14411>.
 11. Caskey M, Schoofs T, Gruell H, Settler A, Karagounis T, Kreider EF, Murrell B, Pfeifer N, Nogueira L, Oliveira TY, Learn GH, Cohen YZ, Lehmann C, Gillor D, Shimeliovich I, Unson-O'Brien C, Weiland D, Robles A, Kümmerle T, Wyen C, Levin R, Witmer-Pack M, Eren K, Ignacio C, Kiss S, West AP, Mouquet H, Zingman BS, Gulick RM, Keler T, Bjorkman PJ, Seaman MS, Hahn BH, Fätkenheuer G, Schlesinger SJ, Nussenzweig MC, Klein F. 2017. Antibody 10-1074 suppresses viremia in HIV-1-infected individuals. *Nat Med* 23:185–191. <https://doi.org/10.1038/nm.4268>.
 12. Lynch RM, Boritz E, Coates EE, DeZure A, Madden P, Costner P, Enama ME, Plummer S, Holman L, Hendel CS, Gordon I, Casazza J, Conan-Cibotti M, Migueles SA, Tressler R, Bailer RT, McDermott A, Narpala S, O'Dell S, Wolf G, Lifson JD, Freemire BA, Gorelick RJ, Pandey JP, Mohan S, Chomont N, Fromentin R, Chun T-W, Fauci AS, Schwartz RM, Koup RA, Douek DC, Hu Z, Capparelli E, Graham BS, Mascola JR, Ledgerwood JE. 2015. Virologic effects of broadly neutralizing antibody VRC01 administration during chronic HIV-1 infection. *Sci Transl Med* 7:319ra206. <https://doi.org/10.1126/scitranslmed.aad5752>.
 13. Mendoza P, Gruell H, Nogueira L, Pai JA, Butler AL, Millard K, Lehmann C, Suárez I, Oliveira TY, Lorenzi J, Cohen YZ, Wyen C, Kümmerle T, Karagounis T, Lu C-L, Handl L, Unson-O'Brien C, Patel R, Ruping C, Schlotz M, Witmer-Pack M, Shimeliovich I, Kremer G, Thomas E, Seaton KE, Horowitz J, West AP, Bjorkman PJ, Tomaras GD, Gulick RM, Pfeifer N, Fätkenheuer G, Seaman MS, Klein F, Caskey M, Nussenzweig MC. 2018. Combination therapy with anti-HIV-1 antibodies maintains viral suppression. *Nature* 561:479–484. <https://doi.org/10.1038/s41586-018-0531-2>.
 14. Bar KJ, Sneller MC, Harrison LJ, Justement JS, Overton ET, Petrone ME, Salantes DB, Seamon CA, Scheinfeld B, Kwan RW, Learn GH, Proschan MA, Kreider EF, Blazkova J, Bardsley M, Refsland EW, Messer M, Clarridge KE, Tustin NB, Madden PJ, Oden K, O'Dell SJ, Jarocki B, Shiakolas AR, Tressler RL, Doria-Rose NA, Bailer RT, Ledgerwood JE, Capparelli EV, Lynch RM, Graham BS, Moir S, Koup RA, Mascola JR, Hoxie JA, Fauci AS, Tebas P, Chun T-W. 2016. Effect of HIV antibody VRC01 on viral rebound after treatment interruption. *N Engl J Med* 375:2037–2050. <https://doi.org/10.1056/NEJMoa1608243>.
 15. Scheid JF, Horwitz JA, Bar-On Y, Kreider EF, Lu C-L, Lorenzi JCC, Feldmann A, Braunschweig M, Nogueira L, Oliveira TY, Shimeliovich I, Patel R, Burke LA, Cohen YZ, Hadrihan S, Settler A, Witmer-Pack M, West AP, Jr, Juelg B, Keler T, Hawthorne T, Zingman BS, Gulick RM, Pfeifer N, Learn GH, Seaman MS, Bjorkman PJ, Klein F, Schlesinger SJ, Walker BD, Hahn BH, Nussenzweig MC, Caskey M. 2016. HIV-1 antibody 3BNC117 suppresses viral rebound in humans during treatment interruption. *Nature* 535:556–560. <https://doi.org/10.1038/nature18929>.
 16. Ahmad ZA, Yeap SK, Ali AM, Ho WY, Alitheen NBM, Hamid M. 2012. scFv antibody: principles and clinical application. *Clin Dev Immunol* 2012: 980250–980215. <https://doi.org/10.1155/2012/980250>.
 17. Chen W, Dimitrov DS. 2009. Human monoclonal antibodies and engineered antibody domains as HIV-1 entry inhibitors. *Curr Opin HIV AIDS* 4:112–117. <https://doi.org/10.1097/COH.0b013e328322f95e>.
 18. West AP, Galimidi RP, Gnanapragasam PNP, Bjorkman PJ. 2012. Single-chain Fv-based anti-HIV proteins: potential and limitations. *J Virol* 86: 195–202. <https://doi.org/10.1128/JVI.05848-11>.
 19. Wang S, Zheng C, Liu Y, Zheng H, Wang Z. 2008. Construction of multiform scFv antibodies using linker peptide. *J Genet Genomics* 35: 313–316. [https://doi.org/10.1016/S1673-8527\(08\)60045-4](https://doi.org/10.1016/S1673-8527(08)60045-4).
 20. Colcher D, Pavlinkova G, Beresford G, Booth BJ, Choudhury A, Batra SK. 1998. Pharmacokinetics and biodistribution of genetically-engineered antibodies. *Q J Nucl Med* 42:225–241.
 21. Yokota T, Milenic DE, Whitlow M, Schlom J. 1992. Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res* 52:3402–3408.
 22. Unverdorben F, Richter F, Hutt M, Seifert O, Malinge P, Fischer N, Kontermann RE. 2016. Pharmacokinetic properties of IgG and various Fc fusion proteins in mice. *MAbs* 8:120–128. <https://doi.org/10.1080/19420862.2015.1113360>.
 23. Holliger P, Hudson PJ. 2005. Engineered antibody fragments and the rise of single domains. *Nat Biotechnol* 23:1126–1136. <https://doi.org/10.1038/nbt1142>.
 24. Bivi N, Moore T, Rodgers G, Denning H, Shockley T, Swearingen CA, Gelfanova V, Calderon B, Peterson DA, Hodsdon ME, Siegel RW, Higgs RE, Konrad RJ. 2019. Investigation of pre-existing reactivity to biotherapeutics can uncover potential immunogenic epitopes and predict immunogenicity risk. *MAbs* 11:861–869. <https://doi.org/10.1080/19420862.2019.1612699>.
 25. Khan L, Kumar R, Thiruvengadam R, Parry HA, Makhdoom MA, Kumar S, Aggarwal H, Mohata M, Hussain AW, Das R, Varadarajan R, Bhattacharya J, Vajpayee M, Murugavel KG, Solomon S, Sinha S, Luthra K. 2017. Cross-neutralizing anti-HIV-1 human single chain variable fragments (scFvs) against CD4 binding site and N332 glycan identified from a recombinant phage library. *Sci Rep* 7:45163. <https://doi.org/10.1038/srep45163>.
 26. Burton DR, Barbas CF, Persson MA, Koenig S, Chanock RM, Lerner RA. 1991. A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. *Proc Natl Acad Sci U S A* 88:10134–10137. <https://doi.org/10.1073/pnas.88.22.10134>.
 27. Reference deleted.
 28. Doria-Rose NA, Bhiman JN, Roark RS, Schramm CA, Gorman J, Chuang G-Y, Pancera M, Cale EM, Erandes MJ, Louder MK, Asokan M, Bailer RT, Druz A, Fraschilla IR, Garrett NJ, Jarosinski M, Lynch RM, McKee K, O'Dell S, Pegu A, Schmidt SD, Staupé RP, Sutton MS, Wang K, Wibmer CK, Haynes BF, Abdool-Karim S, Shapiro L, Kwong PD, Moore PL, Morris L, Mascola JR. 2016. New member of the V1V2-directed CAP256-VRC26 lineage that shows increased breadth and exceptional potency. *J Virol* 90:76–91. <https://doi.org/10.1128/JVI.01791-15>.
 29. Walker LM, Huber M, Doores KJ, Falkowska E, Pejchal R, Julien J-P, Wang S-KS-K, Ramos A, Chan-Hui P-Y, Moyle M, Mitcham JL, Hammond PW, Olsen OA, Phung P, Fling S, Wong C-H, Phogat SK, Wrin T, Simek MD, Principal Investigators PG, Koff WC, Wilson IA, Burton DR, Poignard P, Walker LM, Huber M, Doores KJ, Falkowska E, Pejchal R, Chan-Hui P-Y, Moyle M, Mitcham JL, Hammond PW, Olsen OA, Phung P, Principal Investigators PG, Koff WC, Wilson IA, Burton DR, Poignard P, Protocol G Principal Investigators. 2011. Broad neutralization coverage of HIV by multiple highly potent antibodies. *Nature* 477:466–470. <https://doi.org/10.1038/nature10373>.
 30. Scheid JF, Mouquet H, Ueberheide B, Diskin R, Klein F, Oliveira TY, Pietzsch J, Fenyo D, Abadir A, Velinzon K, Hurley A, Myung S, Boulard F, Poignard P, Burton DR, Pereyra F, Ho DD, Walker BD, Seaman MS, Bjorkman PJ, Chait BT, Nussenzweig MC. 2011. Sequence and structural convergence of broad and potent HIV antibodies that mimic CD4 binding. *Science* 333:1633–1637. <https://doi.org/10.1126/science.1207227>.
 31. Kwon YD, Georgiev IS, Ofek G, Zhang B, Asokan M, Bailer RT, Bao A, Caruso W, Chen X, Choe M, Druz A, Ko S-Y, Louder MK, McKee K, O'Dell S, Pegu A, Rudicell RS, Shi W, Wang K, Yang Y, Alger M, Bender MF, Carlton K, Cooper JW, Blinn J, Eudailey J, Lloyd K, Parks R, Alam SM, Haynes BF, Padte NN, Yu J, Ho DD, Huang J, Connors M, Schwartz RM, Mascola JR, Kwong PD. 2016. Optimization of the solubility of HIV-1-neutralizing antibody 10E8 through somatic variation and structure-based design. *J Virol* 90:5899–5914. <https://doi.org/10.1128/JVI.03246-15>.
 32. Julien J-P, Sok D, Khayat R, Lee JH, Doores KJ, Walker LM, Ramos A, Diwanji DC, Pejchal R, Cupo A, Katpally U, Depetris RS, Stanfield RL, McBride R, Marozsan AJ, Paulson JC, Sanders RW, Moore JP, Burton DR, Poignard P, Ward AB, Wilson IA. 2013. Broadly neutralizing antibody PGT121 allosterically modulates CD4 binding via recognition of the HIV-1 gp120 V3 base and multiple surrounding glycans. *PLoS Pathog* 9:e1003342. <https://doi.org/10.1371/journal.ppat.1003342>.
 33. Anthony C, York T, Bekker V, Matten D, Selhorst P, Ferreria R-C, Garrett NJ, Karim SSA, Morris L, Wood NT, Moore PL, Williamson C. 2017. Cooperation between strain-specific and broadly neutralizing responses limited viral escape and prolonged the exposure of the broadly neutralizing epitope. *J Virol* 91:e00828-17. <https://doi.org/10.1128/JVI.00828-17>.
 34. Deshpande S, Patil S, Kumar R, Hermanus T, Murugavel KG, Srikrishnan AK, Solomon S, Morris L, Bhattacharya J. 2016. HIV-1 clade C escapes broadly neutralizing autologous antibodies with N332 glycan specificity by distinct mechanisms. *Retrovirology* 13:60. <https://doi.org/10.1186/s12977-016-0297-2>.
 35. McGuire AT, Hoot S, Dreyer AM, Lippy A, Stuart A, Cohen KW, Jardine J, Menis S, Scheid JF, West AP, Schief WR, Stamatatos L. 2013. Engineering HIV envelope protein to activate germline B cell receptors of broadly

- neutralizing anti-CD4 binding site antibodies. *J Exp Med* 210:655–663. <https://doi.org/10.1084/jem.20122824>.
36. Bricault CA, Yusim K, Seaman MS, Yoon H, Theiler J, Giorgi EE, Wagh K, Theiler M, Hraber P, Macke JP, Kreider EF, Learn GH, Hahn BH, Scheid JF, Kovacs JM, Shields JL, Lavine CL, Ghantous F, Rist M, Bayne MG, Neubauer GH, McMahan K, Peng H, Chéneau C, Jones JJ, Zeng J, Ochsenbauer C, Nkolola JP, Stephenson KE, Chen B, Gnanakaran S, Bonsignori M, Williams LD, Haynes BF, Doria-Rose N, Mascola JR, Montefiori DC, Barouch DH, Korber B. 2019. HIV-1 neutralizing antibody signatures and application to epitope-targeted vaccine design. *Cell Host Microbe* 25: 59–72. <https://doi.org/10.1016/j.chom.2018.12.001>.
 37. Liu Q, Lai YT, Zhang P, Louder MK, Pegu A, Rawi R, Asokan M, Chen X, Shen CH, Chuang GY, Yang ES, Miao H, Wang Y, Fauci AS, Kwong PD, Mascola JR, Lusso P. 2019. Improvement of antibody functionality by structure-guided paratope engraftment. *Nat Commun* 10:721. <https://doi.org/10.1038/s41467-019-08658-4>.
 38. Schreiber G, Haran G, Zhou H-X. 2009. Fundamental aspects of protein–protein association kinetics. *Chem Rev* 109:839–860. <https://doi.org/10.1021/cr800373w>.
 39. Pan AC, Borhani DW, Dror RO, Shaw DE. 2013. Molecular determinants of drug-receptor binding kinetics. *Drug Discov Today* 18:667–673. <https://doi.org/10.1016/j.drudis.2013.02.007>.
 40. Miller DC, Lunn G, Jones P, Sabnis Y, Davies NL, Driscoll P. 2012. Investigation of the effect of molecular properties on the binding kinetics of a ligand to its biological target. *Med Chem Commun* 3:449. <https://doi.org/10.1039/c2md00270a>.
 41. Klasse PJ. 2007. Modeling how many envelope glycoprotein trimers per virion participate in human immunodeficiency virus infectivity and its neutralization by antibody. *Virology* 369:245–262. <https://doi.org/10.1016/j.virol.2007.06.044>.
 42. Chien MP, Lin CH, Chang DK. 2009. Recruitment of HIV-1 envelope occurs subsequent to lipid mixing: a fluorescence microscopic evidence. *Retrovirology* 6:20. <https://doi.org/10.1186/1742-4690-6-20>.
 43. Cohen FS, Melikyan GB. 2004. The energetics of membrane fusion from binding, through hemifusion, pore formation, and pore enlargement. *J Membr Biol* 199:1–14. <https://doi.org/10.1007/s00232-004-0669-8>.
 44. Louder MK, Sambor A, Chertova E, Hunte T, Barrett S, Ojong F, Sanders-Buell E, Zolla-Pazner S, McCutchan FE, Roser JD, Gabuzda D, Lifson JD, Mascola JR. 2005. HIV-1 envelope pseudotyped viral vectors and infectious molecular clones expressing the same envelope glycoprotein have a similar neutralization phenotype, but culture in peripheral blood mononuclear cells is associated with decreased neutralization sensitivity. *Virology* 339:226–238. <https://doi.org/10.1016/j.virol.2005.06.003>.
 45. Cohen YZ, Lorenzi JCC, Seaman MS, Nogueira L, Schoofs T, Krassnig L, Butler A, Millard K, Fitzsimons T, Daniell X, Dizon JP, Shimeliovich I, Montefiori DC, Caskey M, Nussenzweig MC. 2017. Neutralizing activity of broadly neutralizing anti-HIV-1 antibodies against clade B clinical isolates produced in peripheral blood mononuclear cells. *J Virol* 92:e01883-17. <https://doi.org/10.1128/JVI.01883-17>.
 46. Janda A, Bowen A, Greenspan NS, Casadevall A. 2016. Ig constant region effects on variable region structure and function. *Front Microbiol* 7:22. <https://doi.org/10.3389/fmicb.2016.00022>.
 47. Monnier P, Vigouroux R, Tassew N. 2013. In vivo applications of single chain Fv (variable domain) (scFv) fragments. *Antibodies* 2:193–208. <https://doi.org/10.3390/antib2020193>.
 48. Labrijn AF, Poignard P, Raja A, Zwick MB, Delgado K, Franti M, Binley JM, Vivona V, Grundner C, Huang C-C, Venturi M, Petropoulos C, Wrin T, Dimitrov DS, Robinson JE, Kwong PD, Wyatt RT, Sodroski JG, Burton DR. 2003. Access of antibody molecules to the conserved coreceptor binding site on glycoprotein gp120 is sterically restricted on primary human immunodeficiency virus type 1. *J Virol* 77:10557–10565. <https://doi.org/10.1128/jvi.77.19.10557-10565.2003>.
 49. Zhang MY, Shu Y, Sidorov I, Dimitrov DS. 2004. Identification of a novel CD4i human monoclonal antibody Fab that neutralizes HIV-1 primary isolates from different clades. *Antiviral Res* 61:161–164. <https://doi.org/10.1016/j.antiviral.2003.09.009>.
 50. Tanaka K, Kuwata T, Alam M, Kaplan G, Takahama S, Valdez KPR, Roitburd-Berman A, Gershoni JM, Matsushita S. 2017. Unique binding modes for the broad neutralizing activity of single-chain variable fragments (scFv) targeting CD4-induced epitopes. *Retrovirology* 14:44. <https://doi.org/10.1186/s12977-017-0369-y>.
 51. Kumar S, Kumar R, Khan L, Makhdooni MA, Thiruvengadam R, Mohata M, Agarwal M, Lodha R, Kabra SK, Sinha S, Luthra K. 2017. CD4-binding site directed cross-neutralizing scFv monoclonals from HIV-1 subtype C infected Indian children. *Front Immunol* 8:1568. <https://doi.org/10.3389/fimmu.2017.01568>.
 52. Reference deleted.
 53. Zwick MB, Labrijn AF, Wang M, Spennlehauser C, Saphire EO, Binley JM, Moore JP, Stiegler G, Kattinger H, Burton DR, Parren P. 2001. Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. *J Virol* 75:10892–10905. <https://doi.org/10.1128/JVI.75.22.10892-10905.2001>.
 54. Irimia A, Sarkar A, Stanfield RL, Wilson IA. 2016. Crystallographic identification of lipid as an integral component of the epitope of HIV broadly neutralizing antibody 4E10. *Immunity* 44:21–31. <https://doi.org/10.1016/j.immuni.2015.12.001>.
 55. Irimia A, Serra AM, Sarkar A, Jacak R, Kalyuzhnyi O, Sok D, Saye-Francisco KL, Schiffner T, Tingle R, Kubitz M, Adachi Y, Stanfield RL, Deller MC, Burton DR, Schief WR, Wilson IA. 2017. Lipid interactions and angle of approach to the HIV-1 viral membrane of broadly neutralizing antibody 10E8: insights for vaccine and therapeutic design. *PLoS Pathog* 13: e1006212. <https://doi.org/10.1371/journal.ppat.1006212>.
 56. Lee JH, Ozorowski G, Ward AB. 2016. Cryo-EM structure of a native, fully glycosylated, cleaved HIV-1 envelope trimer. *Science* 351:1043–1048. <https://doi.org/10.1126/science.1245050>.
 57. Zhang MY, Borges AR, Ptak RG, Wang Y, Dimitrov AS, Alam SM, Wiczorek L, Bouma P, Fouts T, Jiang S, Polonis VR, Haynes BF, Quinnan GV, Montefiori DC, Dimitrov DS. 2010. Potent and broad neutralizing activity of a single chain antibody fragment against cell-free and cell-associated HIV-1. *MAbs* 2:266–274. <https://doi.org/10.4161/mabs.2.3.11416>.
 58. Naso MF, Tomkowicz B, Perry WL, Strohl WR. 2017. Adeno-associated virus (AAV) as a vector for gene therapy. *BioDrugs* 31:317–334. <https://doi.org/10.1007/s40259-017-0234-5>.
 59. Balazs AB, Chen J, Hong CM, Rao DS, Yang L, Baltimore D. 2011. Antibody-based protection against HIV infection by vectored immunoprophylaxis. *Nature* 481:81–86. <https://doi.org/10.1038/nature10660>.
 60. Lin A, Balazs AB. 2018. Adeno-associated virus gene delivery of broadly neutralizing antibodies as prevention and therapy against HIV-1. *Retrovirology* 15:66. <https://doi.org/10.1186/s12977-018-0449-7>.
 61. van den Berg FT, Makoah NA, Ali SA, Scott TA, Mapengo RE, Mutsunguma LZ, Mkhize NN, Lambson BE, Kgagudi PD, Crowther C, Abdool Karim SS, Balazs AB, Weinberg MS, Ely A, Arbutnot PB, Morris L. 2019. AAV-mediated expression of broadly neutralizing and vaccine-like antibodies targeting the HIV-1 envelope V2 region. *Mol Ther Methods Clin Dev* 14:100–112. <https://doi.org/10.1016/j.omtm.2019.06.002>.
 62. Wagh K, Bhattacharya T, Williamson C, Robles A, Bayne MG, Garrity J, Rist M, Rademeyer C, Yoon H, Lapedes A, Gao H, Greene K, Louder MK, Kong R, Karim SA, Burton DR, Barouch DH, Nussenzweig MC, Mascola JR, Morris L, Montefiori DC, Korber B, Seaman MS. 2016. Optimal combinations of broadly neutralizing antibodies for prevention and treatment of HIV-1 clade C infection. *PLoS Pathog* 12:e1005520. <https://doi.org/10.1371/journal.ppat.1005520>.
 63. Doria-Rose NA, Louder MK, Yang Z, O'Dell S, Nason M, Schmidt SD, McKee K, Seaman MS, Bailer RT, Mascola JR. 2012. HIV-1 neutralization coverage is improved by combining monoclonal antibodies that target independent epitopes. *J Virol* 86:3393–3397. <https://doi.org/10.1128/JVI.06745-11>.
 64. Kong R, Louder MK, Wagh K, Bailer RT, DeCamp A, Greene KM, Gao H, Taft JD, Gazumyan A, Liu C, Nussenzweig MC, Korber BT, Montefiori DC, Mascola JR. 2015. Improving neutralization potency and breadth by combining broadly reactive HIV-1 antibodies targeting major neutralization epitopes. *J Virol* 89:2659–2671. <https://doi.org/10.1128/JVI.03136-14>.
 65. Liu Z, Chen O, Wall JBJ, Zheng M, Zhou Y, Wang L, Ruth Vaseghi H, Qian L, Liu J. 2017. Systematic comparison of 2A peptides for cloning multi-genes in a polycistronic vector. *Sci Rep* 7:2193. <https://doi.org/10.1038/s41598-017-02460-2>.
 66. Portolano N, Watson PJ, Fairall L, Millard CJ, Milano CP, Song Y, Cowley SM, Schwabe JW. 2014. Recombinant protein expression for structural biology in HEK 293F suspension cells: a novel and accessible approach. *J Vis Exp* 92:e51897. <https://doi.org/10.3791/51897>.
 67. Reference deleted.
 68. Reference deleted.
 69. Gastgeier E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A. 2009. Protein identification and analysis tools on the ExPASy server, p 571–607. In Walker JM (ed), *The proteomics protocols handbook*. Humana Press, Totowa, NJ.

70. Mascola JR, D'Souza P, Gilbert P, Hahn BH, Haigwood NL, Morris L, Petropoulos CJ, Polonis VR, Sarzotti M, Montefiori DC. 2005. Recommendations for the design and use of standard virus panels to assess neutralizing antibody responses elicited by candidate human immunodeficiency virus type 1 vaccines. *J Virol* 79:10103–10107. <https://doi.org/10.1128/JVI.79.16.10103-10107.2005>.
71. Seaman MS, Janes H, Hawkins N, Grandpre LE, Devoy C, Giri A, Coffey RT, Harris L, Wood B, Daniels MG, Bhattacharya T, Lapedes A, Polonis VR, McCutchan FE, Gilbert P, Self SG, Korber BT, Montefiori DC, Mascola JR. 2010. Tiered categorization of a diverse panel of HIV-1 Env pseudoviruses for assessment of neutralizing antibodies. *J Virol* 84:1439–1452. <https://doi.org/10.1128/JVI.02108-09>.
72. Todd CA, Greene KM, Yu X, Ozaki DA, Gao H, Huang Y, Wang M, Li G, Brown R, Wood B, D'Souza MP, Gilbert P, Montefiori DC, Sarzotti-Kelsoe M. 2012. Development and implementation of an international proficiency testing program for a neutralizing antibody assay for HIV-1 in TZM-bl cells. *J Immunol Methods* 375:57–67. <https://doi.org/10.1016/j.jim.2011.09.007>.
73. Gray ES, Madiga MC, Hermanus T, Moore PL, Wibmer CK, Tumba NL, Werner L, Mlisana K, Sibeko S, Williamson C, Abdool Karim SS, Morris L. 2011. The neutralization breadth of HIV-1 develops incrementally over four years and is associated with CD4+ T cell decline and high viral load during acute infection. *J Virol* 85:4828–4840. <https://doi.org/10.1128/JVI.00198-11>.
74. Moore PL, Sheward DJ, Nonyane M, Ranchobe N, Hermanus T, Gray ES, Abdool Karim SS, Williamson C, Morris L. 2013. Multiple pathways of escape from HIV broadly cross-neutralizing V2-dependent antibodies. *J Virol* 87:4882–4894. <https://doi.org/10.1128/JVI.03424-12>.
75. Doria-Rose NA, Schramm CA, Gorman J, Moore PL, Bhiman JN, DeKosky BJ, Erandes MJ, Georgiev IS, Kim HJ, Pancera M, Staupe RP, Altae-Tran HR, Bailer RT, Crooks ET, Cupo A, Druz A, Garrett NJ, Hoi KH, Kong R, Louder MK, Longo NS, McKee K, Nonyane M, O'Dell S, Roark RS, Rudicell RS, Schmidt SD, Sheward DJ, Soto C, Wibmer CK, Yang Y, Zhang Z, Mullikin JC, Binley JM, Sanders RW, Wilson IA, Moore JP, Ward AB, Georgiou G, Williamson C, Abdool Karim SS, Morris L, Kwong PD, Shapiro L, Mascola JR. 2014. Developmental pathway for potent V1V2-directed HIV-neutralizing antibodies. *Nature* 509:55–62. <https://doi.org/10.1038/nature13036>.
76. Montefiori DC. 2009. Measuring HIV neutralization in a luciferase reporter gene assay, p 395–405. *In* Prasad VR, Kalpana GV (ed), *HIV protocols. Methods in molecular biology*. Humana Press, Totowa, NJ.
77. Sarzotti-Kelsoe M, Bailer RT, Turk E, Lin C, Bilska M, Greene KM, Gao H, Todd CA, Ozaki DA, Seaman MS, Mascola JR, Montefiori DC. 2014. Optimization and validation of the TZM-bl assay for standardized assessments of neutralizing antibodies against HIV-1. *J Immunol Methods* 409:131–146. <https://doi.org/10.1016/j.jim.2013.11.022>.
78. Lee JH, Andrabi R, Su C-Y, Yasmeen A, Julien J-P, Kong L, Wu NC, McBride R, Sok D, Pauthner M, Cottrell CA, Nieuwma T, Blattner C, Paulson JC, Klasse PJ, Wilson IA, Burton DR, Ward AB. 2017. A broadly neutralizing antibody targets the dynamic HIV envelope trimer apex via a long, rigidified, and anionic β -hairpin structure. *Immunity* 46:690–702. <https://doi.org/10.1016/j.immuni.2017.03.017>.
79. Klein F, Diskin R, Scheid JF, Gaebler C, Mouquet H, Georgiev IS, Pancera M, Zhou T, Incesu RB, Fu BZ, Gnanapragasam PNP, Oliveira TY, Seaman MS, Kwong PD, Bjorkman PJ, Nussenzweig MC. 2013. Somatic mutations of the immunoglobulin framework are generally required for broad and potent HIV-1 neutralization. *Cell* 153:126–138. <https://doi.org/10.1016/j.cell.2013.03.018>.

8.5 Letter of confirmation for publications



HIV Virology Section, Centre for HIV & STI's

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17 December 2020

Postgraduate Office
Faculty of Health Sciences
University of the Witwatersrand

To whom it may concern,

Letter of Confirmation: Rebecca van Dorsten (1340837) - Manuscripts

We are writing to confirm Rebecca van Dorsten's substantial role in the below manuscripts. One has been formally published in the Journal of Virology and two are in preparation. Rebecca generated the data for these papers, analysed the data and generated all figures, and is/will be first author of these manuscripts.

- Rebecca T van Dorsten, Bronwen E Lambson, Constantinos K Wibmer, Marc S Weinberg, Penny L Moore, Lynn Morris. 2020. Neutralization Breadth and Potency of Single-Chain Variable Fragments Derived from Broadly Neutralizing Antibodies Targeting Multiple Epitopes on the HIV-1 Envelope. *Journal of Virology* 94 (2). <https://doi.org/10.1128/JVI.01533-19>.
- Rebecca T van Dorsten, Kitsch Wagh, Penny L Moore, Lynn Morris. 2020. Combinations of scFv made from HIV broadly neutralizing antibodies demonstrate high potency and breadth against a multiclade panel of viruses. (In preparation, to be submitted to Journal of Virology)
- Rebecca T van Dorsten, Lucia Reh, Alexandra Trkola, Penny L Moore, Lynn Morris. Prevention of cell-associated transmission of HIV by scFv of bNABs. (in preparation, to be submitted to Journal of Virology)

Please contact us should you require any further information.

Yours Sincerely

Prof. Lynn Morris

Head: HIV Virology Section: Centre for HIV & STI's, NICD
Research Professor: University of the Witwatersrand
Director: Antibody Immunity Research Unit (AIRU), SAMRC
Honorary Senior Scientist: CAPRISA, UKZN

Prof. Penny Moore

Reader: University of the Witwatersrand
DST/NRF South African Research Chair of Virus-Host Dynamics
Honorary Senior Scientist: CAPRISA, UKZN
Adjunct Member: Institute of Infectious Disease and Molecular Medicine, UCT

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Practice number: 5200296

8.6 Ethics clearance certificate

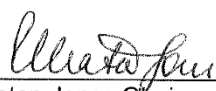


R14/49 Ms Rebecca van Dorsten et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M160341

NAME: Ms Rebecca van Dorsten et al
(Principal Investigator)
DEPARTMENT: Virology
National Institute for Communicable Diseases
PROJECT TITLE: Exploring the Potential of Engineered Antibodies
to Prevent HIV cell-cell Transmission
DATE CONSIDERED: 01/04/2016
DECISION: Approved unconditionally
CONDITIONS:
SUPERVISOR: Prof Lynn Morris

APPROVED BY: 
Professor P Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 15/04/2016

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004, 10th floor, Senate House/2nd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.** The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in March and will therefore be due in the month of March each year.

Principal Investigator Signature _____

Date _____

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

8.7 Turn it in report

Turnitin Originality Report					
Processed on: 22-Dec-2020 11:33 AM SAST ID: 1480468571 Word Count: 38067 Submitted: 1	<table border="1"> <tr> <td>Similarity Index</td> <td>Similarity by Source</td> </tr> <tr> <td>14%</td> <td> Internet Sources: 8% Publications: 11% Student Papers: 4% </td> </tr> </table>	Similarity Index	Similarity by Source	14%	Internet Sources: 8% Publications: 11% Student Papers: 4%
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