

# UNDERSTANDING THE INTERPLAY BETWEEN HIV-1 DIVERSITY, HUMORAL IMMUNE RESPONSES AND VIRAL FITNESS

---

**GAMA PETULO BANDAWE**



This dissertation is submitted in fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in the Division of Medical Virology, Department of Clinical And Laboratory Sciences in the Faculty of Health Sciences at the University Of Cape Town

**Supervisor: Professor Carolyn Williamson**

**Co-supervisor: Professor Lynn Morris**

**FEBRUARY 2014**

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

**Yo, we the reflection of our ancestors We'd like to thank you for the building blocks you left us Cause your spirit possessed us Yo, you blessed us, Thank you very much**

Talib Kweli, Reflection Eternal

**Instead, test everything. Hold on to what is good**

1 Thessalonians 5:21

**Galu wamkota sakandira pachabe**

Chichewa proverb

# CONTENTS

<b>CONTENTS</b> .....	<b>i</b>
<b>ABSTRACT</b> .....	<b>v</b>
<b>ABBREVIATIONS</b> .....	<b>vii</b>
<b>DECLARATION</b> .....	<b>x</b>
<b>ACKNOWLEDGEMENTS</b> .....	<b>xi</b>
<b>Chapter 1: Introduction and literature review</b> .....	<b>1</b>
<b>1.1 Introduction</b> .....	<b>1</b>
<b>1.2 Diversity and Global Distribution of HIV</b> .....	<b>3</b>
<b>1.3 HIV envelope structure and function</b> .....	<b>6</b>
<b>1.3.1 HIV Genome</b> .....	<b>6</b>
<b>1.3.2 Gp120</b> .....	<b>7</b>
<b>1.3.3 Gp41</b> .....	<b>12</b>
<b>1.4 Transmission and acute infection</b> .....	<b>14</b>
<b>1.4.1 Mucosal transmission</b> .....	<b>14</b>
<b>1.4.2 Characteristics of transmitted viruses</b> .....	<b>15</b>
<b>1.5 Responses to HIV infection</b> .....	<b>17</b>
<b>1.5.1 Cytotoxic T-lymphocyte (CTL) immune responses</b> .....	<b>17</b>
<b>1.5.2 Humoral responses to HIV-1 infection</b> .....	<b>20</b>
<b>1.5.3 Antibody responses and HIV-1 subtype</b> .....	<b>22</b>
<b>1.5.4 Broadly cross-neutralizing antibodies</b> .....	<b>23</b>
<b>1.6 Antibody Dependent Cell-Mediated Cytotoxicity</b> .....	<b>26</b>
<b>1.6.1 ADCC</b> .....	<b>26</b>
<b>1.6.2 ADCC in primates, humans and vaccines</b> .....	<b>27</b>
<b>1.6.3 Measurement of ADCC</b> .....	<b>28</b>
<b>1.6.4 ADCC Epitopes and escape from ADCC</b> .....	<b>30</b>
<b>1.7 Fitness and selection in viral evolution</b> .....	<b>33</b>
<b>1.8 Study rationale</b> .....	<b>35</b>
<b>Chapter 2: Differences in HIV-1 Neutralization Breadth in Two Geographically Distinct Cohorts in Africa</b> .....	<b>37</b>
<b>ABSTRACT</b> .....	<b>38</b>
<b>2.1 Introduction</b> .....	<b>39</b>
<b>2.2 Materials and Methods</b> .....	<b>40</b>
<b>2.2.1 Ethics statement</b> .....	<b>40</b>
<b>2.2.2 Participants</b> .....	<b>41</b>
<b>2.2.3 Estimation of duration of infection</b> .....	<b>41</b>

2.2.4	RT-PCR amplification and sequencing .....	42
2.2.5	Characterization of infecting virus.....	42
2.2.6	Panel viral isolates.....	43
2.2.7	Cloning of envelope genes and neutralization assays .....	43
2.2.8	Mapping of HISIS_605 plasma specificity.....	44
2.2.9	Calculation of breadth and potency scores.....	44
2.2.10	Statistical analyses .....	45
2.3	Results .....	45
2.3.1	Characteristics of the two cohorts and their infecting viruses .....	45
2.3.2	HISIS participants had greater neutralization breadth and potency .....	46
2.3.3	Preferential recognition of subtype C viruses by HISIS participants.....	47
2.3.4	Early viral loads were strong predictors of breadth at 2 years in both cohorts	48
2.3.5	HISIS cohort had higher viral loads .....	49
2.3.6	Viral characteristics and development of neutralization breadth.....	50
2.3.7	Development of autologous neutralizing antibody responses .....	51
2.3.8	Development of neutralizing antibody breadth in HISIS_605 .....	52
2.3.9	Specificity of neutralizing antibody response in HISIS_605.....	52
2.4	Discussion.....	54
<b>Chapter 3: Neutralizing Antibody Escape Pathways Constrained by Antibody-Dependent Cellular Cytotoxicity (ADCC) Responses .....</b>		<b>56</b>
Abstract .....		56
3.1	Introduction.....	58
3.2	Materials and methods .....	59
3.2.1	Study participant samples .....	59
3.2.2	Single genome amplification and sequencing .....	59
3.2.3	Sequence analysis .....	60
3.2.4	Identification of sites under positive selection pressure in CAP63.....	60
3.2.5	Cloning and generation of pseudoviruses .....	60
3.2.6	Cell lines.....	61
3.2.7	HIV-1 <i>env</i> pseudovirus production and titration.....	61
3.2.8	Neutralizing antibody assays .....	61
3.2.9	Construction of infectious molecular clones (IMCs) .....	62
3.2.10	Mutagenesis of envelope and infectious molecular clones.....	62
3.2.11	Generation of virus stocks from infectious molecular clones .....	62
3.2.12	Titration of virus stocks from infectious molecular clones .....	62
3.2.13	Infection of CEM.NKR <sub>CCR5</sub> Cell Line with HIV-1 IMC .....	63
3.2.14	Intracellular p24 staining of infected CEM NKR CCR5 cells.....	63

3.2.15 Recombinant gp120 HIV-1 Proteins .....	64
3.2.16 ADCC-GranToxiLux (ADCC-GTL) assay.....	64
3.3 Results.....	65
3.3.1 Evidence of early changes in Env .....	65
3.3.2 Early adaptations in CAP63 .....	67
3.3.3 Early neutralizing antibody responses in CAP63 .....	70
3.3.4 Early ADCC responses in CAP63 .....	70
3.3.5 Changes in V4 affected neutralization sensitivity .....	72
3.3.6 V4 neutralizing antibody mutations facilitate escape from ADCC responses in CAP63 .....	74
3.3.7 The 6D_391 deletion escapes neutralizing antibody responses but becomes highly sensitive to ADCC.....	75
3.3.8 ADCC activity constrains pathways of escape from neutralizing antibody responses .....	77
3.4 Discussion .....	78
<b>Chapter 4: The effects of early evolution on viral fitness.....</b>	<b>83</b>
Abstract .....	84
4.1 Introduction.....	85
4.2 Materials and methods .....	85
4.2.1 Mutagenesis of cloned envelopes .....	85
4.2.2 Cell-cell fusion assay .....	86
4.2.3 Titration of viral stocks for entry and replication assays by beta gal staining.....	86
4.2.4 Replication assay .....	87
4.2.5 p24 ELISA .....	87
4.3 Results.....	88
4.3.1 Viruses from CAP63 show reduced fusion capacity within 11 weeks relative to the T/F virus .....	88
4.3.2 Deletions in V4 contribute to reduced fusion capacity in CAP63 .....	89
4.3.4 Point mutations in the gp41 are preferentially fixed outside the LLP domains and have no effect on fusion capacity.....	90
4.3.5 Mutations in the V4 region of CAP63 result in reduced infectivity of virus stock.....	91
4.3.6 Adaptations in the V4 region of CAP63 affect viral replicative capacity in PBMC.....	92
4.4 Discussion .....	94
<b>Chapter 5: Conclusions .....</b>	<b>96</b>
<b>APPENDICES .....</b>	<b>98</b>
Appendix A: Standard RNA and DNA techniques.....	99
Appendix B: Reagents and buffers.....	102
Appendix C: Viral load data .....	103

<b>Appendix D: Neutralization assay data .....</b>	<b>104</b>
<b>Appendix E: HIV-1 subtype C envelope, full length and sequencing primers .....</b>	<b>105</b>
<b>Appendix F: Mutagenesis primers .....</b>	<b>107</b>
<b>Appendix G: Titration of IMC virus stocks for infection of CEM NKR<sub>CCR5</sub> cells .....</b>	<b>108</b>
<b>Appendix H: Determination of the optimal length of infection for target cells with IMCs .....</b>	<b>110</b>
<b>Appendix I: Cell-cell fusion capacity of CAPRISA envelopes from acute and chronic infection .....</b>	<b>111</b>
<b>Appendix J: Replication assay slope calculations .....</b>	<b>112</b>
<b>REFERENCES.....</b>	<b>113</b>

## ABSTRACT

HIV-1 antibody dependent cell cytotoxicity (ADCC) and neutralizing antibody (nAb) responses are both thought to be important responses to elicit through vaccination. This thesis characterises neutralizing response in two cohorts in Africa, and in a detailed study of one individual, elucidates the interplay between ADCC and nAb responses in early infection, and the impact of humoral escape on viral fitness.

It remains an open question whether different geographically distinct population groups vary in their neutralization responses to HIV-1. We compared neutralizing antibody responses in two African cohorts and found 35% of the Tanzanians in the HIV Superinfection Study (HISIS) cohort had neutralization breadth (neutralized >50% of panel viruses) at two years post infection compared to only 9% in the Centres for AIDS Program of Research in South Africa (CAPRISA) cohort. Cumulative viral loads between 3 and 12 months post infection were strongly associated with neutralization breadth ( $p < 0.001$ ), and were higher in the Tanzanian cohort ( $p = 0.046$ ). No association was found between breadth and dual infection, subtype or features of the envelope. One elite neutralizer was identified in the HISIS cohort with responses targeting the CD4 binding site.

While neutralizing antibodies are considered central for protection from infection, ADCC activity correlated with reduced risk of HIV-1 acquisition in the RV144 trial. There is limited understanding of the overlapping ADCC and neutralizing antibody functions in early infection. We investigated the kinetics and targets of both responses in one individual from CAPRISA. ADCC responses were detected 4 weeks post infection, with nAbs responses emerging at 7 weeks post infection. We identified five neutralization escape patterns in the V4 region of the envelope by 11 weeks post-infection. Four of these also conferred ADCC escape; however the fifth neutralization escape variant resulted in increased sensitivity to ADCC. This variant was eliminated in vivo by 29 weeks post infection.

Finally, we studied the effect of neutralizing and ADCC antibody escape mutations on the virus' ability to mediate fusion, infectivity and replicative fitness. Envelopes bearing immune escape adaptations had lower cell-cell fusion ability compared to the T/F virus. The mutations also resulted in reduced infectivity of infectious molecular clone virus stocks. However, only

the largest deletion in the V4 caused reduced growth in peripheral blood mononuclear cells (PBMC).

In conclusion, the study finds that neutralizing antibody responses are influenced by community viral loads. The study defined the first ADCC epitope reported in the V4 region, and describes overlapping targets for ADCC and neutralizing antibodies. Where neutralization escape resulted in increased ADCC sensitivity, this was a dead end escape pathway. Finally we find that early V4 escape from both ADCC and nAb responses had a fitness impact on the virus. We thus demonstrate a mechanism through which ADCC and neutralizing antibodies can synergistically influence viral evolution and potentially produce a protective immune response.

**This study has resulted in the following publication:**

\*Nofemela, A., \***Bandawe, G. P.**, Thebus, R., Marais, J., Wood, N., Maboko, L., Hoelscher, M., Woodman, Z., Williamson, C. Defining the Human Immunodeficiency Virus type 1 transmission genetic bottleneck in a region with multiple circulating subtypes and recombinant forms *Virology* 415 (2011) 107–113

(\* These two authors contributed equally to this work)

The remainder of the work herein is currently in preparation for publication in the form of two manuscripts, one encompassing chapter 2 and the other comprising chapters 3 and 4.

**ABBREVIATIONS**

aa	amino acid
ADCC	antibody-dependent cellular cytotoxicity
AIDS	acquired immune deficiency syndrome
AUC	area under the curve
bp	base pair(s)
°C	degrees Celsius
CAPRISA	Centre for the AIDS Programme of Research in South Africa
CCR5	chemokine receptor 5
cDNA	copy DNA
CRF	circulating recombinant form
CRFs	circulating recombinant forms
CTL	cytotoxic T lymphocytes
CXCR4	CXC chemokine receptor
DEAE	diethylaminoethyl
dH <sub>2</sub> O	distilled water
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dN	number of non-synonymous substitutions
dS	number of synonymous substitutions

ds	double stranded
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Env	envelope
Gp120	120kDa envelope glycoprotein
Gp41	41kDa envelope glycoprotein
HEK	human embryonic kidney
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HTA	heteroduplex tracking assay
IFN	interferon
IL	interleukin
IMC	infectious molecular clone
kb	kilobases
kDa	kilodaltons
LB	Luria-Bertani broth
LLP	lentiviral lytic peptides
LTNP	long-term non-progressor
M	Molar
µg	micrograms
µL	microliters
mg	milligrams
MgCl <sub>2</sub>	magnesium chloride
MHA	multi-region hybridization assay
MHC	major histocompatibility complex
min	minutes
mL	millilitres
mM	millimolar
MPER	membrane proximal external region
mRNA	messenger RNA
NaOH	sodium hydroxide
NaCl	sodium chloride
ng	nanograms

NICD	National Institute for Communicable Diseases
NK cells	natural killer cells
NKR	natural killer cell resistant
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	hydrogen potential
PHA	phytohaemagglutinin
PNGs	N-linked glycosylation site
pol	polymerase
pVL	plasma viral load
RLU	relative luminescence units
RNase	ribonuclease
rpm	revolutions per minute
SGA	single genome amplification
SIV	simian immunodeficiency virus
ss	single stranded
Tat	transcriptional transactivator protein
TAE	Tris-acetic acid EDTA (Tris acetate)
T/F	transmitted/founder
TM	transmembrane
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
UCT	University of Cape Town
UNAIDS	Joint United Nations Programme on HIV/AIDS
USA	United States of America
UV	ultraviolet
V	volts
Vpu	viral protein u
Vpr	viral protein r
VL	viral load
YT	yeast-tryptone

## **DECLARATION**

The work presented in this thesis was done at the Division of Medical Virology, Institute for Infectious Disease and Molecular Medicine at the University of Cape Town (UCT) under the supervision of Professor Carolyn Williamson and that neither the whole work nor any part of it has been, is being or is to be submitted for another degree in this or any other university. This work is all original and my own. Where use has been made of work of others, their contribution has been acknowledged in the text.

I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signed by candidate

Gama Petulo Bandawe

February 2014

## **ACKNOWLEDGEMENTS**

I would like to extend my deepest gratitude to Professor Carolyn Williamson. I have learned and grown immeasurably under her guidance both as my employer and my supervisor. It has been a privilege and a blessing to come under the mentorship of someone as competent, accomplished and generous.

I am grateful to my co-supervisor Professor Lynn Morris for your helpful and candid input, advice and support. I wish to thank Dr Darren Martin for his assistance in reading the manuscript as well as his encouragement. I wish to thank Melissa-Rose Abrahams, Ruwayhida Thebus, Penny Moore, Daniel Sheward, Jinny Marais and Florette Treurnicht for their assistance with much of the lab and technical work. To Andile Nofemela, thank you for walking much of this path hand in hand with me.

I wish to thank Dr Guido Ferrari for allowing me to spend time in his laboratory. He was an amazingly kind and gracious host and left a lasting impression. My thanks also go to Dr Justin Pollara and Kaylan Whitaker for their assistance at Duke. I am grateful to Jenifer L Spencer as well as Hongmei Gao and Kelli Greene for their assistance.

To my mother Angelina Thandiwe Bandawe and my father Gamaliel Petro Bandawe, thank you for all that I am. Your greatest sacrifice was to put the education of your children first and I am here as a result of that. Thank you for having faith in my abilities and for allowing me to be my true self, always. Professor Chiwoza Bandawe, thanks for your presence and strength in my darkest hour. To Mayola Bandawe and Tapuwa Bandawe, thank you for the love and friendship only a brother can provide. To all my friends scattered across the world, I cannot thank you enough.

I would like to also thank the following groups and organizations without whom, this work would not have been possible. Participants from the Centre for AIDS Programme of Research (CAPRISA) Acute Infection Study, Participants from the HIV Superinfection (HISIS) Study in Mbeya Tanzania, The Poliomyelitis Research Foundation (PRF), The Bill and Melinda Gates Foundation, The Carnegie Corporation of New York, The Clinical Infectious Diseases Research Initiative (CIDRI), The Canada AIDS Prevention Trials (CAPT) Network and the Columbia University-Southern Africa Fogarty AIDS Research Training Programme.

Last but not least, thank you to **Linda Malilo** my fiancée, best friend and partner for life.  
This is ours.

## Chapter 1: Introduction and literature review

<b>1.1 Introduction.....</b>	<b>1</b>
<b>1.2 Diversity and Global Distribution of HIV .....</b>	<b>3</b>
<b>1.3 HIV envelope structure and function .....</b>	<b>6</b>
1.3.1 HIV Genome .....	6
1.3.2 Gp120.....	7
1.3.3 Gp41.....	12
<b>1.4 Transmission and acute infection .....</b>	<b>14</b>
1.4.1 Mucosal transmission.....	14
1.4.2 Characteristics of transmitted viruses.....	15
<b>1.5 Responses to HIV infection .....</b>	<b>17</b>
1.5.1 Cytotoxic T-lymphocyte (CTL) immune responses.....	17
1.5.2 Humoral responses to HIV-1 infection .....	20
1.5.3 Antibody responses and HIV-1 subtype.....	22
1.5.4 Broadly cross-neutralizing antibodies .....	23
<b>1.6 Antibody Dependent Cell-Mediated Cytotoxicity .....</b>	<b>26</b>
1.6.1 ADCC .....	26
1.6.2 ADCC in primates, humans and vaccines .....	27
1.6.3 Measurement of ADCC.....	28
1.6.4 ADCC Epitopes and escape from ADCC.....	30
<b>1.7 Fitness and selection in viral evolution.....</b>	<b>33</b>
<b>1.8 Study rationale .....</b>	<b>35</b>

## **1.1 Introduction**

The human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS). Over 33 million people worldwide are infected and over 4 million new infections occur every year (UNAIDS, 2012). Although antiretroviral therapy is saving many lives, new infections are greatly outpacing the number of people able to be treated through current global efforts. There is a critical need for developing a vaccine to prevent new infections. The development of a safe and effective HIV vaccine poses one of the greatest medical and scientific challenges of the 21st century. Difficulties facing the search for a vaccine include the lack of correlates of protective immunity, the capacity of the virus to integrate its genome into cells of the immune system, and the unprecedented hyper-variability of HIV (Barouch & Korber, 2010). While cellular immune response may be important in protecting individuals from HIV infection and disease, most successful vaccines to date are thought to protect through induction of neutralizing antibodies (Girard & Plotkin, 2012). Due to the variability of HIV, a vaccine will need to induce broadly neutralizing antibodies (bNAbs) capable of blocking many different HIV subtypes. Recent correlates of risk analysis on the RV144 trial, the first HIV vaccine trial to show protection, have also suggested a role for non-neutralizing antibodies in preventing infection (Haynes, Gilbert, et al., 2012).

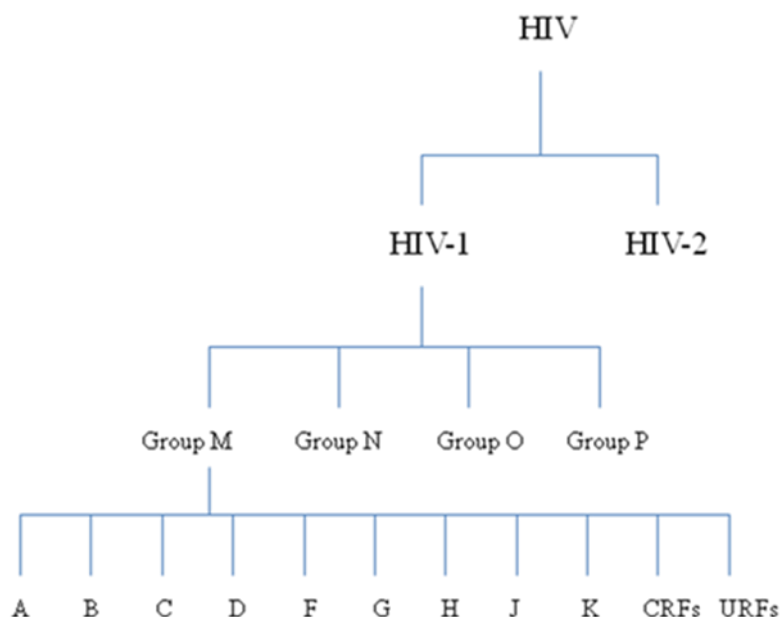
There currently are no candidates in the pipeline that elicit bNAbs against HIV, and the path towards the development of such a candidate appears to be a very long one. Neutralizing antibody responses have been well studied in subtype C infections from Southern Africa and in subtype B infections from the US and Europe. However, the development of bNAbs in other regions with non-subtype B and non-subtype C infections have been understudied. The role of non-neutralizing antibodies in protection and viral control has also been highlighted by the abovementioned vaccine trial and in infection but little is known about how these responses affect viral evolution in natural infection. Understanding the very earliest responses associated with control of viral replication and looking at patterns of escape in the virus may provide further information on the role of neutralizing and non-neutralizing antibodies in viral evolution.

This study seeks to begin to address both of the abovementioned issues and in so doing, the first aim of this thesis is to compare the evolution of neutralizing antibody responses in an exclusively subtype C infected South African cohort with that of a Tanzanian cohort infected with multiple HIV subtypes and recombinants. The second aim is to investigate early humoral responses associated with changes in the viral envelope of a subtype C infected individual and investigate the role of both neutralizing and non-neutralizing antibody responses in driving HIV evolution in early infection. Finally, this study aims to assess the effect of changes induced by early humoral responses on viral fitness. Consequently, the study aims to investigate the interplay between HIV diversity, humoral immune responses and viral fitness as understanding how these factors affect viral evolution and development of antibody responses during the crucial early stages of infection following transmission is critical for informing vaccine design.

This review will provide a basic overview of HIV diversity, the structure and function of the envelope, early immune responses to HIV infection with emphasis on antibodies, and will also discuss some concepts relating to viral fitness.

## 1.2 Diversity and Global Distribution of HIV

The human immunodeficiency viruses (HIV) have diverse origins with HIV-1 thought to have been transmitted to humans from chimpanzees (*Pan troglodytes troglodytes*) found in central Equatorial Africa. HIV-2 came from sooty mangabey monkeys (*Cercocebus atys atys*) from West Africa (Sharp & Hahn, 2010). There have been at least four zoonotic events involving HIV-1 resulting in groups M, N, O (from chimpanzees) and P (from gorillas) (Sharp & Hahn, 2010). HIV-1 group M has given rise to the AIDS pandemic that has infected around 60 million individuals to date (UNAIDS, 2012). It is estimated that the most recent common ancestor of HIV-1 group M was introduced into humans approximately 100 years ago as a founder infection in a single individual (Korber et al., 2001). It gave rise to the HIV-1 group M subtypes (also called clades) A1, A2, B, C, D, F1, G, H, J, and K. These subtypes subsequently gave rise to unique recombinant forms (URFs) as well as circulating recombinant forms (CRFs), some of which became established very early in the epidemic (e.g. CRF01\_AE, CRF02\_AG, CRF04\_cpx; Figure 1.1; Sharp & Hahn, 2010).



**Figure 1. 1:** Classification of HIV into two types HIV-1 and HIV-2, with HIV-1 divided into 4 groups with the Group M comprising of 9 subtypes and 55 circulating recombinant forms (CRFs) and unique recombinant forms (URFs; World Health Organization 'HIV/AIDS' 2011).

Subtypes or clades are classified based on phylogenetic clustering such that the individual genes throughout the viral genome are more closely related to each other than sequences from other subtypes. Proteins expressed by HIV can vary by 5-25% within a single HIV clade and by 10-40% between different clades (HIV sequence compendium; Kiuken et al., 2010). In the viral envelope, sequences from the same subtype can have up to 30% genome diversity (Robertson et al., 2000). Full-length genome sequencing has highlighted the degree to which recombination contributes to subtype diversity globally. Like other retroviruses, HIV undergoes frequent genetic recombination when two strains infect the same host cell. This process has given rise to new epidemic viruses caused by CRFs which resemble subtypes but have hybrid or mosaic genomes whose origins are designated by two or more parental subtypes (Hemelaar et al., 2011).

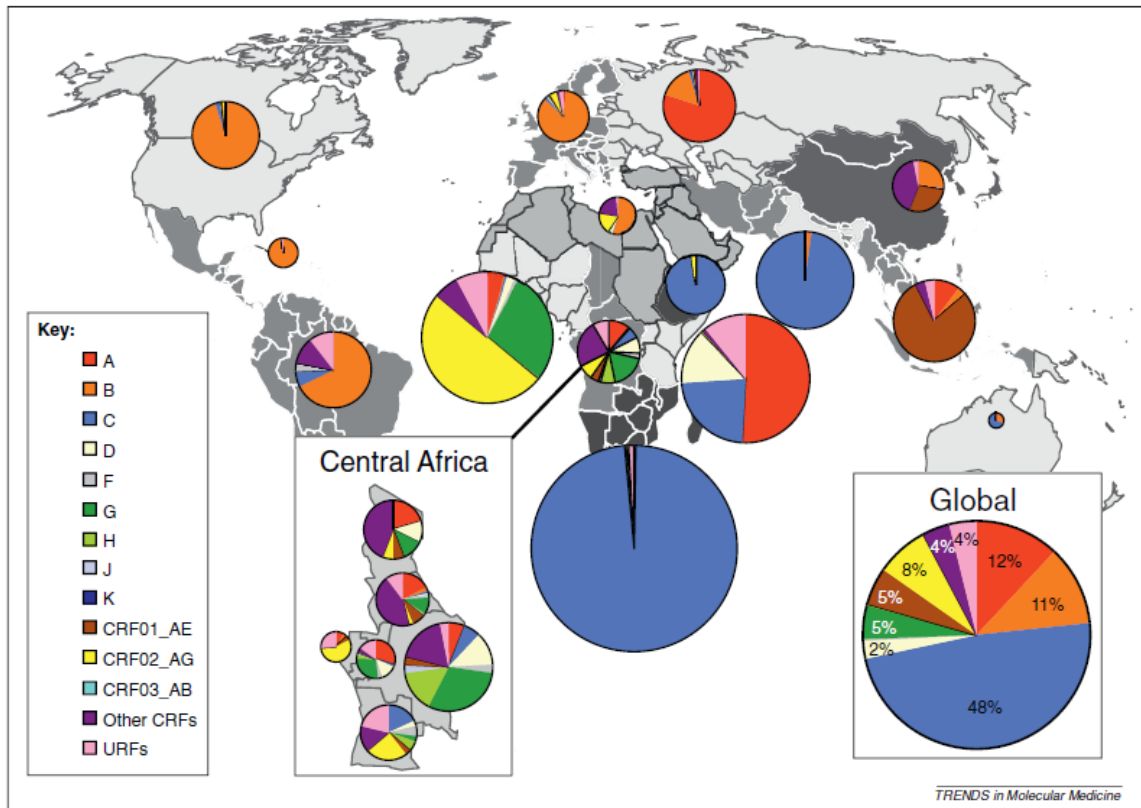
A virus is termed a CRF if it can be identified in at least three epidemiologically unlinked individuals (McCutchan, 2006; Peeters, 2001; Robertson et al., 2000). At least 55 CRFs have been identified to date (Los Alamos National Laboratory HIV Sequence Database, 2013). They are identified by number in ascending order according to the time of discovery, followed by the letters of the parental subtypes. Where recombinant sequences from only a single individual is available, the virus is designated a unique recombinant form (URF;

Ragupathy et al., 2011; Robertson et al., 2000). When viruses are comprised of more than two subtypes, they are termed complex CRFs or URFs (CRF<sub>cpx</sub> and URF<sub>cpx</sub>). These recombinants are common in regions where multiple subtypes are circulating in the local epidemic such as West Central Africa and East Africa, the latter of which is the location of one of the cohorts studied in this thesis.

With the exception of Africa, which is home to every subtype, other parts of the globe show a very clear distribution of HIV-1 subtypes (Hemelaar et al., 2006; Hemelaar et al., 2011). These patterns are mainly attributed to founder effects with migration routes where a single virus is introduced and subsequently spread. This pattern is being increasingly changed by global travel and the mixing of different epidemics (Buonaguro et al., 2008; Perrin et al., 2003). The most prevalent subtypes in the world are subtypes A, B and C, with C alone being responsible for approximately 50% of global infections (Hemelaar, 2012); subtypes A and B account for approximately 12% and 11% of infections respectively (Figure 1.2). Subtype A is predominant in West, Central and East Africa (Kenya, Uganda, Tanzania, Rwanda and Burundi) as well as Eastern Europe (Bobkov et al., 2004). Subtype B is the main subtype in Western and Central Europe, North and South America, and Australia. Subtype B is also prevalent in Southeast Asia, North Africa and the Middle East. In South Africa during the late 1990s, subtype B was mainly found among men who have sex with men (MSM). This is changing where there is evidence that the MSM and heterosexual epidemics are mixing (Middelkop et al., 2013 submitted). Subtype C is predominant in southern Africa and India, which together are home to 80% of the epidemic (Hemelaar, 2012). Of the less prevalent subtypes, subtype G accounts for 5% of the global infections, while subtype D makes up about 2% and is found in Central and East Africa including in Tanzania. The remaining subtypes F, H, J and K together account for less than 1% of global infections (Figure 1.2).

Recombinant viruses have become increasingly prevalent in the global HIV-1 pandemic with CRFs accounting for 20% of infections worldwide (Hemelaar, 2012). Two CRFs, CRF01\_AE and CRF02\_AG, are predominant in Southeast Asia and West Central Africa respectively and account for about half of all global recombinant infections (Tebit & Arts, 2011). However, in regions where multiple subtypes circulate, 30% of infections are made up of unique recombinants (Peeters et al., 1999, reviewed by Tebit & Arts, 2011). The global distribution of CRFs is broadly stable. However, because recombination and mutation are features of the

virus, increased ease of global travel could maintain or increase the importance of recombinant viruses in the epidemic.



**Figure 1.2:** Global distribution of HIV-1 subtypes and recombinants. Pie charts represent the distribution of HIV-1 subtypes and recombinants from 2004 to 2007 in each region. The relative surface areas of the pie charts correspond to the relative numbers of people living with HIV in the regions. The colours representing the different HIV-1 subtypes and recombinants are indicated. The HIV-1 subtype distributions found around the world and within Central African countries are shown in the insets of the main figure, as indicated. Reproduced and adapted with permission from (Hemelaar et al., 2011).

## 1.3 HIV envelope structure and function

### 1.3.1 HIV Genome

The HIV genome is made of single-stranded RNA. It is approximately 9.8 kilobases long and is composed of nine genes that code for 15 proteins. There are three structural or enzyme polyproteins whose products are essential components of the viral particle: Gag, Env, and Pol. The Gag polyprotein is cleaved into p17, p24, p7 and p6. The Env polyprotein is further processed into gp120 and gp41. The Pol polyprotein is processed into four enzymes, namely

integrase, reverse transcriptase, protease and RNase-H. Two regulatory proteins (Tat and Rev) and four accessory proteins (Nef, Vif, Vpr and Vpu) (HIV sequence compendium 2008) are also encoded in the genome. The genome has two long terminal repeat units (LTR) at the unique 5' (U5) and unique 3' (U3) ends that have control elements required for transcription of the viral genome (el Kharroubi & Martin, 1996).

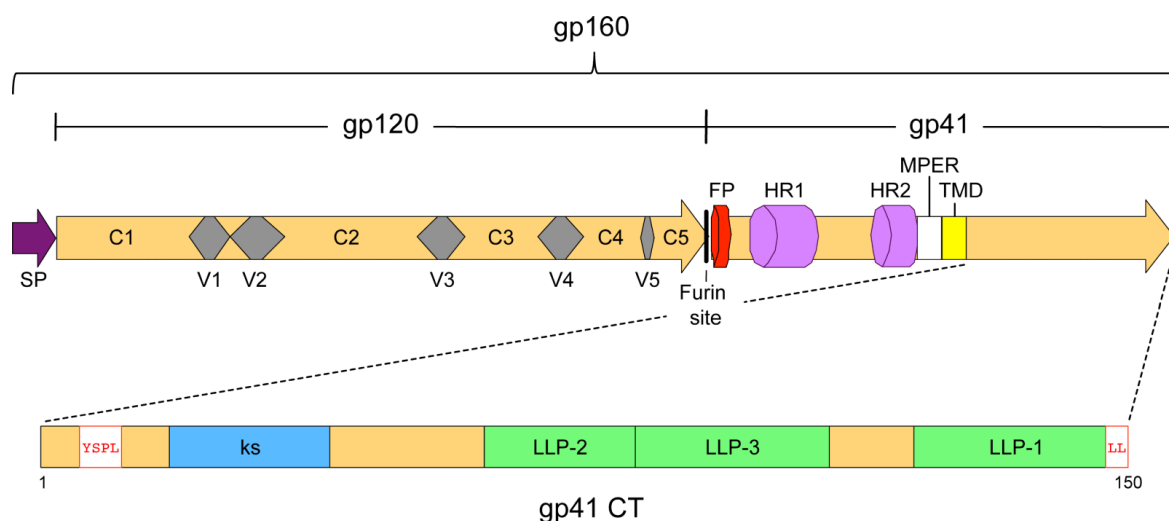
The *env* gene evolves at a particularly high rate (1–2% per year) at the population level (Korber et al. 2000). Consequently, *env* is highly genetically diverse, posing a significant challenge to vaccine development. The HIV envelope mediates viral tropism, determines viral fitness (Marozsan et al., 2005) and is targeted by humoral immune responses which include non-neutralizing and neutralizing antibodies. These humoral immune responses are important drivers of *env* evolution and are the main selective pressures analysed in this study.

### 1.3.2 Gp120

The surface of HIV contains Env spikes that engage the cellular receptors for entry into target cells. Each envelope spike consists of a trimer of gp160 proteins, which in turn makes a gp120 exterior subunit and a gp41 transmembrane unit (Wyatt & Sodroski, 1998). The gp120 subunit of the *env* gene has five variable domains (V1-V5) which are interspersed within five relatively constant domains (C1-C5, Fig. 1.3; Starcich et al., 1986; Willey et al., 1986).

About 18 highly conserved cysteine residues located throughout gp120 and gp41 form nine intramolecular disulfide bonds that are crucial to the formation of Env tertiary structure (Leonard et al., 1990) and its antigenicity (Gao et al., 1996; Jobes et al., 2006). Two disulphide bonds separate V1 from V2 and V3 from V4 (Leonard et al., 1990).

The envelope glycoprotein is a highly glycosylated protein with up to half of the molecular mass of gp120 composed of *N*-linked glycans (Allan et al., 1985), with a small additional contribution from *O*-linked sugars (Bernstein et al., 1994). A gp120 molecule has between 20 and 35 *N*-linked glycosylation sites, while gp41 has 3-5. Glycans serve several functions including masking Env from host immune recognition (Montefiori et al., 1988), contributing to Env folding (Li et al., 2008) and helping virions bind to the host cell surface (Raska & Novak, 2010).



**Figure 1.3:** Domains of HIV-1 Env. Precursor gp160 contains the signal peptide (SP), which is cleaved during translation. The remaining precursor is cleaved at the furin site into the surface subunit (gp120) and transmembrane subunit (gp41) in the Golgi complex. Subunit gp120 contains five variable domains (V1-V5) and five constant domains (C1-C5). Subunit gp41 consists of the fusion peptide (FP), heptad-repeats (HR1 and HR2), the membrane-proximal external region (MPER), a transmembrane domain (TMD), and a cytoplasmic tail (CT). An enlarged representation of the gp41 CT is shown to highlight several motifs: the internalization signal YSPL, the Kennedy sequence (KS), the amphipathic  $\alpha$ -helices LLP-1, -2, -3, and a C-terminal dileucine motif (LL) involved in endocytosis and intracellular distribution of Env (Checkley et al., 2012).

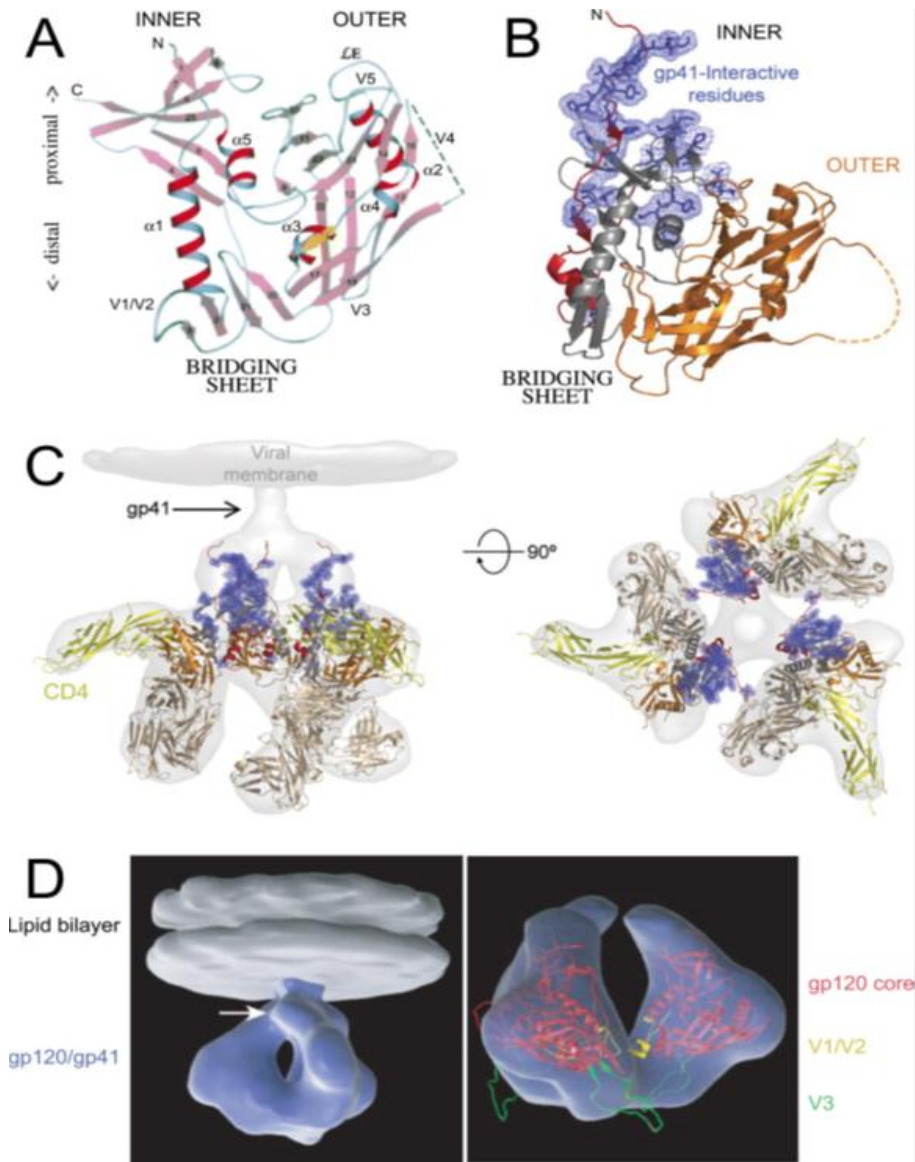
The V1V2 domain is the most variable in loop length and number of glycosylation sites (Chohan et al., 2005; Kitrinis et al., 2003; Masciotra et al., 2002; Palmer et al., 1996; Sagar et al., 2006; Shioda et al., 1997). The V1V2 loop can range in length from 50 to 90 amino acids (aa), while the length variation of V4 and V5 loops ranges from 19 to 44 aa and 14 to 36 aa, respectively. Compared to V1V2, V4 and V5, the V3 loop and C2, C3, and C4 domains show relatively little length variation (Checkley et al., 2012).

HIV enters cells via the CD4 receptor (Figure 1.4 C; McDougal et al., 1986) and a co-receptor, usually CCR5 (Alkhatib et al., 1996) or CXCR4 (Doranz et al., 1996). The crucial CD4 receptor binding site (CD4bs) consists of a conserved pocket-like structure formed from conserved residues in discontinuous segments that are folded into proximity in the Env tertiary structure between the three gp120 units. It is flanked by variable loops and the chemokine co-receptor binding site for CXCR4 and/or CCR5 that is exposed and/or formed

after virus attachment to cells (Benjelloun et al., 2012). The conserved C1, C3 and C4 domains within gp120 are the principle Env determinants that bind CD4 (Lasky et al., 1987). CD4 is observed to directly contact 26 residues (Kwong et al., 1998) that are widely dispersed in gp120 (Cordonnier et al., 1989; Kowalski et al., 1987; Olshevsky et al., 1990). The exposed variable domains on the gp120 surface do not have a direct role in CD4 binding. The gp120 variants with deleted V1, V2, and V3 domains still bind to CD4 with high affinity (Pollard et al., 1992; Wyatt et al., 1993).

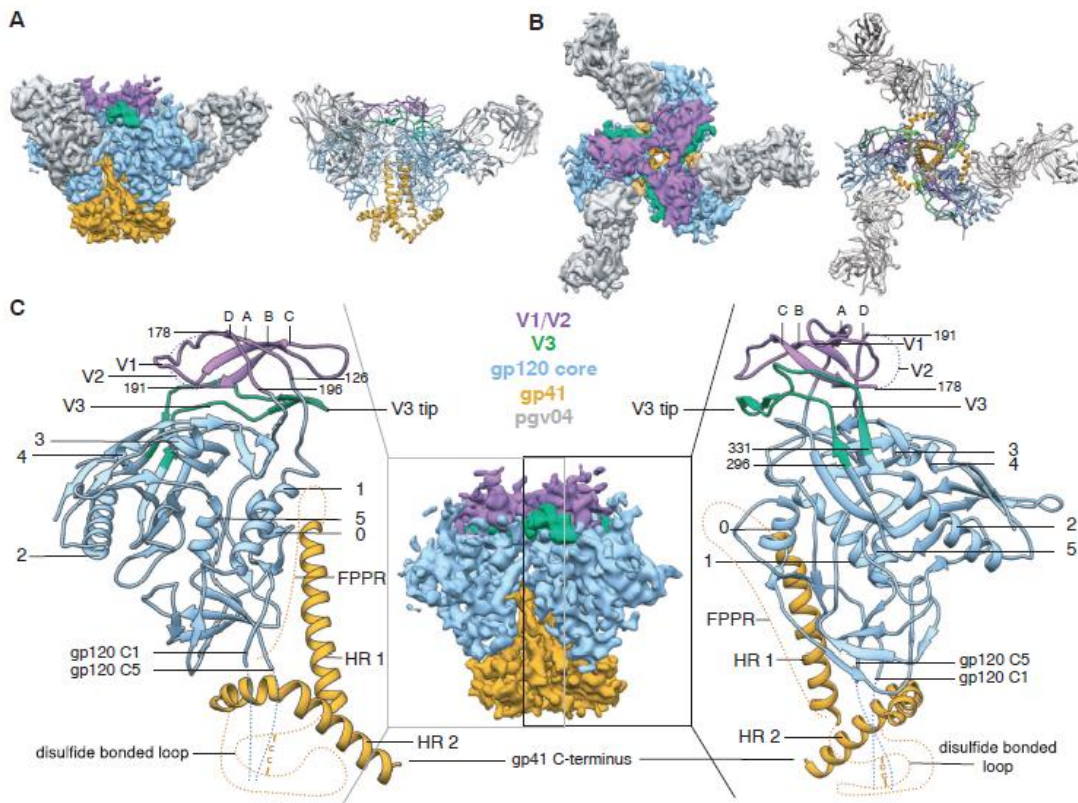
The V3 loop is important for membrane fusion (Freed et al., 1991) and co-receptor specificity (Cann et al., 1992; Chesebro et al., 1991; Hwanget al., 1991; O'Brien et al., 1990; Shioda et al., 1991). It also contains dominant epitopes recognized by neutralizing antibodies (Goudsmit et al., 1988; Matsushita et al., 1988; Palker et al., 1988; Rusche et al., 1988). Transmitted viruses almost always have the ability to utilize the CCR5 co-receptor (R5 viruses), however some viruses switch to using the CXCR4 co-receptor (X4). The switch from R5 to X4 tropism is driven by V3 mutations and has been linked to an increase in the net positive charge of V3, allowing an interaction with the negatively charged surface of CXCR4 (Fouchier et al., 1992; Pollakis et al., 2004; T Shioda et al., 1994).

The structure of gp120 has been solved largely through X-ray crystallography of the HIV-1 gp120 “core” (from which N- and C-termini and V1/V2 and V3 variable loops were deleted) either “unliganded” or in complex with various ligands or fragments such as CD4, V3 loop, various binding and neutralizing antibodies and the gp120-interacting portion of gp41 (Chen et al., 2005; Chen et al., 2009; Huang et al., 2005; Kwong et al., 1998; Mao et al., 2013; Pancera et al., 2010; Wu et al., 2010; Zhou et al., 2007). The overall fold of CD4-bound gp120 consists of an “inner” domain and an “outer” domain (based on their orientation in the trimeric complex) linked by a four-stranded “bridging sheet” (Fig. 4A, B; Kwong et al., 1998). A comparison of CD4-bound and unliganded gp120 structures suggests that gp120 undergoes a remarkable degree of refolding following CD4 binding. This large CD4-induced conformational change results in the formation of the coreceptor binding surface from residues that were spatially well separated prior to CD4 binding (Chen et al., 2005).



**Fig 1.4:** HIV-1 Env gp120 and gp41 structures. (A) Ribbon diagram of gp120 core containing  $\alpha$  - helices ( $\alpha$  1-5) and  $\beta$  - strands (1-25) with relative positions of variable loops (V1-V5) and N and C-termini shown. The orientation of gp120 in this diagram places the viral membrane toward the top and the cell membrane toward the bottom. When gp120 is bound to CD4 it forms a “bridging sheet” consisting of four  $\beta$  -strands, which separates the inner and outer domains of gp120 relative to their orientation in the trimeric complex. Image reprinted by permission from Macmillan Publishers Ltd: Nature, copyright 2008. (B) Ribbon diagram of gp120 core (as in panel A) with N- terminus (red) and gp41 interaction site (blue) shown. The inner domain is shown in red and grey and the outer domain is shown in orange. The bridging sheet, which shares elements from both inner and outer domains, is in grey and orange. (C) Trimeric gp120 (same colours as in panel B) bound to three molecules of CD4 (yellow) and Fab from neutralizing antibody 17b (brown), used to stabilize the gp120 structure, superimposed onto the electron density observed by cryoelectron tomography (light grey). Orientation of this structure rotated 90°, which places the viral membrane in the plane of the page, is also shown on the right. Images (panels B, C) reproduced from Pancera *et al.*, with permission. (D) Three-dimensional representation of HIV-1 Env in its CD4- bound conformation. (Left) A trimeric Env spike (blue) anchored in the lipid bilayer of the viral membrane (grey) is shown. The white arrow indicates the predicted location of gp41. (Right) Ribbon diagram of gp120 core (red) superimposed on the density map (blue) with V1/V2 loop (yellow) and V3 loop (green) shown. Reprinted by permission from Macmillan Publishers Ltd: Nature, copyright 2008.

Recently, the structure of the Env trimer has been resolved (Figure 1.5). Sanders et al., (2002) developed gp140 trimers from the BG505 phenotype truncated at residue 664, which are antigenically near-native, soluble, proteolytically cleaved trimer variants stabilized by the presence of an engineered intermolecular disulfide bond (termed SOS). This binds gp120 and gp41 and creates a single residue change in I559P within gp41 (termed IP). These structures known as BG505 SOSIP.664 gp140 trimers have enabled independent, fine resolution of the closed (unbound) pre-fusion native structures of the trimer by X-ray crystallography (Julien et al., 2013) and cryo-electron microscopy (Bartesaghi, 2013). Previously uncharacterized elements in the trimer were observed on these structures, such as the gp41 helices, the V1/V2/V3 loops and various glycans (Julien et al., 2013; Lyumkis et al., 2013; Figure 1.5).



**Figure 1.5:** 5.8 Å EM reconstruction and model of Env trimer in complex with PGV04. (A and B) Side (A) and top (B) views of BG505 SOSIP trimer EM reconstruction (left) and corresponding model (right). Segmentation and color coding: gray, PGV04; blue, gp120; orange, gp41; purple, V1/V2; green, V3. (C) The center panel shows a side view of the EM map along with the Fab density removed. The outer panels show the modeled portion corresponding to the boxed region in the middle panel. The viral membrane would be at the bottom of the figure (Lyumkis et al., 2013).

### 1.3.3 Gp41

The gp41 transmembrane (TM) glycoprotein mediates fusion of the viral envelope with host cell membrane. The gp41 subunit is comprised of around 345 amino acids and is organized into three major domains: an extracellular domain (or ectodomain), a transmembrane domain (TMD), and a C-terminal cytoplasmic tail (CT) (reviewed by Checkley et al., 2012; Fig.1.2). The extracellular domain contains the major fusion determinants. These include: an N-terminal hydrophobic region known as the fusion peptide (Bosch et al., 1989; Freed et al., 1992; Freed et al., 1990); a polar region; two hydrophobic regions that form  $\alpha$ -helical coiled-coil structures referred to as the heptad-repeat regions HR1 and HR2 (also known as N-helix and C-helix, respectively; Chan et al., 1997; Dubay et al., 1992; Pancera et al., 2010; Tan et al., 1997; Weissenhorn., 1997); and a Tryptophan-rich domain referred to as the membrane-proximal external region (MPER; Muñoz-Barroso et al., 1999; Salzwedel et al., 1999). HR1 and HR2 are connected by a disulfide-bridge within a hydrophilic C-C loop and the fusion process is largely driven by their interaction. The fusion peptide is lodged deep in the envelope spike where gp120 and gp41 interact but becomes exposed following binding of gp120 to CD4 and a coreceptor. This binding changes the structure of the envelope spike allowing penetration into target cell membranes by the fusion peptide of gp41. This causes membrane destabilization and formation of the fusion pore. Three HR1 motifs form a core bundle in parallel and fold over a hydrophobic groove antiparallel to three HR2 domains within each trimer, thus forming a stable six-helix bundle that brings the viral and cell membranes into close enough proximity for fusion to occur (Chan et al., 1997 reviewed by Checkley et al., 2012).

The membrane proximal external region (MPER) is made up of the last 24 amino acids of the gp41 extracellular domain. This region is highly conserved and is required for fusogenicity and virus infectivity (Muñoz-Barroso et al., 1999; Pountourios et al., 1995; Salzwedel et al., 1999). The gp41 trans-membrane domain (TMD) situated downstream of the MPER consists of ~25 highly conserved amino acids anchoring Env in the lipid bilayer. The gp41 TMD plays an important role in Env function. Mutations in the “core” region of the TMD have a strong influence on Env-mediated fusion (Kondo et al., 2010; Shang & Hunter, 2010; Shang et al., 2008) The early models for gp41 anchored in the cellular membrane proposed that the TMD was an  $\alpha$ -helix that spanned the membrane once and the CT was inside the virus particle. However, based on recognition of an epitope within the gp41 CT by neutralizing

monoclonal Abs, an alternative topology has been proposed in which gp41 spans the membrane three times (Chanh et al., 1986; Cleveland, 2003; Hollier & Dimmock, 2005; Kennedy et al., 1986).

The transmembrane subunit of most lentiviral Env glycoproteins has a very long cytoplasmic tail compared to those found in other retroviruses. The CT of HIV-1 gp41 influences multiple properties of the gp120/gp41 glycoprotein complex, such as Env incorporation into virus particles, virus infectivity, cell-surface Env expression, gp120 shedding, and Env induced fusion (Affranchino & González, 2006; Freed & Martin, 1996a; Gabuzda et al., 1992; Kalia et al., 2003; Lee et al., 1989; Murakami & Freed, 2000a, 2000b; Wilk et al., 1992; Wyss et al., 2001; Yu et al., 1993). A membrane-proximal tyrosine-based sorting signal with the consensus sequence YxxL mediates endocytosis of Env from the plasma membrane (Berlioz-Torrent et al., 1999; Ohno et al., 1997; Rowell et al., 1995). The signal also mediates the release of virions from some cells (Lodge et al., 1997), cell-cell transmission in T cells (Deschambeault et al., 1999) and particle infectivity (Day, Münk, & Guatelli, 2004; West et al., 2002). A dileucine motif in the gp41 CT regulates the intracellular distribution of Env (Berlioz-Torrent et al., 1999; Byland et al., 2007; Wyss et al., 2001) and regulates endocytosis (Byland et al., 2007).

Three amphipathic,  $\alpha$ -helical segments are present in the central and C-terminal regions of the gp41 CT (Fig. 2, 5) (Eisenberg & Wesson, 1990; Miller et al., 1993, 1991; Venable et al., 1989). These segments are highly conserved among lentiviruses (Miller et al., 1991) and are referred to as lentiviral lytic peptides (LLP-1, LLP-2, and LLP-3). LLP-1 and LLP-2 segments are highly positively charged due to the presence of arginine residues on one face of the  $\alpha$ -helix. LLP-3 is located between the other two segments. LLP domains have been implicated in Env fusogenicity (Kalia et al., 2003), protein stability (Lee et al., 2002), multimerization (Lee et al., 2000), cell surface expression (Bültmann et al., 2001), and incorporation (Cosson, 1996; Murakami & Freed, 2000a; Piller et al., 2000). Studies of LLP fragments have shown that they bind to and perturb membranes (Chen et al., 2001; Chernomordik et al., 1994; Comardelle et al., 1997; Gawrisch et al., 1993) causing cytolysis, hence the name “lytic peptides” (Miller et al., 1993). Truncation of LLP-1 led to a decrease in localization of Env to lipid rafts, suggesting that the LLP-1 segment contains the determinants for lipid raft association (Yang et al., 2010). The LLP-2 region in gp41 CT may be transiently exposed on the cell surface during cell-cell fusion (Lu et al., 2008).

Because the biological functions of the gp41 CT are diverse, it is relatively well conserved. This makes it a potentially productive target for therapies and vaccines. However, investigation of its exact role in cell-cell transfer, and the identification of cellular interaction partners is still on-going (Checkley et al., 2012)

## **1.4 Transmission and acute infection**

### **1.4.1 Mucosal transmission**

Although the risk of transmission is higher for individual acts of anal intercourse (0.65% to 1.7%; Boily et al., 2009; Jin et al., 2010) than for individual acts of vaginal intercourse (0.03% to 0.5%; Ariën et al., 2011; Boily et al., 2009), the AIDS pandemic has arisen largely through transmission during unprotected heterosexual contact (UNAIDS, 2012; Ariën et al., 2011). Studies of HIV sero-discordant couples show the probability of infection for each encounter with the virus is quite small, i.e. less than 0.0014 (Gray et al., 2001; Piot et al., 2001; Pope & Haase, 2003; Wawer et al., 2005). This is most likely a consequence of low amounts of virus inoculum, restricted access to target cells and selective transmission. However, this value represents the lower bound of the range, as a single value fails to reflect the variation associated with numerous co-factors that increase the risk of transmission. STIs, young age, and lack of circumcision increase susceptibility with early- and late-stage index infection amplifying transmissibility (Powers et al., 2008). Studies of cumulative HIV incidence have provided mixed evidence in support of differences between male-to-female and female-to-male transmission (Guthrie et al., 2007) with one meta-analysis suggesting that there is no meaningful difference by direction of transmission at the per-contact level (Powers et al., 2008). However, in at least two detailed studies, the risk of HIV acquisition during receptive penile vaginal intercourse is double that seen in insertive penile vaginal intercourse (Alcantara et al., 2009; Leynaert et al., 1998; Varghese et al., 2002).

In heterosexual transmission, HIV gains entry into the body mainly by crossing epithelial barriers covering the mucosal surfaces of the female or male genital tracts as well as the anal/rectal epithelia. An intact vaginal epithelium consists of several thick layers of stratified squamous epithelial cells and lamina propria which provide a mucosal barrier to HIV infection (Pope and Haase, 2003). Female genital tract virus transmissions involve early capture by epidermal Langerhans cells (LCs) in the vagina and endocervix stratified epithelia.

Langerhans cells bind the envelope gp120 of HIV through their C-type unique Langerin, a process that, at low viral loads, might lead to the degradation of HIV (de Witte et al., 2007; Figdor et al., 2002). On the contrary, if the protective effect of Langerin is inhibited due to higher viral concentrations, the internalized virus will be transferred to T cells leading to a productive infection of these cells within the mucosae or in draining lymph nodes following migration of LCs to secondary lymphoid organs (Hladik & McElrath, 2008; Hladik et al., 2007; Merad et al., 2008). Sexually transmitted infections, micro-ulcerations, and hormone-induced changes in the vaginal epithelium during the menstrual cycle (Hu et al., 2000) can cause disruption to the mucosal barrier which can lead to inflammation. This results in the influx of CD4<sup>+</sup> CCR5<sup>+</sup> T cells to the submucosa, enabling HIV infection and then rapid dissemination to the lymphoid tissues.

In the 10 days that follow sexual mucosal exposure to HIV, viral RNA is not detectable in the circulation at the lowest threshold of 100 copies of viral RNA per milliliter (mL) of plasma. This time frame has been referred to as the eclipse phase (reviewed by Haase, 2011). Based on SIV models, HIV crosses the mucosal epithelial barrier to establish a founder population of infected cells within hours of infection (Miller et al., 2005; Zhang et al., 2004). Local expansion of this population during the first week of infection is followed by the establishment of a self-propagating systemic infection in the secondary lymphoid organs (Miller et al., 2005). The increased virus access to more susceptible target cells results in a significant increase in replication in the lymphatic tissues. The establishment of peak viremia in blood and tissue happens during the second week of infection before declining to stable levels around four weeks post exposure (Haase, 2010). Long term reservoirs for virus production and storage (as well as harbouring of proviruses) are established in latently infected cells in the lymphatic tissues (Haase, 1999 reviewed by Tebit et al., 2012).

#### **1.4.2 Characteristics of transmitted viruses**

Based on evidence from cross-sectional studies of individuals with acute and early infections, it is clear that transmission is associated with a population bottleneck; a single virion is responsible for establishment in approximately 80% of heterosexual HIV-1 transmissions (Abrahams et al., 2009; Keele et al., 2008). Therefore, a detailed molecular understanding of the transmission and the early evolution of HIV, including a precise description of the

transmitted or early founder virus, is critical for the development of microbicides as well as a potential HIV vaccine which need to target these viruses (Keele et al., 2008).

A very large number of transmitted, founder and acute Envs have been examined across a number of different studies and investigative groups (reviewed by Shaw & Hunter, 2012). The only major genetic or phenotypic signature that is consistently required for transmission appears to be the use of CCR5 (Berger et al., 1999; Parrish et al., 2013; Scarlatti et al., 1997; Zhu et al., 1993). Enhanced virus transmission has been associated with several genetic traits, including but not limited to shorter variable loops and reduced numbers of amino-linked glycosylation sites (Chohan et al., 2005; Derdeyn et al., 2004; Sagar et al., 2006; Wu et al., 2006) at least for subtypes A (Chohan et al., 2005) and C (Haaland et al., 2009). These signatures, which constitute relatively subtle alterations of Env structure and function in the context of the native Env trimer, could provide sufficient selective advantage during the eclipse phase of HIV-1 transmission (Shaw & Hunter, 2012). A recent study of the phenotypic properties of infectious molecular clones of transmitted/founder viruses by Parrish et al., (2013) found that relative to chronic control viruses, transmitted/founder viruses are enriched for higher Env content, enhanced cell-free infectivity, improved dendritic cell interaction, and relative IFN- $\alpha$  resistance. Asmal et al., (2011) have found signatures near the CD4 and CCR5 binding sites, the gp41 cytoplasmic domain, and the signal peptide. They found a significantly high frequency of a histidine residue at position 12 of the leader sequence amongst transmitted viral sequences. This study showed that viruses that contain this residue are more efficient at transporting the envelope glycoprotein to the endoplasmic reticulum; those with the residue have higher envelope expression and incorporation compared to those without the residue.

The recent finding that HIV-1 gp120 binds to the CD4<sup>+</sup> T cell gut homing integrin  $\alpha 4\beta 7$  has suggested that this may be important in allowing an infecting virus to target cells capable of trafficking to the gut associated lymphoid tissue (Arthos et al., 2008). It has been reported that  $\alpha 4\beta 7$ -expressing CD4<sup>+</sup> T cells are more susceptible to productive infection by HIV-1 than those expressing low levels of the integrin. This is in part because this subset is enriched for activated CD4<sup>+</sup> cells, and in part because  $\alpha 4\beta 7^{\text{hi}}$  cells express high levels of CCR5 and low levels of CXCR4 (Cicala et al., 2009). Interestingly, a small sample of acute subtype A and C virus Envs bound  $\alpha 4\beta 7$  with high affinity, and in some cases, later virus strains showed significantly reduced binding (Nawaz et al., 2011). This finding is consistent with an early

requirement for infection of  $\alpha 4\beta 7$ -expressing cells that is dispensable once infection in the gut mucosa has been established (Shaw & Hunter, 2012). However, a recent study by Parrish et al. (2012) found that transmitted viruses did not differ in their ability to bind CD4 and CCR5 compared to chronic viruses. They also found that anti- $\alpha 4\beta 7$  antibodies were not effective at inhibiting infection. This study concluded that the transmission bottleneck does not result in the selection of viruses that utilize CD4, CCR5, and  $\alpha 4\beta 7$ . Therefore, the role of the intergrin  $\alpha 4\beta 7$  during HIV mucosal transmission is yet to be fully understood, and the effect of the blockage of gp120- $\alpha 4\beta 7$  interaction on HIV replication is not yet known (Parrish et al., 2012). Given that HIV-1 transmission is inherently inefficient and likely represents the most vulnerable point in the natural history of HIV-1 infection, identifying unique properties and potential vulnerabilities of transmitted/founder viruses remains an important objective.

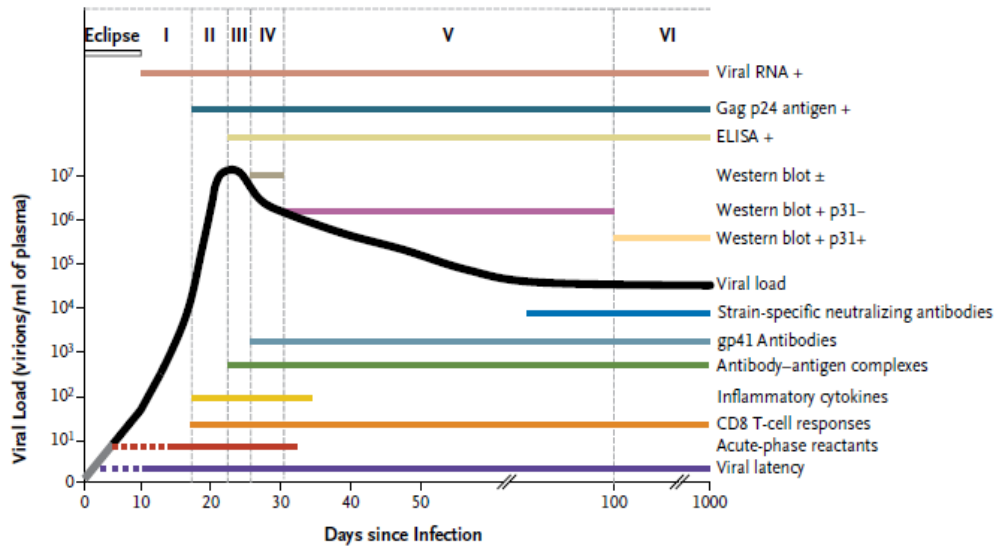
## **1.5 Responses to HIV infection**

### **1.5.1 Cytotoxic T-lymphocyte (CTL) immune responses**

Among the most important and effective immune responses to HIV infection is the cytotoxic T-lymphocyte (CTL) response. CTLs play an important role in suppressing viral replication, during both acute and chronic infection, and they are seen as early as two to three weeks after infection (Goonetilleke et al., 2009; Lichterfeld et al., 2004; Mlotshwa et al., 2010), where they are believed to contribute to a decline in viremia until the viral set point is achieved (Altfeld et al., 2001; Dalod et al., 1999; Figure 1.6). In an infected cell, viral peptides are processed inside the endoplasmic reticulum before being transported to the cell membrane for presentation by the Major Histocompatibility Complex (MHC) class I molecules. The MHC is known as human leukocyte antigen (HLA) in humans. The presentation of the viral peptide on the surface of the cell triggers a response by the CTLs. The T cell receptors (TCR) that are expressed on the surface of CTLs bind to the MHC class I molecules and its peptide on the surface of the virus-infected cells. The binding induces the release of perforins and proteases that lyse the infected cells (Lamas et al., 1998).

Non-mutational and mutational mechanisms of avoiding recognition by CTLs have evolved in HIV-1. The non-mutational escape of CTLs by HIV is achieved by a Nef mediated mechanism that involves the downregulation of the MHC class I molecules in the HIV infected cells (Greenberg et al., 1998). Nef causes the MHC class 1 molecules to accumulate

in clathrin coated vesicles in the Golgi apparatus resulting in the lack of recognition of the infected cell by the CTLs (Tomiyama et al., 2002).



**Figure 1.6:** The timing of CTL and antibody responses during acute stage of HIV infection (Cohen et al., 2011). The Fiebig stages are indicated in Roman numerals with the detection of viral RNA and viral antigens indicated (Fiebig et al, 2003). Individuals classified as being in Stage I/II were viral RNA positive, EIA antibody negative; those in Stage III were EIA antibody positive, but negative by Western blot; those in Stage IV had an indeterminate Western blot; those in Stage V were Western blot positive but without reactivity to the p31 integrase band; and those in Stage VI were Western blot positive with a p31 band present.

Mutational escape is effected through amino acid changes in the viral peptides or epitopes that reduce binding to the MHC class I molecules and/or alter the interaction with the T-cell receptors. These changes result in a loss of recognition by CTLs (Goulder & Watkins, 2004; Klenerman et al., 1994; Le Gall et al., 2007; Tenzer et al., 2009). Additionally, mutational escape by HIV-1 may involve interfering with the intracellular processing of the viral peptides, which prevents these epitopes from being presented at the cell surface in the context of MHC class I molecules for recognition by CTLs (reviewed by Sewell et al., 2000).

In addition to the genetic diversity of HIV-1, the complexity of the epidemic is further increased by the diverse genetic backgrounds of HLA among individuals from different populations. HLAs are grouped into subfamilies of closely related variants. HLA corresponding to MHC class I has 3 major genes (HLA-A, HLA-B, HLA-C) and 3 minor genes (HLA-E, HLA-F and HLA-G; (Parham et al., 1995).

Genome wide association studies (GWAS) is an approach involving the identification of genetic variations in human genomes that are associated with any disease outcome. These studies have shown that variation attributed to HLA types is associated with differential disease progression. The pressure that CTLs exert on the virus results in the selection of certain viruses that can successfully avoid recognition by the CTLs. Therefore, the polymorphism of HLA molecules determines how different individuals can respond to specific immunodominant epitopes depending on their HLA background. Mutational escape occurs along generally reproducible pathways that are predictable based on host HLA allele expression (Allen et al., 2005; Brumme et al., 2007, 2009; C. B. Moore et al., 2002; Rousseau et al., 2008 reviewed by Chopera et al., 2011).

Because an individual's HLA determines disease outcome, various types of CTL response will be much more effective at controlling viral replication than others. HLA alleles such as HLA B\*27:05, HLA B\*57:01, HLA B\*58, B\*44, B\*14/Cw\*08:02, A\*25 and HLA B\*63 have been strongly associated with immune control of HIV, resulting in slower disease progression (Gao et al., 2001; Kaslow et al., 1996; Kiepiela et al., 2004; Migueles et al., 2000; Pereyra et al., 2010; Zhang et al., 2013). The long-term benefit of these 'protective' HLA class I alleles is probably the result of their ability to mediate early, robust CTL responses against highly conserved HIV epitopes, most notably in the structural p24 Gag protein which results in lower viral loads (Altfeld et al., 2006; Crawford et al., 2007; Goulder et al., 1996). GWAS comparing individuals that control viral loads at less than 200 copies/mL (HIV controllers) and rapid progressors have found the presence of single nucleotide polymorphisms (SNPs) associated with viral control in the HLA region of chromosome 6. Further analysis in the HLA B gene found five amino acids that are critical for the binding of the viral peptide to the HLA binding groove (Fellay et al., 2007). On the other hand, HLA alleles such HLA B\*35, Cw\*07, HLA B\*53 and HLA B\*5802 have been associated with rapid disease progression (Carrington et al., 1999; Carrington & O'Brien, 2003; Ngumbela et al., 2008; Pereyra et al., 2010). Despite the abundance of studies that have identified a central role for HLA-B alleles in influencing control of HIV infection there is evidence from a subtype C infection cohort of interdependent protective effects of the HLA-Cw, and HLA-A haplotypes that cannot be explained solely by linkage to a protective HLA-B allele (Leslie et al., 2010).

During acute infection, very narrow CTL responses are observed, with the Gag, Pol, Nef and Env regions being frequently targeted (Abrahams et al., 2013; Borrow et al., 1997; Goonetilleke et al., 2009; Goulder & Watkins, 2004; Lichterfeld et al., 2004). Over the first 6 months of infection Nef, Gag, and Pol are mostly targeted (Goonetilleke et al., 2009; Gray et al., 2009; Mlotshwa et al., 2010). During chronic infection, the Gag and Env-specific CTL response have been found to be associated with low and high viral loads respectively, suggesting that Gag, and not Env, is critical in CTL-mediated viral control (Geldmacher et al., 2007; Kiepiela et al., 2007). The escape mutations in Gag, particularly in the p24 capsid region, carry a fitness cost to the virus as these mutations may affect the conformation of the viral capsid (Martinez-Picado et al., 2006; Schneidewind et al., 2008). A study by Martinez-Picado et al., (2006) identified a mutation in the TW10 epitope in Gag that restricted in HLA B57 individuals to reduce the viral replicative capacity.

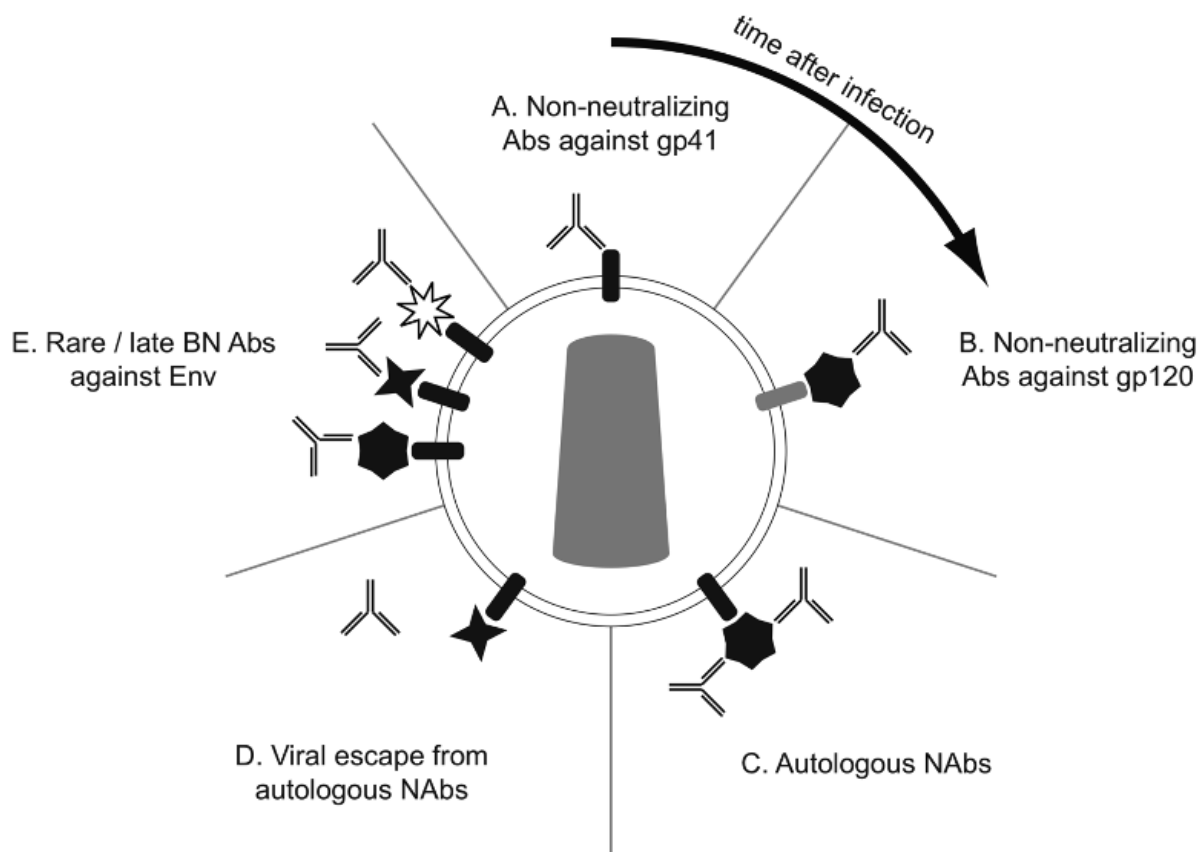
Several studies have shown that individuals with protective alleles harbor viruses with lower replicative capacity during acute infection than those lacking such alleles. The levels of viral replication are broadly correlated with the presence of HLA B-associated polymorphisms (Brockman et al., 2010; Goepfert et al., 2008; Wright et al., 2011).

This immune mediated attenuation of HIV-1 may be exploited to design an HIV-1 vaccine capable of stimulating effective CTL responses against highly conserved, mutationally constrained viral regions, where immune escape could only occur at substantial functional costs. Such a vaccine might ‘channel’ HIV-1 evolution towards a less-fit state, thus lowering viral load set points, attenuating the infection course and potentially reducing the risk of transmission (Chopera et al., 2011). However, major barriers to this approach include the virus’ ability to restore its fitness by means of compensatory mutations (Crawford et al., 2007; Goepfert et al., 2008; Schneidewind et al., 2007, 2008) and the likely accumulation of immune escape variants at the population level, which could lead to the loss of immunogenic CTL epitopes and diminished vaccine-induced cellular immune responses as the epidemic progresses (Chopera et al., 2011).

### **1.5.2 Humoral responses to HIV-1 infection**

The first detectable B-cell response to HIV infection is in the form of immune complexes 8 to 20 days after plasma virus detection (Figure 1.6). The first free, plasma, anti-HIV-1

antibodies are directed to gp41 and appear 13 days after the appearance of plasma virus (Figure 1.7). In contrast, envelope gp120-specific antibodies are delayed an additional 14



**Figure 1.7.** The antibody response to human immunodeficiency virus type 1 (HIV-1) occurs in stages, shown here in a clockwise direction starting at the top. *A:* Initial antibody response to HIV-1 is non-neutralizing and directed at gp41. *B:* Non-neutralizing antibodies directed against gp120 arise soon thereafter. *C:* After a delay of weeks to months, autologous neutralizing antibodies (NAbs) arise that apply selection pressure on the virus. *D:* Viral mutation results in neutralization escape by HIV-1, represented here by a change in the shape of gp120. *E:* In some patients, antibodies that can neutralize a wide range of HIV-1 isolates arise (bnAbs, broadly neutralizing antibodies), represented here by a variety of shapes of gp120. Mixing of envelope shapes on a single virus particle is shown for illustrative purposes only (Alter & Moody, 2010).

days and these antibodies target the V3 loop (Tomaras et al., 2008a). These first antibodies are by themselves incapable of stopping the virus from infecting new cells and are thus ineffective in controlling viremia. Antibodies that are able to prevent the virus from infecting cells are called neutralizing antibodies. These usually appear a few months post-infection (Gray et al., 2007; Li et al., 2006; Wei et al., 2003). These early neutralizing antibodies are effective against the autologous virus but are usually strain-specific and unable to neutralize

divergent HIV strains from other hosts (Gray et al., 2007). The virus can easily escape these early neutralizing antibodies because they are so narrowly focused and target variable regions of Env. Additionally, early neutralizing antibodies only reach biologically relevant levels long after the initial exposure to the infecting virus (Davis et al., 2009; Moore, Gray, & Morris, 2009; Rong et al., 2009; Wei et al., 2003). Escape is achieved by disruption of the epitopes recognized through point mutations, insertions and deletions, and recombination. While all three mechanisms are able to directly alter the epitope, they are also used indirectly to achieve structural shielding of the epitopes through shifting N-glycosylation sites that prevent access of the antibodies to the epitopes. Deletions and recombination are also used to alter the lengths of the variable domains of the envelope (Herrera et al., 2003; Overbaugh & Rudensey, 1992; Richman et al., 2003).

Certain regions in the viral envelope protein are preferentially targeted by the first autologous neutralizing antibodies. These are mainly on the extensively N-glycosylated outer surface of the virus. The V1V2 region (Overbaugh et al., 1991; Overbaugh & Rudensey, 1992), the alpha-2 helix in the C3 region, and the V4 region are all major targets of neutralizing antibodies in subtype C (Moore et al., 2009; Rong et al., 2007a; Rong et al., 2007b). Also, neutralizing antibody escape is driven by multiple regions in gp120, which include the V3-V5 region, and the V1V2 region in association with the gp41 region (Rong et al., 2009). The V1V2 loops together with the V3 region have also been identified as targets during subtype B infections particularly the N-glycosylation sites within these regions (Tang et al., 2011).

### **1.5.3 Antibody responses and HIV-1 subtype**

It has proven difficult to clearly define the relationship between subtype and neutralization serotype. Numerous early studies found that there was little or no relationship between the genetic subtypes and what was observed in neutralization assays (Beirnaert et al., 2000; Kostrikis et al., 1996; Kostrikis et al., 1996; J. P. Moore et al., 1996; Nyambi et al., 1996; Weber et al., 1996 reviewed in Moore et al., 2001). However, later evidence from improved analyses of subtype B and C infections showed that there are some shared regional and subtype-specific determinants of neutralization. There was also a significant trend toward increased neutralization sensitivity when the virus and HIV-1 plasma or plasma pool were of the same clade (Bures et al., 2002; Lakhashe et al., 2007; Rademeyer et al., 2007; Seaman et

al., 2010; van Gils et al., 2010). Although the genetic subtypes do not precisely correspond to serotypes (Moore et al., 2001), they do impact epitope structure and variability and thus are related to antibody susceptibility patterns (Binley et al., 2004; Brown et al., 2008; Li et al., 2006). Differences in the immunogenicity of different subtypes have also been implicated in the strength of elicited antibody responses. Several studies have reported that infection with subtype C and A viruses results in greater breadth and potency than subtype B viruses (Brown et al., 2008; Bures et al., 2002; Dreja et al., 2010; Li et al., 2006). Similar findings have also been shown in plasmas from individuals infected with subtype D, CRF02\_AG and CRF01\_AE, although the effect was less pronounced (Brown et al., 2008; Dreja et al., 2010). There is very little data available on how antibody responses differ in populations with a variety of local epidemics. Similarly, little is known about how the increasing global diversity presence of CRFs and URFs may affect the development of antibody responses.

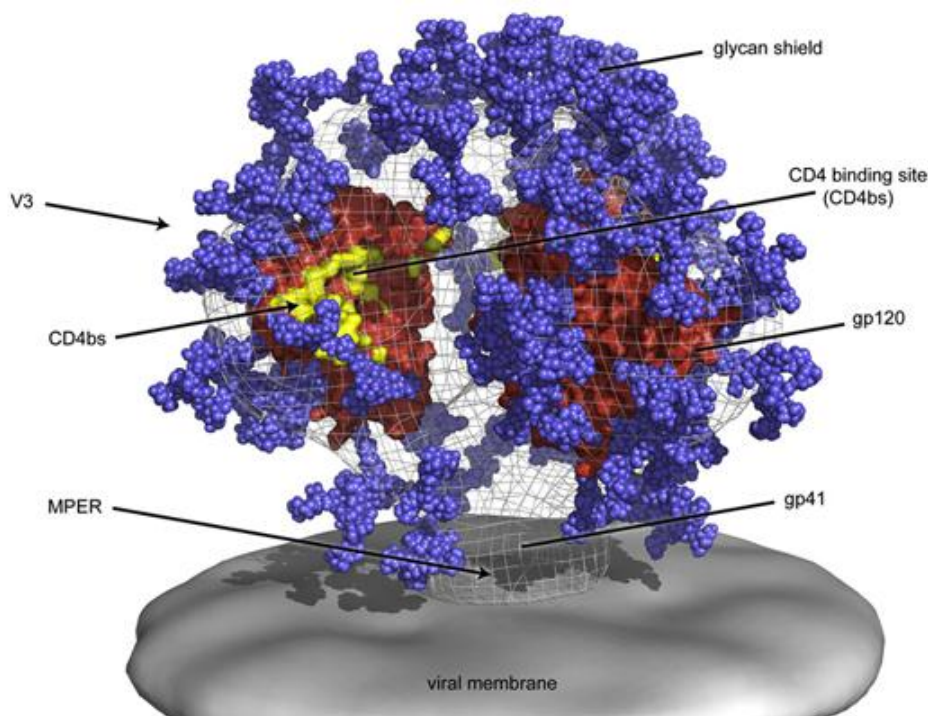
#### **1.5.4 Broadly cross-neutralizing antibodies**

Antibodies that are able to neutralize a range of viruses are called broadly cross-neutralizing antibodies (bnAbs). Any successful vaccine for HIV will have to induce a neutralizing antibody response of some breadth to be effective. Approximately 10 – 30 % of individuals are able to develop broadly neutralizing antibodies after years of infection (Doria-rose et al., 2010; Gray et al., 2011; Keele et al., 2008; Sather et al., 2009a; Simek et al., 2009). The rarity of bnAbs is most likely related to the unusual features of the Env protein and the capabilities of the virus. These include Env's extensive glycan 'shield' (Binley et al., 2010; Richman et al., 2003; Wei et al., 2003), rapid neutralizing Ab selection of escape mutants (Richman et al., 2003; Wei et al., 2003), transient epitope expression (Frey et al., 2008), steric hindrance (Labrijn et al., 2003; Schief et al., 2009), and inability to overcome Ab-binding entropic barriers (Kwong et al., 2002). Another potential reasons for the rarity of broadly neutralizing antibody responses is that HIV-1 particles bear non-functional gp120/gp41 monomers and gp120-depleted gp41 stumps (Moore et al., 2006). The highly immunogenic epitopes present on non-native Env structures may hinder bnAb responses by inducing dominant Ab responses that are non-neutralizing or neutralizing but strain-specific (Haynes & McElrath, 2013; Labrijn et al., 2003).

It is still unclear why certain individuals can develop these antibodies and others cannot. Longer periods of infection and high levels of viremia (thus persistent viral stimulation) are associated with the development of neutralization breadth (Euler et al., 2011; Gray et al., 2011; Piantadosi et al., 2009; Sather et al., 2009a). The extent of neutralizing breadth has also been correlated with CD4(+) T cell decline and CD4(+) T cell count at 6 months post infection (Gray et al., 2011). In addition to infection with a non-B virus (Brown et al., 2008; Dreja et al., 2010), high envelope diversity during early infection (Sather et al., 2009b) as well as superinfection (Cortez et al., 2012), particularly with a very divergent virus (Powell, Kinge, & Nyambi, 2010), has been associated with increased breadth of neutralizing antibody responses. This again raises the question of how a highly diverse local epidemic and the presence of multiple unique or circulating recombinant forms might affect the development of broadly neutralizing antibody responses in a population.

A number of cross-reactive monoclonal neutralizing antibodies and the epitopes that they target have been identified. Initially, IgGb12 (which targets the CD4 binding site), 4E10, Z13 (carboxy terminal MPER), 2F5 (amino-terminal MPER), and 2G12 (gp120) were isolated and represented the prototypic protective antibodies an HIV-1 vaccine should elicit (Burton et al., 1994; Muster et al., 1993; Stiegler et al., 2001; Trkola, Purtscher, et al., 1996; Zwick et al., 2001 reviewed by Verkoczy et al., 2011). Recently developed high-throughput recombinant antibody technology has enabled the identification of many new bnAbs, some with higher potency than those of the first generation. These include PG9, PG16, VRC01, PGT121 and PGT128 antibodies which target the gp120 region of the HIV-1 envelope (Corti et al., 2010; Walker et al., 2009a; Zhou et al., 2010). Following extensive and in depth analysis of available antibodies, four distinct Env regions have been identified (Figure 1.8). Each of these represents a potential Achilles' heel for HIV-1 that could be targeted by an antibody-based vaccine: the gp41 Membrane Proximal External Region (MPER), the gp120 CD4 binding site, quaternary V2/V3 loop epitopes, and Env carbohydrates (reviewed by McElrath & Haynes, 2010). These studies have also revealed an interesting pattern in which all bnAbs share at least one of the following three unusual characteristics: a) self-/polyreactivity, b) elongated and highly hydrophobic (and/or charged) heavy chain third complementarity determining regions (HCDR3), and c) high numbers of Ig somatic mutations (McElrath & Haynes, 2010). Thorough investigation of the traits associated with bnAb may provide clues about potential mechanisms controlling the induction, specificity and neutralization potential of bnAb.

Recent studies have highlighted the significance of the interplay between early antibodies and viral escape in the evolution of conserved bnAb epitopes. Moore et al., (2012) showed, in two HIV-1 infected individuals who developed bnAbs targeting the glycan at Asn332 on the gp120 envelope, that this glycan was absent on the initial infecting virus. The glycan evolved within 6 months, as a consequence of immune escape from earlier strain-specific antibodies



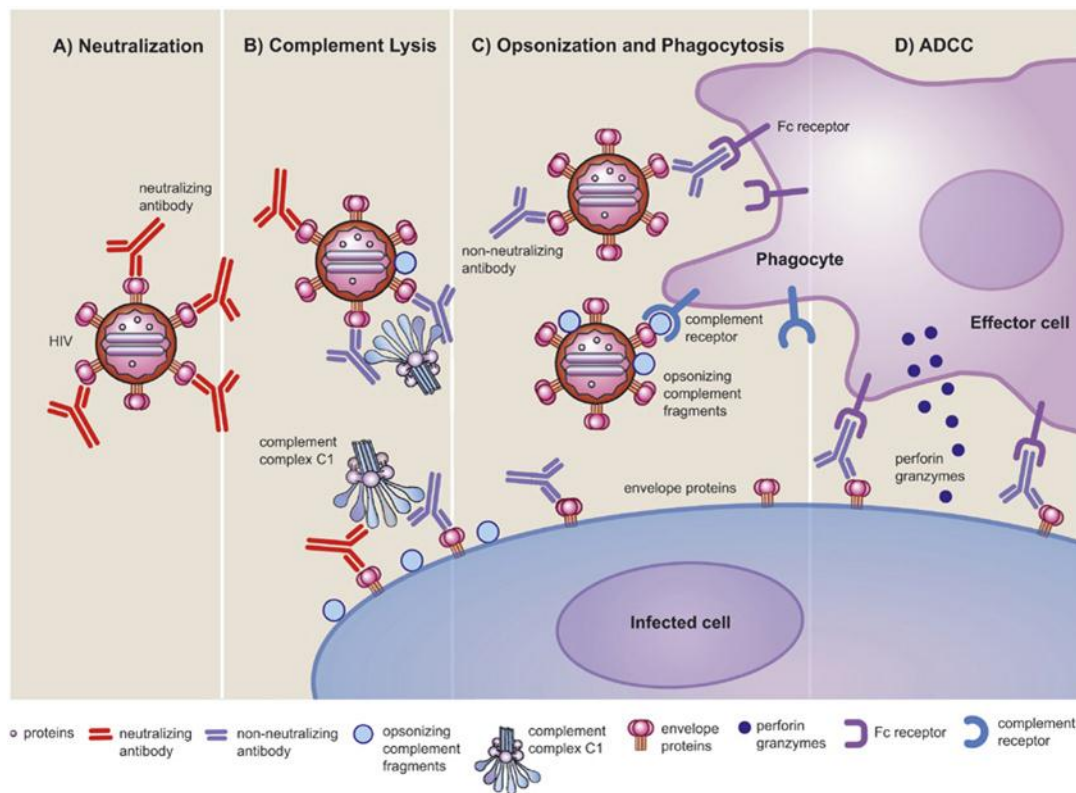
**Figure 1.8:** Model of the structure and Antibody Recognition of the HIV Envelope Spike (Schief et al., 2009) adapted from a cryo-electron tomographic structure of the HIV trimer (Liu et al., 2008). The crystal structure of the b12-bound monomeric gp120 core (red) has been fitted into the density map (Zhou et al., 2007). Glycans are shown in purple. The CD4 binding site is shown in yellow. The approximate locations of the epitopes targeted by existing bnMAbs are indicated with arrows. The figure is adapted from a review by (Burton et al., 2012).

that resulted in a shift of a glycan to position 332. Liao et al., (2013) demonstrated that the binding of a transmitted/founder Env to an unmutated common ancestral B-cell receptor of a bnAb lineage was responsible for the induction of broad neutralizing antibodies, thus providing a logical starting place for vaccine-induced CD4-binding site bnAb clonal activation and expansion.

The induction of bnAb in a vaccine setting remains a major challenge in the field. It is important to remain hopeful, however, as a number of studies have shown that neutralizing antibodies can provide sterilizing immunity in macaques in a SHIV model. These studies highlight the potential importance of an early and potent neutralizing response during that window of virus vulnerability immediately after transmission when low genetic diversity reduces the chance of immune escape (Baba et al., 2000; Emini et al., 1992; Hofmann-Lehmann et al., 2001; Mascola et al., 1999, 2000; Parren et al., 2001; Shibata et al., 1999; Xu et al., 2002; Moldt et al., 2012).

## 1.6 Antibody Dependent Cell-Mediated Cytotoxicity

### 1.6.1 ADCC



**Figure 1.9:** How antibodies combat HIV-1. (a) Neutralization of free virus by antibodies, (b) complement-mediated lysis of free virus and infected cells triggered by antibodies, (c) opsonization of virus particles by antibodies and phagocytosis of virus particles via Fc- or complement-receptors, (d) antibody-dependent cellular cytotoxicity (ADCC) against infected cells. Neutralizing antibodies (red), non-neutralizing (blue), Fc-receptors (violet), complement components (light-blue), complement-receptors (black).

In addition to neutralization, antibodies mediate antiviral immunity through numerous functions (Alpert, Heyer, et al., 2012; Figure 1.9). Antibody coating of HIV may lead to complement opsonization and viral lysis (Aasa-chapman et al., 2005) or phagocytosis (Kedzierska et al., 2001, 2013). The constant (Fc) region of IgG interacts with Fc receptors expressed on leukocytes and with complement. These interactions can contribute to antiviral immunity via several mechanisms. These include orchestrating the homing of effector cells (Ottoneo et al., 1999; Rot & von Andrian, 2004; von Andrian & Mackay, 2000), inhibiting virus replication through antibody dependent cellular viral inhibition (ADCVI: Ackerman et al., 2011; Kottlil et al., 2003; Oliva et al., 1998; Trkola, et al., 1996), and killing virus-infected cells by complement-dependent cytotoxicity (CDC; Spear et al., 1990) as well as antibody-dependent cell-mediated cytotoxicity (ADCC; Koup et al., 1989; Lanier et al., 1986; Rook et al., 1987). These non-neutralizing effector functions are mainly associated with the release of chemokines (Chung et al., 2009; Stratov et al., 2008), which are important components of antiviral immunity (Hessell et al., 2007). These functions have been implicated in protective immune responses against HIV-1 (Baum et al., 1996; Forthal et al., 1999; Ljunggren et al., 1990). While macrophages, neutrophils and eosinophils can mediate ADCC, classical ADCC is mediated by Fc binding to the Fc $\gamma$ -Receptor (FcR) IIIa also known as CD16 on the surface of natural killer (NK) cells (Clynes et al., 2000). Activation of NK cells through the binding of antibody to FcRs leads to ADCC. If the antibody binds to a viral antigen on the surface of infected cells (Figure 1.9 B), it leads to perforin, granzyme and Fas ligand release and death of the infected cell (Brown et al., 2012).

### **1.6.2 ADCC in primates, humans and vaccines**

A distinct advantage of non-neutralizing Ab-mediated ADCC and ADCVI is their ability to harness the innate immune system early on in acute HIV infection, specifically through NK cells. These cells destroy infected cells expressing antibody-bound antigen or mediate virus inhibition having bound antibody-antigen complexes (Ahmad & Menezes, 1996; Asmal et al., 2011). NK cell activity is dramatically elevated during acute infection before the CD8<sup>+</sup> T cell response and is the dominant cytolytic effector population during acute HIV infection (Alter & Altfeld, 2009; Alter et al., 2007; Altfeld et al., 2012). In part, this is due to the presence of antigen-specific Abs in mucosal secretions and also to the large number of potential ADCC effector cells in the peripheral circulation (Battle-Miller et al., 2002; Nag et al., 2004). This

differs from CTL responses which often do not have mucosal homing ability. These innate effector cells could be utilized by vaccines to gain more effective control of HIV (Berger & Alter, 2011).

Several studies have reported active and passive immunization that protected non-human primates from SIV or SHIV infection. The mechanism of protection was related at least in part, to ADCC- and ADCVI-mediating antibodies (Alpert et al., 2012; Florese et al., 2009; Gómez-Román et al., 2005; Hidajat et al., 2009; Xiao et al., 2010). In three studies, ADCC responses are associated with delayed acquisition or reduced acute and chronic viral load, indicating that antibodies play an important antiviral role even after acquisition (Hidajat et al., 2009; Xiao et al., 2010, 2012). ADCC responses are elicited by several candidate vaccines in humans recently including virus-like particles and recombinant gp120 (Moody et al., 2012; Pastori et al., 2012). The recent emphasis on the importance of antibody effector functions is further emphasized by the recent results of the RV144 Thai trial. This trial used an ALVAC recombinant DNA canarypox vector vaccine with two boosters of AIDSVAX recombinant gp120 protein. Statistical analysis showed 31% vaccine efficacy (Rerks-Ngarm et al., 2009) and subsequent immune correlate analysis found that V1V2 binding but not neutralizing antibodies correlated inversely with infection risk (Haynes, Gilbert, et al., 2012; Rolland et al., 2012). An earlier trial using the same vaccine, VAX004, gave no protection but supported the later finding that recombinant gp120 can elicit antibodies with antiviral activity against clinical HIV-1 (Forthal et al., 2007). This activity required the presence of FcR-bearing effector cells, further suggesting a role for ADCC/ADCVI in an HIV vaccine.

### **1.6.3 Measurement of ADCC**

Several laboratory methods exist for determining the efficacy of antibodies or effector cells in eliciting ADCC. Essentially, a labelled target primary cell (or cell line expressing a certain surface-exposed antigen) is incubated with antibody specific for that antigen. When measuring HIV specific ADCC activity, target cells may be coated with gp120, gp140, or peptides (Asmal et al., 2011; Barouch et al., 2012; Chung et al., 2011; Ferrari et al., 2011; Fouda et al., 2011; Matthews et al., 2004). Chronically infected T-cell lines can also be used (Gómez-Román et al., 2005; Pollara et al., 2011). After washing, effector cells expressing Fc receptor CD16 are co-incubated with the antibody-labelled target cells. Target cell lysis or

effector cell functions are subsequently measured by one of several methods. Early ADCC assays were based on readouts of  $^{51}\text{Cr}$  release from labelled target cells by a scintillation counter or spectrophotometry (Alsmadi et al., 1997). This method measured cell death due to ADCC but suffered from high levels of non-specificity and background due to leakiness of cells. The study of ADCC was held back due to the laborious technical requirements and insensitive nature of the assay. There are often variations in the overall levels of antigens present on the surface of the target cells, as well as variations to the particular antigenic epitopes available (CD4 binding site, CD4 inducible, gp120 monomers/trimers). This means that ADCC assays based on target cells coated with recombinant forms of Env, or some chronically infected T-cell lines will measure antibodies that may not direct ADCC against cells infected with primary isolates compromising the actual biological relevance of the assays (Pollara et al., 2011).

Several improved ADCC assays have recently become available. A fluorescent killing assay termed “rapid fluorescent ADCC” (RFADCC) assay (Gómez-Román et al., 2006) which relies on the release of intracellular fluorescent dye has been developed. This assay is simpler and more quantitative than chromium release assays. Forthal et al. (2005) have used assays of *in vitro* viral replication inhibition to illustrate the effectiveness of HIV- and SIV-specific ADCC responses (Forthal et al., 2005; Hessel et al., 2007). Alpert et al. (2012) developed a novel ADCC assay based on immortalized cell lines - an NK cell line that expresses either human or rhesus macaque CD16, and a CD4+ target cell line that expresses luciferase from a Tat-inducible promoter upon HIV-1 or SIV infection. The dose-dependent loss of luciferase in the presence of NK cells and serial dilutions of plasma or serum indicates the killing of virus-infected cells by ADCC. Stratov et al. (2008) explored an intracellular cytokine staining (ICS) based assay by studying NK cell expression of effector molecules following activation by HIV Ags and Abs. Here, small quantities of whole blood are incubated with HIV peptide antigens. Gated NK cells are then monitored for the expression of cytokines, such as IFN- $\gamma$ , or degranulation markers, such as the expression of CD107a or the loss of intracellular granzyme B. This assay is simpler than killing assays and can measure a variety of functions of NK cells triggered by HIV-specific Ab.

The measurement of ADCC-mediating Abs by effector cells has been limited by the lack of a quantitative technique that allowed for specific and high-throughput analysis of target cell killing at the single cell level. Pollara et al. (2011) developed a flow cytometry-based assay

that takes advantage of the ability to reproducibly detect the proteolytic activity of Granzyme B (GzB) after its delivery into target cells, initiated by Ab recognition of viral antigens on the target cell membrane. This method has enabled the detailed study of ADCC responses in human and non-human primate vaccine studies.

#### **1.6.4 ADCC Epitopes and escape from ADCC**

Detailed sequence analysis of single HIV genomes reveals that most changes acquired during the first months of infection align with sites of CTL or nAb escape mutations (Abrahams et al., 2013; Goonetilleke et al., 2009) or are related to APOBEC (Wood et al., 2009). However, a few mutations do not map clearly to these sites, suggesting that they may be reversions from escape in the previous host (Song et al., 2012) or escape from immune responses, such as ADCC.

Relatively few ADCC epitopes have been identified to date within HIV. ADCC antibody responses are generally thought to target viral surface proteins presented on the surface of infected cells. As a consequence of this, the majority of identified ADCC epitopes are within Env glycoproteins, gp120 (Alsmadi et al., 1997; Alsmadi & Tilley, 1998; Koup et al., 1991) and gp41 (Duval et al., 2008; Tyler et al., 1990; Ziegner et al., 1992). Epitopes directed against Env have been reported as either linear or conformational/discontinuous and have been well described in a recent review by Pollara et al., (2013) (Table 1.1). Within gp120, several monoclonal antibodies that bind to epitopes which are induced by conformational changes that follow the binding of CD4 to gp120 have demonstrated ADCC activity (Bonsignori et al., 2012; Ferrari et al., 2011; Friedman et al., 2012; Guan et al., 2013). One of these, A32, binds to a conformational epitope involving the C1 region (Finnegan et al., 2001). While it cannot bind the Env expressed on infectious virions, it is capable of broad and potent recognition and binding of cell surface expressed Env in the presence of CD4 (Ferrari et al., 2011). This is indicative of differences in the expression of Env in these two contexts and thus differences in the requirements of antibody activity for neutralization and ADCC (Pollara et al., 2013). Several epitopes have been described in the V2 region of Env (Liao, Bonsignori, et al., 2013), the CD4 binding site (Alsmadi & Tilley, 1998; Ferrari et al., 2011; Forthal et al., 1995; Hezareh et al., 2001; Koup et al., 1991; Posner et al., 1992), the V3 region (Alsmadi & Tilley, 1998; Bonsignori et al., 2012; Forthal et al., 1995). In gp41,

ADCC epitopes have been described in cluster I (Moog et al., 2014; Tyler et al., 1990), HR2 (Forthal et al., 1995; Tyler et al., 1990) and the MPER (Forthal et al., 1995; Moog et al., 2014; Tudor & Bomsel, 2011; Tyler et al., 1990).

ADCC responses to viral peptides derived from internal HIV-1 proteins such as Tat, Nef, Vpu and Pol have also been identified (Florese et al., 2009; Isitman et al., 2011; Stratov et al., 2008; Yamada & Iwamoto, 1999; Yamada et al., 2004). These proteins are not targeted to the cell membrane and it is yet to be shown how epitopes on the surface of cells would be presented to ADCC antibodies. Furthermore, it is not clear whether ADCC antibodies to such internal proteins can actually recognize HIV-infected cells *in vitro* in a biologically relevant way. One possible explanation is that ADCC recognition of viral debris on the surface of healthy neighbouring cells may trigger non-cytolytic activity from NK cells that could limit HIV-1 spread in a local environment (Isitman et al., 2012).

ADCC responses forcing immune escape has only been demonstrated recently (reviewed by Isitman et al., 2012). Demonstration of viral escape from ADCC responses strongly suggests that ADCC responses exert significant pressure on the virus. Evidence of immune escape was found in one study in the Env protein of HIV-1 using peptides with known ADCC epitopes and an NK-cell activation assay (A. W. Chung et al., 2011). Selective pressure, most likely from ADCC has been demonstrated to act on amino acid position 169 in the V2 region of the envelope of transmitted/founder virus sequences from the breakthrough infections in RV144 (Rolland et al., 2012). Escape has also been shown in an epitope of the highly conserved protein Pol (Crowe et al., 2001; Isitman et al., 2011). However, this and other work likely underestimates the number of ADCC epitopes targeted by each HIV positive subject, since linear epitopes are readily mapped and dissected and consensus subtype B overlapping peptides are used for screening. Conformational ADCC antibodies are also likely to elicit escape, but to map such responses and identify escape patterns will be difficult and will require large numbers of mutant, whole, Env proteins (Isitman et al., 2012). Furthermore, many ADCC epitopes have clearly been demonstrated to overlap with neutralizing antibody epitopes (Pollara et al., 2013) and therefore their escape mechanisms will likewise inevitably overlap. ADCC-forced mutations could theoretically incur some “fitness cost” to viral replicative capacity. Constructing replicating viruses with ADCC-induced mutations will allow testing of this hypothesis.

**Table 1.1:** Defined Regions of the HIV-Envelope Glycoprotein Targeted by Human mAbs with ADCC Activity. The table is taken from the review by (Pollara et al., 2013)

HIV-1 Envelope Glycoprotein	Envelope Region	mAb	Discontinuous or Linear Epitope	Neutralizing or Non-Neutralizing	ADCC References
gp41	Cluster I	246-D	Linear	Non-Neutralizing	(Forthal et al., 1995; Moog et al., 2014)
		4B3	Linear	Non-Neutralizing	(Moog et al., 2014)
		98-43	Linear	Non-Neutralizing	(Tyler et al., 1990)
		50-69	Discontinuous	Non-Neutralizing	(Tyler et al., 1990)
	Cluster II (HR2)	98-6	Discontinuous	Non-Neutralizing	(Forthal et al., 1995; Tyler et al., 1990)
		126-50	Discontinuous	Non-Neutralizing	(Tyler et al., 1990)
	Not defined	31710B	not defined		(Alsmadi et al., 1998)
	MPER	120-16	Linear	Non-Neutralizing	(Forthal et al., 1995; Tyler et al., 1990)
		2F5	Linear	Neutralizing	(Moog et al., 2014; Tudor et al., 2011)
		4E10	Linear	Neutralizing	(Moog et al., 2014)
gp120	CD4i C1 region (Cluster A)	A32	Discontinuous	Non-Neutralizing	(Ferrari et al., 2011)
	CD4i CoRBS (Cluster C)	17B	Discontinuous	Neutralizing	(Ferrari et al., 2011)
	CD4i	CH08	Discontinuous	Neutralizing	(Friedman et al., 2012)
	CD4i Cluster A	C11, L9-i1, N5-i5, L9-i2, N12-i3, N26-i1	Discontinuous	Non-Neutralizing	(Guan et al., 2013)
	CD4i Cluster B	N12-i15	Discontinuous	Non-Neutralizing	(Guan et al., 2013)
	CD4i Cluster C.1	L9-i3, N5-i1, N5-i3, N5-i4, N5-i8, N10-i1.1, N10-15.3, N12-i1, N12-i2, N12-i4, N12-i5, N12-i7, N12-i8	Discontinuous	Neutralizing	(Guan et al., 2013)
	CD4i Cluster C.2	N12-i10, N12-i17, N12-i18, N12-i19	Discontinuous	Neutralizing	(Guan et al., 2013)
	CD4i Cluster C.3	N5-i2, N5-i6, N5-i9, N5-i14, N5-i7, N5-i12, N10-i3.1, N12-i12, N12-i9, N12-i11	Discontinuous	9/10 Neutralizing	(Guan et al., 2013)
	CD4i Cluster C.4	L9-i4, N5-i10.1, N5-i13, N10-i2, N12-i14, N12-i16	Discontinuous	2/6 Neutralizing	(Guan et al., 2013)
	CD4i	N10-i4, N10-i6.1	Discontinuous	Non-Neutralizing	(Guan et al., 2013)
	CD4i C1 region	CH20, CH29, CH38, CH40, CH49, CH51, CH52, CH53, CH54, CH55, CH57, CH77, CH78, CH80, CH81, CH90, CH91, CH92, CH94	Discontinuous	Non-Neutralizing	(Bonsignori et al., 2012)
	C2, C3, C4, V4 glycansites	2G12	Discontinuous	Neutralizing	(Trkola et al., 1996)
	C5	670-D	Discontinuous	Neutralizing	(Forthal et al., 1995)
		750-D	not defined	Non-Neutralizing	(Forthal et al., 1995)
		42F, 43F	Linear	Non-Neutralizing	(Alsmadi et al., 1997)
	CD4 binding site	15e	Discontinuous	Neutralizing	(Koup et al., 1991)
		F105	Discontinuous	Neutralizing	(Posner et al., 1992)
		448-D	Discontinuous	Neutralizing	(Forthal et al., 1995)
		1125H, 5145A	Discontinuous	Neutralizing	(Alsmadi et al., 1998)
		b12	Discontinuous	Neutralizing	(Hezareh et al., 2001)
		VRC01	Discontinuous	Neutralizing	(Ferrari et al., 2011)
	V2	CH58, CH59, HG107, HG120	Linear	Neutralizing	(Liao et al., 2013)
PG9		Discontinuous	Neutralizing	(Biedma ME, 2013)	
V3	694/98D	Linear	Neutralizing	(Forthal et al., 1995)	
	4117C, 41148D	Linear	Neutralizing	(Alsmadi et al., 1998)	
	CH22, CH23	Linear	Neutralizing	(Bonsignori et al., 2012)	

## **1.7 Fitness and selection in viral evolution**

Viral fitness is defined as “the capacity of a virus to produce infectious progeny in a given environment” (Domingo & Holland, 1997). A more accurate and specific term commonly in use today is replicative fitness (reviewed by Wargo & Kurath, 2012). Due to host immune clearance of viruses and finite host lifespan, viruses must be transmitted to new hosts to survive. As such, transmission fitness is an important component of overall fitness. Ultimately, replication and transmission contribute to the prevalence of viral genetic material at a population level in the field over time. The capacity of a virus (a serotype, clade, or variant) to become dominant in the field (relative to other serotypes, clades, or variants of the same virus) has been defined as epidemiologic fitness (Domingo, 2010). Resistance fitness is the ability of a virus to resist antiretroviral drugs and escape surveillance and destruction by host immune responses, notably T cells, neutralizing antibodies, or host genetic resistance mechanisms.

Replicative fitness of HIV-1 can be measured in cultured cells, tissue explants, or within individual hosts. It is often assessed by comparing viral replication in parallel hosts or cell cultures infected with single viral variants. However, the assessment of fitness in mixed infections of two viral variants is a more sensitive and possibly more valid measure of viral fitness differences (Domingo & Holland, 1997). Unfortunately, competition assays face the challenge of superinfection and recombination which would result from the presence of multiple viruses infecting one cell and production of chimeric viruses bearing components of both “parent” viruses. Transmission fitness studies of HIV-1 have proved extremely difficult to conduct due to practical and ethical considerations. Data on transmission fitness of HIV-1 have thus relied mainly on non-human primate models. Data from other simpler systems have shown that replicative fitness does not always match transmission fitness. Viral genotypes with the greatest replication success are not always those that have the greatest transmission to new hosts (Duan et al., 2010; Seibert et al., 2010).

Resistance fitness is strongly associated with replicative fitness costs. This means resistant variants show reduced replication ability compared to wild-type strains in the absence of the selective drug or host immune pressure. This has massive implications for the development of a vaccine and for the epidemic in general. Primary HIV-1 isolates in the epidemic appear to

have undergone a reduction in replicative fitness over time comparing historical (1986–1989) and more recent samples (2002–2003; Ariën et al., 2005). Several studies on intra-patient HIV-1 replicative fitness show that it tends to progressively increase through the course of infection in the absence of ART (Quiñones-Mateu et al., 2000; Barbour et al., 2004; Troyer et al., 2005). Consecutive genetic bottlenecks with each transmission event may reset the fitness for each new infecting virus. The resulting reduction in fitness may be greater than the replicative fitness increase within an infected individual prior to new transmission, thereby attenuating HIV-1 in the human population over time. It is well known that successive transmission events of RNA viruses may cause deleterious genetic bottlenecks which result in profound fitness losses (i.e., Muller's ratchet; Peris et al., 2010). Upon transmission, there is an absence of selective pressure prior to host responses or there may be differences in early host responses between the donor and recipient. This removes any need for transmission and resistance fitness and provides an opportunity for reversion to optimal replicative fitness. Herbeck et al. (2006) demonstrated reversion to ancestral states upon transmission driven by a need to re-establish replication efficiency. Reversion from CTL induced mutations with fitness cost has also been shown in numerous studies (Allen et al., 2004; Leslie et al., 2004).

Fitness acts on viral evolution through selection. Selection will act to either preserve or eliminate changes in the genome that result in different mutational fitness effects. As would be expected, the majority of mutations have negative impacts on fitness, often resulting in nonviable viruses. However, mutations with positive fitness effects also occur. These changes are sought after through the exploration of sequence space and are preserved or selected for the benefit of the virus (Wargo & Kurath, 2012). The very high replication rate of HIV-1 coupled with an extremely error-prone reverse transcriptase gene of one error in every two to four thousand bases (Preston et al., 1988) translates into a large and diverse quasispecies population, which is an extremely efficient exploration of sequence space. For HIV-1, increases in quasispecies heterogeneity were associated with fitness recovery *in vitro*, even in the absence of changes in the consensus sequence (Bordería et al., 2010).

Statistical models of codon evolution have been developed and applied to protein-coding sequences from viral genomes pathogens for the detection of evidence of diversifying selection acting on protein coding DNA sequences (Muse & Gaut, 1994; Nielsen & Yang, 1998; Z. Yang et al., 2000). Positive selection is frequently inferred by comparing the rate of non-synonymous substitutions per non-synonymous site (dN) to the rate of synonymous

substitutions per synonymous site (dS). The ratio of these two rates is often represented by the symbol  $\omega$ . Under the assumption that synonymous substitutions are neutral and that the synonymous substitution rate therefore approximates the neutral rate of evolution, diversifying selection can be inferred when  $\omega$  is greater than one (Tajima, 1989). HIV-1 has several examples of sequence motifs within protein-coding sequences that are expected to be under purifying selection at the nucleotide level. Many of these are involved in regulating gene expression and have been shown to evolve under the influence of strong, purifying selection pressure at the nucleotide level (Ngandu et al., 2008). Analysis of the type of selection acting on a gene or codon gives insight into the type of selection pressure under which it is evolving. Mathematical analyses of selection are confounded by recombination (Anisimova et al., 2003; Scheffler et al., 2006; Shriner et al., 2003).

Recombination occurs when the reverse transcriptase switches RNA templates during replication, thereby forming progeny viruses that are representative of different parent viruses (Wooley et al., 1997). HIV has one of the highest recombination rates compared to other organisms, with approximately 2.8 recombination events occurring per genome per replication cycle (Zhuang et al., 2002). The recombination rate therefore exceeds the mutation rate per replication. Recombination events can occur between viruses from different subtypes (intersubtype recombination) and also between viruses derived from the same subtype (intrasubtype recombination; Takehisa et al., 1999). This second type of recombination is much harder to identify but is important to take into account. Publically available tools for site specific inferences of purifying and diversifying selection such as Datamonkey make use of a recombination detection algorithm (GARD) (Kosakovsky Pond et al., 2006).

## 1.8 Study rationale

The diversity of HIV-1 remains a massive challenge in the development of an effective vaccine. Most of the biologically relevant variation observed *in vivo* is the result of genetic variation and competitive selection together with random events acting on a large mass of replicative units. The aim of all interventions and therapies is to prevent infections or oppose increases of viral load or viral fitness. Replication rate, viral load, genetic heterogeneity, viral fitness and disease progression are all strongly interconnected. Thorough understanding of

the interplay between these factors and their influence on both the virus and the host immune response is essential for the development of effective interventions.

A priority for vaccine design is to elicit humoral responses that provide sterilizing immunity. Broadly neutralizing antibodies are central to this effort. Despite the presence of evidence of subtype-specific determinants of neutralization, only a few studies have related the development of breadth to the specific subtype of the infecting virus. Viral load and time since infection are very strongly associated with the increased breadth of neutralizing antibody responses. Controlling for these factors is essential for the accurate and meaningful understanding of the role of subtypes and viral diversity in the development of broadly neutralizing antibodies in natural infection. Events occurring during early viral evolution are likely to have a disproportionately large effect on the development of broadly neutralizing antibodies. The role of effector functions of neutralizing and non-neutralizing antibodies, particularly antibody dependent cell-mediated cytotoxicity (ADCC), in viral control and evolution is still very unclear. Looking at early patterns of escape and identification of selective pressures driving early changes should provide insights into how these factors drive viral evolution.

The specific objectives of the project were:

1. To compare the development of broadly neutralizing antibody responses after two years of infection in two cohorts of African women with very different circulating, local HIV-1 epidemics: the CAPRISA cohort based in the KwaZulu Natal region of South Africa and the HISIS cohort based in the Mbeya region of Tanzania.
2. To study the earliest humoral responses as selective pressures driving the viral evolution and diversification process in an individual infected with HIV-1 with an emphasis on understanding the interplay of neutralizing and ADCC antibodies.
3. To study the cost on viral fitness of early adaptations to humoral responses during HIV-1 infection.

Development of effective interventions against HIV-1 is likely to result from successful integration of multiple approaches to achieve optimal results. This will therefore depend on a significantly improved understanding of the interplay between different aspects of the disease than we currently possess.

## Chapter 2: Differences in HIV-1 Neutralization Breadth in Two Geographically Distinct Cohorts in Africa

<b>ABSTRACT</b> .....	<b>38</b>
<b>2.1 Introduction</b> .....	<b>39</b>
<b>2.2 Materials and Methods</b> .....	<b>40</b>
2.2.1 Ethics statement.....	40
2.2.2 Participants.....	41
2.2.3 Estimation of duration of infection.....	41
2.2.4 RT-PCR amplification and sequencing.....	42
2.2.5 Characterization of infecting virus.....	42
2.2.6 Panel viral isolates.....	43
2.2.7 Cloning of envelope genes and neutralization assays.....	43
2.2.8 Mapping of HISIS_605 plasma specificity.....	44
2.2.9 Calculation of breadth and potency scores.....	44
2.2.10 Statistical analyses.....	45
<b>2.3 Results</b> .....	<b>45</b>
2.3.1 Characteristics of the two cohorts and their infecting viruses.....	45
2.3.2 HISIS participants had greater neutralization breadth and potency.....	46
2.3.3 Preferential recognition of subtype C viruses by HISIS participants.....	47
2.3.4 Early viral loads were strong predictors of breadth at 2 years in both cohorts.....	48
2.3.5 HISIS cohort had higher viral loads.....	49
2.3.6 Viral characteristics and development of neutralization breadth.....	50
2.3.7 Development of autologous neutralizing antibody responses.....	51
2.3.8 Development of neutralizing antibody breadth in HISIS_605.....	52
2.3.9 Specificity of neutralizing antibody response in HISIS_605.....	52
<b>2.4 Discussion</b> .....	<b>54</b>

## **ABSTRACT**

It remains an open question whether different geographically distinct population groups, vary in their neutralization responses to HIV-1. Here we compared the development of HIV-1 neutralizing antibody responses, over the first two years of infection, in two African cohorts. Serum samples from 20 women from the HISIS cohort in Tanzania infected with subtypes A, C, D or unique recombinant forms, and 22 women from the CAPRISA cohort in South Africa infected with subtype C, were assayed against a multi-subtype 12-virus panel in the TZM-bl assay. There were significantly higher neutralizing antibody responses among the Tanzanian participants, 35% of whom could neutralize >50% of the panel viruses by two years post infection compared to only 9% in the CAPRISA cohort ( $p=0.0131$ ). Accumulative viral loads between 3 and 12 months post infection were strongly associated with neutralization breadth ( $p<0.0001$ ,  $r^2=0.4747$ ) and were higher in the Tanzanian cohort ( $p=0.0460$ ). The HISIS cohort also had higher autologous neutralization titers at 12 months post infection (6649.76) than the CAPRISA cohort (1336.69:  $p=0.0327$ ). No association was found between the neutralizing antibody breadth and dual infection, infection by intersubtype recombinant variable loop length. One elite neutralizer was identified in the HISIS cohort in whom breadth was likely attributed to antibodies directed toward the CD4 binding site. Our results show that the Tanzanian cohort had higher viral loads and an increased frequency of cross-neutralizing responses compared to the South African cohort suggesting that community viral loads play a role in the development of broadly neutralizing antibodies in different populations.

## **2.1 Introduction**

The unprecedented viral diversity seen in the HIV-1 pandemic poses a major challenge to the development of an effective preventive vaccine. Studies on HIV-infected humans with broadly neutralizing antibody (bNAb) responses may provide important insights into how to elicit these types of responses through vaccination. Broadly neutralizing responses generally take 2-4 years to develop, and have been strongly associated with high viral loads and low CD4 counts in early infection (Gray et al., 2011; Piantadosi et al., 2009; Sather et al., 2009b). Only 10-30% of individuals develop bNAb responses (Dhillon et al., 2007; Doria-rose et al., 2010; Euler et al., 2010; Gray et al., 2011; Yuxing Li et al., 2007; McElrath & Haynes, 2010; Sather et al., 2009b; Simek et al., 2009), with approximately 1% of individuals classified as elite neutralizers (Simek et al., 2009). There have been limited studies that have directly compared neutralization responses between cohorts to determine if these responses vary between different populations infected with different subtypes.

This study compares kinetics and potency of neutralization responses in two cohorts in Africa: one in South Africa (Centre for the AIDS programme of research in South Africa, CAPRISA) exclusively infected with subtype C; and one in Tanzania (HIV superinfection study, HISIS) infected with multiple subtypes or recombinant forms. Although subtype has also been implicated in the potency and breadth of neutralizing responses (Bures et al. 2002; Brown et al. 2008; Dreja et al. 2010; M. Li et al. 2006), further investigation is warranted as these studies were cross-sectional and did not control for confounders such as viral load or time since infection. In addition, other viral envelope properties may influence the development of breadth including high viral diversity in early infection (Euler et al., 2012; Piantadosi et al., 2009; Sather et al., 2009b), infection by viruses with shorter variable loops, differences in glycosylation motifs, and amino acid changes (Rademeyer et al., 2007; van den Kerkhof et al., 2013). Furthermore, dual infection with two distinct HIV strains has also been associated with broader neutralizing antibody responses (Cortez et al., 2012; Powell et al., 2010).

The targets of HIV-specific bNAbs are common between subtypes and have been mapped to four sites of vulnerability: the CD4 binding site, membrane proximal external region (MPER), V1V2 domain, and the coreceptor/V3 region of gp120 (reviewed by Haynes et al

2013; Walker et al. 2009; Walker et al. 2011; Stamatatos et al. 2009; McElrath & Haynes 2010; Mascola & Montefiori 2010; Pejchal et al. 2012). However, studies have shown broader neutralizing antibody responses against viruses of the same subtype as the infecting virus that elicited the response (Bures et al., 2002; Lakhashe et al., 2007; Rademeyer et al., 2007; van Gils et al., 2010) suggesting that there are subtype-specific epitope variants (i.e. that some epitopes are less variable within a subtype compared to between subtypes) (Binley et al., 2004; Brown et al., 2008; Li et al., 2006). Inter-subtype recombinant viruses in the form of circulating recombinant forms (CRF) potentially contain epitopes from discordant subtypes or provide a different scaffold for the known sites of vulnerability, and it is not clear to what extent this may impact on the development of neutralizing antibody response in natural infection.

To understand the evolution of bNAb responses in different populations in Africa, we compared the kinetics and breadth of responses in a South African and Tanzanian cohort. This study showed that participants in the Tanzanian cohort had higher viral loads and significantly broader neutralizing responses at two years post infection compared to the South African cohort.

## **2.2 Materials and Methods**

### **2.2.1 Ethics statement**

All laboratory and cohort work done in the HISIS study was reviewed and approved by the research ethics committees of the Ministry of Health, Tanzania; the Ludwig-Maximilians-University, Munich, Germany; and the University of Cape Town, South Africa. The CAPRISA Acute Infection study was reviewed and approved by the research ethics committees of the University of KwaZulu- Natal, the University of Cape Town, and the University of the Witwatersrand. All participants provided written informed consent for study participation.

### **2.2.2 Participants**

Twenty-two participants from the CAPRISA 002 Acute Infection study were included in this study. CAPRISA 002 is a cohort in KwaZulu-Natal, South Africa that recruits from both urban Durban and rural Vulindlela. For the CAPRISA cohort, high risk HIV negative women monitored either monthly or three monthly for recent HIV-1 infection using two HIV-1 rapid antibody tests and PCR (Roche Amplicor v1.5). HIV-1 infection was confirmed using an enzyme immunoassay (EIA) test. Following diagnosis of HIV-1 infection, women were followed weekly for a month, bi-weekly until 3 months post-infection, monthly for one year, and 3-monthly thereafter (van Loggerenberg et al., 2008).

Twenty participants from the HIV Superinfection Study (HISIS) were included in this study. HISIS is a cohort of 600 women at high risk of HIV infection working in modern and traditional bars, guesthouses and hotels, established in Mbeya Region, south west Tanzania (Riedner et al., 2003). For the HISIS cohort, high risk seronegative women were followed three monthly and were screened for HIV infection using EIA and PCR (Roche Amplicor v1.5). Recently HIV-1 infected HISIS participants were followed up 3-monthly for up to 2 years post infection.

HIV-1 plasma RNA load for both cohorts was determined with the Roche Amplicor HIV-1 Monitor Test version 1.5 (Roche Diagnostics). The range of quantitation was  $\geq 400$  to  $\leq 750,000$  virus copies per millilitre (Saathoff et al., 2010; van Loggerenberg et al., 2008). All women were antiretroviral treatment naïve for the duration of the study.

### **2.2.3 Estimation of duration of infection**

The estimated duration of infection for both cohorts was categorized into five stages based on evolving HIV-1 RNA or antibody profiles (Fiebig et al., 2003). Duration of infection was estimated for those in Fiebig stage I/II as 14 days; and Fiebig stages III/IV as 30 days. For participants in Fiebig stage V or VI stage, duration of infection was estimated as the midpoint between the last HIV-1 negative visit (PCR negative and seronegative) and first seropositive visits, or as 45 days prior to the first seropositive visit, whichever was closer.

#### 2.2.4 RT-PCR amplification and sequencing

HIV-1 RNA was purified from plasma using the Qiagen Viral RNA kit, and reverse transcribed to cDNA using Superscript III Reverse Transcriptase (Invitrogen, CA). Single genome amplification (SGA) or end-point dilution was carried out on plasma from the first seropositive visit in HISIS and on the first HIV positive (serum or RNA positive) visit in CAPRISA (Salazar-Gonzalez et al., 2008). Amplicons were directly sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA) and resolved on an ABI 3100 automated genetic analyzer. The full-length *env* sequences were assembled and edited using Sequencher v.4.0 software (Genecodes, Ann Arbor, MI). Multiple sequence alignments were performed using Clustal X (ver. 1.83) and edited by eye with BioEdit (ver. 5.0.9). Pairwise DNA distances and neighbor joining trees were computed using Mega 4 (Tamura, Dudley, Nei, & Kumar, 2007). Trees generated in MEGA were visualized using Dendroscope (ver. 2.3) (Huson et al., 2007).

#### 2.2.5 Characterization of infecting virus

Infecting virus sequences in the CAPRISA cohort were subtyped using the REGA subtyping tool available at <http://bioafrica.mrc.ac.za/reg-a-genotype/html/subtypinghiv.html> (Alcantara et al., 2009; de Oliveira et al., 2005). The infecting HIV-1 virus/viruses in the HISIS cohort are described in full by Nofemela, Bandawe et al. (2011).

The presence of multiple subtype infections in the HISIS cohort was previously determined by the Mbeya Medical Research Programme and Ludwig-Maximilians-University team one year after infection by the Multi-region Hybridization Assay (MHA), using subtype A, C and D-specific fluorescent probes in five genomic regions in a real-time PCR format (Geldmacher et al., 2007; Hoelscher et al., 2002). Detection of multiple subtype C infections (dual infection) in CAPRISA was defined previously using heteroduplex mobility assay (HMA) and confirmed using single genome amplification (SGA) and sequencing (Woodman et al., 2011).

### 2.2.6 Panel viral isolates

Molecularly cloned full-length *env* genes for HIV-1 Env pseudovirus production were obtained as follows: four representative clones from the standard panel of HIV-1 subtype B reference strains (AC10.0.29, CAAN5342.A2, QH0692.42 and PVO.4: Li et al. 2005; Li et al. 2006) and the standard panel of HIV-1 subtype C reference strains (Du156.12, Du422.1, ZM214M.PL15, and ZM109F.PB4: Li et al. 2007) as well as four Kenyan acute/early subtype A *env* clones (Q168ENVa2, Q461ENVe2, Q842ENVd12, and Q23ENV17: Long et al. 2002; Poss & Overbaugh 1999) that have been previously described were obtained from the NIH AIDS Research and Reference Reagent Program (ARRRP).

### 2.2.7 Cloning of envelope genes and neutralization assays

For 16 HISIS participants, we generated functional *env* clones whose sequences were identical to or most closely matched the consensus of SGA-derived sequences from the earliest RNA positive visit. These molecular clones were assumed to represent the T/F virus responsible for clinical infection. First-round PCR products were used as templates to amplify full-length envelope genes. The 3-kb PCR fragments, generated using the *envA*-rx (5'-CACCGGCTTAGGCATCTCCTATAGCAGGAAGAA-3') and *envM* primers (Keele et al., 2008), were cloned into the pCDNA 3.1-TOPO vector (Invitrogen) and screened as previously described (Haaland et al., 2009).

The JC53bl-13 (TZM-bl) cell line was obtained from the NIH AIDS Research and Reference Reagent Program. 293T cells were obtained from G. Shaw. Both cell lines were cultured in DMEM (Gibco BRL Life Technologies) containing 10% heat-inactivated FBS and 50 µg/mL gentamicin (Sigma). Cell monolayers were disrupted at confluency by treatment with 0.25% trypsin in 1mM EDTA.

Env-pseudotyped viruses were obtained by co-transfecting the Env plasmid with pSG3ΔEnv (Li et al., 2005), into HEK293T cells using Fugene transfection reagent (Roche). Pseudovirus-containing supernatant was harvested 24 h following transfection and clarified by centrifugation and 0.45 µm filtration. Single-use aliquots (1.0 mL) were stored at -80°C. The 50% tissue culture infectious dose (TCID<sub>50</sub>) for each pseudovirus preparation was

determined by infection of TZM-bl cells as previously described (Gray et al. 2007; Li et al. 2005). Virus neutralization was measured using a luciferase based assay in TZM.bl cells as previously described (Gray et al. 2007). In brief, three-fold serial dilutions of plasma samples were performed in duplicate (96-well flat bottom plate) in 10% D-MEM growth medium (100  $\mu$ L/well). An amount of 200 TCID<sub>50</sub> of virus was added to each well in a 50  $\mu$ L volume, and the plates were incubated for 1 h at 37°C. TZM.bl cells were then added ( $1 \times 10^4$ /well in a 100 $\mu$ L volume) in 10% D-MEM growth medium containing DEAE-dextran (Sigma, St. Louis, MO) at a final concentration of 11  $\mu$ g/mL. Assay controls included replicate wells of TZM.bl cells alone (cell control) and TZM.bl cells with virus (virus control) as well as wells containing VSV-G pseudotyped virus to ensure inhibition is HIV-1 specific. Following 48-h incubation at 37°C, 150  $\mu$ L of assay medium was removed from each well and 100  $\mu$ L of Bright-Glo luciferase reagent (Promega, Madison, WI) was added. The cells were allowed to lyse for 2 min, and then 150  $\mu$ L of the cell lysate was transferred to a 96-well black solid plate, and luminescence was measured using a Victor 3 luminometer (Perkin Elmer). Titres were calculated as the 50% inhibitory dilution (ID<sub>50</sub>) which is the serum dilution that caused a 50% reduction in relative luminescence units (RLU) compared to the level in the virus control wells after subtraction of cell control RLU.

### **2.2.8 Mapping of HISIS\_605 plasma specificity**

The consensus C (ConC) plasmid pConCgp160-opt (catalog no. 11405) was obtained through the NIH ARRRP, provided by Beatrice Hahn (University of Alabama). The following ConC mutants, containing mutations in known broadly neutralizing epitopes, were screened for neutralization activity; (V2) N160A, K169E; (V3) I307A, H330Y, N332A; (CD4 binding site, CD4bs) K360V, E362N, L369P, T372V/T373M, S375M; (V4) T408A, T415I; (C4) R416A; (V5) F468V (Mufhandu et al., 2012). Neutralization assays were conducted and the shift in the ID<sub>50</sub> titre of the mutant relative to the wild-type pseudovirus was calculated.

### **2.2.9 Calculation of breadth and potency scores**

Breadth and potency scores were calculated for each plasma sample to compare neutralization between samples according to the method of Blish et al. (2008). A median ID<sub>50</sub> value was assigned to each panel virus, based on the median of all the ID<sub>50</sub> values from every

plasma sample tested against that virus. To define a breadth score for an individual plasma sample, the plasma/virus combinations in which the ID<sub>50</sub> was above the median ID<sub>50</sub> defined for that virus with all plasma samples were given a score of 1, and those below were scored as 0. The breadth score within each subtype was calculated and the overall breadth score was determined by summing these numbers for all 12 viruses tested. The potency score was derived by dividing the ID<sub>50</sub> value of a given plasma/virus combination by the median virus ID<sub>50</sub> value. As with the breadth score, the potency scores against all 12 panel viruses were added to obtain an overall potency score.

### **2.2.10 Statistical analyses**

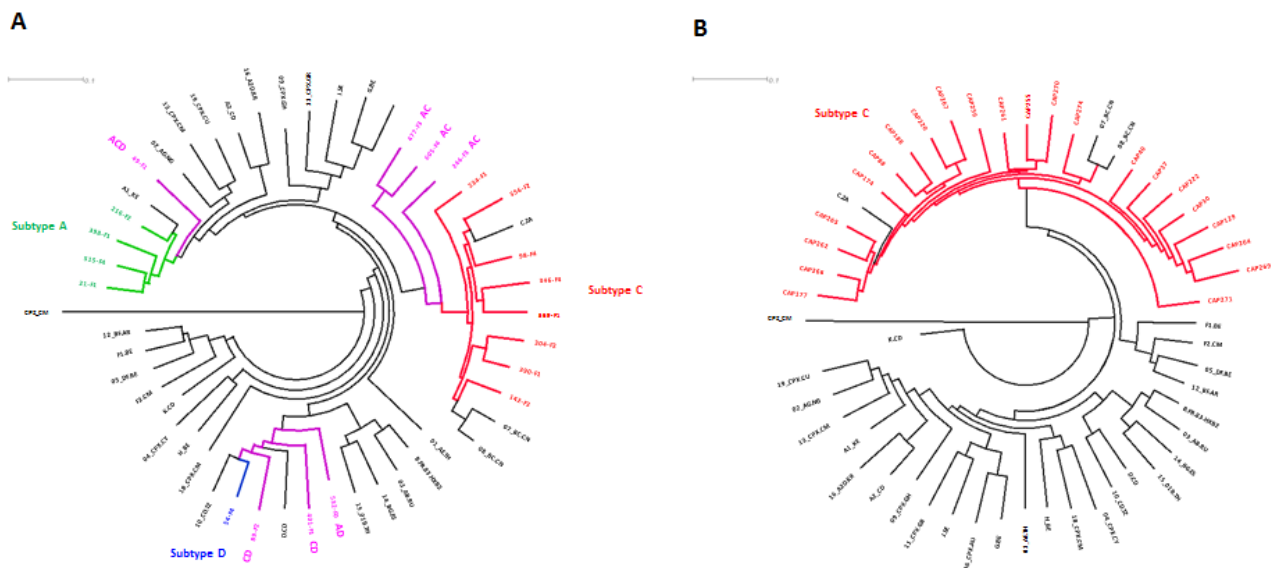
To compare the viral load measurements between the CAPRISA and HISIS cohorts, the lower and upper detection limits of those viral loads measured in the CAPRISA cohort were set artificially at 399 copies/mL and 750 001 copies/mL, respectively. For both cohorts, viral loads were measured regularly up to 4 years post infection. Generation of LOESS smoothing lines and Wilcoxon rank sum and Mann Whitney tests were performed by Lise Werner (UKZN) using Graphpad Prism. Linear mixed model analyses fitted to viral load and corrections accounting for repeated measures were performed by Lise Werner using SAS version 9.3 (SAS Institute Inc., Cary).

## **2.3 Results**

### **2.3.1 Characteristics of the two cohorts and their infecting viruses**

This study compared the neutralization breadth at two years post infection in 22 participants in the CAPRISA cohort (van Loggerenberg et al., 2008) recruited from rural and urban areas in KwaZulu/Natal Province, South Africa; and 20 participants in the HISIS cohort (Hoelscher et al., 2002) recruited from Mbeya region on the south-western border of Tanzania. All participants were recruited within a mean 34 days of estimated time of infection (range of 14 to 45 days), and were followed for a minimum of 2 years (range 24-27 months). Both cohorts consisted of high risk female participants who had acquired HIV via heterosexual transmission and remained anti-retroviral treatment naïve during the 2 years of follow up. Infections in the both cohorts reflected the HIV diversity of their respective local epidemics

with HISIS participants infected with subtypes A (n=4), C (n=8), D (n=1) and recombinant viruses (n=7) [2 AC, 2 ACD, 1 AD and 2 CD] (Nofemela et al., 2011); while CAPRISA participants were all infected with subtype C viruses (van Loggerenberg et al., 2008) (Figure 1A and 1B). A description of each of the unique inter-subtype mosaic viruses from the HISIS cohort was published previously (Nofemela, Bandawe et al., 2011). Mean inter-participant pair-wise distances between infecting viruses was significantly higher for HISIS viruses at 15.63% than for the CAPRISA viruses at 10.68% ( $p < 0.0001$  Mann-Whitney).



**Figure 1 A and B:** Circular maximum likelihood trees showing consensus derived infecting viruses sequences from the HISIS (A) and CAPRISA (B) cohorts. Thirty reference sequences including 18 CRF sequences and a SIVcpz out-group are included (in black). Branch tips for subtype A are green, subtype B blue subtype C red, and recombinants purple.

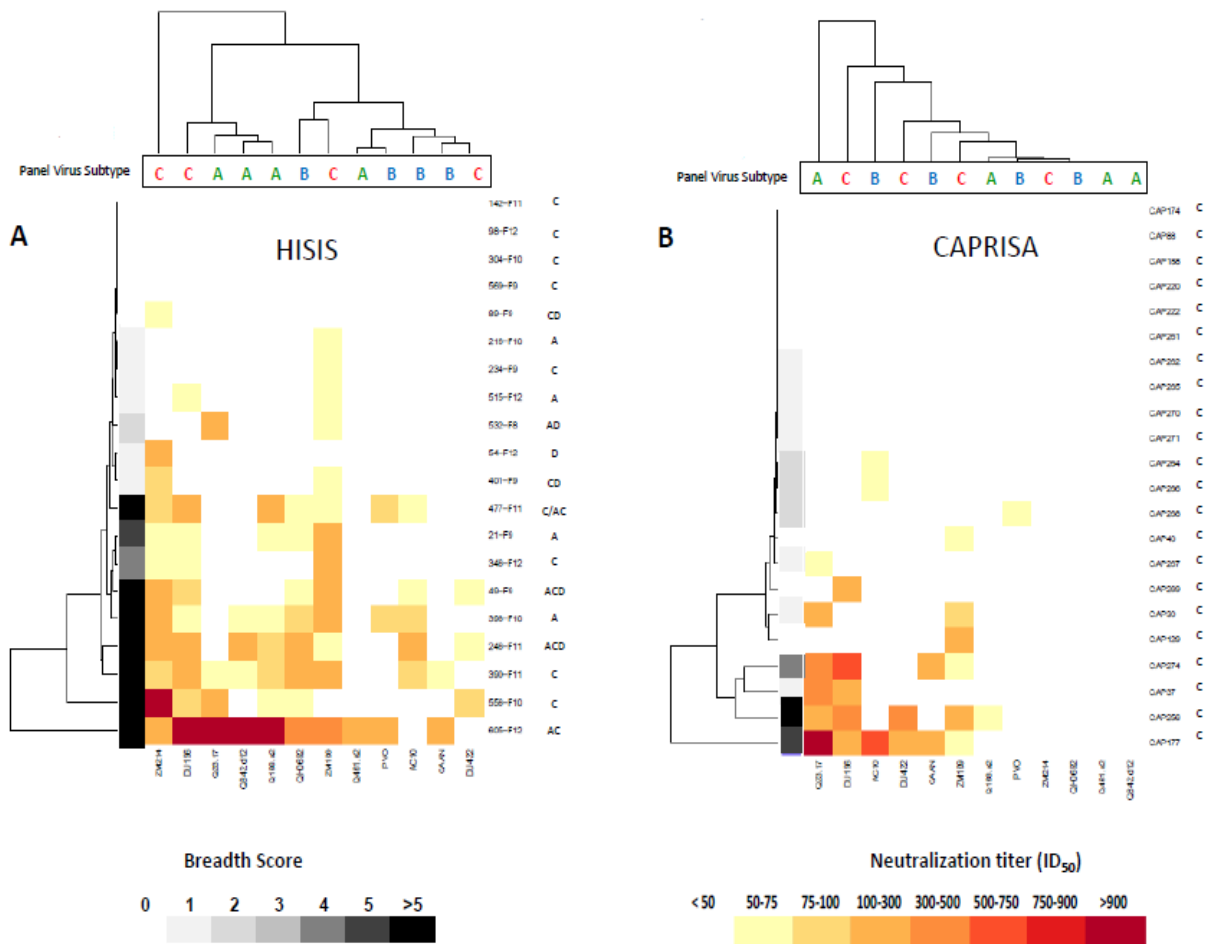
### 2.3.2 HISIS participants had greater neutralization breadth and potency

Neutralization assays were conducted using a 12 pseudovirus panel consisting of four subtype A (one tier 1B; three tier 2), four subtype C (one tier 1B; three tier 2) and four subtype B viruses (1 tier 3; three tier 2: Seaman et al. 2010: Appendix D). Each plasma sample was assigned a breadth and potency score which takes into consideration the overall neutralization titre and susceptibility of each panel virus. At two years post infection, there was significantly higher breadth of neutralizing responses among the Tanzanian participants of whom 35% (7/20) developed broadly neutralizing responses (bNAbs: could neutralize >50% of the panel viruses at titres of over 1:50,  $ID_{50} > 50$ ) compared to only 9% (2/22) in the CAPRISA cohort ( $p = 0.0139$ : Appendix D). Potency scores did not differ between the two cohorts but

participants in the HISIS cohort had a significantly higher mean breadth score (3.35) compared to the CAPRISA participants (0.95) ( $p=0.0131$ ).

### 2.3.3 Preferential recognition of subtype C viruses by HISIS participants

The subtype C viruses in the panel were more commonly recognised by HISIS participants with a mean breadth scores of 1.65 against subtype C, compared to 0.8 and 0.9 against subtype A and B viruses respectively (Appendix D; Figure 2.2). CAPRISA participants, where fewer individuals developed bNAbs, did not show preferential recognition of a subtype with mean breadth scores of 0.36, 0.27 and 0.32 against subtype A, B and C respectively. Interestingly, one subtype C virus, ZM214, was neutralized by plasma from 12 HISIS participants, but was not neutralized by plasma from any individual in the CAPRISA cohort.



**Figure 2.2:** Hierarchical clustering of ID<sub>50</sub> neutralization titres of HISIS (A) and CAPRISA (B) donor sera and twelve panel viruses. The dendrograms on the left cluster sera according to their neutralization capacity while the dendrograms at the top cluster the panel viruses according to their neutralization susceptibilities.

### 2.3.4 Early viral loads were strong predictors of breadth at 2 years in both cohorts

To determine the time point at which viral load was most predictive, breadth scores were divided into three categories: no breadth (0 breadth score); cross-neutralization (1-4 breadth score); and broadly cross-neutralizing (5+ breadth score). A multinomial regression model was fitted to breadth (as a 3-level categorical outcome) at 4, 7, 13, 19 and 25 months post infection in order to determine the mean viral load most predictive of breadth at 2 years.

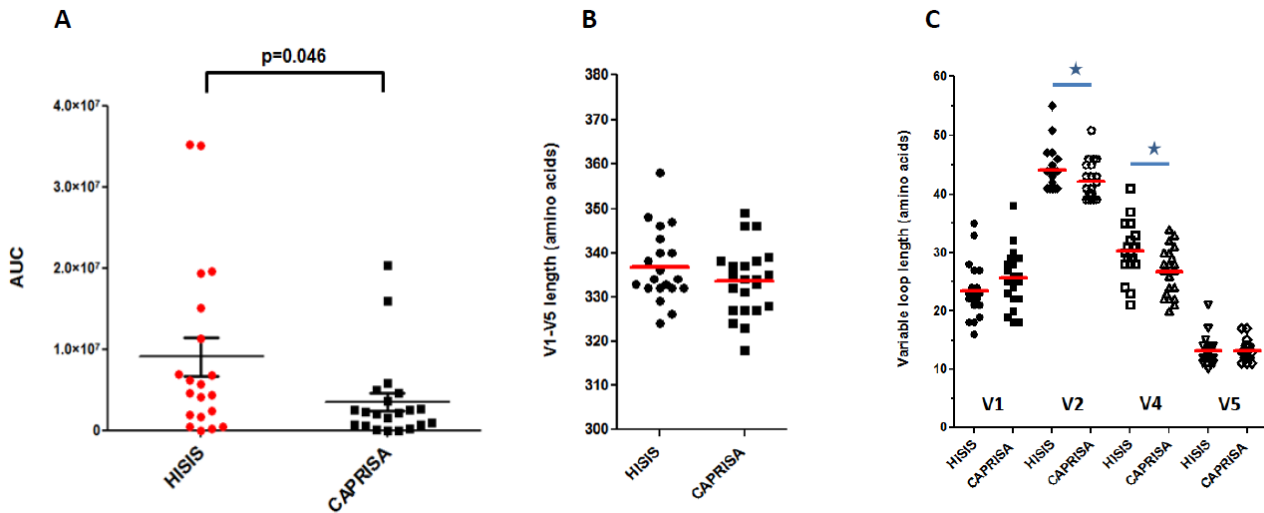
**Table 2.1:** Odds of developing a breadth of 1-4 or 5+ versus no breadth at 2 years, for every 1 log increase in log viral load at each of the individual time points (multinomial regression model performed by Lise Werner).

Variable	Breadth 1-4 vs Breadth 0		Breadth 5+ vs Breadth 0	
	Odds ratio (95% CI)	p-value	Odds ratio (95% CI)	p-value
Log viral load				
4 months	0.93 (0.40 – 2.16)	0.8576	4.55 (1.05 – 19.63)	0.0424
7 months	1.02 (0.39 – 2.66)	0.9671	32.41 (1.35 – 776.49)	0.0318
13 months	0.92 (0.38 – 2.23)	0.8449	2.97 (0.71 – 12.31)	0.1345
19 months	0.55 (0.23 – 1.33)	0.1864	1.59 (0.44 – 5.77)	0.4775
25 months	0.94 (0.38 – 2.34)	0.8883	3.19 (0.83 – 12.21)	0.09

Using combined data from both cohorts, viral loads at 4 and 7 months post infection, were significantly associated with bNAb responses ( $p = 0.0424$  and  $p = 0.0318$ ) (Table 2.1). At 4 months, for every 1 log increase in viral load, there was a 4.55 times increased likelihood of having a bNAb response compared to not having a breadth response (OR 4.55; 95% CI 1.05 – 19.63;  $p=0.0424$ ). Stronger associate was detected at 7 months where, for every 1 log increase in viral load, there was a 32 times increased likelihood for a bNAb response compared to not having a breadth response (OR 32.41; 95% CI 1.35 – 776.49;  $p=0.0318$ ). However, accumulative viral exposure as measured by plasma viral load area under the curve (pVL AUC) between 3 and 12 months was the strongest predictor of breadth at 2 years post infection ( $p<0.0001$ ;  $r^2=0.4747$ ). Interestingly, despite being predictive of breadth scores greater than 5, viral load was not predictive of the development of breadth scores of 1 to 4 (Table 1).

### 2.3.5 HISIS cohort had higher viral loads

To determine if viral load was associated with increased breadth observed in the HISIS cohort, the mean pVL AUC values was compared between the CAPRISA and HISIS cohorts. The mean pVL AUC for HISIS was significantly higher than that in CAPRISA ( $p=0.046$ ; Figure 2.3A).



**Figure 2.3:** **A:** Cumulative plasma viral load measured by the area under the curve (pVL AUC) between 3 and 12 months post infection for each individual in CAPRISA and HISIS (Mann-Whitney test). **B:** Comparison of total V1–V5 amino acid loop lengths of derived transmitted/founder virus sequences from HISIS and CAPRISA cohorts. Horizontal bars indicating median lengths are in red. **C:** Comparison of individual variable loop amino acid lengths of derived transmitted/founder virus sequences from HISIS and CAPRISA cohorts. Horizontal bars indicating median lengths are in red.

To determine how much of the differences in neutralizing responses could be attributed to factors other than viral load, we fitted a multinomial model after adjusting for pVL AUC. In this adjusted model, we found that HISIS cohort participants were 10.21 (95% CI 0.65-160.88  $p=0.0986$ ) times at greater odds of developing bNAb responses compared to the CAPRISA participants. This compared to only 5.88 greater odds in the unadjusted model (OR 5.88, 95% CI 0.90 – 33.3;  $p=0.0644$ ). Although this difference was not statistically significant, this result indicated that factors, in addition to elevated viral loads, may have contributed to the development of greater breadth in the HISIS cohort.

### **2.3.6 Viral characteristics and development of neutralization breadth**

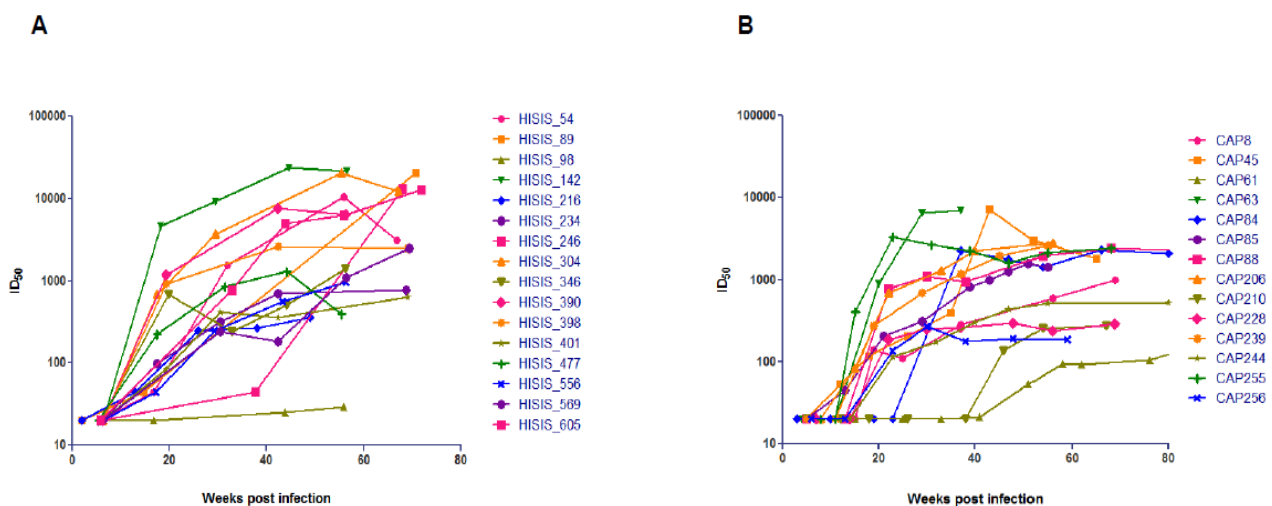
Of the 20 participants in the HISIS cohort, eight (40%) were infected with subtype C, and seven (35%) were infected with an intersubtype recombinant virus. To investigate the influence of subtype C and recombinant viruses on the development of breadth, HISIS participants were partitioned into subtype C and “other” infections (non-C and recombinants) as well as into “pure” HIV subtype versus inter-subtype recombinant infections. Subtype C infections had lower mean breadth scores compared to other infections (2.25 and 4.08 respectively) but this was not statistically significant ( $p=0.1577$ ). The mean breadth score for recombinant virus infections was higher compared to pure subtypes (4.71 and 2.62 respectively), but this difference was also not statistically significant ( $p=0.1840$ : Wilcoxon rank sums exact test) both before and after accounting for viral load. While viral subtype did not appear to be a major factor in the development of breadth in the HISIS cohort, a larger sample size is needed to address the effect of subtype on neutralization breadth.

Dual infections have been associated with breadth of neutralizing antibody responses (Cortez et al., 2012; Powell et al., 2010). Dual infection data was available for participants in the HISIS and CAPRISA cohort (Herbinger et al., 2006; Woodman et al., 2011). In the HISIS cohort, 7/20 (35%) were dually infected compared to 4/22 (18%) in the CAPRISA cohort (Appendix D). Dual infection was not associated the development breadth in either of the cohorts nor in a combined analysis (using a Wilcoxon ranks sum test and multinomial regression model).

We then investigated if there were other genotypic features of infecting viruses that may affect the development of the neutralizing antibody response. A comparison of variable loops between the cohorts found that there was no significant difference in V1-V5 length (Figure 2.3B), but that HISIS viruses in early infection had significantly longer V2 ( $p=0.03$ ) and V4 ( $p=0.0133$ ) loops than CAPRISA viruses (Figure 2.3C). However, unlike previous studies (Rademeyer et al., 2007), there was no association between loop length of the infecting virus and development of breadth in this small cohort study.

### 2.3.7 Development of autologous neutralizing antibody responses

We then compared the kinetics of autologous neutralizing antibody responses in the CAPRISA cohort with those of the HISIS cohort (Figure 2.4). Previously published data from the CAPRISA cohort showed that individuals had a median time to first detection of autologous neutralization of 19 weeks (Gray et al. 2007). Twelve of the fourteen participants studied here had developed responses with titres over 100 at 20 weeks post infection. In the HISIS cohort, fourteen of the sixteen participants tested also developed responses by 20 weeks post infection. Most participants (11/16) could neutralize their infecting virus with titres over 1,000 at 12 months with four participants, HISIS\_54, HISIS\_142, HISIS\_304 and HISIS\_605, reaching extremely high titres of over 10,000. No CAPRISA participant had reached titres of 10,000. Median autologous neutralizing antibody titers at 12 months post infection were higher in HISIS (6649.76) than in CAPRISA (1336.69) ( $p=0.0327$ , Wilcoxon signed rank test). HISIS\_98 developed a very weak neutralization response to its infecting virus, and was unable to neutralize any heterologous virus at 2 years post infection.



**Figure 2.4:** The development of autologous neutralizing antibody responses in HISIS (A) and CAPRISA (B). A functional envelope clone representing the transmitted/founder or an early virus for each of 16 HISIS and 14 CAPRISA participants was tested against longitudinal samples of autologous plasma in an Env-pseudotyped virus neutralization assay. Results are shown as neutralization titres (ID<sub>50</sub>). CAPRISA assays were performed by Penny Moore (NICD).

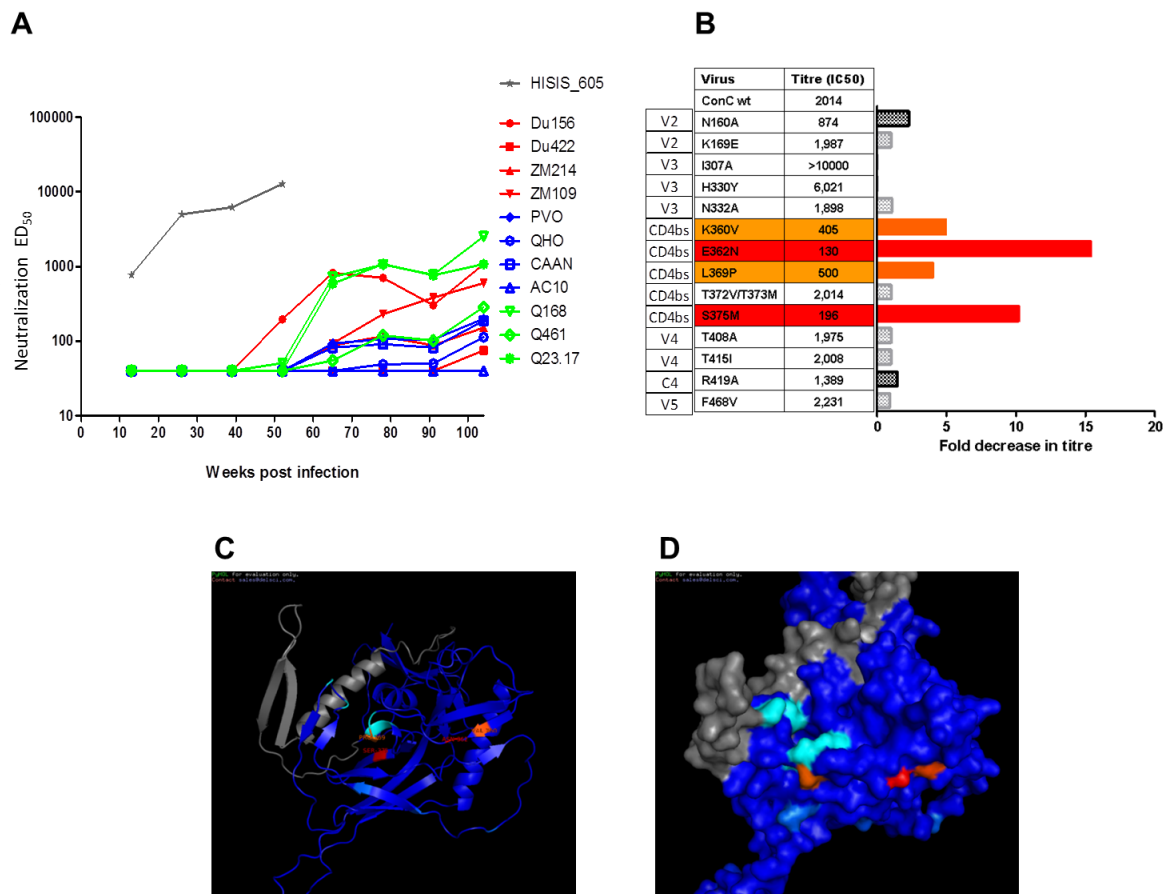
### 2.3.8 Development of neutralizing antibody breadth in HISIS\_605

HISIS\_605 showed the broadest response and neutralized 83% of the virus panel which is highly unusual after only 2 years of infection (Figure 2.2). This participant was infected with a subtype A/C recombinant virus (Nofemela, Bandawe et al. 2011) and neutralized 3 subtype A viruses at ID<sub>50</sub> values of over 1,000, two subtype Cs and one subtype B and subtype D virus with ID<sub>50</sub> of over 300 fulfilling the definition of an elite neutralizer (Simek et al., 2009). There was no cross reactivity at 36 weeks (9 months), and some cross neutralization was detected at 52 weeks (12 months) post infection with strong neutralization of Du156 and some weak neutralization of Q168 (Figure 2.5A). By 15 months post infection, HISIS\_605 was able to neutralize 72% of the panel viruses. This was significantly faster than the most potent neutralizer in the CAPRISA cohort, namely CAP256, who was able to neutralize 50% of the panel after 2 years and 83% of the panel after 3 years of infection through the development of antibodies to the V1V2 loop (Gray et al., 2011).

### 2.3.9 Specificity of neutralizing antibody response in HISIS\_605

To identify the major target of this elite neutralizer we focussed on sites that we found to be significantly more conserved in neutralization sensitive viruses relative to neutralization resistant viruses based on the entropy at each gp160 position in the 12 panel viruses. Twenty two sites were identified, of which 14 were located in the V2, V3, CD4 binding site (CD4-bs), and V4, C4 and V5 regions. We performed mapping studies with pseudoviruses with mutations in that would abrogate recognition of the well-known bNab targets (provided by Morris laboratory, NICD). Reductions in the ability of plasma to neutralize the ConC virus were observed with the CD4-bs mutations K360V, E362N, L369P and S375M (Figure 2.4B). For position 362 there was a 15-fold (93%) reduction in titre from 1:2,014 to 1:130 while at position 375 there was a reduction in titre to 1:196 (90%). Mutations at positions 360 and 369 also reduced the titres to 1:405 (80%) and 1:500 (75%) respectively (Figure 2.5B). These residues overlap the CD4 binding loop (Figure 2.4C and D) with residues Asp368, Glu370 and Lle371 involved in direct interactions with CD4 (Huang et al., 2004). Residues at positions 360 and 362 overlap with the binding sites of VRC01 mAb (Zhou et al., 2010). The V3 mutant I307A, shown to make viruses very sensitive to CD4-Ig as well as monoclonal

antibody b12 (Li et al., 2011), was extremely sensitive to the HISIS\_605 plasma at an elevated titre of >10,000.



**Figure 2.5 A:** Kinetics of development of neutralizing antibody breadth in HISIS\_605 over time. Sequential serum samples were tested against 9 of the panel viruses. Subtype A viruses are in green, subtype B viruses blue, subtype C viruses red, and autologous transmitted/founder AC recombinants in grey. **B:** Identification of residues involved in neutralization of ConC by HISIS\_605 plasma. Residues that most affected neutralization are shown in red (E362N: 15-fold reduction and S375M: 11 fold reduction) and others in orange (K360V: 5-fold reduction and L369P: 4-fold reduction). **C and D:** Three dimensional model showing residues involved in the specificity associated with neutralization by HISIS\_605 plasma. The inner domain of gp120 is in grey while the outer domain is in blue. Residues directly involved in CD4 binding are highlighted in cyan and residues that affect the recognition of ConC by HISIS\_605 plasma are in red and orange. The ribbon structure (C) as well as the surface view (D) is shown.

## **2.4 Discussion**

In this study, we showed different frequencies of broadly neutralizing antibody responses in two female cohorts in Africa, with the HISIS participants from Tanzania having broader neutralizing antibody responses at two years post infection compared to the CAPRISA participants from South Africa. Participants in the HISIS cohort also had more potent autologous neutralizing antibody responses at 12 months post infection. We found that VL was predictive of the development of breadth with cumulative VL between 3 and 12 months being most strongly associated with breadth at 2 years. The HISIS cohort had higher VL accounting for much of the difference observed in breadth between the two populations. However, other factors may still play a role as the odds of developing breadth in HISIS remained higher than in CAPRISA even after correction for viral load. The role of subtype effects, dual infection and other virological characteristics of the infecting virus could not be established, mainly because of the limited size of the study. However, we demonstrate an important role for the VL in shaping the neutralizing antibody responses in different geographical regions.

The association between VL and neutralization breadth has been established by several groups (Doria-rose et al., 2010; Ferreira et al., 2013; Mikell et al., 2011; Sajadi et al., 2011; Sather et al., 2009b) and like others we found a strong association. VL is an important factor in transmission of HIV-1 and higher community VL in sub-Saharan Africa has been identified as a major driver of the explosive epidemics observed in the general populations of this region when compared to other regions across the globe (Abu-raddad et al., 2012). Our study finds that community VL differs within the sub-Saharan region with the higher VL in the East African cohort compared to South Africa. Higher VL in the East African cohort could be driven by the presence of tropical co-infections such as malaria (Abu-Raddad et al., 2006; Cuadros et al., 2011), a factor which absent in South Africa (Snow et al., 2005). There are also substantial genetic variations between African populations which may provide another explanation for differences in VL. The lack of an incremental association of breadth and VL may be explained by the fact that there is an optimal range within which VL positively affects the development of breadth. Sajadi et al., (2011) noted that at VLs of below 100 HIV-1 RNA copies/ml and above 10000 HIV-1 RNA copies /ml the development of

breadth was affected either by insufficient antigenic stimulation or B-cell dysfunction respectively

Because of the small sample size of our study, we were unable to fully address any possible effect of subtype on the development of breadth. Subtype specific differences in ability to elicit breadth of responses have been reported for subtypes A, C, D, CRF02\_AG and CRF01\_AE in comparison with subtype B infections (Brown et al., 2008; Dreja et al., 2010), however, these studies were cross sectional and thus were unable to account for time since infection. Additionally, there remains a paucity of data on how recombinant forms (CRFs or URFs) affect the development of breadth of neutralizing antibody responses. This and the role of other virological factors remains an important question in the field requiring a much larger study.

Breadth develops incrementally over two to four years (Gray et al., 2011) and cross neutralizing antibodies develop, on average, after 2.5 years with a rare few individuals showing early breadth at one year (Mikell et al., 2011). In this study, one individual from the HISIS cohort developed cross-neutralizing responses as early as one year of infection, and was classified as an elite neutralizer by 2 years post infection with neutralization data from mutational analysis suggesting they targeted the CD4 binding site on the HIV envelope. In agreement with several other studies (Li et al., 2007; Lynch et al., 2012; Mikell et al., 2011; Sather et al., 2009b; Zhang et al., 2012) we observed that broad and potent neutralizing antibody responses that bind to the CD4-bs of the HIV envelope can emerge early during infection.

In conclusion, the CAPRISA and HISIS cohorts have enabled us to directly compare the development of breadth in neutralizing antibody responses in populations with different local epidemics. Differences in the local epidemics, particularly the presence of co-infections and genetic differences which may influence viral loads and thus the development of bNAb responses need to be considered as they may have implications for the continued efforts to understand how to elicit these responses through vaccination.

## Chapter 3: Neutralizing Antibody Escape Pathways Constrained by Antibody-Dependent Cellular Cytotoxicity (ADCC) Responses

<b>Abstract .....</b>	<b>56</b>
<b>3.1 Introduction.....</b>	<b>58</b>
<b>3.2 Materials and methods .....</b>	<b>59</b>
3.2.1 Study participant samples.....	59
3.2.2 Single genome amplification and sequencing .....	59
3.2.3 Sequence analysis.....	60
3.2.4 Identification of sites under positive selection pressure in CAP63 .....	60
3.2.5 Cloning and generation of pseudoviruses .....	60
3.2.6 Cell lines .....	61
3.2.7 HIV-1 <i>env</i> pseudovirus production and titration .....	61
3.2.8 Neutralizing antibody assays.....	61
3.2.9 Construction of infectious molecular clones (IMCs) .....	62
3.2.10 Mutagenesis of envelope and infectious molecular clones .....	62
3.2.11 Generation of virus stocks from infectious molecular clones .....	62
3.2.12 Titration of virus stocks from infectious molecular clones .....	62
3.2.13 Infection of CEM.NKR <sub>CCR5</sub> Cell Line with HIV-1 IMC.....	63
3.2.14 Intracellular p24 staining of infected CEM NKR CCR5 cells .....	63
3.2.15 Recombinant gp120 HIV-1 Proteins .....	64
3.2.16 ADCC-GranToxiLux (ADCC-GTL) assay .....	64
<b>3.3 Results.....</b>	<b>65</b>
3.3.1 Evidence of early changes in Env .....	65
3.3.2 Early adaptations in CAP63 .....	67
3.3.3 Early neutralizing antibody responses in CAP63 .....	70
3.3.4 Early ADCC responses in CAP63.....	70
3.3.5 Changes in V4 affected neutralization sensitivity .....	72
3.3.6 V4 neutralizing antibody mutations facilitate escape from ADCC responses in CAP63 .....	74
3.3.7 The 6D_391 deletion escapes neutralizing antibody responses but becomes highly sensitive to ADCC .....	75
3.3.8 ADCC activity constrains pathways of escape from neutralizing antibody responses .....	77
<b>3.4 Discussion .....</b>	<b>78</b>

## **Abstract**

While neutralizing antibodies are considered central to the development of an HIV vaccine, ADCC activity correlated with reduced risk of HIV-1 acquisition in the RV144 trial. There is limited understanding of the overlapping ADCC and neutralizing antibody functions in early infection. This study investigated the kinetics and targets of neutralizing and ADCC antibody responses in early infection.

We analysed 16 individuals from HISIS and 14 from CAPRISA for evidence of early immune escape in gp160. Six individuals showed evidence of early changes and in three individuals changes were detectable before neutralizing antibody responses. One individual was selected and characterised in detail. In this individual (CAP63) ADCC responses were present at enrolment (4 weeks post infection) and neutralizing antibody responses emerged at 7 weeks post infection. By 11 weeks, five neutralization escape patterns were identified by sequence analysis and confirmed through mutagenesis and pseudovirion neutralization assays. One virus escaped through point mutations and four viruses escaped through deletions of 5 to 9 amino acids at various positions starting at codons 391-399 in the V4 region.

Four of these neutralizing antibody escape adaptations in CAP63 were also confirmed to mediate escape from ADCC via a GranToxilux ADCC assay. One (6D\_391) rendered the virus more sensitive to ADCC mediated by autologous plasma as well as two control plasma samples and a monoclonal antibody A32. Viruses containing the 6D\_391 deletion were absent from the viral population sampled at 29 weeks post infection.

This study defines the first ADCC epitope reported in the V4 region and demonstrates a common epitope recognised by antibodies with ADCC and neutralizing activity. The elimination of the neutralization resistant/ADCC sensitive variant suggests that antibody effector functions apply additional pressure on the virus which may constrain neutralization escape pathways.

### 3.1 Introduction

It is generally accepted that broadly neutralizing antibodies against HIV-1 will be important responses to elicit through vaccination. However non-human primate SIV/SHIV models suggest that the other antibody effector functions, such as antibody-dependent cell cytotoxicity (ADCC), may also play a role both in protection from infection, and in the modification of disease progression (Alpert, Harvey, et al., 2012; Florese et al., 2009; Gómez-Román et al., 2005; Hidajat et al., 2009; Xiao et al., 2010, 2012). In humans, the V2 region of HIV-1 is a major target of broadly neutralizing antibodies (Haynes & McElrath, 2013) but the immune correlate analysis of the RV144 vaccine trial showed that, V2 binding antibodies and low levels of plasma IgA Env antibodies in association with high levels of ADCC, were inversely correlated with infection risk (Haynes, Tomaras, et al., 2012). Furthermore, in natural infection, ADCC antibodies in breast milk have been correlated with reduced vertical transmission from viremic mothers (Mabuka et al., 2012).

Cytotoxic T lymphocytes (CTLs) are associated with the control of HIV replication during acute infection (Goonetilleke et al., 2009; Koup et al., 1994). In contrast, the first antibodies to arise following infection are binding antibodies targeting gp41, and these are thought to have little or no impact on the virus (reviewed by Alter & Moody, 2010). CTL escape accounts for some, but not all, of the changes seen in early *env* evolution (Abrahams et al., 2013; Liu et al., 2013; Wood et al., 2009). A recent study found evidence of low levels of neutralizing antibodies as early as two weeks post seroconversion which were sufficient to result in viral escape (Bar et al., 2012). However, little is known about escape from effector mediated antibody functions, such as ADCC, which generally arise before neutralizing antibodies.

The relationship between the neutralization and ADCC antibody functions has been difficult to unravel due to lack of clearly defined ADCC epitopes. This gap is in part due to many different methods used to study ADCC (or other Fc receptor-mediated activity) which may be measuring slightly different functions. These methods have relied on targets coated with peptides or recombinant forms of Env (reviewed in chapter 1), whereas mutant whole Env proteins are required to accurately map escape from ADCC responses (Isitman et al., 2012). Due to the high plasticity of the viral genome, identification of escape mutations through

sequencing is a sensitive tool for evaluating immune pressure acting on the virus. Single genome amplification based approaches have enabled the identification of the transmitted/founder viruses (Keele et al., 2008). Tracking of their evolving progeny thereby allowing us to determine the selective pressures that are acting on the virus through the footprints of these pressures on the viral quasispecies (Abrahams et al., 2013; Bar et al., 2012). Specifically, this approach can be used to investigate how ADCC responses influence viral evolution in natural infection and provide further insights into the importance of ADCC responses in HIV infection.

Broadly reactive and effective ADCC activity has been demonstrated for non-neutralizing monoclonal antibodies such as A32 (Ferrari et al., 2011). However, neutralizing and ADCC functions can also be mediated by the same IgG response and their epitope recognition footprint often overlaps (Alpert, Harvey, et al., 2012; Fouda et al., 2011; Pollara et al., 2013; Smalls-Mantey et al., 2012). In such cases, it is possible that the antibody effector functions apply additional pressure on the virus. Such pressure could result in the generation of ADCC-induced mutations that could influence susceptibility to neutralizing antibodies, or *vice versa*. In this chapter, we investigate the pattern of early escape in an individual from the CAPRISA cohort in whom we detected significant changes to the viral envelope within 13 weeks of infection. We characterised the timing and effects of these changes in the Envelope protein on neutralizing and ADCC antibody recognition. We aimed to determine how the interplay between the neutralizing and effector mediated functions of early antibody responses affects viral evolution in natural HIV-1 infections.

## **3.2 Materials and methods**

### **3.2.1 Study participant samples**

Longitudinal plasma samples were obtained from the HISIS and CAPRISA cohorts as detailed in Chapter 2.2.2.

### **3.2.2 Single genome amplification and sequencing**

Viral sequences were generated using the single genome amplification method as detailed in Chapter 2.2.4.

### 3.2.3 Sequence analysis

Sequence alignments, amino acid identity plots and participant consensus sequences were generated using BioEdit version 7.0.8.0 (Hall, 1999). The transmitted full-length envelope virus sequence for each participant was taken as the consensus of all sequences generated from the earliest sampled timepoint (Keele et al., 2008). Maximum likelihood trees were generated using Mega version 4 (Tamura et al., 2007). The Los Alamos Highlighter tool ([www.hiv.lanl.gov/content/sequence/HIGHLIGHT/highlighter](http://www.hiv.lanl.gov/content/sequence/HIGHLIGHT/highlighter)) was used to generate plots of synonymous and non-synonymous nucleotide changes.

### 3.2.4 Identification of sites under positive selection pressure in CAP63

Site-specific non-synonymous to synonymous substitution (dN/dS) rate ratios across each gene were estimated using the MG94xHKY85 codon model (Kosakovsky Pond & Frost, 2005). We allowed dS to vary across codon sites and employed the Dual model which takes into account that dS may vary independently of dN (Kosakovsky Pond & Muse, 2005). The models were implemented within the HyPhy package (Kosakovsky Pond et al., 2005) and ensured that the correct phylogenetic relationships were used for sequence regions that were separated by recombination breakpoints (Scheffler, Martin & Seoighe, 2006). For graphical representation of sites under selection, the  $(p\text{-value})^{-10}$  is used enabling lower p-values to be displayed as taller bars.

### 3.2.5 Cloning and generation of pseudoviruses

PCR amplification of HIV-1 *env* genes was done using the SGA approach previously described (Abrahams et al., 2009; Keele et al., 2008) or a limiting dilution PCR. The second round PCR reaction was repeated using the high fidelity Phusion Hot Start DNA Polymerase (Finnzymes), together with, 0.2 mM dNTPs (Roche), 4  $\mu$ M of Env 1A-Rx (5' CAC CGG CTT AGG CAT CTC CTA TAG CAG GAA GAA 3') and EnvN (5' CTG CCA ATC AGG GAA AGT AGC CTT GT 3') in a final volume 50  $\mu$ L. The cycling conditions for this reaction were as follows: denaturing at 94 °C for 5 minutes, 45 cycles of 94 °C for 30 seconds; 55 °C for 30 seconds; 72 °C for 4 minutes, and final extension step at 72 °C for 10 minutes. The amplicons were gel purified and cloned into the mammalian expression vectors pcDNA3.1D/V5-His-TOPO (Invitrogen) or pTarget (Promega, US) according to the

manufacturer's instructions. Functional envelope clones were selected using a 96-well plate format pseudovirion entry efficiency assay based on relative luminescence units (RLU) that is 2.5 times above background.

### 3.2.6 Cell lines

TZM-bl cells, also known as JC53bl-13 cells (Montefiori et al., 2004) were obtained from the NIH AIDS Research and Reference Reagent Program Division of AIDS, NIAID, NIH. 293T/17 cells were obtained from Dr George Shaw (University of Alabama, Birmingham, AL). Both cell lines were cultured in D-MEM (Gibco BRL Life Technologies) containing 10% heat-inactivated fetal bovine serum (FBS) and 50 µg/mL gentamicin (Sigma). Cell monolayers were disrupted at confluency by treatment with 0.25% trypsin in 1 mM EDTA. CEM.NKR<sub>CCR5</sub> and CEM.NKR<sub>LUC</sub> cells from Dr. Alexandra Trkola were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, and NIH.

### 3.2.7 HIV-1 *env* pseudovirus production and titration

Stocks of HIV-1 *env* pseudovirus were produced by co-transfecting 293T/17 cells ( $1.7 \times 10^7$  cells per T75 flask) with 4 µg of an HIV-1 *rev/env* expression plasmid and 8 µg of an *env*-deficient HIV-1 backbone plasmid (pSG3ΔEnv) (M. Li et al., 2005) using the PolyFect Transfection Reagent (QIAGEN). Pseudovirus-containing supernatants were harvested 48h following transfection and clarified by 0.45 µm filtration and adjusted to 20% FBS. Single-use aliquots (1.0 mL) were stored at -80°C. The 50% tissue culture infectious dose (TCID<sub>50</sub>) for each pseudovirus preparation was determined by infection of TZM.bl cells as previously described (Gray et al. 2007; Li et al. 2005).

### 3.2.8 Neutralizing antibody assays

Virus neutralization was measured by luciferase based assay as described in Chapter 2.2.7.

### **3.2.9 Construction of infectious molecular clones (IMCs)**

Infectious molecular clones representing the transmitted/founder virus sequences of CAP45, CAP63, CAP210 and CAP239 were constructed from proviral DNA by Florette Treurnicht (UCT) in collaboration with members of the Kappes laboratory at the University of Alabama at Birmingham (UAB) using the method described in detail by Ochsenbauer et al., (2012).

### **3.2.10 Mutagenesis of envelope and infectious molecular clones**

Site-directed mutagenesis was used to introduce mutations and deletions into the CAP63\_T/F IMC using a QuikChange II site-directed mutagenesis kit (Stratagene). Mutagenesis primers used are listed in appendix F and properties of mutant viruses are listed in Table 3.2. All mutations were confirmed by full length sequencing of the pseudovirus (PSV) or IMC. The following PSV mutants were generated; CAP63\_PSV\_6D-391, CAP63\_PSV\_7D-397, CAP63\_PSV\_9D-396, and CAP63\_PSV\_5D-399. For the GLY\_398\_404 adaptation, two separate pseudoviruses, CAP63\_PSV\_GLY\_398 and CAP63\_PSV\_GLY\_404, were constructed to individually test the effect of each glycan on the neutralization sensitivity of the virus. The following IMC mutants were generated; CAP63\_IMC\_GLY404, CAP63\_IMC\_6D-391, CAP63\_IMC\_7D-397, CAP63\_IMC\_9D-396, and CAP63\_IMC\_5D-399.

### **3.2.11 Generation of virus stocks from infectious molecular clones**

Stocks of live HIV-1 IMC virus were produced by transfecting 293T/17 cells ( $3 \times 10^6$  cells per T75 flask) with 12  $\mu$ g of an HIV-1 IMC plasmid DNA using the FUGENE Transfection Reagent (Roche). IMC virus-containing supernatant was harvested 48h following transfection and clarified by centrifugation and 0.45  $\mu$ m filtration and adjusted to 20% FBS. Single-use aliquots (1.0 mL) were stored at -80°C and the harvest date recorded. To expand the stock, 12mL of Growth Media (GM) was added to the cells and incubated overnight. A second harvest was performed as outlined above and the harvest date recorded.

### **3.2.12 Titration of virus stocks from infectious molecular clones**

The TCID<sub>50</sub> for each IMC virus preparation was determined both by infection of TZM.bl cells and CEM.NKR<sub>LUC</sub> cells as previously described (Gray et al., 2007; Li et al., 2005). 25

$\mu\text{L}$  of virus stock was placed in a total volume of 100  $\mu\text{L}$  of GM and 11 serial 5-fold dilutions were made in a 96 well plate. Virus dilutions were co-incubated with 60,000 cells in 100  $\mu\text{L}$  RPMI-12%-GM containing 16  $\mu\text{g}$  DEAE-Dextran/mL for 4 days. On day 4, 100  $\mu\text{L}$  of the cell suspension was placed in a 96-well solid white plate. 100 $\mu\text{L}$  of BriteLite Plus (Perkin Elmer) was added to the white plate and incubated for 2 minutes. Luminescence was read in a luminometer using the 1.0 sec/well protocol implemented by the computer program Wallac (Perkin Elmer).

### **3.2.13 Infection of CEM.NKR<sub>CCR5</sub> Cell Line with HIV-1 IMC**

HIV-1 IMC virus stocks were titrated on CEM.NKR<sub>CCR5</sub> to determine the input that achieved optimal viral gene expression within 72 h post infection, as measured by detection of intracellular p24 expression. IMC virus stock volumes of 900  $\mu\text{L}$ , 300  $\mu\text{L}$  and 100  $\mu\text{L}$  were used to infect  $2 \times 10^6$  cells for 72 hours and levels of intracellular p24 were assayed. Having determined the volume of stock that gave the highest rates of infection for each IMC by intracellular p24 staining, we subsequently infected  $2 \times 10^6$  cells with each IMC by incubation with the appropriate dose of IMC for 0.5 h at 37°C and 5% CO<sub>2</sub> in the presence of DEAE-Dextran (7.5  $\mu\text{g}/\text{mL}$ ). The cells were subsequently resuspended at  $0.5 \times 10^6/\text{mL}$  and cultured for 2 days in complete medium containing 7.5  $\mu\text{g}/\text{mL}$  DEAE-Dextran. On assay day, the infection was monitored by measuring the frequency of cells expressing intracellular p24. The assays performed using the IMC-infected target cells were considered reliable if the percentage of viable p24+ target cells on assay day was  $\geq 20\%$ . The data are presented after normalizing to the % of target cells positive for intracellular p24.

### **3.2.14 Intracellular p24 staining of infected CEM NKR CCR5 cells**

Following 72 h of infection, cells were washed in PBS, dispensed in 96-well V-bottom plates at  $1 \times 10^5$  viable cells per well, and stained with a vital dye (LIVE/DEAD Fixable Aqua Dead Cell Stain, Invitrogen) to exclude non-viable cells from subsequent analyses. The cells were then washed twice with 250  $\mu\text{L}$  per well of washing buffer (WB; PBS + 1% FBS) and incubated with Cytifix/Cytoperm (BD Bioscience, San Jose, CA) for 20 min at 4°C. The cells were then washed twice with 200  $\mu\text{L}$  of 1% Cytoperm washing buffer. After the final wash, the anti-p24 Ab (clone KC57-RD1; Beckman Coulter) was added to a final dilution

1:400 and the plates were incubated for 30 min at 4°C. The plates were washed twice with WB, and the cells were resuspended in 200 µL 1% formaldehyde-PBS. The samples were acquired within 24 hours using the LSR II flow cytometer. A minimum of 10,000 total singlet events was acquired for each analysis. Gates were set to include singlet and live events. The appropriate compensation beads were used to compensate the spillover signal for the two fluorophores. Data analysis was performed using FlowJo 8.8.4 software (TreeStar, Ashland, OR). The uninfected CEM.NKR<sub>CCR5</sub> and the chronically infected A1953 cell lines were used to titer the vital dye and the anti-p24 Ab, and were used as negative and positive controls, respectively, for the described staining procedure.

### **3.2.15 Recombinant gp120 HIV-1 Proteins**

Recombinant gp120 HIV-1 protein representing the envelope of the HIV-1 subtype C isolate CAP45.2.00 (GenBank No. DQ435682; Immune Technology, New York), was used to coat CEM.NKR<sub>CCR5</sub> target cells by incubating  $1 \times 10^6$  cells in 1mL RPMI media with 20 µg/mL gp120 for 90 min at 48C.

### **3.2.16 ADCC-GranToxiLux (ADCC-GTL) assay**

ADCC activity was detected according to a modification of the GranToxiLux (GTL) cell-mediated cytotoxicity procedure (Liu et al., 2002; Packard et al., 2007) with the assay performed in 96- well plates as follows. Infected and uninfected CEM.NKR<sub>CCR5</sub> target cells were counted, washed, resuspended in R10 at  $1 \times 10^6$  cell/mL, and labelled with a fluorescent target- cell marker (TFL4; OncoImmunit, Gaithersburg, MD) and a viability marker (NFL1; OncoImmunit) for 15 min in a 37°C water bath as specified by the manufacturer. After two washes using 10 mL of R10, viable cells were counted using a Guava PCA (Millipore, Billerica, MA) and adjusted to reach a final viable effector to viable target ratio of 30:1 to PBMC effector cells. Twenty-five µL of each effector and target cell suspension and 75 µL of GzB substrate (OncoImmunit) was dispensed into each well of a 96-well V-bottom plate. After incubation for 5 min at room temperature, 25 µL of the appropriate anti- body or IgG dilutions were added to the target/effector cell suspension and incubated for 15 min at RT. The plates were subsequently centrifuged for 1 min at 300g, and incubated for 1 h at 37°C and 5% CO<sub>2</sub>. After two washes with WB, cells were resuspended in 225 µL of WB, placed at

4°C, and acquired directly from the assay plate with the LSRII (BD Bioscience, San Jose, California) within 6 h using the high-throughput system (HTS, BD Bioscience, San Jose, California). A minimum of  $2.5 \times 10^3$  and  $5 \times 10^3$  events representing viable gp120-coated and infected target cells, respectively, was acquired for each condition. The signal for each fluorophore was detected using: (1) 640 nm/40 mW laser and 660/20 filter for TFL4; (2) 405 nm/50 mW laser and 450/50 filter for NFL1; and (3) 488 nm/20 mW laser and the combination of 505LP with 525/50 filters for the GzB substrate. The spectral properties of the fluorescent molecules utilized in this panel meant no manual compensation of the signals was required to analyse the data, as reported elsewhere (Liu et al., 2002). Data analysis was performed using a dedicated analysis template that reflecting the gating strategy on FlowJo 8.8.4 software (Tree Star Inc). All wells with more than 8% of cells positive for granzyme were considered positive for granzyme activity and the ADCC titer was the highest antibody dilution at which the cut-off 8% GzB level of activity was achieved (Pollara et al., 2011).

### **3.3 Results**

#### **3.3.1 Evidence of early changes in Env**

To identify individuals with evidence of early adaptations in the envelope gene we analysed sequences from 30 individuals infected by a single variant at transmission as identified by Abrahams et al., (2009) and Nofemela, Bandawe et al., (2011). Envelope SGA sequences generated from the first HIV positive visit (< 3months/13 weeks post infection) included: 153 from 16 individuals from the HISIS cohort (Nofemela, Bandawe et al., 2011); and 280 from 14 individuals from the CAPRISA cohort (Abrahams et al., 2009). Escape from neutralizing antibodies in early infection is typically observed as changes in the lengths of the envelope hyper-variable domains resulting from insertions and deletions (indels), as well as shifting carbohydrate moieties in and around the outer surface of the protein (Moore et al., 2008; Rong, Gnanakaran, Decker, Bibollet-Ruche, et al., 2007; Wei et al., 2003). Sequences from four out of 16 participants from the HISIS cohort and two out of 14 participants from the CAPRISA cohort had changes suggestive of neutralization escape (Table 3.1). In three of these individuals, HISIS\_556, CAP63 and CAP210, these adaptations were observed prior to neutralizing antibody responses, which were detected at 26 weeks (Chapter 2), 19 weeks (Gray et al., 2007) and 49 weeks (Abrahams et al., 2013) respectively.

**Table 3.1A:** HISIS cohort: Summary of adaptations observed in gp160 of transmitted/founder virus sequence in the first 13 weeks of infection (Nofemela, Bandawe et al., 2011).

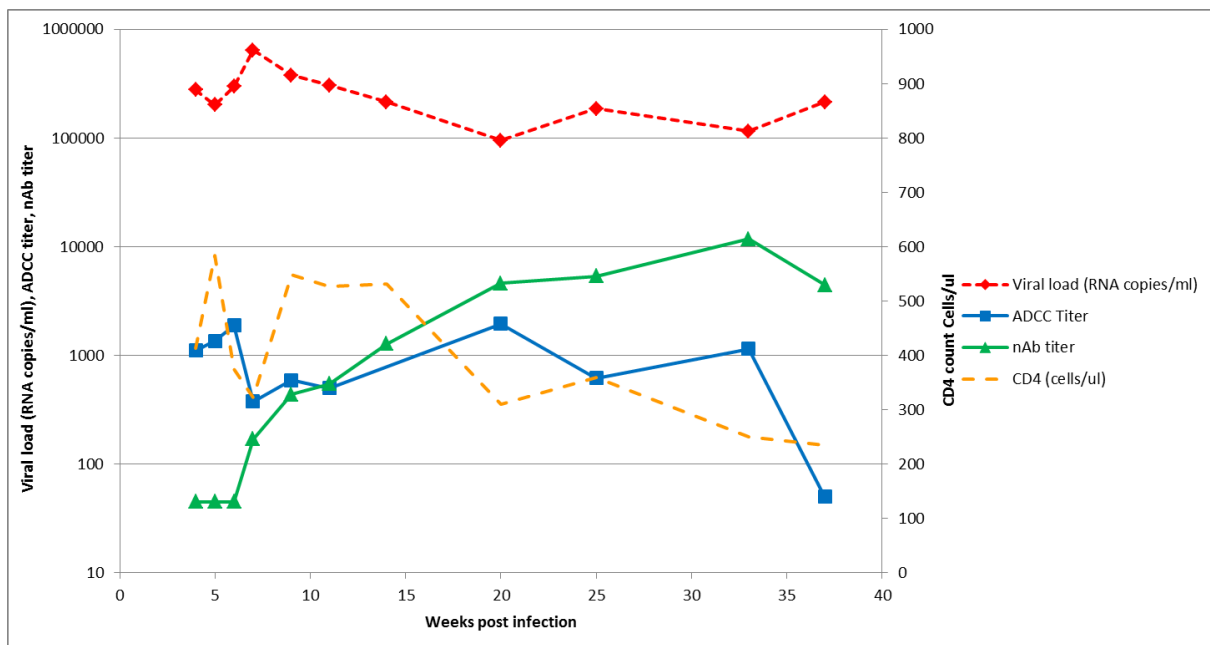
Participant ID	Sequence changes	Timing (weeks)	Change	HXB env position	Env region	Timing of nAbs
HISIS_398	<pre> - --- --- --- --- --- --- --- --- --- ---  GNNSNTNETFRPGG ..... .....S..... .....S..... ..... .....                     </pre>	12	Glycan shift	454	V5	<13 weeks
HISIS_556	<pre> - --- --- --- --- --- --- --- --- --- ---  CTNVNTTNTNDTRSN ..... .....A..... ..... .....A..... ..... .....                     </pre>	12	Deletion	130	V1	
	<pre> - --- --- --- --- --- --- --- --- --- ---  GNGSNNVSGSNNGS ..... .....R..... ..... .....                     </pre>	12	Deletion	193	V2	26 weeks
	<pre> - --- --- --- --- --- --- --- --- --- ---  GNENTTGGNTTNTGKE ..... .....E.EE..... .....E..... .....                     </pre>	12	Deletions	467, 478	V5	
HISIS_477	<pre> - --- --- --- --- --- --- --- --- --- ---  LLKCNKTFNGTG ..... ..... .....A..... .....A.....                     </pre>	12	Glycan loss	230	V2	<13 weeks
	<pre> - --- --- --- --- --- --- --- --- --- ---  WNNSTGNSMEWLNDC .....G..... .....T..... .....G..... .....R..... .....R..... ..... .....T..... .....T..... .....T.....                     </pre>	12	Glycan shift	397, 401	V4	
HISIS_515	<pre> - --- --- --- --- --- --- --- --- --- ---  GDLLELTTHSF ..... ..... ..... .....                     </pre>	7	Deletion	390	V4	ND

**Table 3.1B:** CAPRISA cohort: Summary of adaptations observed in gp160 of transmitted/founder virus sequence in the first 13 weeks of infection (Abrahams et al., 2013).

Participant ID	Sequence changes	Timing (weeks)	Change	HXB env position	Env region	Timing of nAbs
CAP63	<pre> - --- --- --- --- --- --- --- --- --- ---  LFNSTYMPNGIHIPNGASEVI .....T.....T..... .....D..... .....S..... .....SS..... .....S..... .....S.....                     </pre>	11	deletions, glycans	Deletions 391, 396, 397, 399, glycans 398, 404	V4	19 weeks
CAP210	<pre> - --- --- --- --- --- --- --- --- --- ---  ITHNSTDSTVNSTDST ..... ..... ..... .....                     </pre>	12	deletions	391	V4	49 weeks

### 3.3.2 Early adaptations in CAP63

To elucidate interplay between neutralizing and ADCC responses driving these early changes, we studied one individual, CAP63, in detail. This individual was selected as her virus had large deletions in the V4 region at 11 weeks post infection, prior to the detection of neutralizing antibodies at 19 weeks post infection (Abrahams et al., 2013; Gray et al., 2007) (Table 3.1). Previous work had identified that the neutralization epitope was located in the V4 region (Gray et al., 2007). We hypothesized that these changes in V4 were due to escape from either low levels of neutralization antibodies prior to detectable neutralization responses, or from ADCC responses. CAP63 maintained high viral loads throughout infection (Figure 3.1) and progressed rapidly to AIDS within 55 weeks when her CD4+ T-cell count dropped below 200 cells/ $\mu$ L and antiretroviral therapy was initiated according to prevailing guidelines (Republic of South Africa, Department of Health, 2005).



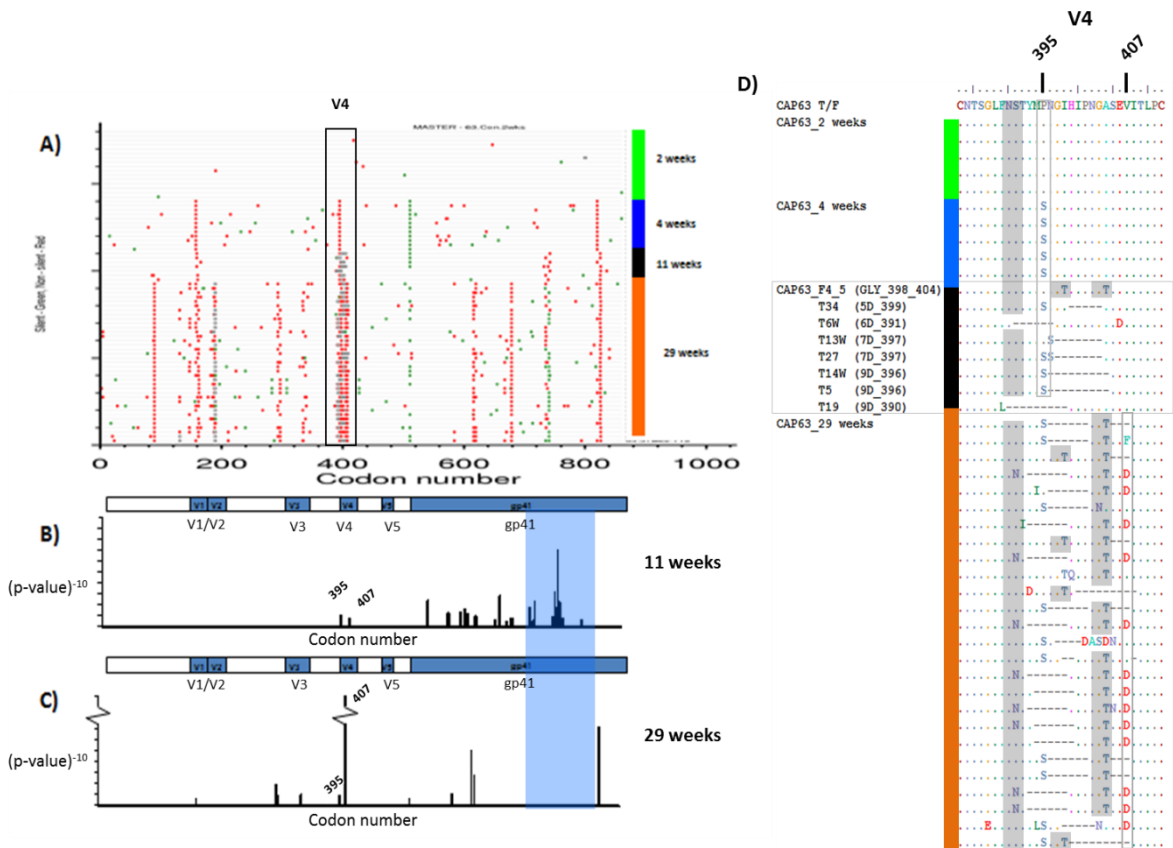
**Figure 3.1:** Viral load, CD4 counts and humoral responses in CAP63 over the 37 weeks of infection. Viral load is shown by the red dotted line, ADCC titres against CEM NKRCCR5 cells infected with CAP63\_T/F\_IMC virus shown by the blue line, neutralizing antibody (nAb) titres ( $ID_{50}$ ) against CAP63\_T/F pseudovirus shown by the green line and are all plotted on the left axis. CD4 count is shown by the orange dotted line and is plotted on the right axis. VL and CD4 counts were performed by the CAPRISA AI Study team (UKZN).

CAP63 was infected with a single founder virus (Abrahams et al., 2009). To identify sites under selective pressure, we obtained a total of 89 full length gp160 sequences from 2 weeks (n=30), 4 weeks (n=11), 11 weeks (n=8), 29 weeks, (n=29) and 37 weeks post infection (n=4) (Figure 3.2 A) that were generated either by single genome amplification or by limiting dilution PCR (Abrahams et al., 2013; Abrahams et al., 2009). We evaluated evidence of early adaptation by (i) investigating sub-lineages with shared mutations compared to the T/F virus, that were consistent selective pressure applied on the virus (Keele et al., 2008); (ii) insertions and deletions as well as changes to the glycosylation pattern in the envelope; (iii) evidence of positive selection over time as detected by the HYPHY (MEME) analysis (Murrell et al., 2012).

Shared codon changes were observed 4 weeks after infection in the V1V2 (A158T), V4 (P395S) and in gp41 (D818N) (Figure 3.2). At 11 weeks post infection, 5 out of 7 sequences contained deletions in the V4 region which resulted in changes to the glycosylation profile. Viruses with deletions in the V4 region compared to the founder virus persisted, with 27 out of 29 viruses sampled at 29 weeks carrying a deletion. There was one sequence where changes in the glycosylation profile were caused by two point mutations resulting in the addition of two potential N-linked glycosylation sites (PNGS).

We identified strong selective pressure on the V4 region, which changed in magnitude over time. During the first 11 weeks of infection, two sites in gp120 (codons 395 and 407) were under positive selection. When analysed over the entire 29 weeks of follow up period the signal at 407 was amplified with a (p-value)<sup>-10</sup> reading of 73.5 (p = 0.00136), while none of the other values exceed 15 (p=0.007) (Figure 3.2C). The amino acid at this position in the transmitted founder was Valine (V), which was maintained until 11 weeks post infection (Figure 3.2 D), and by 29 weeks post infection this position showed high entropy with 4 other amino acids also represented at this position.

During the first 11 weeks, positive selection was also identified in the gp41 region. Sites under selection in gp41 were distributed throughout the gene with a cluster of sites at codons 749, 753, 754 and 757, with a particularly strong signal at codon 754. These sites all fall within the *rev* exon 2 reading frame and results obtained in this region are unreliable due to the overlapping reading frame. After 29 weeks, we also identified sites under positive selection in the V1, C2 and V3 regions of gp120.



**Figure 3.2:** Analysis of CAP63 sequences over 29 weeks of infection.

(A) The highlighter plot illustrating acquired mutations in Env compared to the derived transmitted/founder (T/F) sequence. Red tick: non-synonymous substitutions; green tick: synonymous substitutions; grey, deletions. (B) and (C) Plot of sites under positive selection detected over 11 weeks (B) and 29 weeks (C). Selection detecting by mixed effects model of evolution likelihood of positive selection. The y axis plots the  $(p\text{-value})^{-10}$  with higher bars indicating a higher likelihood of positive selection at that codon position. The area shaded in blue represents the overlapping tat and rev exon 2 reading frames. Results obtained in these regions are not reliable.

(D) Amino acid alignment of the V4 region. Glycosylation sites are shaded in grey. Viruses from 11 weeks post infection carrying the changes under investigation are in the rectangular box. Residues in gp120 under the strongest positive selection in the first 11 weeks (residue 395) and after 11 weeks (residue 407) are marked in boxes.

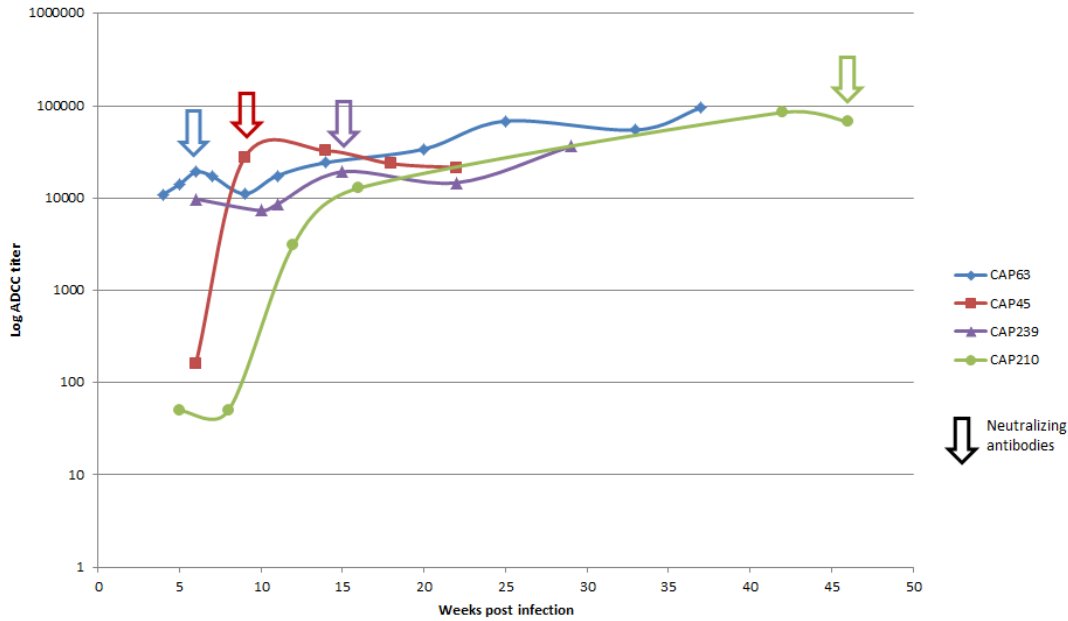
### **3.3.3 Early neutralizing antibody responses in CAP63**

CAP63 was enrolled into the AI cohort at four weeks post-infection, Gray et al., (2007) had timed neutralization responses utilizing pseudotyped envelope clones generated from sequences from this time-point. However sample was available from 2 weeks pre-enrolment (antibody negative, HIV PCR positive) visit (Abrahams et al., 2009) which differed from the derived transmitted/founder in up to 4 amino acid positions (CAP63.2.00.A9.J, Figure 3.5). As these changes may affect neutralization results, we repeated the assay using the T/F virus envelope. We measured autologous neutralizing antibody response to CAP63\_T/F pseudovirus at 7 weeks with an ID<sub>50</sub> of 169 (Figure 3.1), which is earlier than 19 weeks previously identified (Gray et al., 2007) using a later envelope. The neutralizing antibody response was characterized by steadily rising titers against the T/F virus until 33 weeks post infection. A drop in titres was seen between 33 and 37 weeks post infection.

### **3.3.4 Early ADCC responses in CAP63**

We next investigated kinetics of ADCC responses using two approaches, firstly by coating target cells with recombinant GP160 (rGP160) from CAP45.2.00; and secondly, by infecting cells with T/F autologous infectious molecular clones.

Using rgp120 coated target cells, kinetics of ADCC responses in CAP63, was compared with three other CAPRISA participants. The kinetics of ADCC responses differed between participants (Figure 3.3). At the first available visit post HIV diagnosis, CAP63 (4 weeks pi) and CAP293 (6 weeks pi) had potent ADCC activity, while CAP45 and CAP210 high ADCC titres only increased at the second (9 weeks pi) and third (12 weeks pi) visit respectively. In all participants, once established, high titer responses were maintained throughout monitored infection period which ranged from 29 and 67 weeks. In three of the CAPRISA participants studied, ADCC responses were present before detectable neutralizing antibody responses (Gray et al., 2007: Figure 3.3), with responses emerging concurrently in one participant (CAP45).



**Figure 3.3:** The kinetics of ADCC responses to HIV-1 infection in 4 CAPRISA participants. The ADCC activity of the participants is measured against CEM-NKR<sub>CCR5</sub> coated with recombinant CAP45.2.00 gp120. The time-point at which the first autologous neutralizing antibody response with an ID<sub>50</sub> of over 100 is marked with an arrow. Neutralizing antibody data for CAP45, CAP239 and CAP210 was published by Gray et al. (2007) and CAP63 neutralizations were repeated here. Log ADCC titer was the highest antibody dilution at which the cut-off 8% GzB level of activity was achieved.

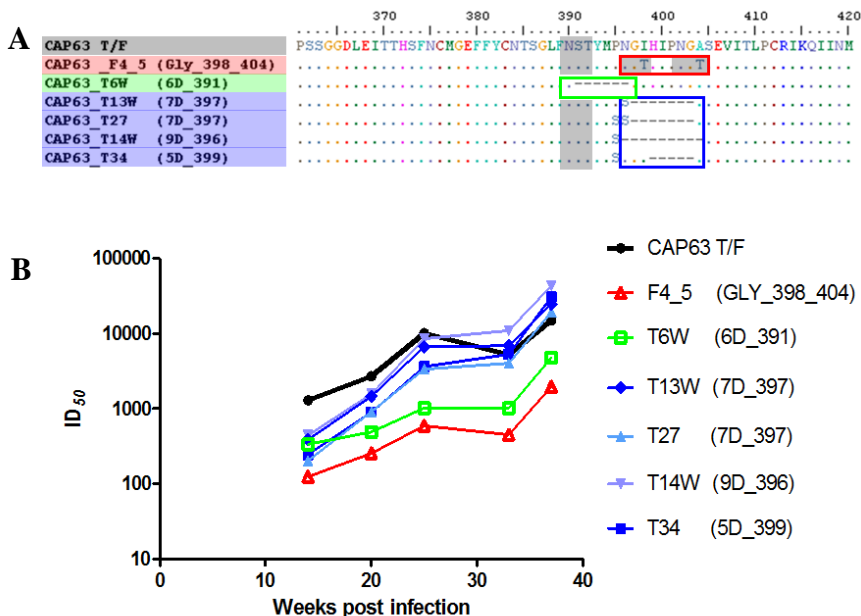
For CAP63, we repeated the ADCC assay using CEM NKR<sub>CCR5</sub> cells infected with IMC virus of the CAP63\_T/F (provided by Melissa-Rose Abrahams). CEM.NKR<sub>CCR5</sub> is a lymphoid cell line transduced with a retroviral vector to express human CCR5. The cells present Env on the surface, however do not secrete infectious virus making them suitable for infection with primary HIV isolates for use in the GranToxiLux assay. This assay may be biologically more relevant as the protein is matched to the infecting virus and its conformation would be more similar to natural infection.

Titration were performed to determine the optimal level of inoculum for infection. A minimum infection level of 20% of p24+ target cells was required for the ADCC assay (Pollara et al., 2011). The highest level of infection was attained using 300 µL of virus stock (Appendix H) and this volume was used in an assay performed over two days. Similar to previous results (Figure 3.3), we found high titre ADCC responses at the earliest time-point (4 weeks post infection), which were sustained until 33 weeks post infection. However, in contrast to the coating target cells, using the infectious assay we found no detectable ADCC activity at 37 weeks post infection (Figure 3.1).

### 3.3.5 Changes in V4 affected neutralization sensitivity

To assay the effect of the changes observed in the 11 week viruses on neutralizing antibody responses, functional *env* clones were constructed. A total of six out of seven *env* SGA clones were functional in pseudovirion assays. The *env* SGA clone, CAP63.2.05\_T19 was non-functional and therefore excluded from the assays (Table 3.2). The T/F pseudovirus and the six 11 week pseudoviruses (PSV) were assayed against plasma samples from between 14 and 37 weeks post infection (Figure 3.4). Relative to the T/F virus, all of the naturally occurring viral variants showed evidence of neutralization escape, with reduced sensitivity to neutralization relative to the T/F virus at 11 and 20 weeks. The two variants (CAP63\_F4\_5 and CAP63\_T6W) that contained changes in or near the codon 407, the codon under strong positive selection (Figure 3.4), showed sustained resistance to neutralization at all the time-points tested.

As there were changes from the T/F virus outside of the V4 region which may have affected neutralization, we introduced the V4 changes into the T/F backbone (Table 3.2, Figure 3.5). In the case of the GLY\_398\_404 adaptation, two separate pseudoviruses, GLY\_398 and GLY\_404, were constructed to individually test the effect of each glycan on the neutralization sensitivity of the virus.

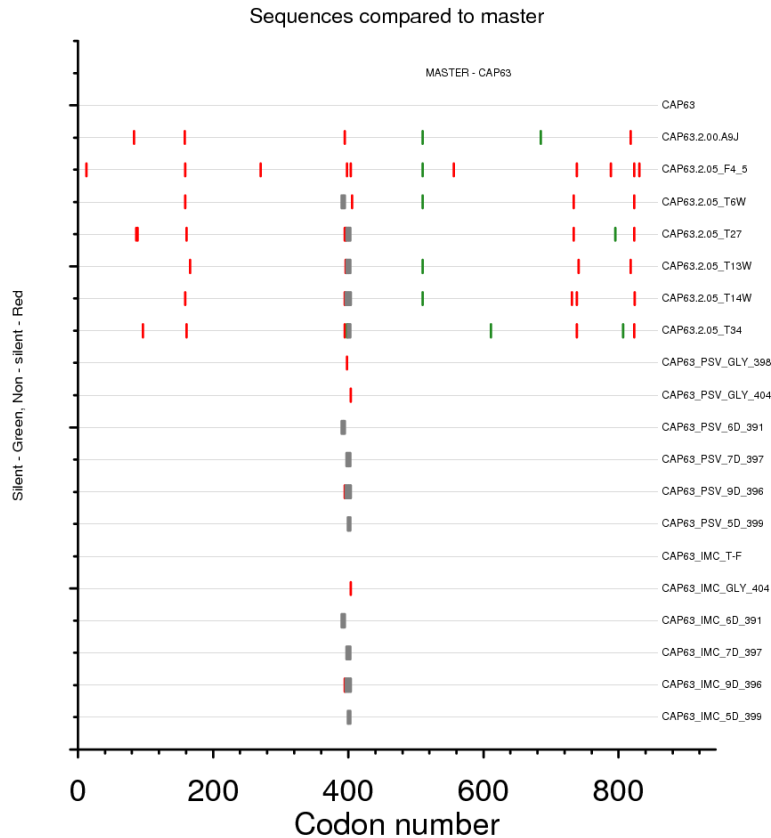


**Figure 3.4:** A) Amino acid alignment of the V4 region of pseudovirus clones of CAP63 T/F and six naturally occurring variants from 11 weeks post infection. The putative escape mutation associated with each virus is listed in brackets. B) Pseudovirus neutralization assay of CAP63 T/F and naturally occurring cloned variants from 11 weeks post infection.

**Table 3.2:** Naturally occurring viruses from 11 weeks post infection and their corresponding constructed pseudoviruses and infectious molecular clones.

Natural sequence pseudovirus	Env and	Changes from T/F in V4	CAP63 Pseudovirus constructs	CAP 63 IMC constructs
CAP63.2.05_F4_5		two point mutations causing the addition of glycosylation sites at positions 398 and 404	PSV_GLY_398 PSV_GLY_404*	IMC_GLY_404
CAP63.2.05_T6W		6 amino acid deletion starting at codon 391	PSV_6D_391	IMC_6D_391
CAP63.2.05_T13W		7 amino acid deletion starting at codon 397	PSV_7D_397	IMC_7D_397
CAP63.2.05_T14W		9 amino acid deletion starting at codon 396	PSV_9D_396	IMC_9D_396
CAP62.2.05_T19*		9 amino acid deletion starting at codon 390	PSV_9D_390**	ND
CAP63.2.05_T27		7 amino acid deletion starting at codon 397	PSV_7D_397	IMC_7D_397
CAP63.2.05_T34		5 amino acid deletion starting at codon 399	PSV_5D_399	IMC_5D_399

\*Two different pseudoviruses were constructed to test the impact of each glycan individually. \*\*For sequence CAP63.2.05\_T19, all pseudotyped viruses subsequently cloned from this sequence were non-functional.



**Figure 3.5:** Highlighter plot showing envelope sequences from pseudoviruses and infectious molecular clones of CAP63. The transmitted/founder pseudovirus clone is the reference line at the top. An early pseudovirus cloned from a virus sampled at 4 weeks post infection (CAP63.2.00\_A9) (Gray et al., 2007) and pseudoviruses constructed from 6 viruses sampled at 11 weeks post infection (CAP63.2.05) are below. Pseudoviruses generated from the introduction of V4 deletions are next (CAP63\_PSV) and these are followed by infectious molecular clones constructed by mutagenesis of the transmitted founder IMC (CAP63\_IMC).

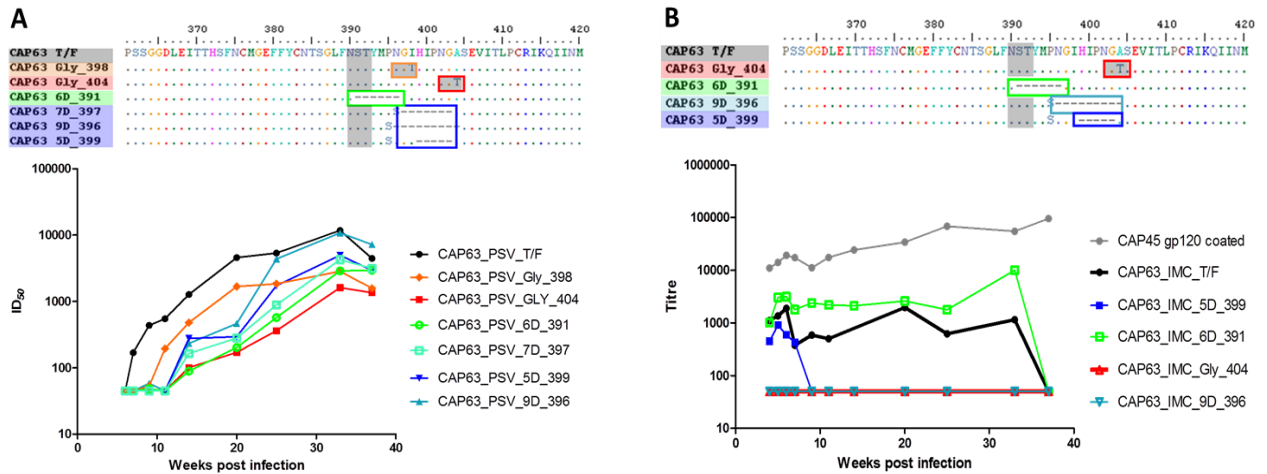
Similar to the wild-type variants, the 11 week plasma did not neutralize five of the six contemporaneous T/F V4 mutant pseudoviruses (Figure 3.6A), confirming that these changes were responsible for neutralization escape. Similarly, the 14 weeks plasma neutralized the earlier 11 week virus, although at much lower titres than the T/F, with ID<sub>50</sub> values of between 89 and 481 compared to T/F virus which is neutralized with an ID<sub>50</sub> of 1276. The introduction of a glycan at amino acid 404, as well as the large V4 deletions, resulted in a decrease in sensitivity to neutralization, while an additional single N-linked glycan at position 398 (which occurs together with the glycan at position 404 in natural infection) caused a smaller reduction in sensitivity to neutralization. In subsequent experiments, the glycan at position 404 is used as this is the modification with the most impact on neutralization.

### **3.3.6 V4 neutralizing antibody mutations facilitate escape from ADCC responses in CAP63**

We were interested in mapping effects of these V4 neutralization escape mutations on ADCC activity. To do this, we introduced these changes into CAP63 T/F IMC and infected a target cell (CEM.NKR<sub>CCR5</sub> cells) (Table 3.2). Four IMC mutants (three with deletions and one with a glycan introduced at position 404) were constructed: CAP63\_IMC\_Gly404, CAP63\_IMC\_6D\_391, CAP63\_IMC\_7D\_397, CAP63\_IMC\_9D\_396 and CAP63\_IMC\_5D\_399 and confirmed by sequencing of the full-length genome (Figure 3.5). Plasma samples were evaluated at 11 time points up to 37 weeks post infection. Titrations were performed to determine the optimal level of inoculum for infection (Appendix H). A minimum infection level of 20% of p24+ target cells was required for the ADCC assay (Pollara et al., 2011). Levels of infection differed between the different IMCs. CAP63\_IMC\_T/F showed the most efficient rates of infection with the 300µL inoculum resulting in 95.6% of the cells staining positive for p24 after 72 hours. The lowest infectivity was CAP63\_IMC\_7D\_397, where the 900µL inoculum resulted in only a 25.7% positive rate (Appendix H).

The IMC clones containing changes in V4 observed at 11 weeks post infection were assayed for recognition by ADCC (Figure 3.6B). Early ADCC activity between 4 and 7 weeks, recognised the T/F and two of the four 11 week mutants: the one mutant, IMC\_6D\_391 (green line), was more sensitive than the T/F IMC to ADCC activity throughout the 33 weeks

post infection suggesting that neutralization escape resulted in increased exposure of the ADCC epitope; while responses to the second mutant, IMC\_5D\_399 (dark blue line), were lost after 7 weeks, suggesting that ADCC activity disappeared when neutralization responses emerged. No ADCC activity was detected against cells infected with IMC\_GLY\_404 (red line) and IMC\_9D\_396 (light blue line) which indicates that the glycan at position 404, and the nine amino acid deletion at 396, completely abrogated ADCC recognition, and that new responses to this variant were not elicited.



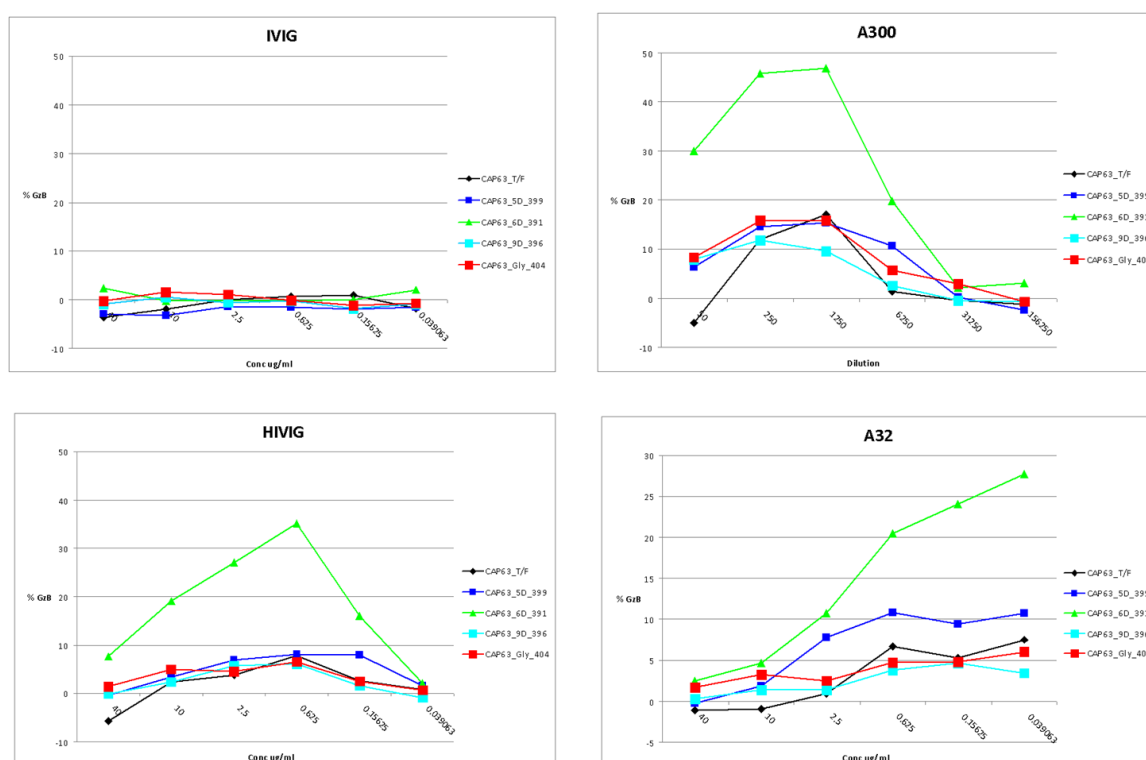
**Figure 3.6:** A: Comparison of autologous neutralization sensitivities of multiple envelope clones from CAP63. Envelope clones from the transmitted/founder virus and 5 viruses with changes observed in viruses sampled at 11 weeks post infection, introduced into the T/F, were tested against autologous serum from various points over 37 weeks of infection in an Env pseudotyped virus neutralization assay. Results are shown as ID<sub>50</sub> values and differences in the viruses are shown in the amino acid alignment above.

B: ADCC responses in CAP63. The results of the ADCC assay with target CEM NKR<sub>CCR5</sub> cells infected with various CAP63 IMCs over 37 weeks of infection. ADCC responses to coated cells are shown in grey and CAP63\_IMC\_T/F infected cells are in black. CAP63\_IMC\_5D\_399 is in blue, CAP63\_IMC\_6D\_391 is green, CAP63\_GLY\_404 is red and CAP63\_IMC\_9D\_396 is in aquamarine. ADCC activity against the T/F IMC is the mean of two separate runs. CAP45 gp120 coated cells were used as control targets and activity against these cells was sustained throughout the period investigated.

### 3.3.7 The 6D\_391 deletion escapes neutralizing antibody responses but becomes highly sensitive to ADCC

The 6D\_391 deletion, which resulted in a loss of a glycosylation site at position 390, enhanced ADCC activity by CAP63 plasma compared to the T/F IMC (Figure 3.6B) while at the same time mediating escape from neutralizing antibodies (Figure 3.6A). To determine if

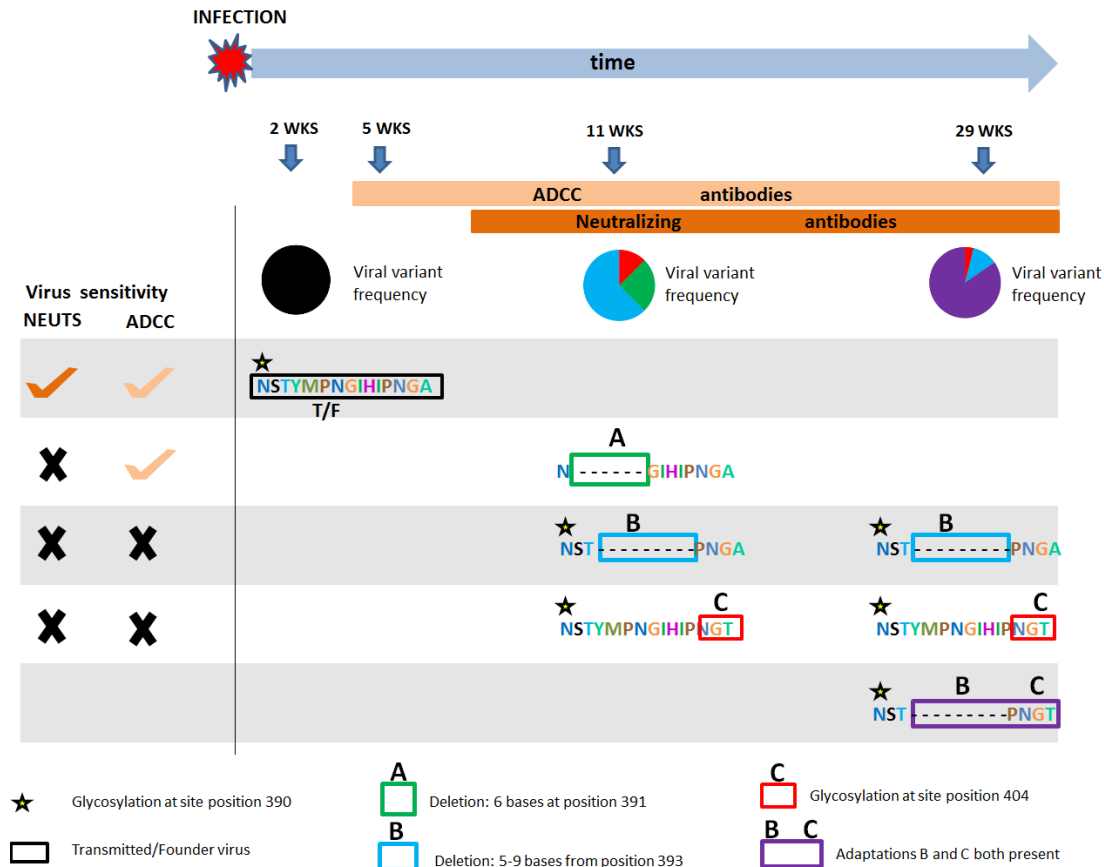
this mutation affected recognition by other ADCC antibodies, this mutant was assessed against a panel of antibodies with known ADCC activity including HIVIG (anti-HIV immunoglobulin prepared from pooled plasma of asymptomatic, HIV antibody positive donors with CD4+ counts above 400 cells/ $\mu$ L); A300 (serum from an HIV-1 infected individual); and the monoclonal antibody A32 (recognizes a conformational epitope that includes the C1 region, Ferrari et al., 2011) (Figure 3.7). Similar to patient derived responses, the ADCC activity against IMC\_6D\_391 infected cells was enhanced for HIVIG, A300 and A32, indicating the 6D\_391 deletion gp120 results in a conformation that promotes recognition and binding by ADCC antibodies of different specificities.



**Figure 3.7:** ADCC activity mediated by control antibodies and plasma against CEM.KNR<sub>CCR5</sub> cells infected with the various CAP63 IMCs. The polyclonal intravenous IgG preparation IVIG, the anti-HIV-1 immunoglobulin HIVIG, the serum from an HIV-1 infected individual A300 and the monoclonal antibody A32 are used to mediate ADCC activity. ADCC activity is shown in percentage GzB positive cells. The CAP63 T/F and all other IMCs with the exception of CAP63\_6D\_391 showed similar levels of ADCC activity. CAP63\_6D\_391 showed significantly elevated peaks of ADCC activity mediated by A300, HIVIG and mAb A32. IVIG was a negative control a polyclonal intravenous IgG preparation. The cut-off value for positivity is 8%.

### 3.3.8 ADCC activity constrains pathways of escape from neutralizing antibody responses

In summary, at 11 weeks post infection there were 5 escape variants which were classified into three types of escape pathways based on their neutralizing and ADCC characteristics (Figure 3.8):



**Figure 3.8** Summary illustrating constrained neutralizing antibody escape pathways by ADCC responses. Three types of escape pathways are designated A (green), B (blue) and C (red) are found at 11 weeks post infection and their persistence in the population at 29 weeks post infection illustrated. The presence of ADCC antibodies is indicated by the light orange bar and sensitivity to ADCC is indicated by a tick. The presence of neutralizing antibodies is indicated by the dark orange bar and sensitivity to neutralization indicated by the same colour tick. The relative proportions of each adaptation in the viral populations at 2, 11 and 29 weeks is represented in the pie charts.

Neutralization escape pathway A, depicted in green in Figure 3.8, is a deletion which knocks out the first glycan at position 389 (6D\_391). This deletion increased sensitivity to ADCC autologous antibodies targeting the V4, as well heterologous antibodies targeting as other regions of the envelope. This mutant with increased susceptibility to ADCC was eliminated

from the viral population at 29 weeks post infection, and as such appears to be a dead-end neutralization escape pathway;

Neutralization escape pathway B, depicted in blue in Figure 3.8, is a series of amino acid deletions of between five and nine amino acids starting at or after codon 392 (5D\_399, 7D\_397 and 9D\_396). This epitope was recognised by ADCC in early infection, however ADCC activity was lost once neutralizing antibodies emerged; and

Neutralization escape pathway C, shown in red, is a point mutation at position 404 that adds a glycosylation site (Gly\_404). This mutation was never recognised by ADCC antibodies, and was poorly recognised by neutralization antibodies.

Escape pathway B or C persisted in the population albeit at low levels, with the majority of the viral population consists of viruses which have acquired mutations from both pathways (displayed in purple in Figure 3.8).

### **3.4 Discussion**

Although neutralizing activity alone may provide protection against HIV the effector functions of antibodies which accelerate the destruction of virus infected cells are likely to be vital components of a completely effective anti-HIV immune response (Picker & Deeks, 2013). Understanding the first responses to HIV-1 infection may provide insights into mechanisms of viral control, and inform vaccine development. In this chapter we analysed infected participants of the HISIS and CAPRISA cohorts and focused the study on one individual, CAP63, to understand the interplay between ADCC and neutralizing antibody responses and how these shape the evolution of HIV-1 during early infection.

In CAP63, ADCC responses were present at the first time-point sampled (four weeks post infection), with autologous neutralizing antibody response to the T/F virus emerging only at seven weeks post infection. Codons in the V4 region were under strong positive selection, and we confirmed that it was the target of both neutralizing and ADCC responses. All the changes in the V4 regions observed at 11 weeks conferred some level of neutralization resistance suggesting that neutralizing antibodies were a major force driving changes in that

region. Prior to neutralization responses, there was a single change from the T/F in the four weeks post infection virus (codon position 395), however this did not appear to be associated with ADCC escape (Figure 3.1D) suggesting that this was not driven by ADCC responses. However it would be of interest to sequence the virus between 4 and 7 weeks post infection to more definitively determine if there is evidence of ADCC driven escape.

ADCC responses are among the earliest responses present during the course of disease and they precede the emergence of autologous neutralizing antibody responses (Forthal, Landucci, & Daar, 2001). The first response to HIV-1 infection involves IgG3 (Yates et al., 2011), and although IgG1 antibodies also arise early, they strengthen and persist and replace the IgG3 response as the disease progresses (Tomaras et al., 2008b). IgG3 and IgG1 both have high affinity for FcγRs and functional potency (IgG3>IgG1) (Yates et al., 2011) but IgG1 responses are associated with neutralization (Banerjee et al., 2010). Although we were unable to differentiate between different IgG subclasses, we hypothesize that the development of the neutralizing antibody response at seven weeks post infection may have coincided with subclass switching, and the loss of IgG3 responses resulting in loss of ADCC responses capable of recognising mutant 5D\_399 (blue line, Figure 3.6B).

Although both neutralizing antibodies and ADCC responses are targeted at the V4 region, some neutralizing escape variants did not confer resistance to ADCC and this difference in recognition pattern and kinetics of responses is indicative of humoral responses with overlapping but unique characteristics. In addition to the fact that ADCC antibodies do not necessarily have neutralizing activity and can recognise different targets, the very same targets on the envelope may also be presented in different conformations. The cell-associated form is often associated with lipid rafts and an uncleaved main structural Gag polyprotein and is mechanically stiff. The mature virion bound form is associated with a mature Gag that has undergone proteolysis subsequent to budding resulting for morphological maturation and enabling infectivity (Chojnacki et al., 2012; Kol et al., 2013; Pang et al., 2013).

Of interest, the one neutralization escape variant where the loss of a glycosylation site at position 389 (6D\_391) that facilitated neutralization escape, resulted in increased sensitivity to ADCC by both CAP63 plasma, and by two control plasmas and A32, which recognizes a CD4 inducible conformational epitope that includes the C1 region (Ferrari et al., 2011). This indicates that the glycan at position 390 is important either for shielding one or several major

ADCC associated epitopes, or affects the conformational integrity of the Env trimer expressed on the surface of infected cells. This adaptation, unlike most other adaptations found at 11 weeks post infection, was not present in sequences from 29 weeks post infection and it is possible that was selected against and removed from the population through the action of ADCC associated antibodies.

In addition to the target cells and the presentation of epitopes, there is an important role for effector cells in the mediation efficient ADCC activity. In vivo as well as in the GTL assay where PBMCs were used as the effector cell source, natural killer (NK) cells and monocytes carry out this function. The type and expression levels of Fc receptors on these cells can play an important role in Fc-receptor binding affinity, as can the Fc portion of antibodies. Infection and activation of these innate immune cells has a great influence on their ability to perform ADCC by reducing their numbers, reducing FcR expression levels and impairing normal function. As such, it is possible to detect ADCC activity in vivo using donor PBMC which would not be present in vitro as a consequence of depletion or impairment of an individual's NK and monocyte population.

In conclusion, our results confirm only partial overlap between the epitopes of neutralizing antibodies and antibodies mediating ADCC in early infection: while some adaptations mediating escape from both neutralization and ADCC, others did not. Relatively few ADCC epitopes have been identified to date and prior to this, there have been no reports of ADCC epitopes in the V4 region (Pollara et al., 2013) and there is even less evidence of immune escape from ADCC (reviewed by Isitman et al., 2012). Furthermore, much of the available evidence is based on linear peptides and gp140 (Chung et al., 2011) which limit our understanding of the full breadth of ADCC activity to that which is mediated by Abs recognizing antigens expressed on the surface of infected cells. This study highlights the complex relationships that exist between neutralizing antibodies and ADCC mediating antibodies in HIV-1 infections, and the role of ADCC in the evolution of the viral quasispecies during the course of disease. We present a novel finding of a dead-end neutralizing antibody escape pathway as a result of its increased sensitivity to ADCC. This increased sensitivity results in the population-wide depletion of mutants carrying the neutralization escape mutation and is the first reported evidence of this type of influence on viral evolution by ADCC activity. While the exact mechanism is yet to be fully elucidated, it

may point to potential methods for disrupting disease progression, with applicability in the development of HIV vaccines or therapeutic interventions.



## Chapter 4: The effects of early evolution on viral fitness

<b>Abstract .....</b>	<b>84</b>
<b>4.1 Introduction.....</b>	<b>85</b>
<b>4.2 Materials and methods .....</b>	<b>85</b>
4.2.1 Mutagenesis of cloned envelopes .....	85
4.2.2 Cell-cell fusion assay .....	86
4.2.3 Titration of viral stocks for entry and replication assays by beta gal staining.....	86
4.2.4 Replication assay.....	87
4.2.5 p24 ELISA .....	87
<b>4.3 Results.....</b>	<b>88</b>
4.3.1 Viruses from CAP63 show reduced fusion capacity within 11 weeks relative to the T/F virus .....	88
4.3.2 Deletions in V4 contribute to reduced fusion capacity in CAP63.....	89
4.3.4 Point mutations in the gp41 are preferentially fixed outside the LLP domains and have no effect on fusion capacity .....	90
4.3.5 Mutations in the V4 region of CAP63 result in reduced infectivity of virus stock .....	91
4.3.6 Adaptations in the V4 region of CAP63 affect viral replicative capacity in PBMC .....	92
<b>4.4 Discussion .....</b>	<b>94</b>

## **Abstract**

The ability of a virus to infect new host cells, replicate and escape host surveillance, all contribute to viral fitness. While escape from cellular immune responses has been associated with a fitness cost through reduced replicative capacity, given the plasticity of env, it is generally thought that escape from humoral responses would not impact on fitness. We studied the effect of neutralizing and ADCC antibody escape mutations on the viral fitness using the virus' ability to mediate fusion, infectivity and replicative fitness as surrogate markers of fitness.

Wild type transmitted/founder (T/F) envelope were compared to 11 week envelopes, and the T/F envelope with the nAb and ADCC escape mutations introduced. The T/F infectious molecular clones (IMCs) were compared to matched T/F constructs with these escape mutations. The ability of cloned envelopes to mediate fusion was tested using a cell-cell fusion assay; infectivity of IMC virus was measured by determining the ratio of number of infectious units (IU) per mL to p24; and replication capacity was measure in growth assays conducted in PBMC from two donors over two weeks.

Wild type envelopes bearing immune escape adaptations had lower cell-cell fusion ability compared to the T/F virus. Introduction of these mutations into T/F env and IMCs showed that these V4 adaptations were responsible for this reduction, and furthermore resulted in reduced infectivity. The largest deletion in the V4 (9D\_396) caused an 11-fold reduction in infectivity of IMC virus stocks and also had reduced growth in PBMCs.

This study shows that escape from humoral responses in early infection resulted in fitness cost affecting fusion, infectivity and replication.

## **4.1 Introduction**

An important focus of HIV research has been to try to understand how patterns of viral diversification and adaptation affect viral fitness as this may assist in identifying sites of vulnerability on the virus. However, the extensive variability and the apparent ability of the virus to tolerate large changes in these regions with minimal fitness cost (Bunnik et al., 2010) have made this difficult. Despite this, there is, a large body of evidence showing that the variable loops of the Env glycoprotein gp120 are important determinants and markers of vital components of viral fitness such as co-receptor tropism and viral pathogenesis (Monno et al., 2011), sensitivity to neutralization by antibody responses (Bunnik et al., 2010; van Gils et al., 2011; Wei et al., 2003), viral fusion (Cavrois et al., 2013) and disease progression (Curlin et al., 2010).

The V4 region of gp120 has been reported to evolve independently, resulting in very high intra-patient diversity (Castro et al., 2008) and it has been proposed that a high frequency of insertions and deletions is achieved through a mechanism involving strand slippage misalignment of long sequence duplications, repeats, and palindromes (Guglietta, Pantaleo, & Graziosi, 2010). V4 is one of the major targets of early autologous neutralizing antibody responses in subtype C infection (Moore et al., 2008; Rong et al., 2009) and deletions of V4 and V5 were shown to disrupt Env cell surface display and viral assembly (Yuan, Li, & Zhang, 2013). We have shown in the preceding chapter that V4 is the target of the neutralizing and ADCC antibody responses in CAP63 and that deletions and glycan shifts in the region enhanced the resistance fitness of the virus. The current study aimed to determine what other fitness effects adaptations in the V4 region may have. We monitored the impact of deletions in the V4 on viral fusion, viral infectivity and replicative capacity.

## **4.2 Materials and methods**

### **4.2.1 Mutagenesis of cloned envelopes**

Site-directed mutations corresponding to naturally-occurring mutations and back mutations to the T/F sequence were introduced with QuickChange site-directed mutagenesis kit (Stratagene). Primers used for mutagenesis are in Appendix F.

#### 4.2.2 Cell-cell fusion assay

HEK 293T cells ( $4 \times 10^5$ ) were seeded in 6 well plates overnight, and co-transfected with 3.75  $\mu\text{g}$  of each individual env plasmid and 3.75  $\mu\text{g}$  of pSVtat72 (NIH AIDS Reagent Programme) for 48 hours using the PolyFect Transfection Reagent (QIAGEN, US) according to the manufacturer's instructions. The cell medium was removed and the cells were lifted using 0.04% EDTA. The 293T cells ( $1 \times 10^4$  cells per well) were added to TZM-bl cells ( $1 \times 10^4$  cells per well) and allowed to incubate at 37°C for 24 hours. The media (100  $\mu\text{L}$ ) was removed from the cells, and 100 $\mu\text{L}$  of Bright-Glo buffer (Promega, US) was added. The plate was incubated for 2 minutes at room temperature with mild shaking. After cell lysis, 150  $\mu\text{L}$  was transferred to a Co-Star black plate, and the plate was read immediately using a Glomax 96 microplate luminometer (Promega, US).

#### 4.2.3 Titration of viral stocks for entry and replication assays by beta gal staining

The  $\beta$ -Gal reporter found in TZM-bl cells allows direct enumeration of infectious viral units by counting  $\beta$ -Gal expression-positive infected cell colonies under a microscope. Briefly,  $1 \times 10^5$  TZM-bl cells in 500 $\mu\text{L}$  of growth media were seeded onto each well of a 24 well plate overnight. On the second day, IMC virus stock was diluted at 1:10, 1:50, 1:250, 1:1250 and 1:6250 in DMEM containing 1% FBS and antibiotics. Media was removed from the cells and 200 $\mu\text{L}$  of fresh media was added. 50 $\mu\text{L}$  of virus dilution and a no-virus control was added to the wells and incubated for 4 hours at 37°C. 500 $\mu\text{L}$  of fresh media was added to each well and samples were incubated for 48 hours at 37°C. On day 4, supernatant was removed from the infected cells and 250 $\mu\text{L}$  of fixing solution (PBS with 0.8% Gluteraldehyde and 2% formaldehyde) and incubated at room temperature for 8 minutes. Fixing solution was removed and the cells washed 3 times with 500 $\mu\text{L}$  PBS per well. 250 $\mu\text{L}$  of staining solution (4mM Potassium Ferricyanide, 4mM Potassium Ferrocyanide, 400 $\mu\text{g}/\text{mL}$   $\text{MgCl}_2$  and 400 $\mu\text{g}/\text{mL}$  X-gal in PBS) was added to each well and incubated at 37°C for 2 hours. The staining solution was removed and 250 $\mu\text{L}$  PBS added to each well. Blue cells were counted under a light microscope and the number of infectious units calculated. Infectivity was calculated by normalizing the virus stock to 7500 IU per mL and determining the number of IU per unit p24 determined by ELISA.

#### **4.2.4 Replication assay**

The kinetics of viral replication was determined in human PBMC. Cells were stimulated for 72 hours in RPMI with 15% FCS with 2mM L-glutamine and gentamycin supplemented with 200U/mL IL2 and phytohemagglutinin (PHA) at 37°C at 5% CO<sub>2</sub>. The cells were then washed twice in RPMI with 15% FCS with 2mM L-glutamine and gentamycin and rested overnight in the same media supplemented with 200U/mL IL-2 (growth media). A 96 well plate was then seeded with 750,000 PHA-IL2 treated cells per well in 100µL of growth media. Virus was added to the PBMC at a multiplicity of infection (MOI) of 0.01 (MOI, IU/cell) in 100µL of media. Cells were incubated at 37°C at 5% CO<sub>2</sub> and 72 µL of culture supernatant was sampled with replacement of media at days 3, 5, 7, 9, 11, and 14 post infection for determination of p24. Each virus inoculum tested was subjected to the same treatment in the absence of cells to determine background levels of p24. The slope of each growth assay was calculated using the LOGEST function in excel on the logarithmic growth phase of the curve.

#### **4.2.5 p24 ELISA**

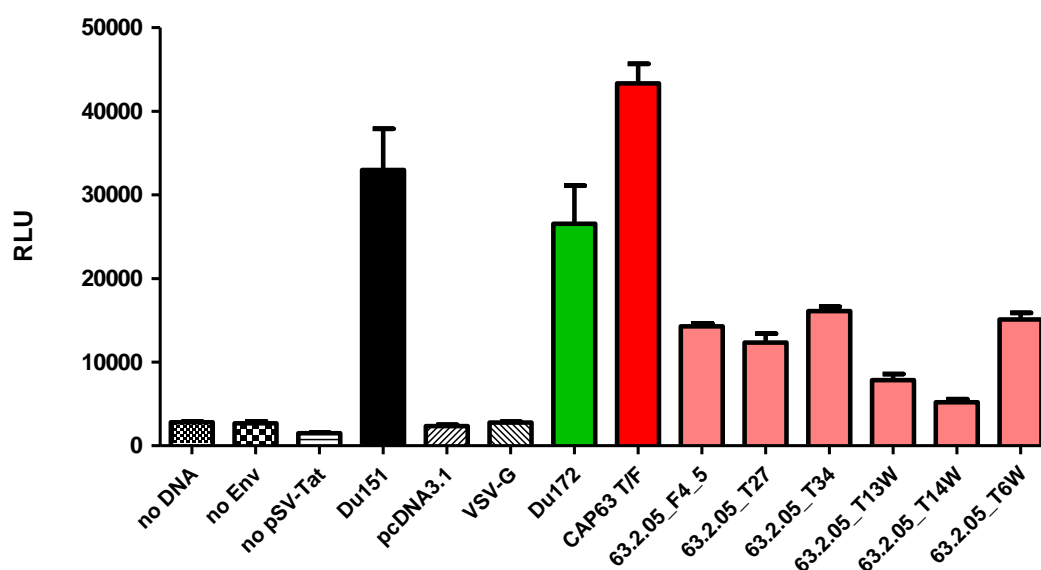
COSTAR white opaque 96-well ELISA plates (Corning) were coated overnight with affinity purified sheep anti HIV-1 p24 gag antibody (Aalto Bio Reagents), washed three times with tris-buffered saline (TBS) and allowed to dry. Plates were stored at -20C for medium term use. A p24 standard dilution series was prepared in binding buffer made up of TBS with 1% Empigen (Sigma) after reconstituting bacterially expressed p24 (Aalto Bio Reagents) in TBS with 1% FCS. Virus containing sample supernatants were also serially diluted in binding buffer and 100µL of samples and p24 standards were placed in pre-coated and blocked plates. Plates were covered and incubated for two hours at room temperature. The plates were then washed three times with TBS. 100µL of a 1 in 12500 dilution of an alkaline phosphatase conjugated sheep anti HIV-1 monoclonal antibody BC 1071-AP (Aalto Biosciences) in TBS with 0.1% TWEEN-20 was added and incubated for 1 hour at room temperature. Wells were washed 4 times with a TBS buffer with TROPIX and 0.1% TWEEN. Bound antibodies were measured by placing 100µL of a solution of CDP Star with Sapphire-II (Life Tech) in TROPIX buffer in each well for half an hour and reading in a luminometer. All reactions were run in triplicate and the equation generated from the linear range of the p24 standard

curve was used to determine the amount of p24 in each sample based on the results of the luminometer readings.

## 4.3 Results

### 4.3.1 Viruses from CAP63 show reduced fusion capacity within 11 weeks relative to the T/F virus

The ability of the viral envelope to mediate fusion is an important aspect of viral fitness. In the previous chapter we identified viruses at 11 weeks post infection which had undergone modifications in the V4 as a result of antibody pressure. To investigate the impact of these modifications on viral fusion, we compared the ability of envelope bearing these adaptations to mediate fusion relative to the T/F virus. To measure fusion, we co-transfected two plasmids into 293T cells, one expressing the envelope of interest and the other expressing Tat, and co-incubated these cells with TZM-bl cells overnight. Cell-cell fusion was measured by luciferase activity induced by Tat resulting from the fusion of the effector 293T and reporter TZM-bl cells.



**Figure 4.1:** Fusion capacities of CAP63 T/F virus and variants from 11 weeks post infection measured by luciferase expression (RLU) in TZM-bl cells. The T/F virus is represented by the red bar while variants from week 11 are shown in pink. Negative controls include a mock transfection (no DNA), transfection with only Tat expressing plasmid (no Env), transfection with only Env expressing plasmid (no Tat), as well as transfection with an empty mammalian expression vector (pcDNA3.1) and a VSV-G Env expressing plasmid. Du151 (black) and Du172 (green) were used as positive controls.

Envelopes from 11 weeks showed markedly reduced fusion capacity relative to the T/F virus envelope (Figure 4.1). All of the variants from 11 weeks post infection had modifications in the V4 region of the envelope and these were studied in detail in the previous chapter (see Figure 3.2 in chapter 3).

### 4.3.2 Deletions in V4 contribute to reduced fusion capacity in CAP63

In addition to shifts in the glycosylation sites and deletions in the V4 region, several other adaptations to the envelopes of viruses from 11 weeks post infection were observed. These included shared point mutations in the V1 region, as well as shared mutations at two locations in the cytoplasmic tail of the gp41 (Figure 3.2 Chapter 3). To exclude the influence of changes outside of V4 on fusion, we introduced V4 mutations into the T/F virus sequence (Chapter 3.2.10). We then compared the fusion capacity of the 11 week wild type virus, the V4 mutant envelopes, and the T/F envelope to determine the influence of any other changes to the sequence (Figure 4.2).

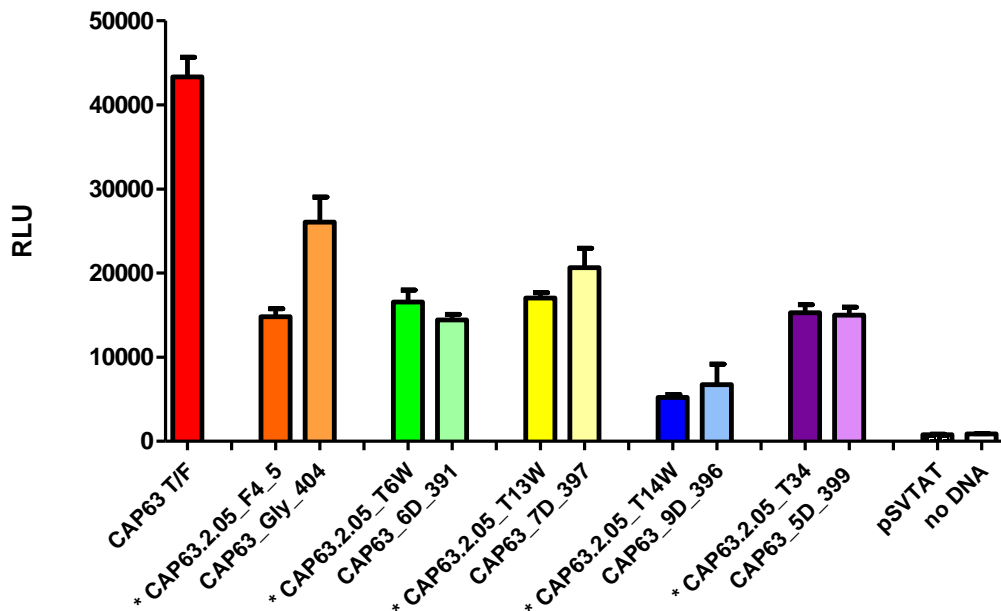


Figure 4.2 Comparison of fusion capacity of the T/F virus (in red) to naturally occurring CAP63 envelopes (in darker shades and marked with \*) and T/F viruses with week 11 adaptations introduced in the V4 region. Error bar is the standard deviation of 3 independent experiments.

The T/F virus had increased fusogenicity compared to all of the variants with V4 adaptations. In four of the five, the wild type week 11 variants had very similar cell-cell fusion capacity compared to the T/F viruses with introduced V4 adaptations, indicating that changes outside the V4 did not affect fusion. In one variant, the wild-type CAP63.2.05\_F4\_5 was less fusogenic than its corresponding mutated T/F virus CAP63\_GLY\_404. These two sequences differ in the V4 region in that the former had two extra glycosylation sites, one at position 398 and the one at 404, while the latter had only the additional glycan at position 404. However there were also changes in gp41 which may have affected fusion. Our results confirm that the immune adaptations to the V4 region caused a reduction in the fusion capacity of the envelopes.

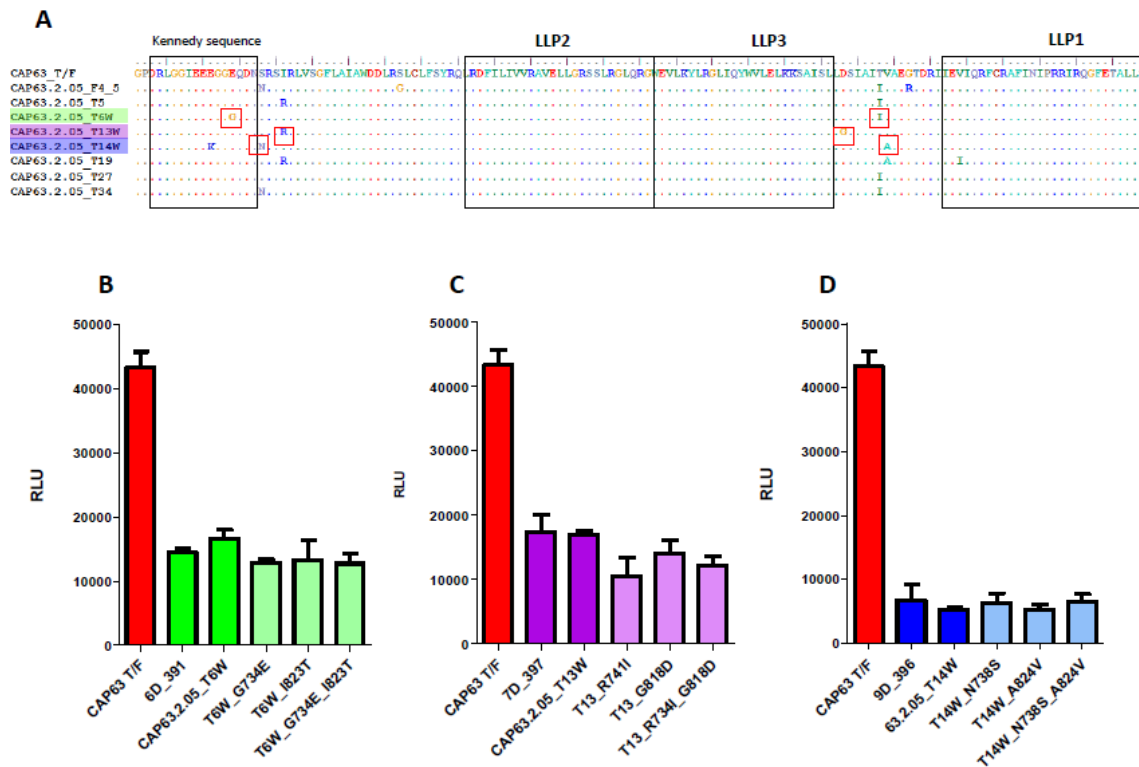
#### **4.3.4 Point mutations in the gp41 are preferentially fixed outside the LLP domains and have no effect on fusion capacity**

Analysis of early evolution in CAP63 showed that there was strong positive selection in the gp41 region of the envelope during the first 11 weeks of infection (Chapter 3). The central and C-terminal regions of the gp41 cytoplasmic tail contain three conserved amphipathic  $\alpha$ -helical segments referred to as lentiviral lytic peptides (LLP-1, LLP-2, and LLP-3) (Miller et al., 1991). These motifs have been associated with cell surface expression (Bültmann et al., 2001), Env fusogenicity (Kalia et al., 2003), and Env incorporation into a virus (Murakami & Freed, 2000; Piller et al., 2000).

Two clusters of changes were observed in viruses at 11 weeks, both in the cytoplasmic tail of the gp41 region, which were shared or closely related to among most variants (Figure 4.3). The first set of changes was between positions 734 and 741 located at the C-terminal end of the Kennedy sequence, with the S738N and I741R mutations being the most prevalent change. A second set of changes were at positions 818 to 824, located between LLP1 and LLP3. The most common mutation there was T823I.

In order to establish whether these mutations affected fusion, we back-mutated CAP63.2.05\_T6W (G734E, I823T), CAP63.2.05\_T13W (R741I, G818D) and CAP63.2.05\_T14W (N738S, A824V) to the T/F sequence. Each of the viruses had two mutations which we reverted to the T/F sequence both individually and in combination. For

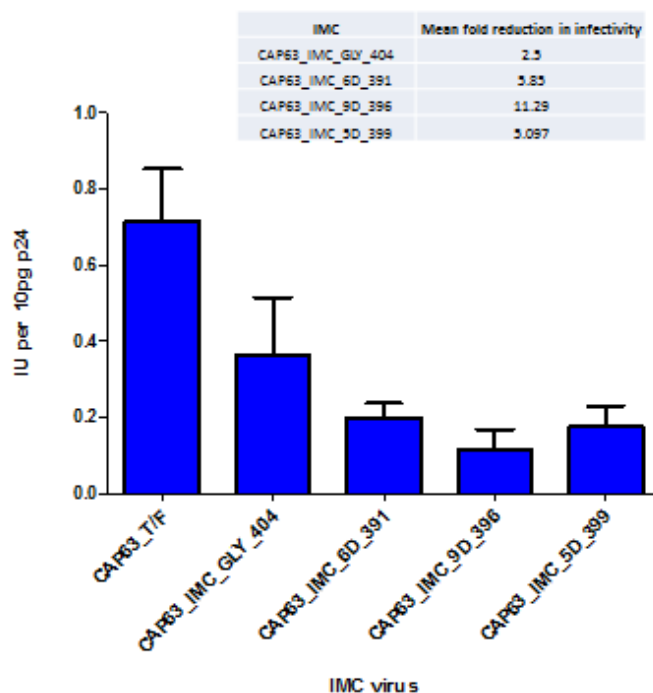
all the viruses, reversion of one or both of the mutations to the T/F sequence had no noticeable effect of the ability of the envelope to mediate fusion (Figure 4.3).



**Figure 4.3:** Mutations in the cytoplasmic tail of gp41 in CAP63 and their effect on cell-cell fusion. A: the positions of mutations relative to the Kennedy sequence and the LLP domains. B: the effect of mutations on the gp41 of CAP63.2.05\_T6W, C: CAP63.2.05\_T13W and D: CAP63.2.05\_T14W. In each case we see the effect of just the V4 mutation, the naturally occurring variant, reversion of the Kennedy sequence mutation, reversion of the C-terminal mutant and reversion of both mutations.

#### 4.3.5 Mutations in the V4 region of CAP63 result in reduced infectivity of virus stock

To investigate the effects of V4 adaptations in CAP63 on infectivity, we compared the CAP63 T/F IMC virus to four variants with adaptations introduced into the V4 region (Chapter 3.3.5). Infectivity was measured by determining the ratio of number of infectious units (IU) per mL to p24. To account for variations between each virus preparation and storage process, three separate virus stock preparations from different days were evaluated (Figure 4.4).



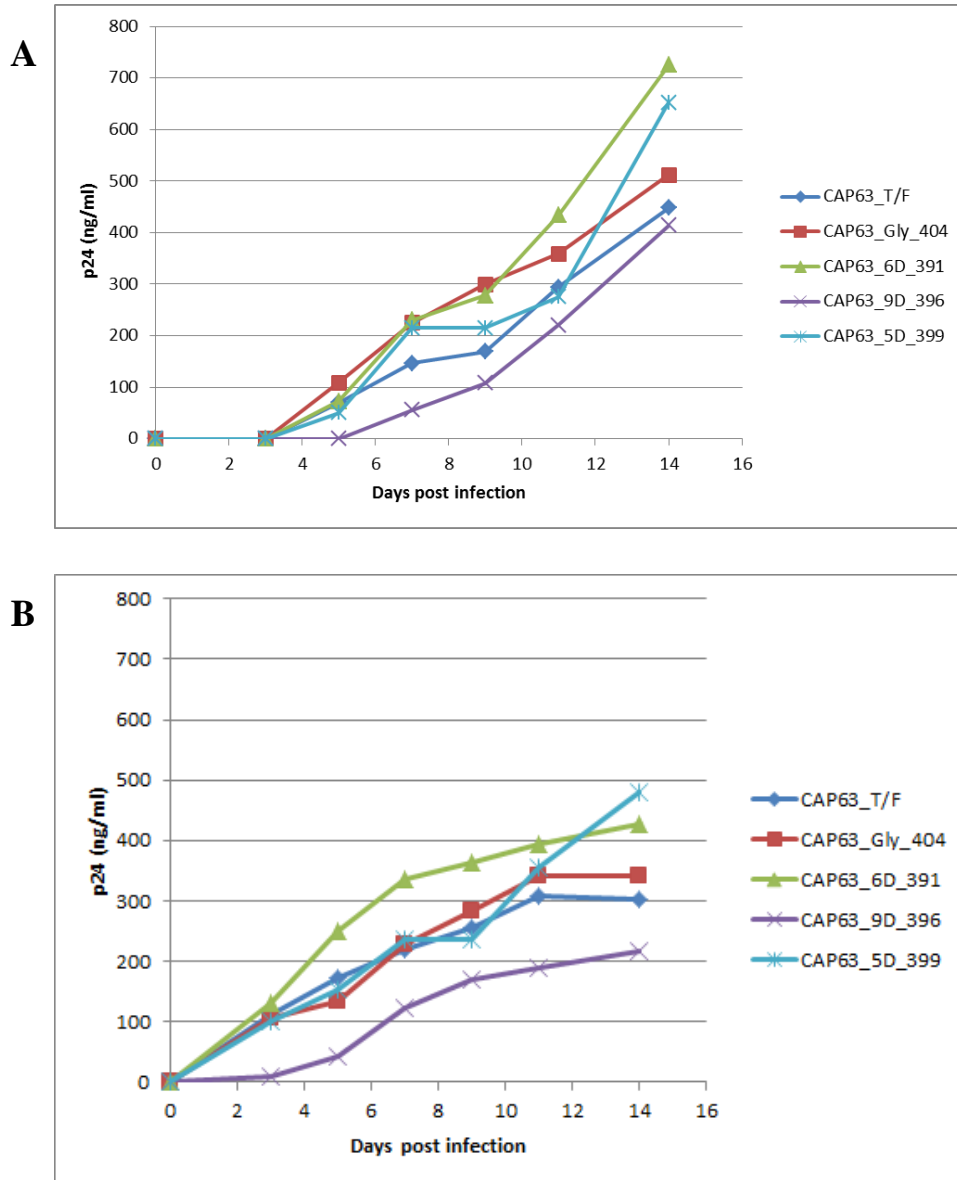
**Figure 4.4:** The average infectivity of viruses and standard deviations (error bars) based on three separate preparations of virus from 293T cells transfected with IMCs. Infectious units were measured by titrating virus preps and infecting TZM-bl cells and staining with beta-galactosidase. The number of infectious units was then normalized to 7500 IU/mL and p24 measured by ELISA. The graph plots infectious units per 10pg p24.

The T/F virus stock was the most infectious compared to the V4 adapted viruses. On average, relative to the infectivity of the T/F virus the Gly<sub>404</sub> mutation caused a 2.5-fold reduction, the 5D<sub>399</sub> and 6D<sub>391</sub> mutations caused 5.097 and 5.85-fold reductions respectively while the 9D<sub>396</sub> deletion caused a 11.29-fold reduction. Larger deletions in the V4 tended to cause greater reductions in infectivity.

#### 4.3.6 Adaptations in the V4 region of CAP63 affect viral replicative capacity in PBMC

Previous experiments evaluated infectivity in TZMbl, and we were interested in determining overall replicative capacity in PBMC. We obtained PBMCs from two donors and evaluated replication for each IMC in triplicate over a 14 day period (Figure 4.6). Replication kinetics of the viruses differed in the two donors, with the natural log slope of production of p24 being significantly greater in donor WP4 than in donor WP1 ( $p=0.007$ ; Appendix J). The slopes of the p24 production by the various IMCs did not differ significantly from each other

within the same donor over the length of the 14 day assay period (Appendix J). However, in both donors, the CAP63\_IMC\_9D\_396 virus was consistently slower in reaching detectable p24 levels, only reaching such levels 2 days later than the rest. The plateau was also much lower than other viruses which suggests a replicative fitness costs associated with this deletion.



**Figure 4.6** Replicative capacities of CAP63\_T/F and V4 mutant viruses from 11 weeks post infection assayed over 14 days in PBMC from 2 donors WP1 (A) and WP4 (B). Each data point represents the mean of triplicate wells.

## **4.4 Discussion**

The impact of neutralization escape on viral fitness is largely unexplored with a recent study showing that in one individual, escape from broadly cross neutralizing antibodies lead to a reduction in the viruses ability to enter and replicate (Sather et al., 2012). Although some studies have implicated early neutralizing antibody responses in controlling viral replication – at least temporarily (Bar et al., 2012; Moore et al., 2009) – these changes are not generally thought to impact viral fitness. In this study, we demonstrate that humoral escape mutations in the V4 region in the first few months of infection resulted in reduction in fusion capacity, infectivity and the variant with the largest deletion also had reduced replication capacity.

The cell-cell fusion assay enables the fusion event to be measured and has been widely used to investigate several aspects of HIV-1 entry into cells including efficiency, kinetics, and number of env trimers needed for fusion (Cheng et al., 2010; Dorr et al., 2005; Guo et al., 2012; Heredia et al., 2008; Lineberger et al., 2002; Markosyan et al., 2009; Melikyan et al., 2000; Mulampaka & Dixit, 2011). Using this assay we demonstrated in one individual that the T/F virus was more fusogenic than the 11 week virus. It has been suggested T/F viruses carry an excess of Env trimers (Parrish et al., 2013) and therefore may generally be more fusogenic. However, we expanded our analysis to a comparison of envelopes from early and chronic infection from eight individuals showed that only four T/F viruses were more fusogenic showing that this is not a property of transmitted viruses (Appendix I). Similar results have been reported in a study by Guo et al., (2012) also conducted in subtype C infected mother-infant pairs.

Changes to the V4 and V5 of gp120 have been shown to disrupt density and structure of cell surface Env and viral assembly (Yuan et al., 2013) and altering the expression levels of envelope trimers greatly affects the efficiency of cell-cell fusion (Lineberger et al., 2002). Several studies have found that gp120/gp41-driven membrane fusion requires the formation of a threshold number of fusion-active intercellular gp120/gp41:CD4:CCR5 complexes (Klasse, 2007; Lineberger et al., 2002; Magnus et al., 2009) and the probability of reaching this threshold is governed, in large part, by the surface density of gp120/gp41 (Lineberger et al., 2002). Only a few functional trimers are packaged on a virus with estimates averaging

eight or nine and ranging from two to 19. Given this low number of Env trimers per virus, small modifications in the distribution of surface Env are likely to translate to significant changes in the packaging and thus infectivity of virions. We found that changes in the V4 had significant impact on the infectivity of virus produced in culture. The virus with lowest infectivity also had an extended lag phase of replication and lower plateau relative to the T/F virus. While we have not measured env density directly we hypothesise the V4 deletions resulted in a reduction of surface expression of Env, and this impacted on the fusion and infectivity. To verify this, future studies should measure the effect of V4 deletion on env expression and presentation using sing flow cytometry and western blot.

In conclusion, adaptations to the V4 brought on by escape from neutralizing and ADCC antibodies result in reductions in fusogenicity and virus particle infectivity probably as a result of changes in the cell surface expression and distribution of Env trimers. Only the immune escape adaptation with largest reduction in particle infectivity was sufficient to cause a change in the replication kinetics of the virus. These results have implications for the understanding of fitness costs associated with escape from humoral responses to HIV and highlight the importance of V4 as a potentially important target on the virus as it has a significant role in viral fitness.

## Chapter 5: Conclusions

After more than three decades there is still no effective vaccine for HIV. The remarkable diversity of HIV and the difficulty associated with eliciting broadly protective antibody responses through vaccination are among the major challenges facing vaccine developers. However recent findings from the RV144 vaccine trial which suggest a role for ADCC in protection underscored the need to fully understand the interplay between ADCC and neutralizing antibodies. We were interested in understanding the interplay between HIV-1 diversity, humoral responses and viral fitness in an effort to further inform vaccine design.

We compared neutralizing antibody responses in two female cohorts in Africa with different local epidemics, the Tanzanian HISIS cohort and South African CAPRISA cohort. We found different frequencies of broadly neutralizing antibody responses, with HISIS participants having broader neutralizing antibody responses at two years post infection compared to the CAPRISA participants. Participants in the HISIS cohort also had more potent autologous neutralizing antibody responses at 12 months post infection. We were unable to establish a role for subtype effects, dual infection and other virological features of the infecting virus in the development of broadly neutralizing antibodies in these two populations. A larger study may be required to fully explore these factors. However, we found that VL was strongly predictive of the development of breadth and that the HISIS cohort had higher VL. The study illustrates a key role for community VL in shaping the neutralizing antibody responses in different geographical regions. The presence of VL-influencing co-infections such as malaria may be one of the drivers as might be the extensive host genetic diversity which is present across the African continent. Both of these factors should be considered in future studies investigating the development of bNAb responses.

To understand the interplay between ADCC and neutralizing antibody responses we analysed one participant, CAP63, in detail. We found that ADCC responses preceded neutralizing antibody responses and that both responses targeted overlapping epitopes in the V4 region of the envelope. Four of the five neutralization escape mutants identified at 11 weeks post infection also mediated escape from ADCC, while one deletion made the virus sensitive to ADCC and was eliminated. Our study showed that certain neutralizing escape pathways were a dead-end, and for viruses to survive they may need to avoid both neutralization and ADCC

responses. This study defined ADCC epitopes based on the functional virus as opposed to peptides or gp140, and was the first study to define an ADCC epitope in the V4 region.

Lastly, the impact of humoral V4 escape mutations on viral fitness was determined by monitoring fusion capacity, viral infectivity and replication capacity. Adaptations to the V4 resulted in reductions in fusogenicity and virus particle infectivity, probably as a result of changes in the cell surface expression and distribution of Env trimers. Replicative capacity of the virus was largely resilient to changes in fusogenicity and infectivity since only the largest reduction in particle infectivity translated to a change in the replication kinetics. These results provide further insights into fitness costs associated with escape from humoral responses to HIV and highlight the importance of V4 as a potentially important target on the virus as it has a significant role in viral fitness. A priority for future studies must be to determine if our findings can be expanded to multiple individuals.

In conclusion, this study was the first to directly compare nAb responses in two cohorts in Africa, showing that differences in community viral loads have a strong influence on frequency of people who develop broadly cross-neutralizing responses. This may in part explain the wide variation reported in the literature. ADCC responses were shown to evolve very early in infection and recognise the same epitope as neutralizing antibodies. This very early pressure by ADCC response prior to neutralizing response could potentially facilitate a rapid rate of escape. Although both nAb and ADCC recognise the same epitopes, *in vivo* they are presented differently (i.e. cell associated or cell free) and it remains to be determined if effective ADCC activity is facilitated by the same antibody with both functions, or two different antibodies. Of interest is the fact that one neutralization escape mutant was actually more susceptible to ADCC and this was an unsuccessful escape pathway for the virus. Importantly, this was the first study to show that change in V4 driven by early autologous responses affects viral fitness. Our study demonstrates and proposes a mechanism through which an effector mediated humoral response can complement the action of neutralizing antibodies in exerting selective pressure, exacting a discernible fitness cost and influencing viral evolution. Given the relative ease with which such responses have been generated, an adjustment in our understanding of what constitutes potentially protective vaccine-induced immunity to include effector mediated humoral responses may offer an additional avenue of hope in the quest for an effective vaccine.

## APPENDICES

Appendix A: Standard RNA and DNA techniques .....	99
Appendix B: Reagents and buffers .....	102
Appendix C: Viral load data .....	103
Appendix D: Neutralization assay data .....	104
Appendix E: HIV-1 subtype C envelope, full length and sequencing primers .....	105
Appendix F: Mutagenesis primers .....	107
Appendix G: Titration of IMC virus stocks for infection of CEM NKR <sub>CCR5</sub> cells .....	108
Appendix H: Determination of the optimal length of infection for target cells with IMCs .....	110
Appendix I: Cell-cell fusion capacity of CAPRISA envelopes from acute and chronic infection .....	111
Appendix J: Replication assay slope calculations .....	112

## **Appendix A: Standard RNA and DNA techniques**

### **A1. Extraction of viral RNA**

RNA was extracted both manually and using the automated method. For automatic extraction using 200µL of plasma, the Magna Pure Compact machine was used (Roche, Mannheim, Germany). RNA was extracted manually using the QIAmp® Viral RNA Mini Kit for the purification of viral RNA from plasma (Qiagen, Valencia CA, USA). A 140-280µL sample aliquot was added to 560µL prepared AVL containing carrier RNA in a 1.5mL microfuge tube, mixed by pulse-vortexing for 15 seconds, incubated at room temperature (15-25°C) for 10 minutes and briefly centrifuged to remove drops from the inside of the lid. Ethanol (560µL;96-100%) was added and mixed by pulse vortexing for 15 seconds and briefly centrifuged to remove drops from the inside of the lid and the solution was carefully applied to the QIAmp spin columns and centrifuged at 6000xg (8000 rpm) for 1 minute. The column was placed in a clean 2mL collection tube and the tube containing filtrate was discarded. Next 500µL Buffer AW1 was added and centrifuged at 6000xg (8000 rpm) for 1 minute, the column placed in a clean collection tube and the tube containing filtrate was discarded. Next 500µL Buffer AW2 was added and centrifuged at 20 000xg (14000 rpm) for 3 minutes and the tube containing filtrate was discarded. The column was then placed in a clean 2mL collection tube and centrifuged for an additional 1 minute at full speed. The sample was then eluted after a 1 minute incubation step with 60µL AVE buffer aliquoted to 10-20µL and either used directly in the cDNA synthesis or stored at -80°C.

### **A2. PCR Purification**

PCR products were purified by the QIAquick Spin Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Briefly PB buffer (5x volume of PCR product) was mixed with PCR product and loaded on the spin column and centrifuged for a minute at 13000rpm. PE buffer (750µL) was used to wash by centrifugation at 13000rpm for one minute. Clean PCR product was eluted by centrifugation at 13000rpm using buffer EB (50µL).

### **A3. Transformation**

### Preparation of competent cells

Both commercial available competent cells (cells supplied with the kits) and E.coli DH5 $\alpha$  cells, prepared according to the dimethyl sulphoxide (DMSO) method (C. T. Chung & Miller, 1988) were used for transformation. A 5mL volume of 2YT broth was inoculated and incubated overnight at 37°C on a shaker. A dilution of 1/100 was made from the culture into 100mL 2YT broth in a litre culture flask and grown on the shaker to early log phase (OD<sub>600</sub> 0.2 to 0.4). The cells were then harvested by centrifugation at 500 rpm for 5 min at 4°C in a Beckman J2-21 centrifuge. The pellet was resuspended in ice cold TSB buffer (Appendix B) and placed on ice for 10 min. Sterile glycerol was added to a final concentration of 10% v/v and 100 $\mu$ L aliquots were stored at -80°C.

### Transformation

Frozen cells were defrosted on ice and 3 $\mu$ L ligation mix was then mixed with the cells and left on ice between 10 to 20 min. the cells were heat shocked at 42°C for 30 seconds. One mL of 2YT medium was added and the cells incubated for about 30 to 45 min at 37°C. A volume of 50 to 100 $\mu$ L was plated on selective plates containing either ampicillin or kanamycin (Appendix B). The plates were then incubated at 37°C and single colonies were either inoculated into 2mL 2YT broth for minipreparations or for colony PCR screening.

### A4. Colony Screening

Following transformation colonies were screened by colony PCR as described by (A. B. Lee & Cooper, 1995). The colonies were picked using a plastic pipette tip, replicated on another plate for future reference and inserted into a 25 to 50 $\mu$ L PCR master mix with primers T7 and Rev17. PCR reactions of 35 cycles were performed and 5 $\mu$ L of PCR product was run on a gel to confirm the presence of the insert. Positive PCR fragments were cleaned up for sequencing and cloning.

### A5. Agarose gel electrophoresis

DNA fragments were visualised in 0.8% agarose gels. Agarose gel electrophoresis was performed using horizontal gel apparatus (Stratagene, La Jolla, USA). The agarose gel was prepared by melting the appropriate weight per volume agarose (Agarose Di LE, Hispanagar, Burgos, Spain) in 1X TAE (Appendix B). Once melted, GelRed (Biotium, Hayward, CA, USA) was added to the gel and the agarose poured into gel setting trays and allowed to cool to room temperature to set. Set gels were placed in a gel apparatus submerged in 1X TAE. Before loading, 1-5 $\mu$ L PCR product mixed with 2 $\mu$ L of 6X agarose gel electrophoresis

loading dye. In order to determine the size of amplicons a DNA molecular weight marker V! (Roche, GmbH, Mannheim, Germany) was included in the first lane of all gels. The gel was electrophoresed at 100 to 120 V according to gel size for 60 minutes or until sufficient separation of bands. The DNA fragments were visualized on a UVP transilluminator (UVP, San Gabriel, CA, USA) at 256nm wavelength and photographed with Kodak ds 1D Electrophoresis Documentation and Analysis System 120 V2.0.3 computerized gel imager.

## **Appendix B: Reagents and buffers**

### **B1. 50x TAE buffer:**

121g Tris base, 28.55mL glacial acetic acid, 50mL 0.5M EDTA pH 8.0, make up to 500mL with deionized H<sub>2</sub>O

### **B2. 0.5M EDTA:**

Add 186.1g disodium ethylenediaminetetraacetate.2H<sub>2</sub>O to ~800mL deionized sterile water. Dissolve by stirring vigorously on a magnetic stirrer while adjusting the pH to 8.0 with ~20g NaOH pellets. The disodium salt of ADTA will only dissolve if the pH of the solution is adjusted to ~8.0.

### **B3. LB broth**

10g NaCl, 5g Yeast extract, 10g Tryptone, up to 1L with deionised water

### **B4. LB agar selection plates**

LB medium was made as described above. 15g agar was added before autoclaving. Agar was cooled to ~55°C. Ampicillin (Sigma, MO, USA) 100µg/mL or 15µg/mL kanamycin (Km; Nova Nordisk, Johannesburg, RSA) and agar was poured into plates and allowed to cool and harden. Plates were inverted and stored in the dark at 4°C.

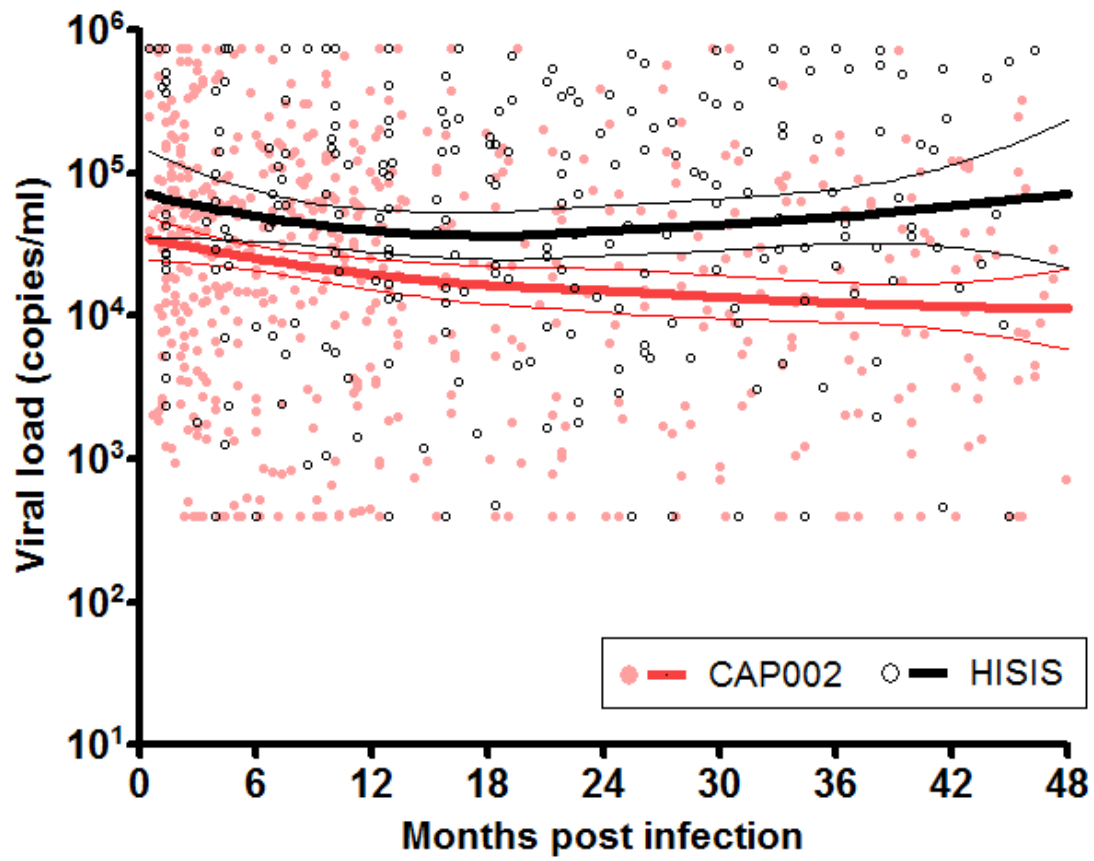
### **B5. Growth media**

Complete growth media for adherent human tissue cell lines consisted of High glucose D-MEM with L-glutamine and 25mM HEPES (Life Technologies, CA, USA) supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin and 100 µg/mL Gentamicin (Life Technologies, Carlsbad, CA, USA)

### **B6. RPMI media**

R10 growth media for suspension human tissue cell lines consisted of RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS, 10 mM HEPES (Life Technologies, Carlsbad, CA, USA), 100 µg/mL Gentamicin (Life Technologies, Carlsbad, CA, USA), and 1% Penicillin-Streptomycin and Glutamine (Life Technologies, Carlsbad, CA, USA),

## Appendix C: Viral load data



**Appendix C:** A scatterplot with a LOESS smoothing line (with 95% confidence intervals) depicting all available viral load measurements for both CAPRISA (AI) and HISIS cohorts over time.

## Appendix D: Neutralization assay data

### D1: Breadth and potency scores HISIS

24 month	Infecting	single/dual	VSV-G	subtype A				IC50 (titre) subtype B				subtype C				% viruses neutralized	Breadth score	Potency score
				Q23.17 (tier 1B)	Q168.a2 (tier 2)	Q461.e2 (tier 2)	Q842.d12 (tier 2)	AC10 (tier 2)	CAAN (tier 2)	PVO (tier 3)	QH0692 (tier 2)	DU156 (tier 2)	DU422 (tier 2)	ZM109 (tier 1B)	ZM214 (tier 2)			
605.F12	AC	Dual	45	>1000	>1000	198	>1000	45	112	187	410	918	45	320	192	83%	10	123.98
390.F11	C	Single	45	50	81	45	72	90	51	45	101	220	45	300	96	75%	9	21.16
246.F11	ACD	Single	45	45	98	45	150	160	45	45	110	200	69	70	210	67%	7	24.4
398.F10	A	Single	45	45	50	45	66	86	45	83	88	50	45	189	148	67%	7	17.2
477.F11	C/JAC	Dual	45	45	120	45	45	55	45	76	70	127	45	64	79	58%	6	18.2
596.F10	C	Single	45	140	50	45	45	45	45	45	66	96	82	45	>1000	50%	6	16.29
49.F9	ACD	Single	45	45	45	45	45	70	45	45	60	80	68	150	130	50%	6	14
21.F9	A	Single	45	45	60	45	45	45	45	45	50	53	45	135	75	42%	4	13.58
532.F8	AD	Dual	45	101	45	45	45	45	45	45	45	45	45	70	45	17%	1	13.24
515.F12	A	Single	45	45	45	45	45	45	45	45	45	63	45	60	45	17%	1	12.4
346.F12	C	Single	45	45	45	45	45	45	45	45	45	50	45	165	73	25%	1	12.11
54.F12	D	Dual	45	45	45	45	45	45	45	45	45	45	45	45	115	8%	1	12
216.F10	A	Dual	45	45	45	45	45	45	45	45	45	45	45	75	45	8%	1	12
401.F9	CD	Single	45	45	45	45	45	45	45	45	45	45	45	51	85	17%	1	12
234.F9	C	Dual	45	45	45	45	45	45	45	45	45	45	45	70	45	8%	0	12
89.F9	CD	Dual	45	45	45	45	45	45	45	45	45	45	45	45	50	8%	0	12
142.F11	C	Single	45	45	45	45	45	45	45	45	45	45	45	45	45	0%	0	12
569.F9	C	Single	45	45	45	45	45	45	45	45	45	45	45	45	45	0%	0	12
304.F10	C	Single	45	45	45	45	45	45	45	45	45	45	45	45	45	0%	0	12
98.F12	C	Single	45	45	45	45	45	45	45	45	45	45	45	45	45	0%	0	12

### D2: Breadth and potency scores for CAPRISA

24 month	Infecting	single/dual	VSV-G	subtype A				IC50 (titre) subtype B				subtype C				% viruses neutralized	Breadth score	Potency score
				Q23.17 (tier 1B)	Q168.a2 (tier 2)	Q461.e2 (tier 2)	Q842.d12 (tier 2)	AC10 (tier 2)	CAAN (tier 2)	PVO (tier 3)	QH0692 (tier 2)	DU156 (tier 2)	DU422 (tier 2)	ZM109 (tier 1B)	ZM214 (tier 2)			
CAP256	C	Dual	50	171	70	45	47	45	45	45	45	394	346	174	45	50%	6	32.71
CAP177	C	Dual	50	1024	45	45	45	695	227	45	45	269	105	64	45	50%	6	58.98
CAP274	C	Single	50	464	45	45	45	45	117	45	45	591	45	60	45	33%	4	35.37
CAP37	C	Dual	50	485	45	45	45	45	45	45	45	218	45	45	45	17%	2	25.63
CAP30	C	Single	50	134	45	45	45	45	45	45	45	45	45	90	45	17%	2	14.97
CAP268	C	Single	50	45	45	45	45	49	45	59	45	45	45	45	45	17%	2	12.39
CAP269	C	Single	50	45	45	45	45	45	45	45	45	129	45	45	45	8%	1	13.86
CAP129	C	Single	50	45	45	45	45	45	45	45	45	45	45	130	45	8%	1	13.89
CAP267	C	Dual	50	71	45	45	45	45	45	45	45	45	45	45	45	8%	1	12.57
CAP40	C	Single	50	45	45	45	45	45	45	45	45	45	45	59	45	8%	1	12.31
CAP264	C	Single	50	45	45	45	45	52	45	45	45	45	45	45	45	8%	1	12.16
CAP266	C	Single	50	45	45	45	45	51	45	45	45	45	45	45	45	8%	1	12.13
CAP88	C	Single	50	45	45	45	45	45	45	45	45	45	45	45	45	0%	0	12
CAP174	C	Single	50	45	45	45	45	45	45	45	45	45	45	45	45	0%	0	12
CAP188	C	Single	50	45	45	45	45	45	45	45	45	45	45	45	45	0%	0	12
CAP220	C	Single	50	45	45	45	45	45	45	45	45	45	45	45	45	0%	0	12
CAP222	C	Single	50	45	45	45	45	45	45	45	45	45	45	45	45	0%	0	12
CAP261	C	Single	50	45	45	45	45	45	45	45	45	45	45	45	45	0%	0	12
CAP262	C	Single	50	45	45	45	45	45	45	45	45	45	45	45	45	0%	0	12
CAP265	C	Single	50	45	45	45	45	45	45	45	45	45	45	45	45	0%	0	12
CAP270	C	Single	50	45	45	45	45	45	45	45	45	45	45	45	45	0%	0	12
CAP271	C	Single	50	45	45	45	45	45	45	45	45	45	45	45	45	0%	0	12

### Appendix D: Neutralization by plasma sampled at 2 years post infection in CAPRISA (D1) and HISIS (D2)

cohorts of a panel of 12 viruses. Values represent the dilution at which 50% neutralization was detected ( $ID_{50}$ ) in a serial dilution range starting at 1:45. The breadth of neutralization is listed on the right hand side showing percentage of panel viruses neutralized with  $ID_{50}$  values greater than 1:45 and 1:100. The infecting subtype and detection of dual infection at any time during the 2 year follow-up since infection as well as viral load at sampling are shown on the left.

## Appendix E: HIV-1 subtype C envelope, full length and sequencing primers

### Appendix E1: PCR primers

Primer	Direction	sequence
VIF1	forward	GGG TTT ATT ACA GGG ACA GCA GAG
OFM19	reverse	GCA CTC AAG GCA AGC TTT ATT GAG GCT TA
EnvA	forward	GGC TTA GGC ATC TCC TAT GGC AGG AAG AA
EnvArx	forward	
EnvN	reverse	CTG CCA ATC AGG GAA GTA GCC TTG TGT

### Appendix E2: Sequencing primers

#### Appendix E2 (A): Envelope sequencing primers

Primer	Direction	HXB2 position	Sequence
EF00	forward	6201	GGG AAA GAG CAG AAG ACA GTG GCA ATG A
For16	forward	7350-7375	TTTAATTGTGGAGGAGAATTTTTCTA
EF200	forward	8092	GGG ATA ACA TGA CCT GGA TGC AGT GGG
EF175	reverse	6401	TTT AGC ATC TGA TGC ACA GAA TAG
EF115	reverse	7374	AGA AAA ATT CTC CTC TAC AAT TAA
EF15	reverse	8445	CTT GCT CTC CAC CTT CTT CTT C
For14	forward	6556-6582	TATGGGACCAAAGCCTAAAGCCATGTG
EF170	forward	7799	AGC AGG AAG CAC TAT GGG
EF260	forward	8520	TTC AGC TAC CAC CGA TTG AGA GAC T
Rev15	reverse	7015-6990	CTGCCATTTAACAGCAGTTGAGTTGA
EF55	reverse	7940	GCC CCA GAC CGT GAG TTG CAA CAT ATG
Rev19	reverse	8820-8797	ACTTTTTGACCACTTGCCACCCAT

**Appendix E2 (B): Full length HIV-1 genome sequencing primers**

<b>primer</b>	<b>Direction</b>	<b>HXB2 position</b>	<b>Sequence</b>
5R(3)C	reverse	7502←7526	CATTGCTCGTCCTACCCCCTGCCAC
13R2C2	reverse	2712←2735	GGCAAATATTGGAGTGTTATATGG
EF15	reverse	8424←8442	CTT GCT CTC CAC CTT CTT CTT C
EF55	reverse	7914←7937	GCC CCA GAC CGT GAG TTG CAA CAT ATG
EF110	forward	7005→7025	CTG TTA AAT GGT AGC CTA GCA GAA
EF00	forward	6204→6228	GGG AAA GAG CAG AAG ACA GTG GCA ATG A
E230	forward	8276→8296	AAT ATT CAT AAT GAT AGT AGG AGG
GF80	forward	1477→1493	AGA GAA CCA AGG GGA AGT GA
G35	reverse	1817←1835	CAT GCT GTC ATC ATT TCT TCT A
P7	forward	4025→4039	AGA AGT AAA CAT AGT AAC
G00	forward	767→782	GAC TAG CGG AGG CTA GAA G
NefOR	reverse	523←540	AGGCAAGCTTTATTGAGG
SQ3R(2)C	reverse	8651←8680	GCTATGGTATCAAGCAGACTAATAGCACTC
SQ4F	forward	2076→2095	ACAGGCTAATTTTTTAGGGA
SQ5F	forward	2613→2637	AAACAATGGCCATTAACAGAAGAGA
SQ6.5FC	forward	3444→3469	GCAGAGTTAGAATTAGCAGAGAACAG
SQ8R	reverse	5963←5988	CTCCGCTTCTTCCTGCCATAGGAGAT
SQ8RC	reverse	4660←4685	TTCTACTACTCCCTGACTTTGGGGAT
SQ9FC3	forward	5074→5099	CAGGTAGACAGGATGAAGATCAGAAC
SQ9'RC	reverse	5341←5366	ATATGAATTAGTTGGTCTGCCAGGCC
SQ10FC	forward	5833→5857	GGAGCCAGTAGATCCTAACCTAGAG
SQ11RC3	reverse	3687←3713	TTTAGGAGTCTTTCCCATATTACTAT
SQ15FC	forward	8561→8580	GAGAGCGGTGGAACCTTCTGG
SQ16F	forward	56→75	CCACACACAAGGCTACTTCC

## Appendix F: Mutagenesis primers

### Appendix F1: CAP63 IMC mutagenesis primers

Primer Name	Primer Sequence
CAP63F4_5_I398T	AGTACATACATGCCTAATGGTACGCACATACCAAATGGTGCAAGC
CAP63F4_5_I398T_antisense	GCTTGCACCATTTGGTATGTGCGTACCATTAGGCATGTATGTACT
CAP63F4_5_A404T	CCTAATGGTATACACATACCAAATGGTACGAGCGAAGTCATCAC
CAP63F4_5_A404T_antisense	GTGATGACTTCGCTCGTACCATTTGGTATGTGTATACCATTAGG
CAP63T6Wdel390-395	GAATTTTTCTATTGCAATACATCAGGACTGTTAATGGTATACACATACCAA
CAP63T6Wdel390-395-antisense	TTTGGTATGTGTATACCATTAAACAGTCCTGATGTATTGCAATAGAAAAATTC
CAP63T13Wdel397-403	GTTTAAACAGTACATACATGCCTAATGCAAGCGAAGTCATCACA
CAP63T13Wdel397-403-antisense	TGTGATGACTTCGCTTGCATTAGGCATGTATGTACTGTAAAC
CAP63T14Wdel396-404	GACTGTTTAAACAGTACATACATGCCTAGCGAAGTCATCAC
CAP63T14Wdel396-404-antisense	GTGATGACTTCGCTAGGCATGTATGTACTGTAAACAGTC
CAP63T19del389-396	CTATTGCAATACATCAGGACTGGGTATACACATACCAAATGGTG
CAP63T19del389-396-antisense	CACCATTTGGTATGTGTATACCCAGTCCTGATGTATTGCAATAG
CAP63T34del399-403	TTAACAGTACATACATGCCTAATGGTATAGCAAGCGAAGTCATC
CAP63T34del399-403-antisense	GATGACTTCGCTTGCTATACCATTAGGCATGTATGTACTGTAA

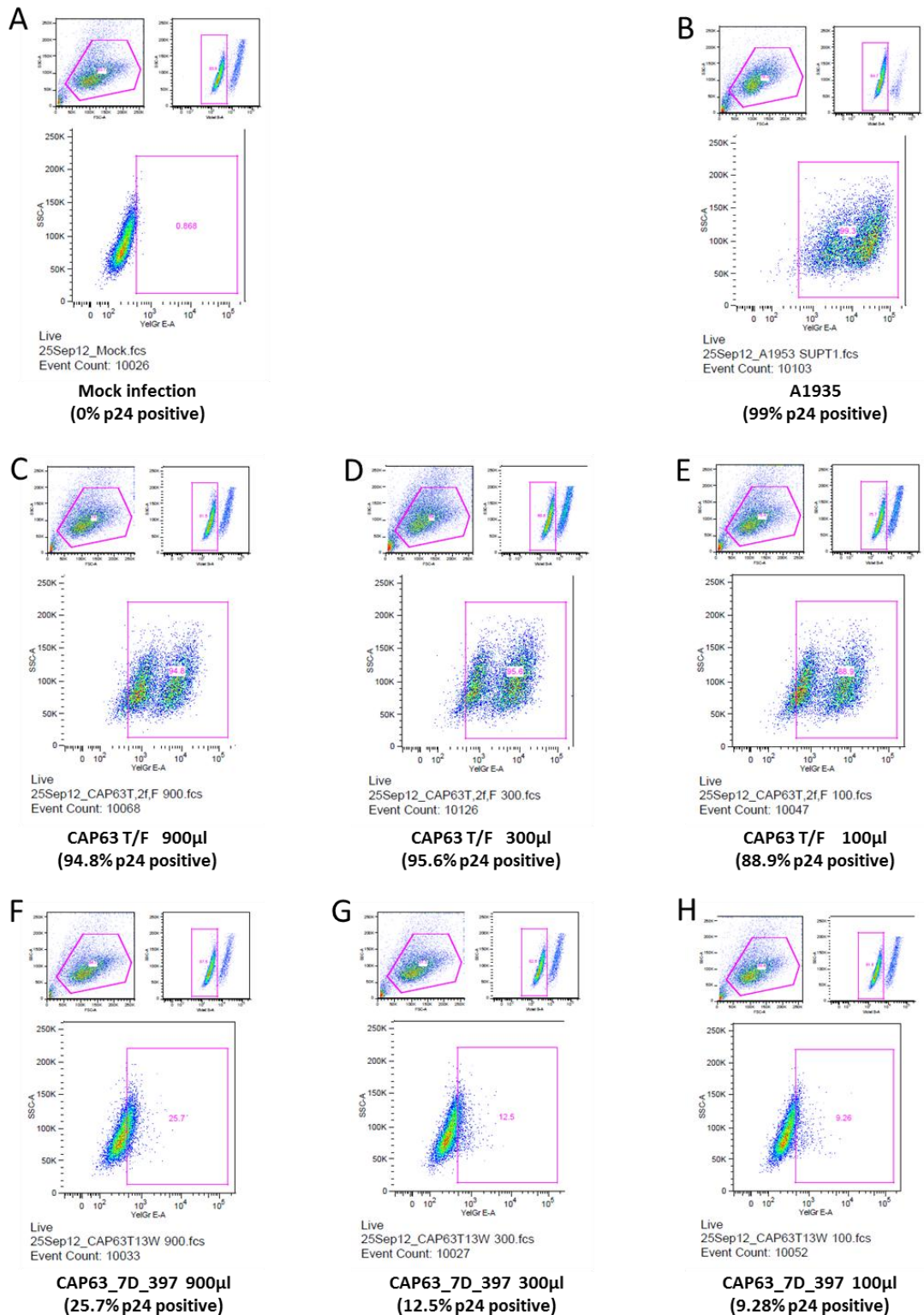
### Appendix F2: CAP63 pseudovirus mutagenesis primers

Primer Name	Primer Sequence
CAP63_G728E	CGAAGAAGAAGGTGGAGAGCAAGACAACAGCAGAT
CAP63_G728E_antisense	ATCTGCTGTTGTCTTGTCTCCACCTTCTTCTTCG
CAP63_I817T	GCTATTAGTTTGCTTGATAGCATAGCAATAACGGTAGCTGAGGGAACAGA
CAP63_I817T_antisense	TCTGTTCCCTCAGCTACCGTTATTGCTATGCTATCAAGCAAATAATAGC
CAP63_N733S	GGTGGAGAGCAAGACAACAGCAGATCCATAAGATTAGTG
CAP63_N733S_antisense	CACTAATCTTATGGATCTGCTGTTGTCTTGTCTCCACC
CAP63_T14W_K722E	ACAGGCTCGGAGGAATCGAAGAAGAGGGTGGAGAGCAAG
CAP63_T14W_K722E_antisense	CTTGCTCTCCACCCTTCTTTCGATTCTCCGAGCCTGT
CAP63_T14W_N729S	GGTGGAGAGCAAGACAACAGCAGATCCATAAGATTAGTG
CAP63_T14W_N729S_antisense	CACTAATCTTATGGATCTGCTGTTGTCTTGTCTCCACC
CAP63_T14W_A815V	CTTGATAGCATAGCAATAACAGTAGCTGAGGGAACAGAT
CAP63_T14W_A815V_antisense	ATCTGTTCCCTCAGCTACTGTTATTGCTATGCTATCAAG
CAP63_G728E	CGAAGAAGAAGGTGGAGAGCAAGACAACAGCAGAT
CAP63_G728E_antisense	ATCTGCTGTTGTCTTGTCTCCACCTTCTTCTTCG
CAP63_T34_N733S	GGTGGAGAGCAAGACAACAGCAGATCCATAAGATTAGTG
CAP63_T34_N733S_antisense	CACTAATCTTATGGATCTGCTGTTGTCTTGTCTCCACC
CAP63_T13W_R734I	CAAGACAACAGCAGATCCATAAGATTAGTGAGCGGATTC
CAP63_T13W_R734I_antisense	GAATCCGCTCACTAATCTTATGGATCTGCTGTTGTCTTG

## Appendix G: Titration of IMC virus stocks for infection of CEM NKR<sub>CCR5</sub> cells

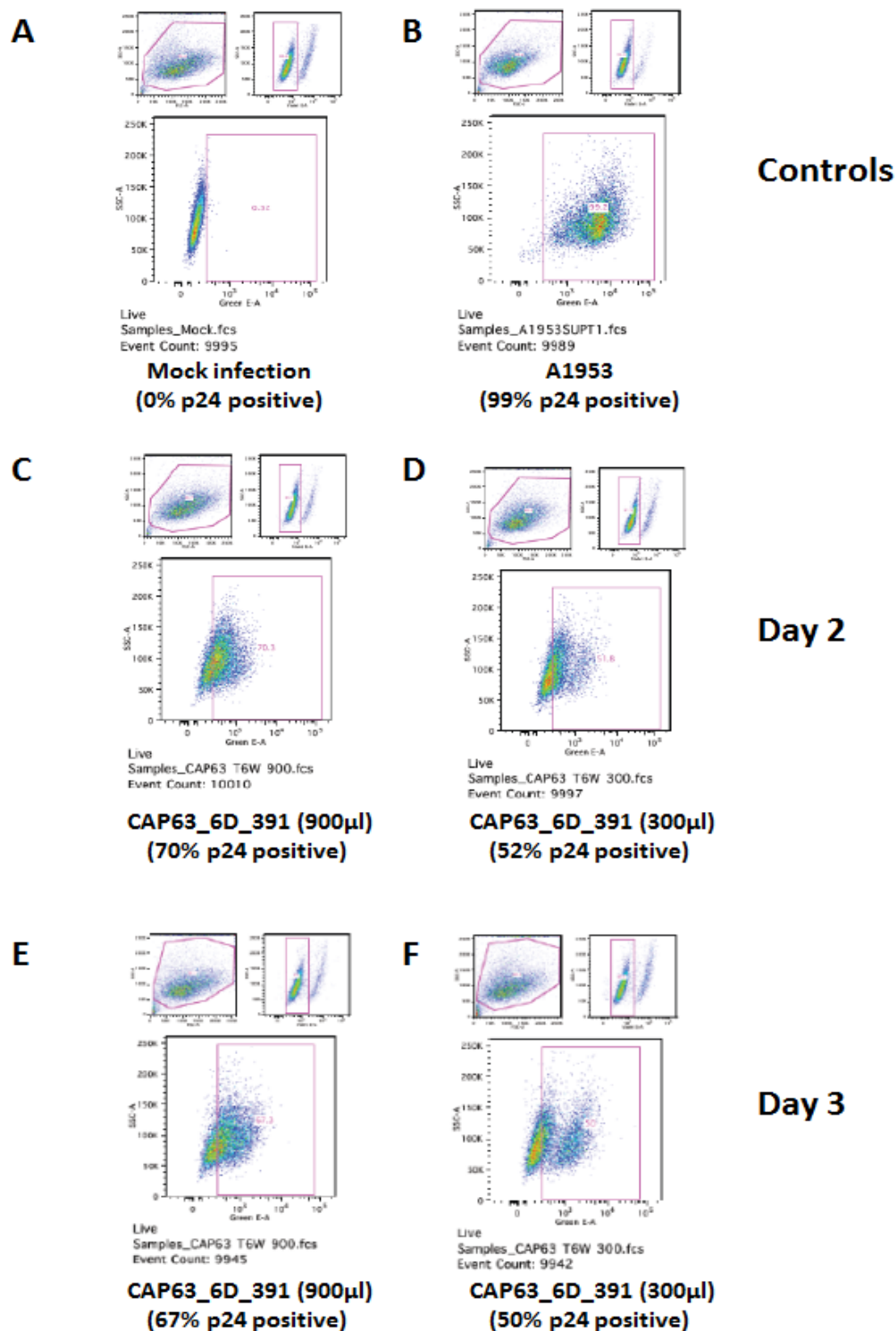
**Appendix G1:** Table showing results of titration of IMC virus inoculum in CEM NKR<sub>CCR5</sub> target cells for determination of optimal inoculum. IMC virus stocks were produced in HEK293T cells and different volumes of the virus stock (900, 300 and 100 $\mu$ L) used to inoculate  $2 \times 10^6$  target cells. Intracellular p24 staining was carried out after 72 hours of infection. Values in the table are %p24 positive cells. Unusable infections (<20% p24 positive) are highlighted in grey.

Clone	Inoculum (microliters)		
	900	300	100
CAP63_IMC_T-F	94.8	95.6	88.9
CAP63_IMC_Gly_404	85.6	85.7	73.1
CAP63_IMC_6D_391	70.3	51.8	20.7
CAP63_IMC_7D_397	25.7	12.5	9.26
CAP63_IMC_9D_396	76.91	48.4	23
CAP63_IMC_5D_399	86.9	86.3	76.7



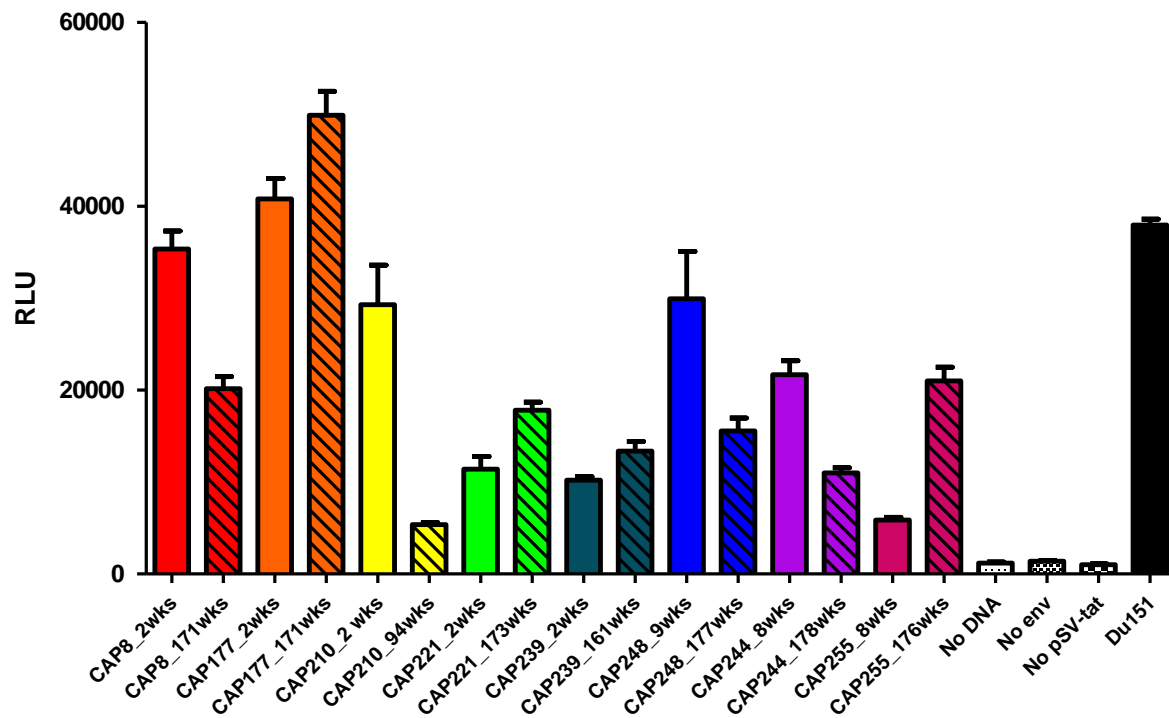
**Appendix G2:** Titration for optimal infection of target cells with IMCs. Intracellular P24 staining of CEM.NKRCCR5 cells 72 hours after infection with 900µL, 300µL and 100µL of HEK293T produced IMC virus. Panel A is a mock infection, panel B is the A1953-CEM-NKR<sub>CCR5</sub> chronically infected cells (A1953) positive control. Panels C, D and E are the CAP63 T/F titrations and panels F, G and H are the CAP63\_7D\_397 titrations. The volume with the best infectivity was chosen. Infections had to exceed 20% to be used for the ADCC assay.

## Appendix H: Determination of the optimal length of infection for target cells with IMCs



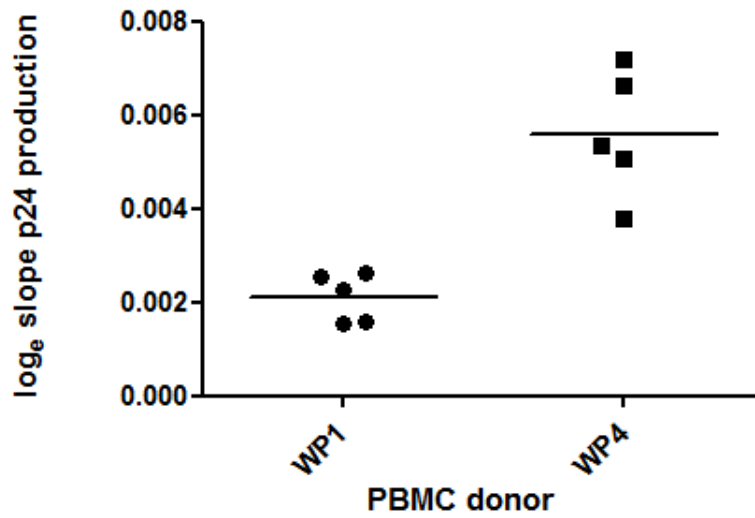
**Appendix H:** Determination of the optimal length of infection for target cells with IMCs. Panel A is a mock infection, panel B is the A1953-CEM-NKR<sub>CCR5</sub> chronically infected cell (A1953) positive control. Panels C through F show the results of intracellular p24 staining of CEM NKR<sub>CCR5</sub> cells after infection for 2 days and 3 days using 900μL (C and E respectively) and 300μL (D and F) of inoculum. The highest levels of intracellular p24 are reached at 2 days post infection.

## Appendix I: Cell-cell fusion capacity of CAPRISA envelopes from acute and chronic infection

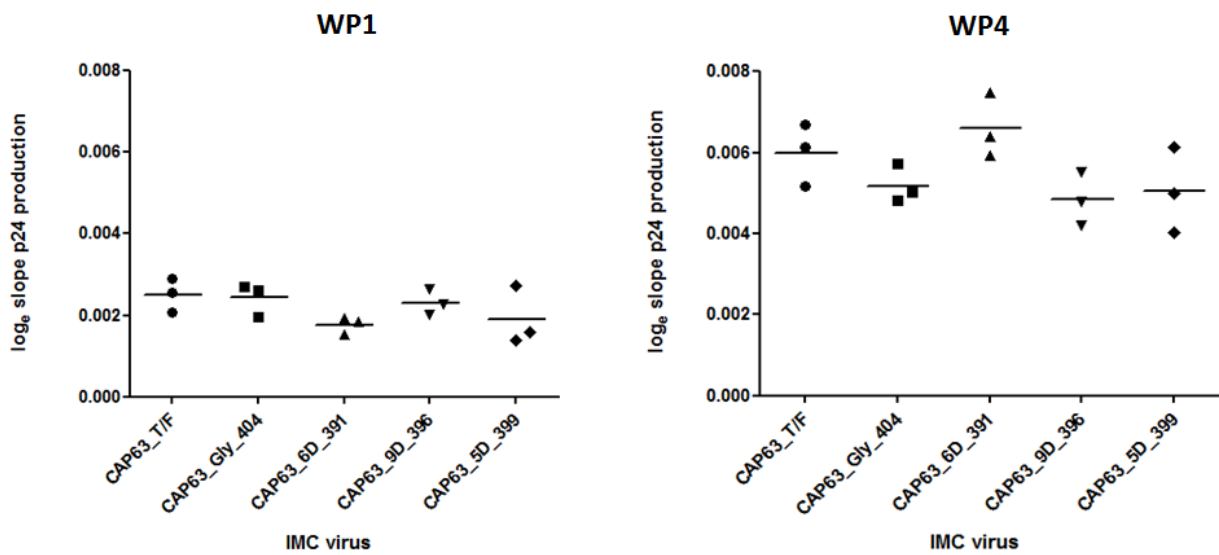


**Appendix I:** Cell-cell fusion capacities of pseudoviruses from acute/early (solid bars) and late/chronic infection (striped bars) in 8 CAPRISA participants. Control reactions with no DNA, no env and no tat and the positive Du151 control are in black.

## Appendix J: Replication assay slope calculations



**Appendix J1:** The mean slope of the growth curves from triplicate replication assays of five viruses in PBMC from two donors (WP1 and WP2) calculated using the LOGEST function in excel. The mean slope of the growth curves was higher in PBMC from WP4 ( $p=0.0079$ ).



**Appendix J2:** The slopes each of the growth curves from triplicate replication assays of five viruses in PBMC from two donors (WP1 and WP2) calculated using the LOGEST function in excel. The mean slope of the growth curves did not differ between viruses.

## REFERENCES

- Aasa-chapman, M. M. I., Holuigue, S., Aubin, K., Wong, M., Jones, N. A., Cornforth, D., ... McKnight, A. (2005). Detection of Antibody-Dependent Complement-Mediated Inactivation of both Autologous and Heterologous Virus in Primary Human Immunodeficiency Virus Type 1 Infection. *Journal of Virology*, 79(5), 2823–2830. doi:10.1128/JVI.79.5.2823
- Abrahams, M.-R., Anderson, J. a, Giorgi, E. E., Seoighe, C., Mlisana, K., Ping, L.-H., ... Williamson, C. (2009). Quantitating the multiplicity of infection with human immunodeficiency virus type 1 subtype C reveals a non-poisson distribution of transmitted variants. *Journal of Virology*, 83(8), 3556–67. doi:10.1128/JVI.02132-08
- Abrahams, M.-R., Treurnicht, F. K., Ngandu, N. K., Goodier, S. A., Marais, J. C., Bredell, H., ... Williamson, C. (2013). Rapid, complex adaptation of transmitted HIV-1 full-length genomes in subtype C-infected individuals with differing disease progression. *AIDS (London, England)*, 27(4), 507–18. doi:10.1097/QAD.0b013e32835cab64
- Abu-raddad, L. J., Barnabas, R. V, Janes, H., Weiss, H. A., Kublin, J. G., Longini, I. M., & Judith, N. (2012). Have the explosive HIV epidemics in sub-Saharan Africa been driven by higher community viral load ?, (November), 1–10. doi:10.1097/QAD.0b013e32835cb927
- Abu-Raddad, L. J., Patnaik, P., & Kublin, J. G. (2006). Dual infection with HIV and malaria fuels the spread of both diseases in sub-Saharan Africa. *Science (New York, N.Y.)*, 314(5805), 1603–6. doi:10.1126/science.1132338
- Ackerman, M. E., Moldt, B., Wyatt, R. T., Dugast, A.-S., McAndrew, E., Tsoukas, S., ... Alter, G. (2011). A robust, high-throughput assay to determine the phagocytic activity of clinical antibody samples. *Journal of Immunological Methods*, 366(1–2), 8–19. doi:http://dx.doi.org/10.1016/j.jim.2010.12.016
- Affranchino, J. L., & González, S. a. (2006). Mutations at the C-terminus of the simian immunodeficiency virus envelope glycoprotein affect gp120-gp41 stability on virions. *Virology*, 347(1), 217–25. doi:10.1016/j.virol.2005.11.032
- Ahmad, A., & Menezes, J. (1996). Antibody-dependent cellular cytotoxicity in HIV infections. *FASEB*, 10, 258–266.
- Alcantara, L. C. J., Cassol, S., Libin, P., Deforche, K., Pybus, O. G., Van Ranst, M., ... de Oliveira, T. (2009). A standardized framework for accurate, high-throughput genotyping of recombinant and non-recombinant viral sequences. *Nucleic Acids Research*, 37(Web Server issue), W634–42. doi:10.1093/nar/gkp455
- Alkhatib, G., Combadiere, C., Broder, C. C., Feng, Y., Kennedy, P. E., Murphy, P. M., & Berger, E. A. (1996). CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science (New York, N.Y.)*, 272(5270), 1955–8.

- Allan, J. S., Coligan, J. E., Barin, F., McLane, M. F., Sodroski, J. G., Rosen, C. A., ... Essex, M. (1985). Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III. *Science*, 228 (4703), 1091–1094. doi:10.1126/science.2986290
- Allen, T. M., Altfeld, M., Geer, S. C., Kalife, E. T., Moore, C., Sullivan, K. M. O., ... Walker, B. D. (2005). Selective Escape from CD8  $\gamma$  T-Cell Responses Represents a Major Driving Force of Human Immunodeficiency Virus Type 1 ( HIV-1 ) Sequence Diversity and Reveals Constraints on HIV-1 Evolution †, 79(21), 13239–13249. doi:10.1128/JVI.79.21.13239
- Allen, T. M., Altfeld, M., Yu, X. G., O’Sullivan, K. M., Lichterfeld, M., Le Gall, S., ... Walker, B. D. (2004). Selection, transmission, and reversion of an antigen-processing cytotoxic T-lymphocyte escape mutation in human immunodeficiency virus type 1 infection. *Journal of Virology*, 78(13), 7069–78. doi:10.1128/JVI.78.13.7069-7078.2004
- Alpert, M. D., Harvey, J. D., Lauer, W. A., Reeves, R. K., Piatak, M., Carville, A., ... Evans, D. T. (2012). ADCC develops over time during persistent infection with live-attenuated SIV and is associated with complete protection against SIV(mac)251 challenge. *PLoS Pathogens*, 8(8), e1002890. doi:10.1371/journal.ppat.1002890
- Alpert, M. D., Heyer, L. N., Williams, D. E. J., Harvey, J. D., Greenough, T., Allhorn, M., & Evans, D. T. (2012). A novel assay for ADCC against HIV-1 or SIV-infected cells reveals incomplete overlap with antibodies measured by neutralization and binding assays. *Journal of Virology*, (August). doi:10.1128/JVI.01650-12
- Alsmadi, O., Herz, R., Murphy, E., Pinter, A., Tilley, S. A., Alsmadi, O., ... Pinter, A. (1997). A novel antibody-dependent cellular cytotoxicity epitope in gp120 is identified by two monoclonal antibodies isolated from a long-term survivor of human immunodeficiency virus type 1 infection . A Novel Antibody-Dependent Cellular Cytotoxicity Epitope in. *Journal of Virology*, 71(2), 925–933.
- Alsmadi, O., & Tilley, S. A. (1998). Antibody-Dependent Cellular Cytotoxicity Directed against Cells Expressing Human Immunodeficiency Virus Type 1 Envelope of Primary or Laboratory-Adapted Strains by Human and Chimpanzee Monoclonal Antibodies of Different Epitope Specificities. *Journal of Virology*, 72 (1), 286–293.
- Alter, G., & Altfeld, M. (2009). NK cells in HIV-1 infection: Evidence for their role in the control of HIV-1 infection. *Journal of Internal Medicine*, 265(1), 29–42. doi:10.1111/j.1365-2796.2008.02045.x.NK
- Alter, G., & Moody, M. A. (2010). The Humoral Response to HIV-1: New Insights, Renewed Focus. *Journal of Infectious Diseases*, 202 (Supplement 2), S315–S322. doi:10.1086/655654
- Alter, G., Teigen, N., Ahern, R., Streeck, H., Meier, A., Rosenberg, E. S., & Altfeld, M. (2007). Evolution of innate and adaptive effector cell functions during acute HIV-1 infection. *The Journal of Infectious Diseases*, 195(10), 1452–60. doi:10.1086/513878

- Altfeld, M., Fadda, L., Frieta, D., & Bhardwaj, N. (2012). DCs and NK cells: critical effectors in the immune response to HIV-1. *Nature Reviews: Immunology*, *11*(3), 176–186. doi:10.1038/nri2935.DCs
- Altfeld, M., Kalife, E. T., Qi, Y., Streeck, H., Lichterfeld, M., Johnston, M. N., ... Walker, B. D. (2006). HLA Alleles Associated with Delayed Progression to AIDS Contribute Strongly to the Initial CD8(+) T Cell Response against HIV-1. *PLoS Medicine*, *3*(10), e403. doi:10.1371/journal.pmed.0030403
- Altfeld, M., Rosenberg, E. S., Shankarappa, R., Mukherjee, J. S., Hecht, F. M., Eldridge, R. L., ... Walker, B. D. (2001). Cellular immune responses and viral diversity in individuals treated during acute and early HIV-1 infection. *The Journal of Experimental Medicine*, *193*(2), 169–80.
- Anisimova, M., Nielsen, R., & Yang, Z. (2003). Effect of recombination on the accuracy of the likelihood method for detecting positive selection at amino acid sites. *Genetics*, *164*(3), 1229–36.
- Ariën, K. K., Jaspers, V., & Vanham, G. (2011). HIV sexual transmission and microbicides. *Reviews in Medical Virology*, *21*(2), 110–133. doi:10.1002/rmv.684
- Ariën, K. K., Troyer, R. M., Gali, Y., Colebunders, R. L., Arts, E. J., & Vanham, G. (2005). Replicative fitness of historical and recent HIV-1 isolates suggests HIV-1 attenuation over time. *AIDS (London, England)*, *19*(15), 1555–64.
- Arthos, J., Cicala, C., Martinelli, E., Macleod, K., Van Ryk, D., Wei, D., ... Fauci, A. S. (2008). HIV-1 envelope protein binds to and signals through integrin alpha4beta7, the gut mucosal homing receptor for peripheral T cells. *Nature Immunology*, *9*(3), 301–9. doi:10.1038/ni1566
- Asmal, M., Hellmann, I., Liu, W., Keele, B. F., Perelson, A. S., Bhattacharya, T., ... Letvin, N. L. (2011). A signature in HIV-1 envelope leader peptide associated with transition from acute to chronic infection impacts envelope processing and infectivity. *PLoS One*, *6*(8), e23673. doi:10.1371/journal.pone.0023673
- Asmal, M., Sun, Y., Lane, S., Yeh, W., Schmidt, S. D., Mascola, J. R., & Letvin, N. L. (2011). Antibody-dependent cell-mediated viral inhibition emerges after simian immunodeficiency virus SIVmac251 infection of rhesus monkeys coincident with gp140-binding antibodies and is effective against neutralization-resistant viruses. *Journal of Virology*, *85*(11), 5465–75. doi:10.1128/JVI.00313-11
- Baba, T. W., Liska, V., Hofmann-Lehmann, R., Vlasak, J., Xu, W., Ayehunie, S., ... Ruprecht, R. M. (2000). Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. *Nature Medicine*, *6*(2), 200–6. doi:10.1038/72309
- Banerjee, K., Klasse, P. J., Sanders, R. W., Pereyra, F., Michael, E., Lu, M., ... Moore, J. P. (2010). IgG subclass profiles in infected HIV type 1 controllers and chronic progressors and in uninfected recipients of Env vaccines. *AIDS Research and Human Retroviruses*, *26*(4), 445–58. doi:10.1089/aid.2009.0223

- Bar, K. J., Tsao, C., Iyer, S. S., Decker, J. M., Yang, Y., Bonsignori, M., ... Shaw, G. M. (2012). Early low-titer neutralizing antibodies impede HIV-1 replication and select for virus escape. *PLoS Pathogens*, 8(5), e1002721. doi:10.1371/journal.ppat.1002721
- Barbour, J. D., Hecht, F. M., Wrin, T., Segal, M. R., Ramstead, C. A., Liegler, T. J., ... Grant, R. M. (2004). Higher CD4+ T Cell Counts Associated with Low Viral pol Replication Capacity among Treatment-Naive Adults in Early HIV-1 Infection. *Journal of Infectious Diseases*, 190 (2), 251–256. doi:10.1086/422036
- Barouch, D. H., & Korber, B. (2010). HIV-1 vaccine development after STEP. *Annual Review of Medicine*, 61, 153–67. doi:10.1146/annurev.med.042508.093728
- Barouch, D. H., Liu, J., Li, H., Maxfield, L. F., Abbink, P., Diana, M., ... Michael, N. L. (2012). NIH Public Access, 482(7383), 89–93. doi:10.1038/nature10766.Vaccine
- Bartesaghi, A., Merk, A., Borgnia, M. J., Milne, J. L. S., & Subramaniam, S. (2013). Prefusion structure of trimeric HIV-1 envelope glycoprotein determined by cryo-electron microscopy. *Nature Structural & Molecular Biology, advance on*. doi:10.1038/nsmb.2711
- Battle-Miller, K., Eby, C. A., Landay, A. L., Cohen, M. H., Sha, B. E., & Baum, L. L. (2002). Antibody-Dependent Cell-Mediated Cytotoxicity in Cervical Lavage Fluids of Human Immunodeficiency Virus Type 1-Infected Women. *Journal of Infectious Diseases*, 185 (4), 439–447. doi:10.1086/338828
- Baum, L. L., Cassutt, K. J., Knigge, K., Khattri, R., Margolick, J., Rinaldo, C., ... Phair, J. (1996). HIV-1 gp120-specific antibody-dependent cell-mediated cytotoxicity correlates with rate of disease progression. *The Journal of Immunology*, 157 (5), 2168–2173.
- Beirnaert, E., Nyambi, P., Willems, B., Heyndrickx, L., Colebunders, R., Janssens, W., & van der Groen, G. (2000). Identification and characterization of sera from HIV-infected individuals with broad cross-neutralizing activity against group M (env clade A-H) and group O primary HIV-1 isolates. *Journal of Medical Virology*, 62(1), 14–24.
- Benjelloun, F., Lawrence, P., Verrier, B., Genin, C., & Paul, S. (2012). Role of human immunodeficiency virus type 1 envelope structure in the induction of broadly neutralizing antibodies. *Journal of Virology*, 86(24), 13152–63. doi:10.1128/JVI.01110-12
- Berger, C. T., & Alter, G. (2011). Natural killer cells in spontaneous control of HIV infection. *Current Opinion in HIV and AIDS*, 6(3), 208–13. doi:10.1097/COH.0b013e3283457798
- Berger, E. A., Murphy, P. M., & Farber, J. M. (1999). Chemokine Receptors as HIV-1 coreceptors: Roles in Viral Entry, Tropism, and Disease. *Annual Review of Immunology*, 17(1), 657–700. doi:10.1146/annurev.immunol.17.1.657
- Berlioz-Torrent, C., Shacklett, B. L., Erdtmann, L., Delamarre, L., Bouchaert, I., Sonigo, P., ... Benarous, R. (1999). Interactions of the cytoplasmic domains of human and simian retroviral transmembrane proteins with components of the clathrin adaptor complexes

- modulate intracellular and cell surface expression of envelope glycoproteins. *Journal of Virology*, 73(2), 1350–61.
- Bernstein, H. B., Tucker, S. P., Hunter, E., Schutzbach, J. S., & Compans, R. W. (1994). Human immunodeficiency virus type 1 envelope glycoprotein is modified by O-linked oligosaccharides. *Journal of Virology*, 68 (1), 463–468.
- Binley, J. M., Ban, Y.-E. A., Crooks, E. T., Eggink, D., Osawa, K., Schief, W. R., & Sanders, R. W. (2010). Role of complex carbohydrates in human immunodeficiency virus type 1 infection and resistance to antibody neutralization. *Journal of Virology*, 84(11), 5637–55. doi:10.1128/JVI.00105-10
- Binley, J. M., Wrin, T., Korber, B., Zwick, M. B., Wang, M., Chappey, C., ... Burton, D. R. (2004). Comprehensive cross-clade neutralization analysis of a panel of anti-human immunodeficiency virus type 1 monoclonal antibodies. *Journal of Virology*, 78(23), 13232–52. doi:10.1128/JVI.78.23.13232-13252.2004
- Blish, C. a, Nguyen, M.-A., & Overbaugh, J. (2008). Enhancing exposure of HIV-1 neutralization epitopes through mutations in gp41. *PLoS Medicine*, 5(1), e9. doi:10.1371/journal.pmed.0050009
- Bobkov, A. F., Kazennova, E. V, Sukhanova, A. L., Bobkova, M. R., Pokrovsky, V. V, Zeman, V. V, ... Erasilo,va, I. B. (2004). An HIV type 1 subtype A outbreak among injecting drug users in Kazakhstan. *AIDS Research and Human Retroviruses*, 20(10), 1134–6. doi:10.1089/aid.2004.20.1134
- Boily, M.-C., Baggaley, R. F., Wang, L., Masse, B., White, R. G., Hayes, R. J., & Alary, M. (2009). Heterosexual risk of HIV-1 infection per sexual act: systematic review and meta-analysis of observational studies. *The Lancet Infectious Diseases*, 9(2), 118–129. doi:http://dx.doi.org/10.1016/S1473-3099(09)70021-0
- Bonsignori, M., Pollara, J., Moody, M. A., Alpert, M. D., Chen, X., Hwang, K.-K., ... Haynes, B. F. (2012). Antibody-dependent cellular cytotoxicity-mediating antibodies from an HIV-1 vaccine efficacy trial target multiple epitopes and preferentially use the VH1 gene family. *Journal of Virology*, 86(21), 11521–32. doi:10.1128/JVI.01023-12
- Bordería, A. V, Lorenzo-Redondo, R., Pernas, M., Casado, C., Alvaro, T., Domingo, E., & Lopez-Galindez, C. (2010). Initial fitness recovery of HIV-1 is associated with quasispecies heterogeneity and can occur without modifications in the consensus sequence. *PloS One*, 5(4), e10319. doi:10.1371/journal.pone.0010319
- Borrow, P., Lewicki, H., Wei, X., Horwitz, M. S., Pfeffer, N., Meyers, H., ... Shaw, G. M. (1997). Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nature Medicine*, 3(2), 205–11.
- Bosch, M. L., Earl, P. L., Fargnoli, K., Picciafuoco, S., Giombini, F., Wong-Staal, F., & Franchini, G. (1989). Identification of the fusion peptide of primate immunodeficiency viruses. *Science*, 244 (4905), 694–697. doi:10.1126/science.2541505

- Brockman, M. A., Brumme, Z. L., Brumme, C. J., Miura, T., Sela, J., Rosato, P. C., ... Allen, T. M. (2010). Early selection in Gag by protective HLA alleles contributes to reduced HIV-1 replication capacity that may be largely compensated for in chronic infection. *Journal of Virology*, *84*(22), 11937–49. doi:10.1128/JVI.01086-10
- Brown, B. K., Wieczorek, L., Kijak, G., Lombardi, K., Currier, J., Wesberry, M., ... Polonis, V. R. (2012). The Role of Natural Killer (NK) Cells and NK Cell Receptor Polymorphisms in the Assessment of HIV-1 Neutralization. *PloS One*, *7*(4). doi:10.1371/journal.pone.0029454
- Brown, B. K., Wieczorek, L., Sanders-Buell, E., Rosa Borges, A., Robb, M. L., Birx, D. L., ... Polonis, V. R. (2008). Cross-clade neutralization patterns among HIV-1 strains from the six major clades of the pandemic evaluated and compared in two different models. *Virology*, *375*(2), 529–38. doi:10.1016/j.virol.2008.02.022
- Brumme, Z. L., Brumme, C. J., Heckerman, D., Korber, B. T., Daniels, M., Carlson, J., ... Harrigan, P. R. (2007). Evidence of differential HLA class I-mediated viral evolution in functional and accessory/regulatory genes of HIV-1. *PLoS Pathogens*, *3*(7), e94. doi:10.1371/journal.ppat.0030094
- Brumme, Z. L., John, M., Carlson, J. M., Brumme, C. J., Chan, D., Brockman, M. A., ... Mallal, S. (2009). HLA-associated immune escape pathways in HIV-1 subtype B Gag, Pol and Nef proteins. *PloS One*, *4*(8), e6687. doi:10.1371/journal.pone.0006687
- Bültmann, A., Muranyi, W., Seed, B., & Haas, J. (2001). Identification of two sequences in the cytoplasmic tail of the human immunodeficiency virus type 1 envelope glycoprotein that inhibit cell surface expression. *Journal of Virology*, *75*(11), 5263–76. doi:10.1128/JVI.75.11.5263-5276.2001
- Bunnik, E. M., Euler, Z., Welkers, M. R. A., Boeser-Nunnink, B. D. M., Grijzen, M. L., Prins, J. M., & Schuitemaker, H. (2010). Adaptation of HIV-1 envelope gp120 to humoral immunity at a population level. *Nature Medicine*, *16*(9), 995–7. doi:10.1038/nm.2203
- Bunnik, E. M., Lobbrecht, M. S. D., van Nuenen, A. C., & Schuitemaker, H. (2010). Escape from autologous humoral immunity of HIV-1 is not associated with a decrease in replicative capacity. *Virology*, *397*(1), 224–230.
- Buonaguro, L., Petrizzo, A., Tagliamonte, M., Vitone, F., Re, M. C., Pilotti, E., ... Buonaguro, F. M. (2008). Molecular and phylogenetic analysis of HIV-1 variants circulating in Italy. *Infectious Agents and Cancer*, *3*(1), 13. doi:10.1186/1750-9378-3-13
- Bures, R., Morris, L., Williamson, C., Ramjee, G., Deers, M., Fiscus, S. A., ... Montefiori, D. C. (2002). Regional Clustering of Shared Neutralization Determinants on Primary Isolates of Clade C Human Immunodeficiency Virus Type 1 from South Africa, *76*(5), 2233–2244. doi:10.1128/JVI.76.5.2233
- Burton, D. R., Ahmed, R., Barouch, D. H., Butera, S. T., Crotty, S., Godzik, A., ... Wyatt, R. (2012). A Blueprint for HIV Vaccine Discovery. *Cell Host & Microbe*, *12*(4), 396–407. doi:10.1016/j.chom.2012.09.008

- Burton, D. R., Pyati, J., Koduri, R., Sharp, S. J., Thornton, G. B., Parren, P. W., ... Nara, P. L. (1994). Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. *Science (New York, N.Y.)*, 266(5187), 1024–7.
- Byland, R., Vance, P. J., Hoxie, J. A., & Marsh, M. (2007). A conserved dileucine motif mediates clathrin and AP-2-dependent endocytosis of the HIV-1 envelope protein. *Molecular Biology of the Cell*, 18(2), 414–25. doi:10.1091/mbc.E06-06-0535
- Cann, A. J., Churcher, M. J., Boyd, M., O'Brien, W., Zhao, J. Q., Zack, J., & Chen, I. S. (1992). The region of the envelope gene of human immunodeficiency virus type 1 responsible for determination of cell tropism. *Journal of Virology*, 66(1), 305–309.
- Carrington, M., Dean, M., Martin, M. P., & O'Brien, S. J. (1999). Genetics of HIV-1 infection: chemokine receptor CCR5 polymorphism and its consequences. *Human Molecular Genetics*, 8(10), 1939–45.
- Carrington, M., & O'Brien, S. J. (2003). The influence of HLA genotype on AIDS. *Annual Review of Medicine*, 54, 535–51. doi:10.1146/annurev.med.54.101601.152346
- Castro, E., Bélair, M., Rizzardi, G. P., Bart, P. A., Pantaleo, G., & Graziosi, C. (2008). Independent evolution of hypervariable regions of HIV-1 gp120: V4 as a swarm of N-Linked glycosylation variants. *AIDS Research and Human Retroviruses*, 24(1), 106–13. doi:10.1089/aid.2007.0139
- Cavrois, M., Neidleman, J., Santiago, M. L., Derdeyn, C. a., Hunter, E., & Greene, W. C. (2013). Enhanced Fusion and Virion Incorporation for HIV-1 Subtype C Envelope Glycoproteins with Compact V1/V2 Domains. *Journal of Virology*, (December). doi:10.1128/JVI.02308-13
- Chan, D. C., Fass, D., Berger, J. M., & Kim, P. S. (1997, April 18). Core Structure of gp41 from the HIV Envelope Glycoprotein. *Cell*. Cell Press.
- Chanh, T. C., Dreesman, G. R., Kanda, P., Linette, G. P., Sparrow, J. T., Ho, D. D., & Kennedy, R. C. (1986). Induction of anti-HIV neutralizing antibodies by synthetic peptides. *The EMBO Journal*, 5(11), 3065–71.
- Checkley, M. A., Luttge, B. G., & Freed, E. O. (2012). NIH Public Access, 410(4), 582–608. doi:10.1016/j.jmb.2011.04.042.HIV-1
- Chen, B., Vogan, E. M., Gong, H., Skehel, J. J., Wiley, D. C., & Harrison, S. C. (2005). Structure of an unliganded simian immunodeficiency virus gp120 core. *Nature*, 433(7028), 834–41. doi:10.1038/nature03327
- Chen, L., Kwon, Y. Do, Zhou, T., Wu, X., O'Dell, S., Cavacini, L., ... Kwong, P. D. (2009). Structural basis of immune evasion at the site of CD4 attachment on HIV-1 gp120. *Science (New York, N.Y.)*, 326(5956), 1123–7. doi:10.1126/science.1175868
- Chen, S. S., Lee, S. F., & Wang, C. T. (2001). Cellular membrane-binding ability of the C-terminal cytoplasmic domain of human immunodeficiency virus type 1 envelope

- transmembrane protein gp41. *Journal of Virology*, 75(20), 9925–38.  
doi:10.1128/JVI.75.20.9925-9938.2001
- Cheng, D.-C., Zhong, G.-C., Su, J.-X., Liu, Y.-H., Li, Y., Wang, J.-Y., ... Zhang, F.-M. (2010). A sensitive HIV-1 envelope induced fusion assay identifies fusion enhancement of thrombin. *Biochemical and Biophysical Research Communications*, 391(4), 1780–4.  
doi:10.1016/j.bbrc.2009.12.155
- Chernomordik, L., Chanturiya, A. N., Suss-Toby, E., Nora, E., & Zimmerberg, J. (1994). An amphipathic peptide from the C-terminal region of the human immunodeficiency virus envelope glycoprotein causes pore formation in membranes. *Journal of Virology*, 68(11), 7115–23.
- Chesebro, B., Nishio, J., Perryman, S., Cann, A., O'Brien, W., Chen, I. S., & Wehrly, K. (1991). Identification of human immunodeficiency virus envelope gene sequences influencing viral entry into CD4-positive HeLa cells, T-leukemia cells, and macrophages. *Journal of Virology*, 65 (11 ), 5782–5789.
- Chohan, B., Lang, D., Sagar, M., Korber, B., Lavreys, L., Richardson, B., & Overbaugh, J. (2005). Selection for Human Immunodeficiency Virus Type 1 Envelope Glycosylation Variants with Shorter V1-V2 Loop Sequences Occurs during Transmission of Certain Genetic Subtypes and May Impact Viral RNA Levels, 79(10), 6528–6531.  
doi:10.1128/JVI.79.10.6528
- Chojnacki, J., Staudt, T., Glass, B., Bingen, P., Engelhardt, J., Anders, M., ... Krausslich, H.-G. (2012). Maturation-Dependent HIV-1 Surface Protein Redistribution Revealed by Fluorescence Nanoscopy. *Science*, 338(6106), 524–528. doi:10.1126/science.1226359
- Chopera, D. R., Wright, J. K., Brockman, M. A., & Brumme, Z. L. (2011). Immune-mediated attenuation of HIV-1. *Future Virology*, 6(8), 917–928. doi:10.2217/fvl.11.68
- Chung, A. W., Isitman, G., Navis, M., Kramski, M., Center, R. J., Kent, S. J., & Stratov, I. (2011). Immune escape from HIV-specific antibody-dependent cellular cytotoxicity (ADCC) pressure. *Proceedings of the National Academy of Sciences of the United States of America*, 108(18), 7505–10. doi:10.1073/pnas.1016048108
- Chung, A. W., Rollman, E., Center, R. J., Kent, S. J., & Stratov, I. (2009). Rapid degranulation of NK cells following activation by HIV-specific antibodies. *Journal of Immunology (Baltimore, Md. : 1950)*, 182(2), 1202–10.
- Chung, C. T., & Miller, R. H. (1988). A rapid and convenient method for the preparation and storage of competent bacterial cells. *Nucleic Acids Research*, 16(8), 3580.
- Cicala, C., Martinelli, E., McNally, J. P., Goode, D. J., Gopaul, R., Hiatt, J., ... Arthos, J. (2009). The integrin  $\alpha 4\beta 7$  forms a complex with cell-surface CD4 and defines a T-cell subset that is highly susceptible to infection by HIV-1. *Proceedings of the National Academy of Sciences*, 106 (49 ), 20877–20882. doi:10.1073/pnas.0911796106
- Cleveland, S. M. (2003). A region of the C-terminal tail of the gp41 envelope glycoprotein of human immunodeficiency virus type 1 contains a neutralizing epitope: evidence for its

- exposure on the surface of the virion. *Journal of General Virology*, 84(3), 591–602. doi:10.1099/vir.0.18630-0
- Clynes, R. A., Towers, T. L., Presta, L. G., & Ravetch, J. V. (2000). Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nature Medicine*, 6(4), 443–6. doi:10.1038/74704
- Comardelle, A. M., Norris, C. H., Plymale, D. R., Gatti, P. J., Choi, B., Fermin, C. D., ... Garry, R. F. (1997). A synthetic peptide corresponding to the carboxy terminus of human immunodeficiency virus type 1 transmembrane glycoprotein induces alterations in the ionic permeability of *Xenopus laevis* oocytes. *AIDS Research and Human Retroviruses*, 13(17), 1525–32.
- Cordonnier, A., Montagnier, L., & Emerman, M. (1989). Single amino-acid changes in HIV envelope affect viral tropism and receptor binding. *Nature*, 340(6234), 571–574.
- Cortez, V., Odem-Davis, K., McClelland, R. S., Jaoko, W., & Overbaugh, J. (2012). HIV-1 superinfection in women broadens and strengthens the neutralizing antibody response. *PLoS Pathogens*, 8(3), e1002611. doi:10.1371/journal.ppat.1002611
- Corti, D., Langedijk, J. P. M., Hinz, A., Seaman, M. S., Vanzetta, F., Fernandez-Rodriguez, B. M., ... Lanzavecchia, A. (2010). Analysis of memory B cell responses and isolation of novel monoclonal antibodies with neutralizing breadth from HIV-1-infected individuals. *PloS One*, 5(1), e8805. doi:10.1371/journal.pone.0008805
- Cosson, P. (1996). Direct interaction between the envelope and matrix proteins of HIV-1. *The EMBO Journal*, 15(21), 5783–8.
- Crawford, H., Prado, J. G., Leslie, A., Hué, S., Honeyborne, I., Reddy, S., ... Goulder, P. J. R. (2007). Compensatory mutation partially restores fitness and delays reversion of escape mutation within the immunodominant HLA-B\*5703-restricted Gag epitope in chronic human immunodeficiency virus type 1 infection. *Journal of Virology*, 81(15), 8346–51. doi:10.1128/JVI.00465-07
- Crowe, S. M., Mills, J., & Mijch, A. (2001). Overview of the Australasian Society for HIV Medicine 12th Annual Conference. *Journal of Clinical Virology*, 22(3), 215–216. doi:http://dx.doi.org/10.1016/S1386-6532(01)00192-5
- Cuadros, D. F., Branscum, A. J., & Crowley, P. H. (2011). HIV-malaria co-infection: effects of malaria on the prevalence of HIV in East sub-Saharan Africa. *International Journal of Epidemiology*, 40(4), 931–9. doi:10.1093/ije/dyq256
- Curlin, M. E., Zioni, R., Hawes, S. E., Liu, Y., Deng, W., Gottlieb, G. S., ... Mullins, J. I. (2010). HIV-1 Envelope Subregion Length Variation during Disease Progression. *PLoS Pathog*, 6(12), e1001228.
- Dalod, M., Dupuis, M., Deschemin, J. C., Sicard, D., Salmon, D., Delfraissy, J. F., ... Guillet, J. G. (1999). Broad, intense anti-human immunodeficiency virus (HIV) ex vivo CD8(+) responses in HIV type 1-infected patients: comparison with anti-Epstein-Barr virus

- responses and changes during antiretroviral therapy. *Journal of Virology*, 73(9), 7108–16.
- Davis, K. L., Gray, E. S., Moore, P. L., Decker, J. M., Salomon, A., Montefiori, D. C., ... Shaw, G. M. (2009). High titer HIV-1 V3-specific antibodies with broad reactivity but low neutralizing potency in acute infection and following vaccination. *Virology*, 387(2), 414–26. doi:10.1016/j.virol.2009.02.022
- Day, J. R., Münk, C., & Guatelli, J. C. (2004). The membrane-proximal tyrosine-based sorting signal of human immunodeficiency virus type 1 gp41 is required for optimal viral infectivity. *Journal of Virology*, 78(3), 1069–79.
- De Oliveira, T., Deforche, K., Cassol, S., Salminen, M., Paraskevis, D., Seebregts, C., ... Vandamme, A.-M. (2005). An automated genotyping system for analysis of HIV-1 and other microbial sequences. *Bioinformatics (Oxford, England)*, 21(19), 3797–800. doi:10.1093/bioinformatics/bti607
- De Witte, L., Nabatov, A., Pion, M., Fluitsma, D., de Jong, M. A. W. P., de Gruijl, T., ... Geijtenbeek, T. B. H. (2007). Langerin is a natural barrier to HIV-1 transmission by Langerhans cells. *Nat Med*, 13(3), 367–371.
- Derdeyn, C. A., Decker, J. M., Bibollet-ruche, F., Mokili, J. L., Muldoon, M., Denham, S. A., ... Hunter, E. (2004). Envelope-Constrained After Heterosexual Transmission, 303(MARCH), 2019–2022.
- Deschambeault, J., Lalonde, J. P., Cervantes-Acosta, G., Lodge, R., Cohen, E. A., & Lemay, G. (1999). Polarized human immunodeficiency virus budding in lymphocytes involves a tyrosine-based signal and favors cell-to-cell viral transmission. *Journal of Virology*, 73(6), 5010–7.
- Dhillon, A. K., Donners, H., Pantophlet, R., Johnson, W. E., Decker, J. M., Shaw, G. M., ... Burton, D. R. (2007). Dissecting the neutralizing antibody specificities of broadly neutralizing sera from human immunodeficiency virus type 1-infected donors. *Journal of Virology*, 81(12), 6548–62. doi:10.1128/JVI.02749-06
- Domingo, E. (2010). Mechanisms of viral emergence. *Veterinary Research*, 41(6), 38. doi:10.1051/vetres/2010010
- Domingo, E., & Holland, J. J. (1997). RNA Virus Mutations and Fitness for Survival. *Annual Review of Microbiology*, 51(1), 151–178. doi:10.1146/annurev.micro.51.1.151
- Doranz, B. J., Rucker, J., Yi, Y., Smyth, R. J., Samson, M., Peiper, S. C., ... Doms, R. W. (1996). A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell*, 85(7), 1149–58.
- Doria-rose, N. A., Klein, R. M., Daniels, M. G., Dell, S. O., Nason, M., Lapedes, A., ... Connors, M. (2010). Breadth of Human Immunodeficiency Virus-Specific Neutralizing Activity in Sera : Clustering Analysis and Association with Clinical Variables , 84(3), 1631–1636. doi:10.1128/JVI.01482-09

- Dorr, P., Westby, M., Dobbs, S., Griffin, P., Irvine, B., Macartney, M., ... Perros, M. (2005). Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity. *Antimicrobial Agents and Chemotherapy*, 49(11), 4721–32. doi:10.1128/AAC.49.11.4721-4732.2005
- Dreja, H., O’Sullivan, E., Pade, C., Greene, K. M., Gao, H., Aubin, K., ... McKnight, A. (2010). Neutralization activity in a geographically diverse East London cohort of human immunodeficiency virus type 1-infected patients: clade C infection results in a stronger and broader humoral immune response than clade B infection. *The Journal of General Virology*, 91(Pt 11), 2794–803. doi:10.1099/vir.0.024224-0
- Duan, S., Boltz, D. A., Seiler, P., Li, J., Bragstad, K., Nielsen, L. P., ... Govorkova, E. A. (2010). Oseltamivir–Resistant Pandemic H1N1/2009 Influenza Virus Possesses Lower Transmissibility and Fitness in Ferrets. *PLoS Pathog*, 6(7), e1001022.
- Dubay, J. W., Roberts, S. J., Brody, B., & Hunter, E. (1992). Mutations in the leucine zipper of the human immunodeficiency virus type 1 transmembrane glycoprotein affect fusion and infectivity. *Journal of Virology*, 66 (8), 4748–4756.
- Duval, M., Posner, M. R., & Cavacini, L. A. (2008). A Bispecific Antibody Composed of a Nonneutralizing Antibody to the gp41 Immunodominant Region and an Anti-CD89 Antibody Directs Broad Human Immunodeficiency Virus Destruction by Neutrophils. *Journal of Virology*, 82(9), 4671–4674. doi:10.1128/JVI.02499-07
- Eisenberg, D., & Wesson, M. (1990). The most highly amphiphilic alpha-helices include two amino acid segments in human immunodeficiency virus glycoprotein 41. *Biopolymers*, 29(1), 171–7. doi:10.1002/bip.360290122
- El Kharroubi, A., & Martin, M. A. (1996). cis-acting sequences located downstream of the human immunodeficiency virus type 1 promoter affect its chromatin structure and transcriptional activity. *Molecular and Cellular Biology*, 16 (6), 2958–2966.
- Emini, E. A., Schleif, W. A., Nunberg, J. H., Conley, A. J., Eda, Y., Tokiyoshi, S., ... Jett, C. M. (1992). Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. *Nature*, 355(6362), 728–30. doi:10.1038/355728a0
- Euler, Z., Bunnik, E. M., Burger, J. a, Boeser-Nunnink, B. D. M., Grijzen, M. L., Prins, J. M., & Schuitemaker, H. (2011). Activity of broadly neutralizing antibodies, including PG9, PG16, and VRC01, against recently transmitted subtype B HIV-1 variants from early and late in the epidemic. *Journal of virology* (Vol. 85, pp. 7236–45). doi:10.1128/JVI.00196-11
- Euler, Z., van den Kerkhof, T. L. G. M., van Gils, M. J., Burger, J. a, Edo-Matas, D., Phung, P., ... Schuitemaker, H. (2012). Longitudinal analysis of early HIV-1-specific neutralizing activity in an elite neutralizer and in five patients who developed cross-reactive neutralizing activity. *Journal of Virology*, 86(4), 2045–55. doi:10.1128/JVI.06091-11

- Euler, Z., van Gils, M. J., Bunnik, E. M., Phung, P., Schweighardt, B., Wrin, T., & Schuitemaker, H. (2010). Cross-reactive neutralizing humoral immunity does not protect from HIV type 1 disease progression. *The Journal of Infectious Diseases*, *201*(7), 1045–53. doi:10.1086/651144
- Fellay, J., Shianna, K. V., Ge, D., Colombo, S., Ledergerber, B., Weale, M., ... Goldstein, D. B. (2007). A whole-genome association study of major determinants for host control of HIV-1. *Science (New York, N.Y.)*, *317*(5840), 944–7. doi:10.1126/science.1143767
- Ferrari, G., Pollara, J., Kozink, D., Harms, T., Drinker, M., Freel, S., ... Haynes, B. F. (2011). An HIV-1 gp120 envelope human monoclonal antibody that recognizes a C1 conformational epitope mediates potent antibody-dependent cellular cytotoxicity (ADCC) activity and defines a common ADCC epitope in human HIV-1 serum. *Journal of Virology*, *85*(14), 7029–36. doi:10.1128/JVI.00171-11
- Ferreira, C. B., Merino-Mansilla, A., Llano, A., Pérez, I., Crespo, I., Llinas, L., ... Sanchez-Merino, V. (2013). Evolution of broadly cross-reactive HIV-1-neutralizing activity: therapy-associated decline, positive association with detectable viremia, and partial restoration of B-cell subpopulations. *Journal of Virology*, *87*(22), 12227–36. doi:10.1128/JVI.02155-13
- Fiebig, E. W., Wright, D. J., Rawal, B. D., Garrett, P. E., Schumacher, R. T., Peddada, L., ... Busch, M. P. (2003). Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection. *AIDS (London, England)*, *17*(13), 1871–9. doi:10.1097/01.aids.0000076308.76477.b8
- Figdor, C. G., van Kooyk, Y., & Adema, G. J. (2002). C-type lectin receptors on dendritic cells and langerhans cells. *Nat Rev Immunol*, *2*(2), 77–84.
- Finnegan, C. M., Berg, W., Lewis, G. K., & DeVico, A. L. (2001). Antigenic properties of the human immunodeficiency virus envelope during cell-cell fusion. *Journal of Virology*, *75*(22), 11096–105. doi:10.1128/JVI.75.22.11096-11105.2001
- Florese, R. H., Demberg, T., Xiao, P., Kuller, L., Larsen, K., Summers, L. E., ... Robert-Guroff, M. (2009). Contribution of Nonneutralizing Vaccine-Elicited Antibody Activities to Improved Protective Efficacy in Rhesus Macaques Immunized with Tat/Env Compared with Multigenic Vaccines. *The Journal of Immunology*, *182* (6), 3718–3727. doi:10.4049/jimmunol.0803115
- Forthal, D. N., Gilbert, P. B., Landucci, G., & Phan, T. (2007). Recombinant gp120 Vaccine-Induced Antibodies Inhibit Clinical Strains of HIV-1 in the Presence of Fc Receptor-Bearing Effector Cells and Correlate Inversely with HIV Infection Rate 1. *The Journal of Immunology*, *178*, 6596–6603.
- Forthal, D. N., Landucci, G., & Daar, E. S. (2001). Antibody from patients with acute human immunodeficiency virus (HIV) infection inhibits primary strains of HIV type 1 in the presence of natural-killer effector cells. *Journal of Virology*, *75*(15), 6953–61. doi:10.1128/JVI.75.15.6953-6961.2001

- Forthal, D. N., Landucci, G., Gorny, M. K., Zolla-Pazner, S., & Robinson, W. E. (1995). Functional activities of 20 human immunodeficiency virus type 1 (HIV-1)-specific human monoclonal antibodies. *AIDS Research and Human Retroviruses*, *11*(9), 1095–9.
- Forthal, D. N., Landucci, G., Haubrich, R., Keenan, B., Kuppermann, B. D., Tilles, J. G., & Kaplan, J. (1999). Antibody-Dependent Cellular Cytotoxicity Independently Predicts Survival in Severely Immunocompromised Human Immunodeficiency Virus-Infected Patients. *Journal of Infectious Diseases*, *180* (4), 1338–1341. doi:10.1086/314988
- Forthal, D. N., Landucci, G., Phan, T. B., & Becerra, J. (2005). Interactions between Natural Killer Cells and Antibody Fc Result in Enhanced Antibody Neutralization of Human Immunodeficiency Virus Type 1. *Journal of Virology*, *79* (4), 2042–2049. doi:10.1128/JVI.79.4.2042-2049.2005
- Fouchier, R. A., Groenink, M., Kootstra, N. A., Tersmette, M., Huisman, H. G., Miedema, F., & Schuitemaker, H. (1992). Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule. *Journal of Virology*, *66* (5), 3183–3187.
- Fouda, G. G., Yates, N. L., Pollara, J., Shen, X., Overman, G. R., Mahlokozera, T., ... Permar, S. R. (2011). HIV-specific functional antibody responses in breast milk mirror those in plasma and are primarily mediated by IgG antibodies. *Journal of Virology*, *85*(18), 9555–67. doi:10.1128/JVI.05174-11
- Freed, E. O., Delwart, E. L., Buchsacher, G. L., & Panganiban, A. T. (1992). A mutation in the human immunodeficiency virus type 1 transmembrane glycoprotein gp41 dominantly interferes with fusion and infectivity. *Proceedings of the National Academy of Sciences*, *89* (1), 70–74.
- Freed, E. O., & Martin, M. a. (1996). Domains of the human immunodeficiency virus type 1 matrix and gp41 cytoplasmic tail required for envelope incorporation into virions. *Journal of Virology*, *70*(1), 341–51.
- Freed, E. O., Myers, D. J., & Risser, R. (1990). Characterization of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41. *Proceedings of the National Academy of Sciences*, *87* (12), 4650–4654.
- Freed, E. O., Myers, D. J., & Risser, R. (1991). Identification of the principal neutralizing determinant of human immunodeficiency virus type 1 as a fusion domain. *Journal of Virology*, *65* (1), 190–194. Retrieved from <http://jvi.asm.org/content/65/1/190.abstract>
- Frey, G., Peng, H., Rits-Volloch, S., Morelli, M., Cheng, Y., & Chen, B. (2008). A fusion-intermediate state of HIV-1 gp41 targeted by broadly neutralizing antibodies. *Proceedings of the National Academy of Sciences*, *105* (10), 3739–3744. doi:10.1073/pnas.0800255105
- Friedman, J., Alam, S. M., Shen, X., Xia, S.-M., Stewart, S., Anasti, K., ... Permar, S. R. (2012). Isolation of HIV-1-neutralizing mucosal monoclonal antibodies from human colostrum. *PloS One*, *7*(5), e37648. doi:10.1371/journal.pone.0037648

- Gabuzda, D. H., Lever, A., Terwilliger, E., & Sodroski, J. (1992). Effects of deletions in the cytoplasmic domain on biological functions of human immunodeficiency virus type 1 envelope glycoproteins. *Journal of Virology*, *66*(6), 3306–15.
- Gao, F., Morrison, S. G., Robertson, D. L., Thornton, C. L., Craig, S., Karlsson, G., ... Hahn, B. H. (1996). Molecular cloning and analysis of functional envelope genes from human immunodeficiency virus type 1 sequence subtypes A through G. The WHO and NIAID Networks for HIV Isolation and Characterization. *Journal of Virology*, *70* (3), 1651–1667. Retrieved from <http://jvi.asm.org/content/70/3/1651.abstract>
- Gao, X., Nelson, G. W., Karacki, P., Martin, M. P., Phair, J., Kaslow, R., ... Carrington, M. (2001). Effect of a single amino acid change in MHC class I molecules on the rate of progression to AIDS. *The New England Journal of Medicine*, *344*(22), 1668–75. doi:10.1056/NEJM200105313442203
- Gawrisch, K., Han, K. H., Yang, J. S., Bergelson, L. D., & Ferretti, J. A. (1993). Interaction of peptide fragment 828-848 of the envelope glycoprotein of human immunodeficiency virus type I with lipid bilayers. *Biochemistry*, *32*(12), 3112–8.
- Geldmacher, C., Currier, J. R., Gerhardt, M., Haule, A., Maboko, L., Birx, D., ... Hoelscher, M. (2007). In a mixed subtype epidemic, the HIV-1 Gag-specific T-cell response is biased towards the infecting subtype. *AIDS (London, England)*, *21*(2), 135–43. doi:10.1097/01.aids.0000247589.77061.f7
- Girard, M. P., & Plotkin, S. A. (2012). HIV vaccine development at the turn of the 21st century. *Current Opinion in HIV and AIDS*, *7*(1), 4–9. doi:10.1097/COH.0b013e32834ddc96
- Goepfert, P. A., Lumm, W., Farmer, P., Matthews, P., Prendergast, A., Carlson, J. M., ... Hunter, E. (2008). Transmission of HIV-1 Gag immune escape mutations is associated with reduced viral load in linked recipients. *The Journal of Experimental Medicine*, *205*(5), 1009–17. doi:10.1084/jem.20072457
- Gómez-Román, V. R., Florese, R. H., Patterson, L. J., Peng, B., Venzon, D., Aldrich, K., & Robert-Guroff, M. (2006). A simplified method for the rapid fluorometric assessment of antibody-dependent cell-mediated cytotoxicity. *Journal of Immunological Methods*, *308*(1–2), 53–67. doi:<http://dx.doi.org/10.1016/j.jim.2005.09.018>
- Gómez-Román, V. R., Patterson, L. J., Venzon, D., Liewehr, D., Aldrich, K., Florese, R., & Robert-Guroff, M. (2005). Vaccine-Elicited Antibodies Mediate Antibody-Dependent Cellular Cytotoxicity Correlated with Significantly Reduced Acute Viremia in Rhesus Macaques Challenged with SIVmac251. *The Journal of Immunology*, *174* (4), 2185–2189.
- Goonetilleke, N., Liu, M. K. P., Salazar-Gonzalez, J. F., Ferrari, G., Giorgi, E., Gansov, V. V., ... McMichael, A. J. (2009). The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. *The Journal of Experimental Medicine*, *206* (6), 1253–1272. doi:10.1084/jem.20090365

- Goudsmit, J., Debouck, C., Meloen, R. H., Smit, L., Bakker, M., Asher, D. M., ... Gajdusek, D. C. (1988). Human immunodeficiency virus type 1 neutralization epitope with conserved architecture elicits early type-specific antibodies in experimentally infected chimpanzees. *Proceedings of the National Academy of Sciences*, 85 (12), 4478–4482.
- Goulder, P. J., Bunce, M., Krausa, P., McIntyre, K., Crowley, S., Morgan, B., ... McMichael, A. J. (1996). Novel, cross-restricted, conserved, and immunodominant cytotoxic T lymphocyte epitopes in slow progressors in HIV type 1 infection. *AIDS Research and Human Retroviruses*, 12(18), 1691–8.
- Goulder, P. J. R., & Watkins, D. I. (2004). HIV and SIV CTL escape: implications for vaccine design. *Nature Reviews. Immunology*, 4(8), 630–40. doi:10.1038/nri1417
- Gray, C. M., Mlotshwa, M., Riou, C., Mathebula, T., de Assis Rosa, D., Mashishi, T., ... Karim, S. A. (2009). Human immunodeficiency virus-specific gamma interferon enzyme-linked immunospot assay responses targeting specific regions of the proteome during primary subtype C infection are poor predictors of the course of viremia and set point. *Journal of Virology*, 83(1), 470–8. doi:10.1128/JVI.01678-08
- Gray, E. S., Madiga, M. C., Hermanus, T., Moore, P. L., Wibmer, C. K., Tumba, N. L., ... Team, and the C. S. (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. *Journal of Virology*, 85 (10), 4828–4840. doi:10.1128/JVI.00198-11
- Gray, E. S., Moore, P. L., Choge, I. A., Decker, J. M., Li, H., Leseka, N., ... Team, S. (2007). Neutralizing Antibody Responses in Acute Human Immunodeficiency Virus Type 1 Subtype C Infection, 81(12), 6187–6196. doi:10.1128/JVI.00239-07
- Gray, R. H., Wawer, M. J., Brookmeyer, R., Sewankambo, N. K., Serwadda, D., Wabwire-Mangen, F., ... Quinn, T. C. (2001). Probability of HIV-1 transmission per coital act in monogamous, heterosexual, HIV-1-discordant couples in Rakai, Uganda. *The Lancet*, 357(9263), 1149–1153. doi:http://dx.doi.org/10.1016/S0140-6736(00)04331-2
- Greenberg, M. E., Iafrate, A. J., & Skowronski, J. (1998). The SH3 domain-binding surface and an acidic motif in HIV-1 Nef regulate trafficking of class I MHC complexes. *The EMBO Journal*, 17(10), 2777–89. doi:10.1093/emboj/17.10.2777
- Guan, Y., Pazgier, M., Sajadi, M. M., Kamin-Lewis, R., Al-Darmarki, S., Flinko, R., ... Lewis, G. K. (2013). Diverse specificity and effector function among human antibodies to HIV-1 envelope glycoprotein epitopes exposed by CD4 binding. *Proceedings of the National Academy of Sciences of the United States of America*, 110(1), E69–78. doi:10.1073/pnas.1217609110
- Guglietta, S., Pantaleo, G., & Graziosi, C. (2010). Long sequence duplications, repeats, and palindromes in HIV-1 gp120: Length variation in V4 as the product of misalignment mechanism. *Virology*, 399(1), 167–175.
- Guo, H., Abrahamyan, L. G., Liu, C., Waltke, M., Geng, Y., Chen, Q., ... Kong, X. (2012). Comparative analysis of the fusion efficiency elicited by the envelope glycoprotein V1-

- V5 regions derived from human immunodeficiency virus type 1 transmitted perinatally. *The Journal of General Virology*, 93(Pt 12), 2635–45. doi:10.1099/vir.0.046771-0
- Guthrie, B. L., de Bruyn, G., & Farquhar, C. (2007). HIV-1-discordant couples in sub-Saharan Africa: explanations and implications for high rates of discordancy. *Current HIV Research*, 5(4), 416–29.
- Haaland, R. E., Hawkins, P. a, Salazar-Gonzalez, J., Johnson, A., Tichacek, A., Karita, E., ... Hunter, E. (2009). Inflammatory genital infections mitigate a severe genetic bottleneck in heterosexual transmission of subtype A and C HIV-1. *PLoS Pathogens*, 5(1), e1000274. doi:10.1371/journal.ppat.1000274
- Haase, A. T. (1999). Population Biology of HIV-1 Infection: Viral and CD4+ T Cell Demographics and Dynamics in Lymphatic Tissues. *Annual Review of Immunology*, 17(1), 625–656. doi:10.1146/annurev.immunol.17.1.625
- Haase, A. T. (2010). Targeting early infection to prevent HIV-1 mucosal transmission. *Nature*, 464(7286), 217–223. Retrieved from <http://dx.doi.org/10.1038/nature08757>
- Haase, A. T. (2011). Early events in sexual transmission of HIV and SIV and opportunities for interventions. *Annual Review of Medicine*, 62, 127–39. doi:10.1146/annurev-med-080709-124959
- Haynes, B. F., Gilbert, P. B., Mcelrath, M. J., Zolla-Pazner, S., Tomaras, G. D., Alam, S. M., ... Kim, J. H. (2012). Immune-Correlates Analysis of an HIV-1 Vaccine Efficacy Trial. *The New England Journal of Medicine*, 366(14), 1275–1286.
- Haynes, B. F., & McElrath, M. J. (2013). Progress in HIV-1 vaccine development. *Current Opinion in HIV and AIDS*, 8(4), 326–32. doi:10.1097/COH.0b013e328361d178
- Haynes, B. F., Tomaras, G. D., Ph, D., Alam, S. M., Evans, D. T., Montefiori, D. C., ... Koup, R. A. (2012). *The New England Journal of Medicine*, 1275–1286.
- Hemelaar, J. (2012). The origin and diversity of the HIV-1 pandemic. *Trends in Molecular Medicine*, 18(3), 182–92. doi:10.1016/j.molmed.2011.12.001
- Hemelaar, J., Gouws, E., Ghys, P. D., & Osmanov, S. (2006). Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004. *AIDS (London, England)*, 20(16), W13–23. doi:10.1097/01.aids.0000247564.73009.bc
- Hemelaar, J., Gouws, E., Ghys, P. D., Osmanov, S., & Characterisation, W.-U. N. for H. I. V. I. and. (2011). Global trends in molecular epidemiology of HIV-1 during 2000–2007. *AIDS*, 25(5).
- Herbeck, J. T., Nickle, D. C., Learn, G. H., Gottlieb, G. S., Curlin, M. E., Heath, L., & Mullins, J. I. (2006). Human Immunodeficiency Virus Type 1 env Evolves toward Ancestral States upon Transmission to a New Host, 80(4), 1637–1644. doi:10.1128/JVI.80.4.1637

- Herbinger, K.-H., Gerhardt, M., Piyasirisilp, S., Mloka, D., Arroyo, M. a, Hoffmann, O., ... Hoelscher, M. (2006). Frequency of HIV type 1 dual infection and HIV diversity: analysis of low- and high-risk populations in Mbeya Region, Tanzania. *AIDS Research and Human Retroviruses*, 22(7), 599–606. doi:10.1089/aid.2006.22.599
- Heredia, A., Latinovic, O., Gallo, R. C., Melikyan, G., Reitz, M., Le, N., & Redfield, R. R. (2008). Reduction of CCR5 with low-dose rapamycin enhances the antiviral activity of vicriviroc against both sensitive and drug-resistant HIV-1. *Proceedings of the National Academy of Sciences of the United States of America*, 105(51), 20476–81. doi:10.1073/pnas.0810843106
- Herrera, C., Spenlehauer, C., Fung, M. S., Burton, D. R., Beddows, S., & Moore, J. P. (2003). Nonneutralizing Antibodies to the CD4-Binding Site on the gp120 Subunit of Human Immunodeficiency Virus Type 1 Do Not Interfere with the Activity of a Neutralizing Antibody against the Same Site. *Journal of Virology*, 77(2), 1084–1091. doi:10.1128/JVI.77.2.1084-1091.2003
- Hessell, A. J., Hangartner, L., Hunter, M., Havenith, C. E. G., Beurskens, F. J., Bakker, J. M., ... Burton, D. R. (2007). Fc receptor but not complement binding is important in antibody protection against HIV. *Nature*, 449(7158), 101–4. doi:10.1038/nature06106
- Hezareh, M., Hessell, A. J., Jensen, R. C., van de Winkel, J. G., & Parren, P. W. (2001). Effector function activities of a panel of mutants of a broadly neutralizing antibody against human immunodeficiency virus type 1. *Journal of Virology*, 75(24), 12161–8. doi:10.1128/JVI.75.24.12161-12168.2001
- Hidajat, R., Xiao, P., Zhou, Q., Venzon, D., Summers, L. E., Kalyanaraman, V. S., ... Robert-Guroff, M. (2009). Correlation of vaccine-elicited systemic and mucosal nonneutralizing antibody activities with reduced acute viremia following intrarectal simian immunodeficiency virus SIVmac251 challenge of rhesus macaques. *Journal of Virology*, 83(2), 791–801. doi:10.1128/JVI.01672-08
- Hladik, F., & McElrath, M. J. (2008). Setting the stage: host invasion by HIV. *Nat Rev Immunol*, 8(6), 447–457.
- Hladik, F., Sakchalathorn, P., Ballweber, L., Lentz, G., Fialkow, M., Eschenbach, D., & McElrath, M. J. (2007). Initial Events in Establishing Vaginal Entry and Infection by Human Immunodeficiency Virus Type-1. *Immunity*, 26(2), 257–270. doi:http://dx.doi.org/10.1016/j.immuni.2007.01.007
- Hoelscher, M., Dowling, W. E., Sanders-Buell, E., Carr, J. K., Harris, M. E., Thomschke, A., ... McCutchan, F. E. (2002). Detection of HIV-1 subtypes, recombinants, and dual infections in east Africa by a multi-region hybridization assay. *AIDS (London, England)*, 16(15), 2055–64.
- Hofmann-Lehmann, R., Rasmussen, R. A., Vlasak, J., Smith, B. A., Baba, T. W., Liska, V., ... Ruprecht, R. M. (2001). Passive immunization against oral AIDS virus transmission: an approach to prevent mother-to-infant HIV-1 transmission? *Journal of Medical Primatology*, 30(4), 190–6.

- Hollier, M. J., & Dimmock, N. J. (2005). The C-terminal tail of the gp41 transmembrane envelope glycoprotein of HIV-1 clades A, B, C, and D may exist in two conformations: an analysis of sequence, structure, and function. *Virology*, *337*(2), 284–296.
- Huang, C., Tang, M., Zhang, M.-Y., Majeed, S., Montabana, E., Stanfield, R. L., ... Kwong, P. D. (2005). Structure of a V3-containing HIV-1 gp120 core. *Science (New York, N.Y.)*, *310*(5750), 1025–8. doi:10.1126/science.1118398
- Huang, C., Venturi, M., Majeed, S., Moore, M. J., Phogat, S., Zhang, M.-Y., ... Kwong, P. D. (2004). Structural basis of tyrosine sulfation and VH-gene usage in antibodies that recognize the HIV type 1 coreceptor-binding site on gp120. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(9), 2706–11. doi:10.1073/pnas.0308527100
- Huson, D. H., Richter, D. C., Rausch, C., DeZulian, T., Franz, M., & Rupp, R. (2007). Dendroscope: An interactive viewer for large phylogenetic trees. *BMC Bioinformatics*, *8*, 460. doi:10.1186/1471-2105-8-460
- Hwang, S. S., Boyle, T. J., Lyerly, H. K., & Cullen, B. R. (1991). Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1. *Science*, *253* (5015), 71–74. doi:10.1126/science.1905842
- Isitman, G., Chung, A. W., Navis, M., Kent, S. J., & Stratov, I. (2011). Pol as a target for antibody dependent cellular cytotoxicity responses in HIV-1 infection. *Virology*, *412*(1), 110–6. doi:10.1016/j.virol.2010.12.044
- Isitman, G., Stratov, I., & Kent, S. J. (2012). Antibody-Dependent Cellular Cytotoxicity and NK Cell-Driven Immune Escape in HIV Infection: Implications for HIV Vaccine Development. *Advances in Virology*, *2012*(Table 1), 637208. doi:10.1155/2012/637208
- Jin, F., Jansson, J., Law, M., Prestage, G. P., Zablotska, I., Imrie, J. C. G., ... Wilson, D. P. (2010). Per-contact probability of HIV transmission in homosexual men in Sydney in the era of HAART. *AIDS (London, England)*, *24*(6), 907–13. doi:10.1097/QAD.0b013e3283372d90
- Jobes, D. V., Daoust, M., Nguyen, V., Padua, A., Michele, S., Lock, M. D., ... Berman, P. W. (2006). High incidence of unusual cysteine variants in gp120 envelope proteins from early HIV type 1 infections from a Phase 3 vaccine efficacy trial. *AIDS Research and Human Retroviruses*, *22*(10), 1014–21. doi:10.1089/aid.2006.22.1014
- Julien, J.-P., Cupo, A., Sok, D., Stanfield, R. L., Lyumkis, D., Deller, M. C., ... Wilson, I. a. (2013). Crystal Structure of a Soluble Cleaved HIV-1 Envelope Trimer. *Science (New York, N.Y.)*, (October), 1–12. doi:10.1126/science.1245625
- Kalia, V., Sarkar, S., Gupta, P., & Montelaro, R. C. (2003). Rational Site-Directed Mutations of the LLP-1 and LLP-2 Lentivirus Lytic Peptide Domains in the Intracytoplasmic Tail of Human Immunodeficiency Virus Type 1 gp41 Indicate Common Functions in Cell-Cell Fusion but Distinct Roles in Virion Envelope Incorporation. *Journal of Virology*, *77*(6), 3634–3646. doi:10.1128/JVI.77.6.3634

- Kaslow, R. A., Carrington, M., Apple, R., Park, L., Muñoz, A., Saah, A. J., ... Mann, D. L. (1996). Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nature Medicine*, 2(4), 405–11.
- Kedzierska, K., Ellery, P., Mak, J., Lewin, S. E., Crowe, S. M., & Jaworowski, A. (2013). HIV-1 Down-Modulates  $\gamma$  Signaling Chain of Fc $\gamma$ R in Human Macrophages: A Possible Mechanism for Inhibition of Phagocytosis. *The Journal of Immunology*, 168, 2895–2903.
- Kedzierska, K., Mak, J., Jaworowski, A., Greenway, A., Violo, A., Chan, H., ... Crowe, S. (2001). nef-deleted HIV-1 inhibits phagocytosis by monocyte-derived macrophages in vitro but not by peripheral blood monocytes in vivo. *AIDS*, 15(February), 945–955.
- Keele, B. F., Giorgi, E. E., Salazar-Gonzalez, J. F., Decker, J. M., Pham, K. T., Salazar, M. G., ... Shaw, G. M. (2008). Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proceedings of the National Academy of Sciences*, 105 (21), 7552–7557. doi:10.1073/pnas.0802203105
- Kennedy, R. C., Henkel, R. D., Pauletti, D., Allan, J. S., Lee, T. H., Essex, M., & Dreesman, G. R. (1986). Antiserum to a synthetic peptide recognizes the HTLV-III envelope glycoprotein. *Science*, 231 (4745), 1556–1559. doi:10.1126/science.3006246
- Kiepiela, P., Leslie, A. J., Honeyborne, I., Ramduth, D., Thobakgale, C., Chetty, S., ... Goulder, P. J. R. (2004). Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. *Nature*, 432(7018), 769–75. doi:10.1038/nature03113
- Kiepiela, P., Ngumbela, K., Thobakgale, C., Ramduth, D., Honeyborne, I., Moodley, E., ... Goulder, P. (2007). CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. *Nature Medicine*, 13(1), 46–53. doi:10.1038/nm1520
- Kitrinos, K. M., Hoffman, N. G., Nelson, J. A. E., & Swanstrom, R. (2003). Turnover of env Variable Region 1 and 2 Genotypes in Subjects with Late-Stage Human Immunodeficiency Virus Type 1 Infection. *Journal of Virology*, 77 (12), 6811–6822. doi:10.1128/JVI.77.12.6811-6822.2003
- Klasse, P. J. (2007). Modeling how many envelope glycoprotein trimers per virion participate in human immunodeficiency virus infectivity and its neutralization by antibody. *Virology*, 369(2), 245–62. doi:10.1016/j.virol.2007.06.044
- Klenerman, P., Rowland-Jones, S., McAdam, S., Edwards, J., Daenke, S., Lalloo, D., ... Edwards, A. (1994). Cytotoxic T-cell activity antagonized by naturally occurring HIV-1 Gag variants. *Nature*, 369(6479), 403–7. doi:10.1038/369403a0
- Kol, N., Tsvitov, M., & Kay, M. S. (2013). Virion stiffness regulates immature HIV-1 entry. *Retrovirology*, 10(4). doi:10.1186/1742-4690-10-4
- Kondo, N., Miyauchi, K., Meng, F., Iwamoto, A., & Matsuda, Z. (2010). Conformational Changes of the HIV-1 Envelope Protein during Membrane Fusion Are Inhibited by the Replacement of Its Membrane-spanning Domain. *Journal of Biological Chemistry*, 285 (19), 14681–14688. doi:10.1074/jbc.M109.067090

- Korber, B., Gaschen, B., Yusim, K., Thakallapally, R., Kesmir, C., & Detours, V. (2001). Evolutionary and immunological implications of contemporary HIV-1 variation. *British Medical Bulletin*, 58, 19–42.
- Kosakovsky Pond, S. L., Posada, D., Gravenor, M. B., Woelk, C. H., & Frost, S. D. W. (2006). GARD: a genetic algorithm for recombination detection. *Bioinformatics (Oxford, England)*, 22(24), 3096–8. doi:10.1093/bioinformatics/btl474
- Kostrikis, L. G., Cao, Y., Ngai, H., Moore, J. P., & Ho, D. D. (1996). Quantitative analysis of serum neutralization of human immunodeficiency virus type 1 from subtypes A, B, C, D, E, F, and I: lack of direct correlation between neutralization serotypes and genetic subtypes and evidence for prevalent serum-dependent infecti. *Journal of Virology*, 70(1), 445–58.
- Kostrikis, L. G., Michalopoulou, Z. H., Cao, Y., Moore, J. P., & Ho, D. D. (1996). Determining neutralization serotypes of HIV type 1 by neural networks. *AIDS Research and Human Retroviruses*, 12(18), 1667–9.
- Kottlilil, S., Chun, T.-W., Moir, S., Liu, S., McLaughlin, M., Hallahan, C. W., ... Fauci, A. S. (2003). Innate immunity in human immunodeficiency virus infection: effect of viremia on natural killer cell function. *The Journal of Infectious Diseases*, 187(7), 1038–45. doi:10.1086/368222
- Koup, R. A., Pikora, C. A., Mazzara, G., Panicali, D., & Sullivan, J. L. (1991). Broadly reactive antibody-dependent cellular cytotoxic response to HIV-1 envelope glycoproteins precedes broad neutralizing response in human infection. *Viral Immunology*, 4(4), 215–23.
- Koup, R. A., Robinson, J. E., Nguyen, Q. V, Pikora, C. A., Blais, B., Roskey, A., ... Sullivan, J. L. (1991). Antibody-dependent cell-mediated cytotoxicity directed by a human monoclonal antibody reactive with gp120 of HIV-1. *AIDS*, 5(11).
- Koup, R. A., Safrit, J. T., Cao, Y., Andrews, C. A., McLeod, G., Borkowsky, W., ... Ho, D. D. (1994). Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. *Journal of Virology*, 68(7), 4650–5.
- Koup, R. A., Sullivan, J. L., Levine, P. H., Brewster, F., Mahr, A., Mazzara, G., ... Panicali, D. (1989). Antigenic specificity of antibody-dependent cell-mediated cytotoxicity directed against human immunodeficiency virus in antibody-positive sera. *Journal of Virology*, 63 (2 ), 584–590.
- Kowalski, M., Potz, J., Basiripour, L., Dorfman, T., Goh, W. C., Terwilliger, E., ... Sodroski, J. (1987). Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. *Science*, 237 (4820 ), 1351–1355. doi:10.1126/science.3629244
- Kwong, P. D., Doyle, M. L., Casper, D. J., Cicala, C., Leavitt, S. A., Majeed, S., ... Arthos, J. (2002). HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. *Nature*, 420(6916), 678–82. doi:10.1038/nature01188

- Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J., & Hendrickson, W. a. (1998). Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature*, *393*(6686), 648–59. doi:10.1038/31405
- Labrijn, A. F., Poignard, P., Raja, A., Zwick, M. B., Delgado, K., Franti, M., ... Burton, D. R. (2003). Access of Antibody Molecules to the Conserved Coreceptor Binding Site on Glycoprotein gp120 Is Sterically Restricted on Primary Human Immunodeficiency Virus Type 1. *Journal of Virology*, *77* (19), 10557–10565. doi:10.1128/JVI.77.19.10557-10565.2003
- Lakhashe, S. K., Kulkarni, S. S., Thakar, M. R., Ghate, M. V., & Paranjape, R. S. (2007). Extensive cross-reactive neutralizing antibody response in Indian patients with limited genetic diversity of HIV-1. *Virology*, *359*(2), 295–301. doi:10.1016/j.virol.2006.09.019
- Lamas, J. R., Brooks, J. M., Galocha, B., Rickinson, A. B., & López de Castro, J. A. (1998). Relationship between peptide binding and T cell epitope selection: a study with subtypes of HLA-B27. *International Immunology*, *10*(3), 259–66.
- Lanier, L. L., Le, A. M., Civin, C. I., Loken, M. R., & Phillips, J. H. (1986). The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *The Journal of Immunology*, *136* (12), 4480–4486.
- Lasky, L. A., Nakamura, G., Smith, D. H., Fennie, C., Shimasaki, C., Patzer, E., ... Capon, D. J. (1987). Delineation of a region of the human immunodeficiency virus type 1 gp120 glycoprotein critical for interaction with the CD4 receptor. *Cell*, *50*(6), 975–985. Retrieved from <http://www.sciencedirect.com/science/article/pii/0092867487905241>
- Le Gall, S., Stamegna, P., & Walker, B. D. (2007). Portable flanking sequences modulate CTL epitope processing. *The Journal of Clinical Investigation*, *117*(11), 3563–75. doi:10.1172/JCI32047
- Lee, A. B., & Cooper, T. A. (1995). Improved direct PCR screen for bacterial colonies: wooden toothpicks inhibit PCR amplification. *BioTechniques*, *18*(2), 225–6.
- Lee, S. F., Wang, C. T., Liang, J. Y., Hong, S. L., Huang, C. C., & Chen, S. S. (2000). Multimerization potential of the cytoplasmic domain of the human immunodeficiency virus type 1 transmembrane glycoprotein gp41. *The Journal of Biological Chemistry*, *275*(21), 15809–19. doi:10.1074/jbc.M000601200
- Lee, S. J., Hu, W., Fisher, A. G., Looney, D. J., Kao, V. F., Mitsuya, H., ... Wong-Staal, F. (1989). Role of the carboxy-terminal portion of the HIV-1 transmembrane protein in viral transmission and cytopathogenicity. *AIDS Research and Human Retroviruses*, *5*(4), 441–9.
- Lee, S.-F., Ko, C.-Y., Wang, C.-T., & Chen, S. S.-L. (2002). Effect of point mutations in the N terminus of the lentivirus lytic peptide-1 sequence of human immunodeficiency virus type 1 transmembrane protein gp41 on Env stability. *The Journal of Biological Chemistry*, *277*(18), 15363–75. doi:10.1074/jbc.M201479200

- Leonard, C. K., Spellman, M. W., Riddle, L., Harris, R. J., Thomas, J. N., & Gregory, T. J. (1990). Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *The Journal of Biological Chemistry*, 265(18), 10373–82.
- Leslie, A. J., Pfafferott, K. J., Chetty, P., Draenert, R., Addo, M. M., Feeney, M., ... Goulder, P. J. R. (2004). HIV evolution: CTL escape mutation and reversion after transmission. *Nature Medicine*, 10(3), 282–9. doi:10.1038/nm992
- Leslie, A., Matthews, P. C., Listgarten, J., Carlson, J. M., Kadie, C., Ndung'u, T., ... Goulder, P. J. R. (2010). Additive contribution of HLA class I alleles in the immune control of HIV-1 infection. *Journal of Virology*, 84(19), 9879–88. doi:10.1128/JVI.00320-10
- Leynaert, B., Downs, A. M., & de Vincenzi, I. (1998). Heterosexual transmission of human immunodeficiency virus: variability of infectivity throughout the course of infection. European Study Group on Heterosexual Transmission of HIV. *American Journal of Epidemiology*, 148(1), 88–96.
- Li, B., Decker, J. M., Johnson, R. W., Bibollet-Ruche, F., Wei, X., Mulenga, J., ... Derdeyn, C. a. (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), 5211–8. doi:10.1128/JVI.00201-06
- Li, H., Chien, P. C., Tuen, M., Visciano, M. L., Cohen, S., Blais, S., ... Hioe, C. E. (2008). Identification of an N-Linked Glycosylation in the C4 Region of HIV-1 Envelope gp120 That Is Critical for Recognition of Neighboring CD4 T Cell Epitopes. *The Journal of Immunology*, 180(6), 4011–4021.
- Li, M., Gao, F., Mascola, J. R., Stamatatos, L., Polonis, V. R., Koutsoukos, M., ... Montefiori, D. C. (2005). Human Immunodeficiency Virus Type 1 env Clones from Acute and Early Subtype B Infections for Standardized Assessments of Vaccine-Elicited Neutralizing Antibodies, 79(16), 10108–10125. doi:10.1128/JVI.79.16.10108
- Li, M., Salazar-Gonzalez, J. F., Derdeyn, C. a, Morris, L., Williamson, C., Robinson, J. E., ... Montefiori, D. C. (2006). Genetic and neutralization properties of subtype C human immunodeficiency virus type 1 molecular env clones from acute and early heterosexually acquired infections in Southern Africa. *Journal of Virology*, 80(23), 11776–90. doi:10.1128/JVI.01730-06
- Li, Y., Migueles, S. A., Welcher, B., Svehla, K., Phogat, A., Louder, M. K., ... Mascola, J. R. (2007). Broad HIV-1 neutralization mediated by CD4-binding site antibodies. *Nature Medicine*, 13(9), 1032–4. doi:10.1038/nm1624
- Li, Y., O'Dell, S., Walker, L. M., Wu, X., Guenaga, J., Feng, Y., ... Mascola, J. R. (2011). Mechanism of neutralization by the broadly neutralizing HIV-1 monoclonal antibody VRC01. *Journal of Virology*, 85(17), 8954–67. doi:10.1128/JVI.00754-11

- Li, Y., Svehla, K., Mathy, N. L., Voss, G., Mascola, J. R., & Wyatt, R. (2006). Characterization of Antibody Responses Elicited by Human Immunodeficiency Virus Type 1 Primary Isolate Trimeric and Monomeric Envelope Glycoproteins in Selected Adjuvants †, *80*(3), 1414–1426. doi:10.1128/JVI.80.3.1414
- Liao, H.-X., Bonsignori, M., Alam, S. M., McLellan, J. S., Tomaras, G. D., Moody, M. A., ... Haynes, B. F. (2013). Vaccine induction of antibodies against a structurally heterogeneous site of immune pressure within HIV-1 envelope protein variable regions 1 and 2. *Immunity*, *38*(1), 176–86. doi:10.1016/j.immuni.2012.11.011
- Liao, H.-X., Lynch, R., Zhou, T., Gao, F., Alam, S. M., Boyd, S. D., ... Young, A. (2013). Co-evolution of a broadly neutralizing HIV-1 antibody and founder virus. *Nature*. doi:10.1038/nature12053
- Lichterfeld, M., Kaufmann, D. E., Yu, X. G., Mui, S. K., Addo, M. M., Johnston, M. N., ... Altfeld, M. (2004). Loss of HIV-1-specific CD8+ T cell proliferation after acute HIV-1 infection and restoration by vaccine-induced HIV-1-specific CD4+ T cells. *The Journal of Experimental Medicine*, *200*(6), 701–12. doi:10.1084/jem.20041270
- Lineberger, J. E., Danzeisen, R., Hazuda, D. J., Simon, A. J., & Miller, M. D. (2002). Altering expression levels of human immunodeficiency virus type 1 gp120-gp41 affects efficiency but not kinetics of cell-cell fusion. *Journal of Virology*, *76*(7), 3522–33.
- Liu, L., Chahroudi, A., Silvestri, G., Wernett, M. E., Kaiser, W. J., Safrit, J. T., ... Feinberg, M. B. (2002). Visualization and quantification of T cell-mediated cytotoxicity using cell-permeable fluorogenic caspase substrates. *Nature Medicine*, *8*(2), 185–9. doi:10.1038/nm0202-185
- Liu, M. K. P., Hawkins, N., Ritchie, A. J., Ganusov, V. V., Whale, V., Brackenridge, S., ... Goonetilleke, N. (2013). Vertical T cell immunodominance and epitope entropy determine HIV-1 escape. *The Journal of Clinical Investigation*, *123*(1), 380–93. doi:10.1172/JCI65330
- Ljunggren, K., Moschese, V., Broliden, P.-A., Giaquinto, C., Quinti, I., Fenyö, E.-M., ... Jondal, M. (1990). Antibodies Mediating Cellular Cytotoxicity and Neutralization Correlate with a Better Clinical Stage in Children Born to Human Immunodeficiency Virus-Infected Mothers. *Journal of Infectious Diseases*, *161* (2), 198–202. doi:10.1093/infdis/161.2.198
- Lodge, R., Lalonde, J.-P., Lemay, G., & Cohen, E. A. (1997). The membrane-proximal intracytoplasmic tyrosine residue of HIV-1 envelope glycoprotein is critical for basolateral targeting of viral budding in MDCK cells. *EMBO J*, *16*(4), 695–705.
- Long, E. M., Rainwater, S. M. J., Lavreys, L., Mandaliya, K., & Overbaugh, J. (2002). HIV type 1 variants transmitted to women in Kenya require the CCR5 coreceptor for entry, regardless of the genetic complexity of the infecting virus. *AIDS Research and Human Retroviruses*, *18*(8), 567–76. doi:10.1089/088922202753747914
- Lu, L., Zhu, Y., Huang, J., Chen, X., Yang, H., Jiang, S., & Chen, Y.-H. (2008). Surface exposure of the HIV-1 env cytoplasmic tail LLP2 domain during the membrane fusion

- process: interaction with gp41 fusion core. *The Journal of Biological Chemistry*, 283(24), 16723–31. doi:10.1074/jbc.M801083200
- Lynch, R. M., Tran, L., Louder, M. K., Schmidt, S. D., Cohen, M., Dersimonian, R., ... Mascola, J. R. (2012). The development of CD4 binding site antibodies during HIV-1 infection. *Journal of Virology*, 86(14), 7588–95. doi:10.1128/JVI.00734-12
- Lyumkis, D., Julien, J.-P., de Val, N., Cupo, A., Potter, C. S., Klasse, P.-J., ... Ward, A. B. (2013). Cryo-EM structure of a fully glycosylated soluble cleaved HIV-1 envelope trimer. *Science (New York, N.Y.)*, 342(6165), 1484–90. doi:10.1126/science.1245627
- Mabuka, J., Nduati, R., Odem-Davis, K., Peterson, D., & Overbaugh, J. (2012). HIV-specific antibodies capable of ADCC are common in breastmilk and are associated with reduced risk of transmission in women with high viral loads. *PLoS Pathogens*, 8(6), e1002739. doi:10.1371/journal.ppat.1002739
- Magnus, C., Rusert, P., Bonhoeffer, S., Trkola, A., & Regoes, R. R. (2009). Estimating the stoichiometry of human immunodeficiency virus entry. *Journal of Virology*, 83(3), 1523–31. doi:10.1128/JVI.01764-08
- Mao, Y., Wang, L., Gu, C., Herschhorn, A., Desormeaux, A., Finzi, A., ... Sodroski, J. G. (2013). Molecular architecture of the uncleaved HIV-1 envelope glycoprotein trimer. *Proceedings of the National Academy of Sciences*. doi:10.1073/pnas.1307382110
- Markosyan, R. M., Leung, M. Y., & Cohen, F. S. (2009). The six-helix bundle of human immunodeficiency virus Env controls pore formation and enlargement and is initiated at residues proximal to the hairpin turn. *Journal of Virology*, 83(19), 10048–57. doi:10.1128/JVI.00316-09
- Marozsan, A. J., Moore, D. M., Lobritz, M. A., Fraundorf, E., Abraha, A., Reeves, J. D., & Arts, E. J. (2005). Differences in the fitness of two diverse wild-type human immunodeficiency virus type 1 isolates are related to the efficiency of cell binding and entry. *Journal of Virology*, 79(11), 7121–34. doi:10.1128/JVI.79.11.7121-7134.2005
- Martinez-Picado, J., Prado, J. G., Fry, E. E., Pfafferott, K., Leslie, A., Chetty, S., ... Goulder, P. (2006). Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. *Journal of Virology*, 80(7), 3617–23. doi:10.1128/JVI.80.7.3617-3623.2006
- Masciotra, S., Owen, S. M., Rudolph, D., Yang, C., Wang, B., Saksena, N., ... Lal, R. B. (2002). Temporal relationship between V1V2 variation, macrophage replication, and coreceptor adaptation during HIV-1 disease progression. *AIDS (London, England)*, 16(14), 1887–98.
- Mascola, J. R., Lewis, M. G., Stiegler, G., Harris, D., VanCott, T. C., Hayes, D., ... Birx, D. L. (1999). Protection of Macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. *Journal of Virology*, 73(5), 4009–18.

- Mascola, J. R., & Montefiori, D. C. (2010). The role of antibodies in HIV vaccines. *Annual Review of Immunology*, 28, 413–44. doi:10.1146/annurev-immunol-030409-101256
- Mascola, J. R., Stiegler, G., VanCott, T. C., Katinger, H., Carpenter, C. B., Hanson, C. E., ... Lewis, M. G. (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), 207–10. doi:10.1038/72318
- Matsushita, S., Robert-Guroff, M., Rusche, J., Koito, A., Hattori, T., Hoshino, H., ... Putney, S. (1988). Characterization of a human immunodeficiency virus neutralizing monoclonal antibody and mapping of the neutralizing epitope. *Journal of Virology*, 62 (6), 2107–2114.
- Matthews, T., Salgo, M., Greenberg, M., Chung, J., DeMasi, R., & Bolognesi, D. (2004). Enfuvirtide: the first therapy to inhibit the entry of HIV-1 into host CD4 lymphocytes. *Nat Rev Drug Discov*, 3(3), 215–225.
- McCutchan, F. E. (2006). Global epidemiology of HIV. *Journal of Medical Virology*, 78(S1), S7–S12. doi:10.1002/jmv.20599
- McDougal, J. S., Kennedy, M. S., Sligh, J. M., Cort, S. P., Mawle, A., & Nicholson, J. K. (1986). Binding of HTLV-III/LAV to T4+ T cells by a complex of the 110K viral protein and the T4 molecule. *Science (New York, N.Y.)*, 231(4736), 382–5. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/3001934>
- McElrath, M. J., & Haynes, B. F. (2010). Induction of immunity to human immunodeficiency virus type-1 by vaccination. *Immunity*, 33(4), 542–54. doi:10.1016/j.immuni.2010.09.011
- Melikyan, G. B., Markosyan, R. M., Hemmati, H., Delmedico, M. K., Lambert, D. M., & Cohen, F. S. (2000). Evidence that the transition of HIV-1 gp41 into a six-helix bundle, not the bundle configuration, induces membrane fusion. *The Journal of Cell Biology*, 151(2), 413–23.
- Merad, M., Romani, N., & Randolph, G. (2008). Langerhans cells at the interface of medicine, science, and industry. *The Journal of Investigative Dermatology*, 128(2), 251–5. doi:10.1038/sj.jid.5701229
- Migueles, S. A., Sabbaghian, M. S., Shupert, W. L., Bettinotti, M. P., Marincola, F. M., Martino, L., ... Connors, M. (2000). HLA B\*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proceedings of the National Academy of Sciences of the United States of America*, 97(6), 2709–14. doi:10.1073/pnas.050567397
- Mikell, I., Sather, D. N., Kalams, S. A., Altfeld, M., Alter, G., & Stamatatos, L. (2011). Characteristics of the earliest cross-neutralizing antibody response to HIV-1. *PLoS Pathogens*, 7(1), e1001251. doi:10.1371/journal.ppat.1001251
- Miller, C. J., Li, Q., Abel, K., Kim, E.-Y., Ma, Z.-M., Wietgreffe, S., ... Haase, A. T. (2005). Propagation and Dissemination of Infection after Vaginal Transmission of Simian

- Immunodeficiency Virus. *Journal of Virology* , 79 (14 ), 9217–9227.  
doi:10.1128/JVI.79.14.9217-9227.2005
- Miller, M. A., Cloyd, M. W., Liebmann, J., Rinaldo, C. R., Islam, K. R., Wang, S. Z., ... Montelaro, R. C. (1993). Alterations in cell membrane permeability by the lentivirus lytic peptide (LLP-1) of HIV-1 transmembrane protein. *Virology*, 196(1), 89–100.  
doi:10.1006/viro.1993.1457
- Miller, M. A., Garry, R. F., Jaynes, J. M., & Montelaro, R. C. (1991). A structural correlation between lentivirus transmembrane proteins and natural cytolytic peptides. *AIDS Research and Human Retroviruses*, 7(6), 511–9.
- Mlotshwa, M., Riou, C., Chopera, D., de Assis Rosa, D., Ntale, R., Treunicht, F., ... Gray, C. M. (2010). Fluidity of HIV-1-specific T-cell responses during acute and early subtype C HIV-1 infection and associations with early disease progression. *Journal of Virology*, 84(22), 12018–29. doi:10.1128/JVI.01472-10
- Moldt, B., Rakasz, E. G., Schultz, N., Chan-Hui, P.-Y., Swiderek, K., Weisgrau, K. L., ... Burton, D. R. (2012). Highly potent HIV-specific antibody neutralization in vitro translates into effective protection against mucosal SHIV challenge in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, 109(46), 18921–5.  
doi:10.1073/pnas.1214785109
- Monno, L., Saracino, A., Scudeller, L., Punzi, G., Brindicci, G., Altamura, M., ... Angarano, G. (2011). Impact of mutations outside the V3 region on coreceptor tropism phenotypically assessed in patients infected with HIV-1 subtype B. *Antimicrobial Agents and Chemotherapy*, 55(11), 5078–84. doi:10.1128/AAC.00743-11
- Montefiori, D. C., Robinson, W. E., & Mitchell, W. M. (1988). Role of protein N-glycosylation in pathogenesis of human immunodeficiency virus type 1. *Proceedings of the National Academy of Sciences* , 85 (23 ), 9248–9252.
- Moody, M. A., Yates, N. L., Amos, J. D., Drinker, M. S., Eudailey, J. A., Gurley, T. C., ... Haynes, B. F. (2012). HIV-1 gp120 Vaccine Induces Affinity Maturation in both New and Persistent Antibody Clonal Lineages. *Journal of Virology* , 86 (14 ), 7496–7507.  
doi:10.1128/JVI.00426-12
- Moog, C., Dereuddre-Bosquet, N., Teillaud, J.-L., Biedma, M. E., Holl, V., Van Ham, G., ... Le Grand, R. (2014). Protective effect of vaginal application of neutralizing and nonneutralizing inhibitory antibodies against vaginal SHIV challenge in macaques. *Mucosal Immunology*, 7(1), 46–56. doi:10.1038/mi.2013.23
- Moore, C. B., John, M., James, I. R., Christiansen, F. T., Witt, C. S., & Mallal, S. A. (2002). Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. *Science (New York, N.Y.)*, 296(5572), 1439–43. doi:10.1126/science.1069660
- Moore, J. P., Cao, Y., Leu, J., Qin, L., Korber, B., & Ho, D. D. (1996). Inter- and intraclade neutralization of human immunodeficiency virus type 1: genetic clades do not correspond to neutralization serotypes but partially correspond to gp120 antigenic serotypes. *Journal of Virology*, 70(1), 427–44.

- Moore, J. P., Parren, P. W., & Burton, D. R. (2001). Genetic subtypes, humoral immunity, and human immunodeficiency virus type 1 vaccine development. *Journal of Virology*, 75(13), 5721–9. doi:10.1128/JVI.75.13.5721-5729.2001
- Moore, P. L., Crooks, E. T., Porter, L., Zhu, P., Cayanan, C. S., Grise, H., ... Irol, J. V. (2006). Nature of Nonfunctional Envelope Proteins on the Surface of Human Immunodeficiency Virus Type 1, 80(5), 2515–2528. doi:10.1128/JVI.80.5.2515
- Moore, P. L., Gray, E. S., Choge, I. A., Ranchobe, N., Mlisana, K., Abdool Karim, S. S., ... Morris, L. (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), 1860–9. doi:10.1128/JVI.02187-07
- Moore, P. L., Gray, E. S., & Morris, L. (2009). Specificity of the autologous neutralizing antibody response. *Current Opinion in HIV and AIDS*, 4(5), 358–63. doi:10.1097/COH.0b013e32832ea7e8
- Moore, P. L., Gray, E. S., Wibmer, C. K., Bhiman, J. N., Nonyane, M., Sheward, D. J., ... Morris, L. (2012). Evolution of an HIV glycan-dependent broadly neutralizing antibody epitope through immune escape. *Nature Medicine*, (October), 1–6. doi:10.1038/nm.2985
- Moore, P. L., Ranchobe, N., Lambson, B. E., Gray, E. S., Cave, E., Abrahams, M.-R., ... Morris, L. (2009). Limited neutralizing antibody specificities drive neutralization escape in early HIV-1 subtype C infection. *PLoS Pathogens*, 5(9), e1000598. doi:10.1371/journal.ppat.1000598
- Mufhandu, H. T., Gray, E. S., Madiga, M. C., Tumba, N., Alexandre, K. B., Khoza, T., ... Khati, M. (2012). UCLA1, a synthetic derivative of a gp120 RNA aptamer, inhibits entry of human immunodeficiency virus type 1 subtype C. *Journal of Virology*, 86(9), 4989–99. doi:10.1128/JVI.06893-11
- Mulampaka, S. N., & Dixit, N. M. (2011). Estimating the threshold surface density of Gp120-CCR5 complexes necessary for HIV-1 envelope-mediated cell-cell fusion. *PLoS One*, 6(5), e19941. doi:10.1371/journal.pone.0019941
- Muñoz-Barroso, I., Salzwedel, K., Hunter, E., & Blumenthal, R. (1999). Role of the Membrane-Proximal Domain in the Initial Stages of Human Immunodeficiency Virus Type 1 Envelope Glycoprotein-Mediated Membrane Fusion. *Journal of Virology*, 73(7), 6089–6092.
- Murakami, T., & Freed, E. O. (2000a). Genetic evidence for an interaction between human immunodeficiency virus type 1 matrix and alpha-helix 2 of the gp41 cytoplasmic tail. *Journal of Virology*, 74(8), 3548–54.
- Murakami, T., & Freed, E. O. (2000b). The long cytoplasmic tail of gp41 is required in a cell type-dependent manner for HIV-1 envelope glycoprotein incorporation into virions. *Proceedings of the National Academy of Sciences of the United States of America*, 97(1), 343–8.

- Murrell, B., Wertheim, J. O., Moola, S., Weighill, T., Scheffler, K., & Kosakovsky Pond, S. L. (2012). Detecting individual sites subject to episodic diversifying selection. *PLoS Genetics*, 8(7), e1002764. doi:10.1371/journal.pgen.1002764
- Muse, S. V., & Gaut, B. S. (1994). A likelihood approach for comparing synonymous and nonsynonymous nucleotide substitution rates, with application to the chloroplast genome. *Molecular Biology and Evolution*, 11 (5), 715–724.
- Muster, T., Steindl, F., Purtscher, M., Trkola, A., Klima, A., Himmler, G., ... Katinger, H. (1993). A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. *Journal of Virology*, 67(11), 6642–7.
- Nag, P., Kim, J., Sapiega, V., Landay, A. L., Bremer, J. W., Laingam, S., ... Baum, L. L. (2004). Women with Cervicovaginal Antibody-Dependent Cell-Mediated Cytotoxicity Have Lower Genital HIV-1 RNA Loads. *Journal of Infectious Diseases*, 190 (11), 1970–1978. doi:10.1086/425582
- Nawaz, F., Cicala, C., Van Ryk, D., Block, K. E., Jelicic, K., McNally, J. P., ... Arthos, J. (2011). The Genotype of Early-Transmitting HIV gp120s Promotes  $\alpha_4\beta_7$ -Reactivity, Revealing  $\alpha_4\beta_7^+$ /CD4<sup>+</sup> T cells As Key Targets in Mucosal Transmission. *PLoS Pathog*, 7(2), e1001301.
- Ngandu, N. K., Scheffler, K., Moore, P., Woodman, Z., Martin, D., & Seoighe, C. (2008). Extensive purifying selection acting on synonymous sites in HIV-1 Group M sequences. *Virology Journal*, 5(1), 160. doi:10.1186/1743-422X-5-160
- Ngumbela, K. C., Day, C. L., Mncube, Z., Nair, K., Ramduth, D., Thobakgale, C., ... Walker, B. D. (2008). Targeting of a CD8 T cell env epitope presented by HLA-B\*5802 is associated with markers of HIV disease progression and lack of selection pressure. *AIDS Research and Human Retroviruses*, 24(1), 72–82. doi:10.1089/aid.2007.0124
- Nielsen, R., & Yang, Z. (1998). Likelihood Models for Detecting Positively Selected Amino Acid Sites and Applications to the HIV-1 Envelope Gene. *Genetics*, 148 (3), 929–936. Retrieved from <http://www.genetics.org/content/148/3/929.abstract>
- Nofemela, A., Bandawe, G., Thebus, R., Marais, J., Wood, N., Hoffmann, O., ... Williamson, C. (2011). Defining the human immunodeficiency virus type 1 transmission genetic bottleneck in a region with multiple circulating subtypes and recombinant forms. *Virology*, 415(2), 107–13. doi:10.1016/j.virol.2010.12.027
- Nyambi, P. N., Nkengasong, J., Lewi, P., Andries, K., Janssens, W., Franssen, K., ... van der Groen, G. (1996). Multivariate analysis of human immunodeficiency virus type 1 neutralization data. *Journal of Virology*, 70(9), 6235–43.
- O'Brien, W. A., Koyanagi, Y., Namazie, A., Zhao, J.-Q., Diagne, A., Idler, K., ... Chen, I. S. Y. (1990). HIV-1 tropism for mononuclear phagocytes can be determined by regions of gp120 outside the CD4-binding domain. *Nature*, 348(6296), 69–73.
- Ochsenbauer, C., Edmonds, T. G., Ding, H., Keele, B. F., Decker, J., Salazar, M. G., ... Kappes, J. C. (2012). Generation of transmitted/founder HIV-1 infectious molecular

- clones and characterization of their replication capacity in CD4 T lymphocytes and monocyte-derived macrophages. *Journal of Virology*, 86(5), 2715–28.  
doi:10.1128/JVI.06157-11
- Ohno, H., Aguilar, R. C., Fournier, M. C., Hennecke, S., Cosson, P., & Bonifacino, J. S. (1997). Interaction of endocytic signals from the HIV-1 envelope glycoprotein complex with members of the adaptor medium chain family. *Virology*, 238(2), 305–15.  
doi:10.1006/viro.1997.8839
- Oliva, A., Kinter, A. L., Vaccarezza, M., Rubbert, A., Catanzaro, A., Moir, S., ... Fauci, A. S. (1998). Natural killer cells from human immunodeficiency virus (HIV)-infected individuals are an important source of CC-chemokines and suppress HIV-1 entry and replication in vitro. *The Journal of Clinical Investigation*, 102(1), 223–231.  
doi:10.1172/JCI2323
- Olshevsky, U., Helseth, E., Furman, C., Li, J., Haseltine, W., & Sodroski, J. (1990). Identification of individual human immunodeficiency virus type 1 gp120 amino acids important for CD4 receptor binding. *Journal of Virology*, 64 (12), 5701–5707.
- Ottonello, L., Corcione, A., Tortolina, G., Airoidi, I., Albesiano, E., Favre, A., ... Dallegri, F. (1999). rC5a Directs the In Vitro Migration of Human Memory and Naive Tonsillar B Lymphocytes: Implications for B Cell Trafficking in Secondary Lymphoid Tissues. *The Journal of Immunology*, 162 (11), 6510–6517.
- Overbaugh, J., & Rudensey, L. M. (1992). Alterations in potential sites for glycosylation predominate during evolution of the simian immunodeficiency virus envelope gene in macaques. *Journal of Virology*, 66 (10), 5937–5948.
- Overbaugh, J., Rudensey, L. M., Papenhausen, M. D., Benveniste, R. E., & Morton, W. R. (1991). Variation in simian immunodeficiency virus env is confined to V1 and V4 during progression to simian AIDS. *Journal of Virology*, 65(12), 7025–31.
- Packard, B. Z., Telford, W. G., Komoriya, A., & Henkart, P. A. (2007). Granzyme B activity in target cells detects attack by cytotoxic lymphocytes. *Journal of Immunology (Baltimore, Md. : 1950)*, 179(6), 3812–20.
- Palker, T. J., Clark, M. E., Langlois, A. J., Matthews, T. J., Weinhold, K. J., Randall, R. R., ... Haynes, B. F. (1988). Type-specific neutralization of the human immunodeficiency virus with antibodies to env-encoded synthetic peptides. *Proceedings of the National Academy of Sciences*, 85 (6), 1932–1936.
- Palmer, C., Balfe, P., Fox, D., May, J. C., Frederiksson, R., Fenyö, E.-M., & Mckeating, J. A. (1996). Functional Characterization of the V1V2 Region of Human Immunodeficiency Virus Type I. *Virology*, 220(2), 436–449.
- Pancera, M., Majeed, S., Ban, Y.-E. A., Chen, L., Huang, C., Kong, L., ... Kwong, P. D. (2010). Structure of HIV-1 gp120 with gp41-interactive region reveals layered envelope architecture and basis of conformational mobility. *Proceedings of the National Academy of Sciences*, 107 (3), 1166–1171. doi:10.1073/pnas.0911004107

- Pancera, M., McLellan, J. S., Wu, X., Zhu, J., Changela, A., Schmidt, S. D., ... Kwong, P. D. (2010). Crystal structure of PG16 and chimeric dissection with somatically related PG9: structure-function analysis of two quaternary-specific antibodies that effectively neutralize HIV-1. *Journal of Virology*, *84*(16), 8098–110. doi:10.1128/JVI.00966-10
- Pang, H.-B., Hevroni, L., Kol, N., Eckert, D. M., Tsvitov, M., Kay, M. S., & Rousso, I. (2013). Virion stiffness regulates immature HIV-1 entry. *Retrovirology*, *10*(1), 4. doi:10.1186/1742-4690-10-4
- Parham, P., Adams, E. J., & Arnett, K. L. (1995). The origins of HLA-A,B,C polymorphism. *Immunological Reviews*, *143*, 141–80.
- Parren, P. W., Marx, P. A., Hessel, A. J., Luckay, A., Harouse, J., Cheng-Mayer, C., ... Burton, D. R. (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), 8340–7.
- Parrish, N. F., Gao, F., Li, H., Giorgi, E. E., Barbian, H. J., Parrish, E. H., ... Hahn, B. H. (2013). Phenotypic properties of transmitted founder HIV-1. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(17), 6626–33. doi:10.1073/pnas.1304288110
- Parrish, N. F., Wilen, C. B., Banks, L. B., Iyer, S. S., Pfaff, J. M., Salazar-Gonzalez, J. F., ... Doms, R. W. (2012). Transmitted/Founder and Chronic Subtype C HIV-1 Use CD4 and CCR5 Receptors with Equal Efficiency and Are Not Inhibited by Blocking the Integrin  $\alpha 4\beta 7$ . *PLoS Pathog*, *8*(5), e1002686.
- Pastori, C., Tudor, D., Diomede, L., Drillet, A. S., Jegerlehner, A., Röhn, T. A., ... Lopalco, L. (2012). Virus like particle based strategy to elicit HIV-protective antibodies to the alpha-helic regions of gp41. *Virology*, *431*(1–2), 1–11. doi:http://dx.doi.org/10.1016/j.virol.2012.05.005
- Peeters, M. (2001). The genetic variability of HIV-1 and its implications. *Transfusion Clinique et Biologique*, *8*(3), 222–225. doi:http://dx.doi.org/10.1016/S1246-7820(01)00131-8
- Peeters, M., Liegeois, F., Torimiro, N., Bourgeois, A., Mpoudi, E., Vergne, L., ... Saragosti, S. (1999). Characterization of a Highly Replicative Intergroup M/O Human Immunodeficiency Virus Type 1 Recombinant Isolated from a Cameroonian Patient. *Journal of Virology*, *73* (9), 7368–7375.
- Pejchal, R., Doores, K. J., Walker, L. M., Khayat, R., Huang, P., Wang, S., ... Wilson, I. A. (2012). NIH Public Access, *334*(6059), 1097–1103. doi:10.1126/science.1213256.A
- Pereyra, F., Jia, X., McLaren, P. J., Telenti, A., de Bakker, P. I. W., Walker, B. D., ... Zhao, M. (2010). The major genetic determinants of HIV-1 control affect HLA class I peptide presentation. *Science (New York, N.Y.)*, *330*(6010), 1551–7. doi:10.1126/science.1195271

- Peris, J. B., Davis, P., Cuevas, J. M., Nebot, M. R., & Sanjuán, R. (2010). Distribution of Fitness Effects Caused by Single-Nucleotide Substitutions in Bacteriophage  $\phi$ 1. *Genetics*, 185(2), 603–609. doi:10.1534/genetics.110.115162
- Perrin, L., Kaiser, L., & Yerly, S. (2003). Travel and the spread of HIV-1 genetic variants. *The Lancet Infectious Diseases*, 3(1), 22–27. doi:http://dx.doi.org/10.1016/S1473-3099(03)00484-5
- Piantadosi, A., Panteleeff, D., Blish, C. a, Baeten, J. M., Jaoko, W., McClelland, R. S., & Overbaugh, J. (2009). Breadth of neutralizing antibody response to human immunodeficiency virus type 1 is affected by factors early in infection but does not influence disease progression. *Journal of Virology*, 83(19), 10269–74. doi:10.1128/JVI.01149-09
- Picker, L. J., & Deeks, S. G. (2013). HIV: Antibodies advance the search for a cure. *Nature*. doi:10.1038/nature12703
- Piller, S. C., Dubay, J. W., Derdeyn, C. A., & Hunter, E. (2000). Mutational analysis of conserved domains within the cytoplasmic tail of gp41 from human immunodeficiency virus type 1: effects on glycoprotein incorporation and infectivity. *Journal of Virology*, 74(24), 11717–23.
- Piot, P., Bartos, M., Ghys, P. D., Walker, N., & Schwartlander, B. (2001). The global impact of HIV/AIDS. *Nature*, 410(6831), 968–973. Retrieved from http://dx.doi.org/10.1038/35073639
- Pollakis, G., Abebe, A., Kliphuis, A., Chalaby, M. I. M., Bakker, M., Mengistu, Y., ... Paxton, W. A. (2004). Phenotypic and Genotypic Comparisons of CCR5- and CXCR4-Tropic Human Immunodeficiency Virus Type 1 Biological Clones Isolated from Subtype C-Infected Individuals. *Journal of Virology*, 78(6), 2841–2852. doi:10.1128/JVI.78.6.2841-2852.2004
- Pollara, J., Bonsignori, M., Moody, M. A., Pazgier, M., Haynes, B. F., & Ferrari, G. (2013). Epitope Specificity of Human Immunodeficiency Virus-1 Antibody Dependent Cellular Cytotoxicity [ ADCC ] Responses Ab-Fab Ab-Fc HIV-1, 378–387.
- Pollara, J., Hart, L., Brewer, F., Pickeral, J., Packard, B. Z., Hoxie, J. a, ... Ferrari, G. (2011). High-throughput quantitative analysis of HIV-1 and SIV-specific ADCC-mediating antibody responses. *Cytometry. Part A : The Journal of the International Society for Analytical Cytology*, 79(8), 603–12. doi:10.1002/cyto.a.21084
- Pollard, S. R., Rosa, M. D., Rosa, J. J., & Wiley, D. C. (1992). Truncated variants of gp120 bind CD4 with high affinity and suggest a minimum CD4 binding region. *The EMBO Journal*, 11(2), 585–91.
- Pope, M., & Haase, A. T. (2003). Transmission, acute HIV-1 infection and the quest for strategies to prevent infection. *Nature Medicine*, 9(7), 847–52. doi:10.1038/nm0703-847

- Posner, M. R., Elboim, H. S., Cannon, T., Cavacini, L., & Hideshima, T. (1992). Functional activity of an HIV-1 neutralizing IgG human monoclonal antibody: ADCC and complement-mediated lysis. *AIDS Research and Human Retroviruses*, 8(5), 553–8.
- Poss, M., & Overbaugh, J. (1999). Variants from the diverse virus population identified at seroconversion of a clade A human immunodeficiency virus type 1-infected woman have distinct biological properties. *Journal of Virology*, 73(7), 5255–64.
- Poumbourios, P., el Ahmar, W., McPhee, D. A., & Kemp, B. E. (1995). Determinants of human immunodeficiency virus type 1 envelope glycoprotein oligomeric structure. *Journal of Virology*, 69(2), 1209–1218.
- Powell, R. L. R., Kinge, T., & Nyambi, P. N. (2010). Infection by discordant strains of HIV-1 markedly enhances the neutralizing antibody response against heterologous virus. *Journal of Virology*, 84(18), 9415–26. doi:10.1128/JVI.02732-09
- Powers, K. A., Poole, C., Pettifor, A. E., & Cohen, M. S. (2008). Rethinking the heterosexual infectivity of HIV-1: a systematic review and meta-analysis. *The Lancet Infectious Diseases*, 8(9), 553–63. doi:10.1016/S1473-3099(08)70156-7
- Preston, B. D., Poiesz, B. J., & Loeb, L. A. (1988). Fidelity of HIV-1 reverse transcriptase. *Science (New York, N.Y.)*, 242(4882), 1168–71.
- Quiñones-Mateu, M. E., Ball, S. C., Marozsan, A. J., Torre, V. S., Albright, J. L., Vanham, G., ... Arts, E. J. (2000). A Dual Infection/Competition Assay Shows a Correlation between Ex Vivo Human Immunodeficiency Virus Type 1 Fitness and Disease Progression. *Journal of Virology*, 74(19), 9222–9233. doi:10.1128/JVI.74.19.9222-9233.2000
- Rademeyer, C., Moore, P. L., Taylor, N., Martin, D. P., Choge, I. a, Gray, E. S., ... Williamson, C. (2007). Genetic characteristics of HIV-1 subtype C envelopes inducing cross-neutralizing antibodies. *Virology*, 368(1), 172–81. doi:10.1016/j.virol.2007.06.013
- Ragupathy, V., Zhao, J., Wood, O., Tang, S., Lee, S., Nyambi, P., & Hewlett, I. (2011). Identification of new, emerging HIV-1 unique recombinant forms and drug resistant viruses circulating in Cameroon. *Virology Journal*, 8(1), 185. doi:10.1186/1743-422X-8-185
- Raska, M., & Novak, J. (n.d.). Involvement of Envelope-Glycoprotein Glycans in HIV-1 Biology and Infection. *Archivum Immunologiae et Therapiae Experimentalis*, 58(3), 191–208. doi:10.1007/s00005-010-0072-3
- Reks-Ngarm, S., Pitisuttithum, P., Nitayaphan, S., Kaewkungwal, J., Chiu, J., Paris, R., ... Kim, J. H. (2009). Vaccination with AIVAC and AIDSVAX to prevent HIV-1 Infection in Thailand. *The New England Journal of Medicine*, 361(23), 2209–2220.
- Richman, D. D., Wrin, T., Little, S. J., & Petropoulos, C. J. (2003). Rapid evolution of the neutralizing antibody response to HIV type 1 infection. *Proceedings of the National Academy of Sciences*, 100(7), 4144–4149. doi:10.1073/pnas.0630530100

- Riedner, G., Rusizoka, M., Hoffmann, O., Nichombe, F., Lyamuya, E., Mmbando, D., ... Grosskurth, H. (2003). Baseline survey of sexually transmitted infections in a cohort of female bar workers in Mbeya Region, Tanzania. *Sexually Transmitted Infections*, 79(5), 382–7.
- Robertson DL, Anderson JP, Bradac JA, Carr JK, Foley B, Funkhouser RK, Gao F, Hahn BH, Kalish ML, Kuiken C, Learn GH, Leitner T, McCutchan F, Osmanov S, Peeters M, Pieniazek D, Salminen M, Sharp PM, Wolinsky S, K. B. (2000). HIV-1 Nomenclature Proposal. *Science*, 288(5463), 55.
- Rolland, M., Edlefsen, P. T., Larsen, B. B., Tovanabutra, S., Sanders-Buell, E., Hertz, T., ... Kim, J. H. (2012). Increased HIV-1 vaccine efficacy against viruses with genetic signatures in Env V2. *Nature*, 1–5. doi:10.1038/nature11519
- Rong, R., Bibollet-Ruche, F., Mulenga, J., Allen, S., Blackwell, J. L., & Derdeyn, C. a. (2007). Role of V1V2 and other human immunodeficiency virus type 1 envelope domains in resistance to autologous neutralization during clade C infection. *Journal of Virology*, 81(3), 1350–9. doi:10.1128/JVI.01839-06
- Rong, R., Gnanakaran, S., Decker, J. M., Bibollet-Ruche, F., Taylor, J., Sfakianos, J. N., ... Derdeyn, C. a. (2007). Unique mutational patterns in the envelope alpha 2 amphipathic helix and acquisition of length in gp120 hypervariable domains are associated with resistance to autologous neutralization of subtype C human immunodeficiency virus type 1. *Journal of Virology*, 81(11), 5658–68. doi:10.1128/JVI.00257-07
- Rong, R., Gnanakaran, S., Decker, J. M., Bibollet-ruche, F., Taylor, J., Sfakianos, J. N., ... Jolla, L. (2007). C a 6 #, (March). doi:10.1128/JVI.00257-07
- Rong, R., Li, B., Lynch, R. M., Haaland, R. E., Murphy, M. K., Mulenga, J., ... Derdeyn, C. A. (2009). Escape from autologous neutralizing antibodies in acute/early subtype C HIV-1 infection requires multiple pathways. *PLoS Pathogens*, 5(9), e1000594. doi:10.1371/journal.ppat.1000594
- Rook, A. H., Lane, H. C., Folks, T., McCoy, S., Alter, H., & Fauci, A. S. (1987). Sera from HTLV-III/LAV antibody-positive individuals mediate antibody-dependent cellular cytotoxicity against HTLV-III/LAV-infected T cells. *The Journal of Immunology*, 138(4), 1064–1067. Retrieved from <http://jimmunol.org/content/138/4/1064.abstract>
- Rot, A., & von Andrian, U. H. (2004). Chemokines in Innate and Adaptive Host Defense: Basic Chemokines Grammar for Immune Cells. *Annual Review of Immunology*, 22(1), 891–928. doi:10.1146/annurev.immunol.22.012703.104543
- Rousseau, C. M., Daniels, M. G., Carlson, J. M., Kadie, C., Crawford, H., Prendergast, A., ... Mullins, J. I. (2008). HLA class I-driven evolution of human immunodeficiency virus type 1 subtype c proteome: immune escape and viral load. *Journal of Virology*, 82(13), 6434–46. doi:10.1128/JVI.02455-07
- Rowell, J. F., Stanhope, P. E., & Siliciano, R. F. (1995). Endocytosis of endogenously synthesized HIV-1 envelope protein. Mechanism and role in processing for association with class II MHC. *The Journal of Immunology*, 155(1), 473–488.

- Rusche, J. R., Javaherian, K., McDanal, C., Petro, J., Lynn, D. L., Grimaila, R., ... Fischinger, P. J. (1988). Antibodies that inhibit fusion of human immunodeficiency virus-infected cells bind a 24-amino acid sequence of the viral envelope, gp120 . *Proceedings of the National Academy of Sciences* , 85 (9 ) , 3198–3202.
- Saathoff, E., Pritsch, M., Geldmacher, C., Hoffmann, O., Koehler, R. N., Maboko, L., ... Hoelscher, M. (2010). Viral and host factors associated with the HIV-1 viral load setpoint in adults from Mbeya Region, Tanzania. *Journal of Acquired Immune Deficiency Syndromes* (1999), 54(3), 324–30.
- Sagar, M., Wu, X., Lee, S., & Overbaugh, J. (2006). Human Immunodeficiency Virus Type 1 V1-V2 Envelope Loop Sequences Expand and Add Glycosylation Sites over the Course of Infection , and These Modifications Affect Antibody Neutralization Sensitivity † , 80(19), 9586–9598. doi:10.1128/JVI.00141-06
- Sajadi, M. M., Guan, Y., DeVico, A. L., Seaman, M. S., Hossain, M., Lewis, G. K., & Redfield, R. R. (2011). Correlation between circulating HIV-1 RNA and broad HIV-1 neutralizing antibody activity. *Journal of Acquired Immune Deficiency Syndromes* (1999), 57(1), 9–15. doi:10.1097/QAI.0b013e3182100c1b
- Salazar-Gonzalez, J. F., Bailes, E., Pham, K. T., Salazar, M. G., Guffey, M. B., Keele, B. F., ... Hahn, B. H. (2008). Deciphering Human Immunodeficiency Virus Type 1 Transmission and Early Envelope Diversification by Single-Genome Amplification and Sequencing. *Journal of Virology* , 82 (8 ) , 3952–3970. doi:10.1128/JVI.02660-07
- Salzwedel, K., West, J. T., & Hunter, E. (1999). A Conserved Tryptophan-Rich Motif in the Membrane-Proximal Region of the Human Immunodeficiency Virus Type 1 gp41 Ectodomain Is Important for Env-Mediated Fusion and Virus Infectivity . *Journal of Virology* , 73 (3 ) , 2469–2480.
- Sanders, R. W., Vesanen, M., Schuelke, N., Master, A., Schiffner, L., Kalyanaraman, R., ... Moore, J. P. (2002). Stabilization of the soluble, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency virus type 1. *Journal of Virology* , 76(17), 8875–89.
- Sather, D. N., Armann, J., Ching, L. K., Mavrantoni, A., Sellhorn, G., Caldwell, Z., ... Stamatatos, L. (2009a). Factors Associated with the Development of Cross-Reactive Neutralizing Antibodies during Human Immunodeficiency Virus Type 1 Infection , 83(2), 757–769. doi:10.1128/JVI.02036-08
- Sather, D. N., Armann, J., Ching, L. K., Mavrantoni, A., Sellhorn, G., Caldwell, Z., ... Stamatatos, L. (2009b). Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection. *Journal of Virology* , 83(2), 757–69. doi:10.1128/JVI.02036-08
- Sather, D. N., Carbonetti, S., Kehayia, J., Kraft, Z., Mikell, I., Scheid, J. F., ... Stamatatos, L. (2012). Broadly neutralizing antibodies developed by an HIV-positive elite neutralizer exact a replication fitness cost on the contemporaneous virus. *Journal of Virology* , 86(23), 12676–85. doi:10.1128/JVI.01893-12

- Scarlati, G., Tresoldi, E., Björndal, A., Fredriksson, R., Colognesi, C., Deng, H. K., ... Lusso, P. (1997). In vivo evolution of HIV-1 co-receptor usage and sensitivity to chemokine-mediated suppression. *Nature Medicine*, 3(11), 1259–65. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9359702>
- Scheffler, K., Martin, D. P., & Seoighe, C. (2006). Robust inference of positive selection from recombining coding sequences. *Bioinformatics (Oxford, England)*, 22(20), 2493–9. doi:10.1093/bioinformatics/btl427
- Schief, W. R., Ban, Y.-E. A., & Stamatatos, L. (2009). Challenges for structure-based HIV vaccine design. *Current Opinion in HIV and AIDS*, 4(5), 431–40. doi:10.1097/COH.0b013e32832e6184
- Schneidewind, A., Brockman, M. A., Sidney, J., Wang, Y. E., Chen, H., Suscovich, T. J., ... Allen, T. M. (2008). Structural and functional constraints limit options for cytotoxic T-lymphocyte escape in the immunodominant HLA-B27-restricted epitope in human immunodeficiency virus type 1 capsid. *Journal of Virology*, 82(11), 5594–605. doi:10.1128/JVI.02356-07
- Schneidewind, A., Brockman, M. A., Yang, R., Adam, R. I., Li, B., Le Gall, S., ... Allen, T. M. (2007). Escape from the dominant HLA-B27-restricted cytotoxic T-lymphocyte response in Gag is associated with a dramatic reduction in human immunodeficiency virus type 1 replication. *Journal of Virology*, 81(22), 12382–93. doi:10.1128/JVI.01543-07
- Seaman, M. S., Janes, H., Hawkins, N., Grandpre, L. E., Devoy, C., Giri, A., ... Mascola, J. R. (2010). Tiered categorization of a diverse panel of HIV-1 Env pseudoviruses for assessment of neutralizing antibodies. *Journal of Virology*, 84(3), 1439–52. doi:10.1128/JVI.02108-09
- Seibert, C. W., Kaminski, M., Philipp, J., Rubbenstroth, D., Albrecht, R. A., Schwalm, F., ... Palese, P. (2010). Oseltamivir-Resistant Variants of the 2009 Pandemic H1N1 Influenza A Virus Are Not Attenuated in the Guinea Pig and Ferret Transmission Models. *Journal of Virology*, 84(21), 11219–11226. doi:10.1128/JVI.01424-10
- Sewell, A. K., Price, D. A., Oxenius, A., Kelleher, A. D., & Phillips, R. E. (2000). Cytotoxic T lymphocyte responses to human immunodeficiency virus: control and escape. *Stem Cells (Dayton, Ohio)*, 18(4), 230–44. doi:10.1634/stemcells.18-4-230
- Shang, L., & Hunter, E. (2010). Residues in the membrane-spanning domain core modulate conformation and fusogenicity of the HIV-1 envelope glycoprotein. *Virology*, 404(2), 158–167.
- Shang, L., Yue, L., & Hunter, E. (2008). Role of the Membrane-Spanning Domain of Human Immunodeficiency Virus Type 1 Envelope Glycoprotein in Cell-Cell Fusion and Virus Infection. *Journal of Virology*, 82(11), 5417–5428. doi:10.1128/JVI.02666-07
- Sharp, P. M., & Hahn, B. H. (2010). The evolution of HIV-1 and the origin of AIDS. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 365(1552), 2487–94. doi:10.1098/rstb.2010.0031

- Shaw, G. M., & Hunter, E. (2012). HIV Transmission. *Cold Spring Harbor Perspectives in Medicine* , 2 (11 ). doi:10.1101/cshperspect.a006965
- Shibata, R., Igarashi, T., Haigwood, N., Buckler-White, A., Ogert, R., Ross, W., ... Martin, M. A. (1999). Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. *Nature Medicine* , 5(2), 204–10. doi:10.1038/5568
- Shioda, T., Levy, J. A., & Cheng-Mayer, C. (1991). Macrophage and T cell-line tropisms of HIV-1 are determined by specific regions of the envelope gp120 gene. *Nature* , 349(6305), 167–169.
- Shioda, T., Oka, S., Ida, S., Nokihara, K., Toriyoshi, H., Mori, S., ... Nagai, Y. (1994). A naturally occurring single basic amino acid substitution in the V3 region of the human immunodeficiency virus type 1 env protein alters the cellular host range and antigenic structure of the virus. *Journal of Virology* , 68 (12 ), 7689–7696.
- Shioda, T., Oka, S., Xin, X., Liu, H., Harukuni, R., Kurotani, A., ... Nagai, Y. (1997). In vivo sequence variability of human immunodeficiency virus type 1 envelope gp120: association of V2 extension with slow disease progression. *Journal of Virology* , 71 (7 ), 4871–4881.
- Shriner, D., Nickle, D. C., Jensen, M. A., & Mullins, J. I. (2003). Potential impact of recombination on sitewise approaches for detecting positive natural selection. *Genetical Research* , 81(2), 115–21.
- Simek, M. D., Rida, W., Priddy, F. H., Pung, P., Carrow, E., Laufer, D. S., ... Koff, W. C. (2009). Human immunodeficiency virus type 1 elite neutralizers: individuals with broad and potent neutralizing activity identified by using a high-throughput neutralization assay together with an analytical selection algorithm. *Journal of Virology* , 83(14), 7337–48. doi:10.1128/JVI.00110-09
- Smalls-Mantey, A., Doria-Rose, N., Klein, R., Patamawenu, A., Migueles, S. a, Ko, S.-Y., ... Connors, M. (2012). Antibody-dependent cellular cytotoxicity against primary HIV-infected CD4+ T cells is directly associated with the magnitude of surface IgG binding. *Journal of Virology* , 86(16), 8672–80. doi:10.1128/JVI.00287-12
- Snow, R. W., Guerra, C. A., Noor, A. M., Myint, H. Y., & Hay, S. I. (2005). The global distribution of clinical episodes of Plasmodium falciparum malaria. *Nature* , 434(7030), 214–7. doi:10.1038/nature03342
- Song, H., Pavlicek, J. W., Cai, F., Bhattacharya, T., Li, H., Iyer, S. S., ... Gao, F. (2012). Impact of immune escape mutations on HIV-1 fitness in the context of the cognate transmitted/founder genome. *Retrovirology* , 9, 89. doi:10.1186/1742-4690-9-89
- Spear, G. T., Landay, A. L., Sullivan, B. L., Dittel, B., & Lint, T. F. (1990). Activation of complement on the surface of cells infected by human immunodeficiency virus. *The Journal of Immunology* , 144 (4 ), 1490–1496.

- Stamatatos, L., Morris, L., Burton, D. R., & Mascola, J. R. (2009). Neutralizing antibodies generated during natural HIV-1 infection: good news for an HIV-1 vaccine? *Nature Medicine*, *15*(8), 866–70. doi:10.1038/nm.1949
- Starcich, B. R., Hahn, B. H., Shaw, G. M., McNeely, P. D., Modrow, S., Wolf, H., ... Wong-Staal, F. (1986). Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. *Cell*, *45*(5), 637–648.
- Stiegler, G., Kunert, R., Purtscher, M., Wolbank, S., Voglauer, R., Steindl, F., & Katinger, H. (2001). A potent cross-clade neutralizing human monoclonal antibody against a novel epitope on gp41 of human immunodeficiency virus type 1. *AIDS Research and Human Retroviruses*, *17*(18), 1757–65. doi:10.1089/08892220152741450
- Stratov, I., Chung, A., & Kent, S. J. (2008). Robust NK Cell-Mediated Human Immunodeficiency Virus (HIV)-Specific Antibody-Dependent Responses in HIV-Infected Subjects. *Journal of Virology*, *82*(11), 5450–5459. doi:10.1128/JVI.01952-07
- Tajima, F. (1989). Statistical Method for Testing the Neutral Mutation Hypothesis by DNA Polymorphism. *Genetics*, *123*(3), 585–595.
- Takehisa, J., Zekeng, L., Ido, E., Yamaguchi-Kabata, Y., Mboudjeka, I., Harada, Y., ... Hayami, M. (1999). Human immunodeficiency virus type 1 intergroup (M/O) recombination in cameroon. *Journal of Virology*, *73*(8), 6810–20.
- Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, *24*(8), 1596–9. doi:10.1093/molbev/msm092
- Tan, K., Liu, J., Wang, J., Shen, S., & Lu, M. (1997). Atomic structure of a thermostable subdomain of HIV-1 gp41. *Proceedings of the National Academy of Sciences*, *94* (23), 12303–12308.
- Tang, H., Robinson, J. E., Gnanakaran, S., Li, M., Rosenberg, E. S., Perez, L. G., ... Montefiori, D. C. (2011). epitopes immediately below the base of the V3 loop of gp120 as targets for the initial autologous neutralizing antibody response in two HIV-1 subtype B-infected individuals. *Journal of Virology*, *85*(18), 9286–99. doi:10.1128/JVI.02286-10
- Tebit, D. M., & Arts, E. J. (2011). Tracking a century of global expansion and evolution of HIV to drive understanding and to combat disease. *The Lancet Infectious Diseases*, *11*(1), 45–56. doi:10.1016/S1473-3099(10)70186-9
- Tebit, D. M., Ndembu, N., Weinberg, A., & Quiñones-Mateu, M. E. (2012). Mucosal transmission of human immunodeficiency virus. *Current HIV Research*, *10*(1), 3–8.
- Tenzer, S., Wee, E., Burgevin, A., Stewart-Jones, G., Friis, L., Lamberth, K., ... Iversen, A. K. N. (2009). Antigen processing influences HIV-specific cytotoxic T lymphocyte immunodominance. *Nature Immunology*, *10*(6), 636–46. doi:10.1038/ni.1728
- Tomaras, G. D., Yates, N. L., Liu, P., Qin, L., Fouda, G. G., Chavez, L. L., ... Haynes, B. F. (2008a). 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), 12449–63. doi:10.1128/JVI.01708-08
- Tomaras, G. D., Yates, N. L., Liu, P., Qin, L., Fouda, G. G., Chavez, L. L., ... Haynes, B. F. (2008b). 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), 12449–63. doi:10.1128/JVI.01708-08
- Tomiyama, H., Akari, H., Adachi, A., & Takiguchi, M. (2002). Different effects of Nef-mediated HLA class I down-regulation on human immunodeficiency virus type 1-specific CD8(+) T-cell cytolytic activity and cytokine production. *Journal of Virology*, 76(15), 7535–43.
- Trkola, A., Dragic, T., Arthos, J., Binley, J. M., Olson, W. C., Allaway, G. P., ... Moore, J. P. (1996). CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. *Nature*, 384(6605), 184–7. doi:10.1038/384184a0
- Trkola, A., Purtscher, M., Muster, T., Ballaun, C., Buchacher, A., Sullivan, N., ... Katinger, H. (1996). Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. *Journal of Virology*, 70(2), 1100–8.
- Troyer, R. M., Collins, K. R., Abraha, A., Moore, D. M., Krizan, R. W., Toossi, Z., ... Fraundorf, E. (2005). Changes in Human Immunodeficiency Virus Type 1 Fitness and Genetic Diversity during Disease Progression Changes in Human Immunodeficiency Virus Type 1 Fitness and Genetic Diversity during Disease Progression †. doi:10.1128/JVI.79.14.9006
- Tudor, D., & Bomsel, M. (2011). The broadly neutralizing HIV-1 IgG 2F5 elicits gp41-specific antibody-dependent cell cytotoxicity in a FcγRI-dependent manner. *AIDS (London, England)*, 25(6), 751–9. doi:10.1097/QAD.0b013e32834507bd
- Tyler, D. S., Stanley, S. D., Zolla-Pazner, S., Gorny, M. K., Shadduck, P. P., Langlois, A. J., ... Weinhold, K. J. (1990). Identification of sites within gp41 that serve as targets for antibody-dependent cellular cytotoxicity by using human monoclonal antibodies. *The Journal of Immunology*, 145 (10), 3276–3282.
- Van den Kerkhof, T. L., Feenstra, K. A., Euler, Z., van Gils, M. J., Rijdsdijk, L. W., Boeser-Nunnink, B. D., ... Sanders, R. W. (2013). HIV-1 envelope glycoprotein signatures that correlate with the development of cross-reactive neutralizing activity. *Retrovirology*, 10(1), 102. doi:10.1186/1742-4690-10-102
- Van Gils, M. J., Bunnik, E. M., Boeser-Nunnink, B. D., Burger, J. a, Terlouw-Klein, M., Verwer, N., & Schuitemaker, H. (2011). Longer V1V2 region with increased number of potential N-linked glycosylation sites in the HIV-1 envelope glycoprotein protects against HIV-specific neutralizing antibodies. *Journal of virology* (Vol. 85, pp. 6986–95). doi:10.1128/JVI.00268-11

- Van Gils, M. J., Edo-Matas, D., Schweighardt, B., Wrin, T., & Schuitemaker, H. (2010). High prevalence of neutralizing activity against multiple unrelated human immunodeficiency virus type 1 (HIV-1) subtype B variants in sera from HIV-1 subtype B-infected individuals: evidence for subtype-specific rather than strain-specific neutralizing activity. *The Journal of General Virology*, *91*(Pt 1), 250–8. doi:10.1099/vir.0.015693-0
- Van Loggerenberg, F., Mlisana, K., Williamson, C., Auld, S. C., Morris, L., Gray, C. M., ... Abdool Karim, S. S. (2008). Establishing a cohort at high risk of HIV infection in South Africa: challenges and experiences of the CAPRISA 002 acute infection study. *PLoS One*, *3*(4), e1954. doi:10.1371/journal.pone.0001954
- Varghese, B., Maher, J. E., Peterman, T. A., Branson, B. M., & Steketee, R. W. (2002). Reducing the risk of sexual HIV transmission: quantifying the per-act risk for HIV on the basis of choice of partner, sex act, and condom use. *Sexually Transmitted Diseases*, *29*(1), 38–43.
- Venable, R. M., Pastor, R. W., Brooks, B. R., & Carson, F. W. (1989). Theoretically determined three-dimensional structures for amphipathic segments of the HIV-1 gp41 envelope protein. *AIDS Research and Human Retroviruses*, *5*(1), 7–22.
- Verkoczy, L., Kelsoe, G., Moody, M. A., & Haynes, B. F. (2011). Role of immune mechanisms in induction of HIV-1 broadly neutralizing antibodies. *Current Opinion in Immunology*, *23*(3), 383–90. doi:10.1016/j.coi.2011.04.003
- Von Andrian, U. H., & Mackay, C. R. (2000). T-Cell Function and Migration — Two Sides of the Same Coin. *New England Journal of Medicine*, *343*(14), 1020–1034. doi:10.1056/NEJM200010053431407
- Walker, L. M., Huber, M., Doores, K. J., Falkowska, E., Pejchal, R., Julien, J.-P., ... Poignard, P. (2011). Broad neutralization coverage of HIV by multiple highly potent antibodies. *Nature*, *477*(7365), 466–70. doi:10.1038/nature10373
- Walker, L. M., Phogat, S. K., Chan-Hui, P.-Y., Wagner, D., Phung, P., Goss, J. L., ... Burton, D. R. (2009a). Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. *Science (New York, N.Y.)*, *326*(5950), 285–9. doi:10.1126/science.1178746
- Walker, L. M., Phogat, S. K., Chan-Hui, P.-Y., Wagner, D., Phung, P., Goss, J. L., ... Burton, D. R. (2009b). Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. *Science (New York, N.Y.)*, *326*(5950), 285–9. doi:10.1126/science.1178746
- Wargo, A. R., & Kurath, G. (2012). Viral fitness: definitions, measurement, and current insights. *Current Opinion in Virology*, *2*(5), 538–45. doi:10.1016/j.coviro.2012.07.007
- Wawer, M. J., Gray, R. H., Sewankambo, N. K., Serwadda, D., Li, X., Laeyendecker, O., ... Quinn, T. C. (2005). Rates of HIV-1 Transmission per Coital Act, by Stage of HIV-1 Infection, in Rakai, Uganda. *Journal of Infectious Diseases*, *191* (9), 1403–1409. doi:10.1086/429411

- Weber, J., Fenyö, E. M., Beddows, S., Kaleebu, P., & Björndal, A. (1996). Neutralization serotypes of human immunodeficiency virus type 1 field isolates are not predicted by genetic subtype. The WHO Network for HIV Isolation and Characterization. *Journal of Virology*, *70*(11), 7827–32.
- Wei, X., Decker, J. M., Wang, S., Hui, H., Kappes, J. C., Wu, X., ... Shaw, G. M. (2003). Antibody neutralization and escape by HIV-1. *Nature*, *422*(6929), 307–12. doi:10.1038/nature01470
- Weissenhorn, W., Dessen, A., Harrison, S. C., Skehel, J. J., & Wiley, D. C. (1997). Atomic structure of the ectodomain from HIV-1 gp41. *Nature*, *387*(6631), 426–430.
- West, J. T., Weldon, S. K., Wyss, S., Lin, X., Yu, Q., Thali, M., & Hunter, E. (2002). Mutation of the dominant endocytosis motif in human immunodeficiency virus type 1 gp41 can complement matrix mutations without increasing Env incorporation. *Journal of Virology*, *76*(7), 3338–49.
- Wilk, T., Pfeiffer, T., & Bosch, V. (1992). Retained in vitro infectivity and cytopathogenicity of HIV-1 despite truncation of the C-terminal tail of the env gene product. *Virology*, *189*(1), 167–77.
- Willey, R. L., Rutledge, R. A., Dias, S., Folks, T., Theodore, T., Buckler, C. E., & Martin, M. A. (1986). Identification of conserved and divergent domains within the envelope gene of the acquired immunodeficiency syndrome retrovirus. *Proceedings of the National Academy of Sciences of the United States of America*, *83*(14), 5038–42.
- Wood, N., Bhattacharya, T., Keele, B. F., Giorgi, E., Liu, M., Gaschen, B., ... Seoighe, C. (2009). HIV evolution in early infection: selection pressures, patterns of insertion and deletion, and the impact of APOBEC. *PLoS Pathogens*, *5*(5), e1000414. doi:10.1371/journal.ppat.1000414
- Woodman, Z., Mlisana, K., Treurnicht, F., Abrahams, M., Thebus, R., Karim, S. A., ... Study, I. (2011). Short Communication Decreased Incidence of Dual Infections in South African Subtype C-Infected Women Compared to a Cohort Ten Years Earlier, *27*(11). doi:10.1089/aid.2010.0162
- Wooley, D. P., Smith, R. A., Czajak, S., & Desrosiers, R. C. (1997). Direct demonstration of retroviral recombination in a rhesus monkey. *Journal of Virology*, *71*(12), 9650–3.
- Wright, J. K., Novitsky, V., Brockman, M. A., Brumme, Z. L., Brumme, C. J., Carlson, J. M., ... Ndung'u, T. (2011). Influence of Gag-protease-mediated replication capacity on disease progression in individuals recently infected with HIV-1 subtype C. *Journal of Virology*, *85*(8), 3996–4006. doi:10.1128/JVI.02520-10
- Wu, S.-R., Löving, R., Lindqvist, B., Hebert, H., Koeck, P. J. B., Sjöberg, M., & Garoff, H. (2010). Single-particle cryoelectron microscopy analysis reveals the HIV-1 spike as a tripod structure. *Proceedings of the National Academy of Sciences*, *107* (44), 18844–18849. doi:10.1073/pnas.1007227107

- Wu, X., Parast, A. B., Richardson, B. A., Nduati, R., John-stewart, G., Mbori-ngacha, D., ... Overbaugh, J. (2006). Neutralization Escape Variants of Human Immunodeficiency Virus Type 1 Are Transmitted from Mother to Infant, *80*(2), 835–844. doi:10.1128/JVI.80.2.835
- Wyatt, R., & Sodroski, J. (1998). The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science (New York, N.Y.)*, *280*(5371), 1884–8.
- Wyatt, R., Sullivan, N., Thali, M., Repke, H., Ho, D., Robinson, J., ... Sodroski, J. (1993). Functional and immunologic characterization of human immunodeficiency virus type 1 envelope glycoproteins containing deletions of the major variable regions. . *Journal of Virology* , *67* ( 8 ), 4557–4565.
- Wyss, S., Berlioz-Torrent, C., Boge, M., Blot, G., Höning, S., Benarous, R., & Thali, M. (2001). The Highly Conserved C-Terminal Dileucine Motif in the Cytosolic Domain of the Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Is Critical for Its Association with the AP-1 Clathrin Adapter . *Journal of Virology* , *75* ( 6 ), 2982–2992. doi:10.1128/JVI.75.6.2982-2992.2001
- Wyss, S., Berlioz-Torrent, C., Boge, M., Blot, G., Höning, S., Benarous, R., & Thali, M. (2001). The highly conserved C-terminal dileucine motif in the cytosolic domain of the human immunodeficiency virus type 1 envelope glycoprotein is critical for its association with the AP-1 clathrin adaptor [correction of adapter]. *Journal of Virology*, *75*(6), 2982–92. doi:10.1128/JVI.75.6.2982-2992.2001
- Xiao, P., Patterson, L. J., Kuate, S., Brocca-Cofano, E., Thomas, M. A., Venzon, D., ... Robert-Guroff, M. (2012). Replicating adenovirus-simian immunodeficiency virus (SIV) recombinant priming and envelope protein boosting elicits localized, mucosal IgA immunity in rhesus macaques correlated with delayed acquisition following a repeated low-dose rectal SIV(mac251) ch. *Journal of Virology*, *86*(8), 4644–57. doi:10.1128/JVI.06812-11
- Xiao, P., Zhao, J., Patterson, L. J., Brocca-Cofano, E., Venzon, D., Kozlowski, P. a, ... Robert-Guroff, M. (2010). Multiple vaccine-elicited nonneutralizing anti-envelope antibody activities contribute to protective efficacy by reducing both acute and chronic viremia following simian/human immunodeficiency virus SHIV89.6P challenge in rhesus macaques. *Journal of Virology*, *84*(14), 7161–73. doi:10.1128/JVI.00410-10
- Xu, W., Hofmann-Lehmann, R., McClure, H. M., & Ruprecht, R. M. (2002). Passive immunization with human neutralizing monoclonal antibodies: correlates of protective immunity against HIV. *Vaccine*, *20*(15), 1956–60.
- Yamada, T., & Iwamoto, a. (1999). Expression of a novel Nef epitope on the surface of HIV type 1-infected cells. *AIDS Research and Human Retroviruses*, *15*(11), 1001–9. doi:10.1089/088922299310511
- Yamada, T., Watanabe, N., Nakamura, T., & Iwamoto, A. (2004). Antibody-dependent cellular cytotoxicity via humoral immune epitope of Nef protein expressed on cell surface. *Journal of Immunology (Baltimore, Md. : 1950)*, *172*(4), 2401–6.

- Yang, P., Ai, L.-S., Huang, S.-C., Li, H.-F., Chan, W.-E., Chang, C.-W., ... Chen, S. S.-L. (2010). The cytoplasmic domain of human immunodeficiency virus type 1 transmembrane protein gp41 harbors lipid raft association determinants. *Journal of Virology*, *84*(1), 59–75. doi:10.1128/JVI.00899-09
- Yang, Z., Nielsen, R., Goldman, N., & Pedersen, A.-M. K. (2000). Codon-Substitution Models for Heterogeneous Selection Pressure at Amino Acid Sites. *Genetics*, *155* (1), 431–449.
- Yates, N. L., Lucas, J. T., Nolen, T. L., Vandergrift, N. A., Soderberg, K. A., Seaton, K. E., ... Tomaras, G. D. (2011). Multiple HIV-1-specific IgG3 responses decline during acute HIV-1: implications for detection of incident HIV infection. *AIDS (London, England)*, *25*(17), 2089–97. doi:10.1097/QAD.0b013e32834b348e
- Yu, X., Yuan, X., McLane, M. F., Lee, T. H., & Essex, M. (1993). Mutations in the cytoplasmic domain of human immunodeficiency virus type 1 transmembrane protein impair the incorporation of Env proteins into mature virions. *Journal of Virology*, *67*(1), 213–21.
- Yuan, T., Li, J., & Zhang, M.-Y. (2013). HIV-1 Envelope Glycoprotein Variable Loops Are Indispensable for Envelope Structural Integrity and Virus Entry. *PLoS ONE*, *8*(8), e69789. doi:10.1371/journal.pone.0069789
- Zhang, M., Jiao, Y., Wang, S., Zhang, L., Huang, Z., Chen, Y., & Wu, H. (2012). Serum neutralizing activities from a Beijing homosexual male cohort infected with different subtypes of HIV-1 in China. *PloS One*, *7*(10), e47548. doi:10.1371/journal.pone.0047548
- Zhang, X., Huang, X., Xia, W., Li, W., Zhang, T., Wu, H., ... Yan, H. (2013). HLA-B\*44 Is Associated with a Lower Viral Set Point and Slow CD4 Decline in a Cohort of Chinese Homosexual Men Acutely Infected with HIV-1. *Clinical and Vaccine Immunology : CVI*, *20*(7), 1048–54. doi:10.1128/CVI.00015-13
- Zhang, Z.-Q., Wietgreffe, S. W., Li, Q., Shore, M. D., Duan, L., Reilly, C., ... Haase, A. T. (2004). Roles of substrate availability and infection of resting and activated CD4+ T cells in transmission and acute simian immunodeficiency virus infection. *Proceedings of the National Academy of Sciences of the United States of America*, *101* (15), 5640–5645. doi:10.1073/pnas.0308425101
- Zhou, T., Georgiev, I., Wu, X., Yang, Z.-Y., Dai, K., Finzi, A., ... Kwong, P. D. (2010). Structural basis for broad and potent neutralization of HIV-1 by antibody VRC01. *Science (New York, N.Y.)*, *329*(5993), 811–7. doi:10.1126/science.1192819
- Zhou, T., Xu, L., Dey, B., Hessel, A. J., Van Ryk, D., Xiang, S.-H., ... Kwong, P. D. (2007). Structural definition of a conserved neutralization epitope on HIV-1 gp120. *Nature*, *445*(7129), 732–737. Retrieved from <http://dx.doi.org/10.1038/nature05580>
- Zhu, T., Mo, H., Wang, N., Nam, D., Cao, Y., Koup, R., & Ho, D. (1993). Genotypic and phenotypic characterization of HIV-1 patients with primary infection. *Science*, *261*(5125), 1179–1181. doi:10.1126/science.8356453

- Zhuang, J., Jetzt, A. E., Sun, G., Yu, H., Klarmann, G., Ron, Y., ... Dougherty, J. P. (2002). Human immunodeficiency virus type 1 recombination: rate, fidelity, and putative hot spots. *Journal of Virology*, 76(22), 11273–82.
- Ziegner, U. H. M., Frank, I., Bernatowicz, A., Starr, S. E., & Streckert, H.-J. (1992). Antibody-Dependent Cellular Cytotoxicity (ADCC) Is Directed Against Immunodominant Epitopes of the Envelope Proteins of Human Immunodeficiency Virus 1 (HIV-1). *Viral Immunology*, 5(4), 273–281. doi:10.1089/vim.1992.5.273
- Zwick, M. B., Labrijn, A. F., Wang, M., Spenlehauer, C., Saphire, E. O., Binley, J. M., ... Parren, P. W. (2001). Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. *Journal of Virology*, 75(22), 10892–905. doi:10.1128/JVI.75.22.10892-10905.2001