T-Cell Receptor Usage in HIV- 2 Infection

Eirini Moysi

St Peter's College

Supervisors
Professor Sarah Rowland-Jones
Dr.Tao Dong
Dr. Guillaume Stewart-Jones

MRC Human Immunology Unit
Weatherall Institute of Molecular Medicine

Nuffield Department of Clinical Medicine

A thesis submitted for the degree of
Doctor of Philosophy
Trinity Term 2012

Στη γιαγιά Όλγα

Acknowledgments

My D.Phil. years were educational in many ways: they taught me how to form questions, design and perform experiments, analyse and present findings. They also taught me how to persevere in harder times and how to find viable solutions to problems that at first seemed insurmountable. More importantly however, throughout this academic journey I came to meet a number of extraordinary people the work and mentoring of which influenced me in one way or another. To all those people I would like to say a wholehearted thank you as without them, I would not have had half the insights and experience, both scientific and personal, that I have today.

To begin with, I would like to thank the scholarship foundations that funded me for this degree: the Bodossaki Foundation, the Wingate Foundation and the Leventis Foundation as well as my undergraduate professors Sotiria Boukouvala, Nicholaos Glykos, Giannoulis Fakis, Raphael Sandaltzopoulos and Leo Stamatatos. Their trust, mentoring and support helped me reach to the point I am today. Thank you Sarah and Tao, for giving me the opportunity to work in the SRJ/TD lab, Peng for teaching me how to make tetramers and for sharing with me the β2m and B*3501 constructs, Gerry for your input on TCR expression and advice throughout my D.Phil. years, Yannis for your never-ending support and friendship, Kati di Gleria and Zhanru Yu for preparing peptides for me, Graig Waugh for your help with cell sorting, Laura Millican for answering my questions regarding TCR purification, John Frankland for performing the DNA sequencing for my thesis and Guillaume for helping me realize the work described in chapter 5.

There are also numerous people outside Oxford to whom I owe a big thank you for offering me advice and support during my project: Dr. Assan Jaye and Carla van Tienen in the MCR Gambia for helping me organise the patient database upon which the patient

recruitment was based and the head of the Caio field station Tim Vincent and the Caio field workers and nurses for assisting me with the patient recruitment and bleeding. A big thank you and immense gratitude goes also to Dr. Danny Douek and Dr. Richard Koup in the Vaccine Research Centre at the NIH who gave me the opportunity to visit their labs to train on the clonotyping method and multicolour flow cytometry. I am especially grateful to Brenna Hill, Jorge Almeida, David Wolinsky and Tiffany Prout for helping me organise the sample shipment and clonotyping analysis and Costas Petrovas for his help with the phenotypic analysis. I would also like to thank the collaborators in Nantes, France, Dr. Sophie Brouard and Nicolas Degauge for performing the spectratyping analysis.

This list however, would not be complete without my dear friends Angelos, Kimon and Vasileia whose friendship, cooking and at times singing kept me optimistic and upbeat when I was racing to finish my degree. Last, but not least, I would like to thank Prof. Edith Sim, my beloved parents Nina and Makis, my sister Anna and Fabi for making sure that I never felt alone during this journey and for reminding me that my dreams come with an inbuilt steering wheel; all I need to do is learn how to drive.

ABSTRACT of THESIS
T-cell Receptor Usage in HIV-2 Infection
Submitted for the degree of Doctor of Philosophy
Eirini Moysi, St Peter's College
Trinity Term 2012

Long-term non-progressors (LTPNs) in HIV infection target the structural protein Gag more frequently than individuals who progress to disease. However, the targeting of Gag *per se* does not always distinguish these two groups. Various factors have been put forth as likely explanations for this discrepancy including differences in the breadth and magnitude of observed responses, the HLA type of the host, the nature of the individual epitopes targeted and the ability of the virus to mutate these antigenic regions.

The purpose of this thesis was to examine, using PBMCs isolated from HIV-2 infected LTNPs and CTL clones established *in vitro,* the clonotypic architecture and quality of an immunodominant HIV-2 Gag-specific response directed towards the HLA-B*3501-restricted epitope NPVPVGNIY (NY9: Gag245-253). The data presented in this thesis show that in spite of the expression of multiple inhibitory receptors on the surface of NY9-specific CD8+ T-cells, the NY9-response, which is a clonotypically 'private' response, bears a signature characterised by an increased cytotoxic sensitivity and the production of an array of cytokines, most notably IFN-γ and MIP-1β. Moreover, the results of this thesis indicate that the NY9-specific CD8+ T-cells are able to cross-recognise and lyse target B-cells pulsed with the corresponding HIV epitope PY9 and its variants at functional avidities (EC_{50}) that are close to those exhibited by PY9-specific T-cells. However, not all mobilised TCR clonotypes are equally sensitive or equally cross-reactive. When individual CTL clones were studied it emerged that dominant clonotypes within the NY9-specific CD8+ T-cell memory pool possessed a higher avidity for tetramer and sensitivity for antigen than subdominant ones and demonstrated a better cross-reactive potential towards variants of the HIV-2 epitope.

Hence, future HIV vaccine strategies may benefit from the inclusion of epitopes like NY9, the presentation of which appears to mobilise CD8+ T-cells with superior functional profiles.

Declaration

I declare that the work presented in this D.Phil. thesis entitled ' T-cell receptor Usage in HIV-2 infection' is entirely my own work except for where the contributions of my collaborators have been clearly acknowledged. No part of my thesis has been submitted for any degree or other qualification in this University of elsewhere.

Trinity Term 2012

Eirini Moysi

MRC Human Immunology Unit
Weatherall Institute of Molecular Medicine

Abbreviations

^{51}Cr	^{51}Chromium
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cell
	Allophycocyanin
ART	Antiretroviral Treatment
bp	base pair
CA	Capsid
CDR	Complementarity Determining Region
CMV	Cytomegalovirus
CTL	Cytotoxic T- cell
D	Diversity
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EBV	Epstein-Barr Virus
ERAAP1	Endoplasmic Reticulum Associated Aminopeptidase -1
ERK	Extracellular signal Regulated Kinase
FITC	Fluorescein Isothiocyanate
FPLC	Fast liquid Protein Chromatography
GPI	Glycophosphatidylinonositol
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
HAART	Highly Active Antiretroviral Treatment
HCV	Hepatitis C Virus
HERV	Human Endogenous Retrovirus
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
HLA	Human Leukocyte Antigen
HSV-1	Herpes Simplex Virus type 1
HTLV-1	Human T- Lymphotropic Virus type 1
ICS	Intracellular Staining
IFN	Interferon
IL	Interleukin
IN	Integrase
IPTG	Isopropyl β-D-1 thiogalactopyranoside
ITAM	Immunoreceptor Tyrosine-based Activation Motif
J	Joining
JNK	c-Jun N-terminal Kinase
KIR	Killer Immunoglobulin like Receptor
LAT	Linker Activator for T-Cells
LCMV	Lymphocytic Choriomeningitis Virus
LFA-1	Lymphocyte Function Associated Antigen -1
LPS	Lipopolysaccharide
LTNP	Long-Term Non-Progressor
LTR	Long Terminal Repeat
MA	Matrix

MCP-1	Monocyte Chemotactic Protein-1
MHC	Major Histocompatibility Complex
MHR	Major Homology Region
MIP	Macrophage Inflammatory Protein
NC	Nucleocapsid
NFAT	Nuclear Factor of Activated T-cells
NK	Natural Killer
NNRTIs	Non-Nucleoside Reverse Transcriptase Inhibitors
ORF	Open Reading Frame
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PD-1	Programmed Death-1
PE	Phycoerythrin
PHA	Phytohaemagglutinin
PMA	Phorbol Myristate Acetate
PR	Protease
PTK	Protein Tyrosine Kinase
RAG	Recombinase Activating Gene
RNA	Ribonucleic Acid
RRE	Rev Response Element
RT	Reverse Transcriptase
SA	Streptavidin
SEB	Staphylococcal Enterotoxin B
SFU	Spot Forming Unit
SIV	Simian Immunodeficiency Virus
SNP	Single Nucleotide Polymorphism
SPR	Surface Plasmon Resonance
TCR	T-cell Receptor
TLR	Toll-like Receptor
TRIM	Tripartite Motif containing protein
V	Variable
VL	Viral Load
WHO	World Health Organisation

Table of Contents

Chapter 1: Introduction

1.1 The HIV-2 virus

The Human Immunodeficiency Virus type-2 (HIV-2) is a human retrovirus that was first identified in 1985 (Barin et al., 1985). It is phylogenetically related to HIV-1, with which it shares a 30-60% sequence homology, depending on the viral region, (Guyader et al., 1987) and is thought to have emerged from the zoonotic entry of HIV-2 from sooty mangabeys (SIVsm) into the human population. To date, eight groups have been described (HIV-2 A-H), the most common of which are clade A in Guinea-Bissau, Senegal and Cape Verde and clade B in Côte d'Ivoire/Ghana (Faria et al., 2012). These clades differ from each other between 12.4-24.3% and 17.7-34.6% in their consensus Gag and Env sequences respectively and each one is thought to represent a unique event of cross-species transmission of the virus from the sooty mangabey to humans (Chen et al., 1997).

The first cross-species transmission might have occurred as early as 1938 (Faria et al., 2012). However, socio-epidemiological and phylogenetic studies associate the main spread of HIV-2 with periods that are chronologically more recent and which coincide with events that might have facilitated the endogenous propagation and geographical dissemination of the virus such as the Guinea-Bissauan war of independence (1963-1974) and civil war (1998-1999) (Mansson et al., 2009; Poulsen et al., 2000; Valadas et al., 2009). It has also been proposed that parenteral iatrogenic transmission associated with pathogen control/eradication campaigns, and tribal rituals, such as excisions, might have assisted this spread among groups of certain age and/or ethnicity (Pepin et al., 2006).

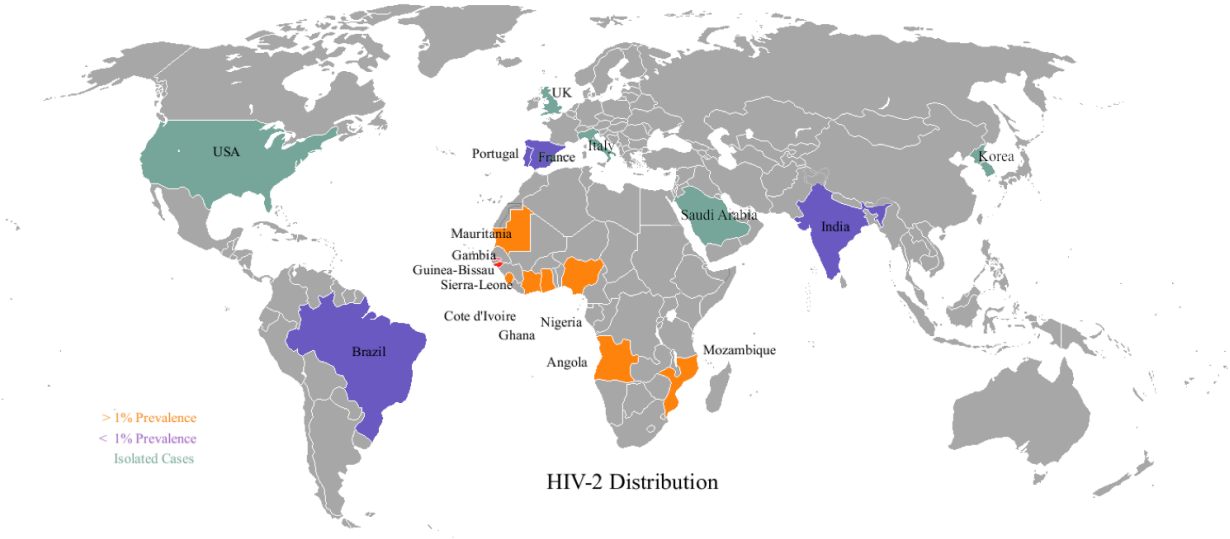


Figure 1.1: HIV-2 Distribution
[Data: CDC HIV-2 Factsheet, http://www.cdc.gov/hiv/topics/basic/#hiv2]

Today, West Africa remains the epicentre of HIV-2 infection with some countries, such as Guinea-Bissau, reporting population infection rates for rural areas as high as 4.7% (Tienen et al., 2010). In Europe, the highest prevalences are found in Portugal and France where HIV-2 infection represented in the years 2007 and 2011 2.3% and 1.8% of the total HIV diagnoses respectively (Barin et al., 2007; Carvalho et al., 2011). A small number of cases has also been described for countries linked to West Africa through migratory routes such as Southern India (Kannangai et al., 2010), Brazil (Broutet et al., 1996), Mozambique (Maueia et al., 2011) and USA (CDC, 2011) (figure 1.1). Hence, HIV-2 represents a virus that is endemic to West Africa and its rate is in steep contrast to that reported for HIV-1: in 2011 alone, 34 million individuals were living with HIV-1, 2.7 of which were new infections (UNAIDS, 2010). Nevertheless, the nature of HIV-2 as the only other circulating human lentivirus apart from HIV-1 and most importantly the introduction of the disease through migration in more countries over the past years, for example Italy (Ciccozzi et al., 2011), Saudi Arabia

(Almaghrabi et al., 2011), United Kingdom (Dougan et al., 2005) and Korea (Kim et al., 2000) have placed this pathogen more prominently on the research agendas of affected countries over the past years (Camacho, 2012). Such emerging trends pose new dilemmas for national screening and treatment strategies, especially as the virus is resistant to some classes of anti-retroviral drugs such as non-nucleoside reverse transcriptase inhibitors (NNRTIs), fusion inhibitors and some protease inhibitors (Camacho, 2012),

1.2 Viral life cycle and proteins

HIV-2 has a genetic organization that is similar to that of HIV-1. The genetic material of the virus is a 9.7kB single-stranded RNA molecule that contains nine overlapping open reading frames (ORFs) that encode structural (Gag, Pol, Env), regulatory (Tat, Rev) and accessory (Vif, Vpr, Vpx, Nef) proteins (figure 1.2). The majority of these HIV-2 proteins have functions identical to their HIV-1 counterparts, with the exception of Vpx which is absent from HIV-1 (Guyader et al., 1987; Laguette et al., 2011).

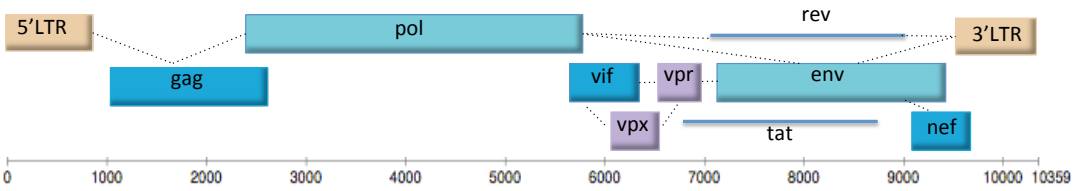


Figure 1.2: HIV-2 genes and ORFs. The HIV-2 genes are flanked by two long-terminal repeats (LTRs) at the 5' and 3' ends respectively. These are short inverted repeats (5'-CTG CAG-3') that contain protein-binding elements, such as the promoter and enhancer that are critical for the regulation of viral transcription after T-cell and/or monocyte activation (Guyader et al., 1987; Markovitz et al., 1990). The remaining genes are organized in three major frames of expression (pol-rev, gag-vif-vpr-env and vpx-tat-nef)

1.2.1 Cell Attachment and Membrane Fusion

HIV-2 infects cells by means of a membrane fusion process, which occurs when its envelope glycoprotein (gp125) interacts with the host glycoprotein CD4 and various other co-receptors on the surface of its target cell (figure 1.3). CD4 consists of four Ig-like domains (D1-D4) the role of which is to mediate the recognition of MHC class II molecules. Gp125 attaches to the cells by interacting with the most amino-terminal of these four domains (D1) through a series of van der Waals contacts and hydrogen bonds that involve the CD4 residues Phe43 and Arg59 and a series of well-conserved envelope amino acids (Kwong et al., 1998). CD4 is not always necessary for infection since CD4-independent infection has been shown to occur for both lab-adapted strains such as HIV-$2_{ROD/B}$ and CBL-20/21/23 and primary isolates (Liu et al., 2000; Reeves et al., 1999).

The viral precursor of the gp125 glycoprotein (gp160) is encoded by the gene *env*. The latter shows only a 41.7% identity to its HIV-1 counterpart in terms of sequence (Guyader et al., 1987) but encodes proteins that despite being different in their predicted structural conformations from those seen in HIV-1 (Barroso et al., 2011), most likely assemble in a similar way to form highly N-glycosylated trimers on the viral surface (Doms et al., 1990). The mature gp125 in HIV-2 is organized into five variable (V1-V5) and five constant regions (C1-C5) with the CD4 binding site emerging from the interaction of the C2 and C3 regions (Barroso et al., 2011).

In terms of co-receptor usage, HIV-2 appears to be more promiscuous compared to HIV-1. The most frequently used co-receptors, especially by CD4-independent strains, are the G-protein coupled receptors (GPCRs) CCR5 and CXCR4. Additional receptors such as CCR1, CCR2b, CCR3, CCR9, BOB/GPR15, STRL33, US28, APJ,

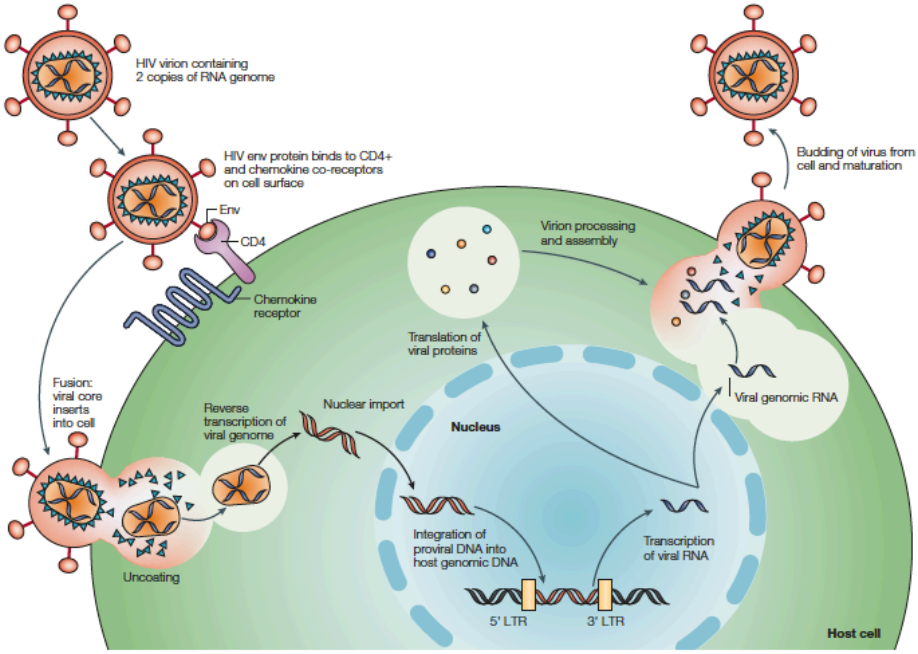


Figure 1.3 Life Cycle of HIV. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics (Rambaut et al., 2004), copyright 2004.

BONZO/CXCR6 and ChemR23 and CCR8 have also been shown to allow infection *in vitro* (Calado et al., 2010; Edinger et al., 1999; Visseaux et al., 2012). CXCR4-tropic strains are more cytopathic *in vitro* than CCR5 and CCR5 to CXCR4 switches are associated with an increased cytopathicity (Schramm et al., 2000). Such changes in co-receptor usage (tropism) are determined by the genotype of the HIV-2 gp125 V3-loop with mutations at positions 18 (K/R), 19 (V>K/R), 24 (insertions) and a global net charge of more than +6 predicting a broadening in tropism and dual co-receptor usage (C5/X4) (Visseaux et al., 2012).

The binding of HIV-2 on the surface of the target cell triggers a cascade of events that culminate in the fusion of the viral envelope with the target cell membrane and the release of the capsid into the cell cytoplasm. For HIV-1, CD4 binding is followed by the formation of the gp120 co-receptor binding site and the induction of a 6-helix bundle arrangement on the viral surface (fusion peptide). This enables the virus

to move closer to its target membrane and ultimately fuse with it (Doms and Moore, 2000).

Similar steps and conformational changes are thought to occur upon CD4 engagement in HIV-2 infection. However, the two viruses do not share the same kinetics of conformational change. The HIV-2 gp125 shows a slower kinetics of co-receptor binding and appears to assume a less stable 6- helix bundle formation than its HIV-1 counterpart (Gallo et al., 2006). In addition, the higher levels of immunogenicity of HIV-2 Env seen in natural infection, and the development of neutralizing antibodies for regions such as the V3-loop, CD4 binding site (CD4bs) and CD4i at levels much higher than those seen for HIV-1 (Kong et al., 2012) strongly suggest structural differences in the assumed conformations (Barroso et al., 2011). This is further supported by the fact that R5-tropic HIV-2 variants also show resistance to β-chemokines such as RANTES and MIP-1β (Blaak et al., 2008) and fusion inhibitors such as the peptide T-20 (enfuvirtide) (Witvrouw et al., 2004).

1.2.2 Uncoating and Reverse Transcription

Once the viral core has been released into the cytoplasm, the process of uncoating begins. This involves the dissociation of the capsid proteins from the core and the release of the viral RNA, inside the host cell (Arhel, 2010). Reverse transcription, is catalysed by the viral enzyme reverse transcriptase (RT) which is a heterodimer composed of two subunits with sizes 68 (p68) and 55 (p55) kDa. The enzyme functions both as a RNA- and DNA- dependent DNA polymerase as well as an RNAse H (Fan et al., 1995). Much like in HIV-1 infection however, the process of reverse transcription in HIV-2 infection is characterised by a low replication fidelity as the HIV-2 RT lacks a

3-5' exonuclease proofreading activity (Kati et al., 1992). As a result, the enzyme shows an high rate of nucleotide misincorporation, which ranges *in vitro* between $0.5x10^{-5}$ to $2.9x10^{-5}$ mutations/bp/cycle (Svarovskaia et al., 2003). The first step in the process of reverse transcription is the conversion of the RNA into DNA which gives rise to a RNA/DNA hybrid. The RNA strand is then hydrolysed by the RNAse H domain of the enzyme and replaced by a second newly synthesized DNA strand. The resulting double-strand DNA is assembled into a pre-integration complex and transported to the nucleus for integration in the host cell DNA (Harrich and Hooker, 2002).

1.2.3 Integration, Activation and Protein Expression

The integration of the viral DNA into the host genome is mediated by the viral enzyme integrase. As for HIV-1, the integration of the HIV-2 provirus occurs mainly within the transcriptional units of the host's genome and only rarely in heterochromatic sites (MacNeil et al., 2006). Initially, the transcription of the integrated viral DNA (proviral DNA) by the cellular RNA polymerase II gives rise to basal levels of Tat protein. This protein then binds to the LTR regions of the provirus (TAR elements) and enhances HIV-2 transcription. As with HIV-1, the early phases of HIV-2 transcription give rise to multiply-spliced mRNAs and expression of regulatory proteins such as Tat, Nef and Rev. It is only later that non-spliced/singly-spliced mRNAs such as those encoding the viral RNA and proteins such as Env, Gag and Pol are produced and transported in the cytoplasm for translation (Sierra et al., 2005). The viral protein Rev, a small, phosphorylated protein that is highly conserved among the family of retroviruses, assists this migration to the cytoplasm. Rev localizes to the nucleolus of the infected cells where it binds unspliced/incompletely spliced viral mRNAs that possess the Rev-

response element (RRE) for cytoplasmic transport. Additionally, the protein contributes to the expression of structural genes such as *gag, env* and *pol* (Cochrane et al., 1990) and is also thought to mediate functions relating to the polyadenylation of viral mRNAs, RNA stability, translation and encapsidation in HIV-1 infection. However, to what extend these additional functionalities are also shared by HIV-2 Rev is still unknown (Groom et al., 2009).

1.2.4 Assembly, Budding & Release

The translation of the *env*, *gag* and *pol* mRNAs in the cytoplasm gives rise to the viral proteins protease (PR), reverse transcriptase (RT), RNase H and integrase (IN) (Weiss, 2000). Once this process has been completed, the Env proteins, full-length RNA molecules and viral enzymes migrate to the cellular membrane to form budding virions.

1.2.4.1 Env

The release of the new progeny out of the infected cells is assisted by the Env proteins, which apart from giving rise to the gp125 and gp35 glycoproteins of the viral spikes, also act to redirect tetherin (BST-2), an α- interferon-inducible host cell restriction protein that prevents the release of newly formed virions, away from the cell membrane where it is normally found (Hauser et al., 2010). These proteins originate from envelope precursors that are produced and glycosylated in the endoplasmic reticulum and transported to the cell surface through a secretory pathway. As they transit through the Golgi apparatus and endosomal compartments, they are cleaved by furin-like proteases to give rise to their mature forms which then modulate the membrane trafficking of tetherin (Le Tortorec and Neil, 2009).

1.2.4.2 Gag

Gag is a viral protein that has also a key role in the assembly and budding of the nascent virions out of the infected cells.. It is available in abundance early in infection and is critical for the viral fitness and therefore highly conserved in terms of structure and function among different HIV strains (Borghans et al., 2007). Even though it is not currently known how many copies of Gag are packed within each immature HIV-2 particle, studies on HIV-1 estimate the number to be close to 5000 copies/virion (Briggs et al., 2004). Gag is processed enzymatically at the end of the budding process by the viral protease to give rise to the structural components matrix (p17), capsid (p26), nucleocapsid (p7) and p6 (figure 1.4) of the virus. This step is necessary for the maturation and infectivity of the HIV-2 progeny (Sierra et al., 2005).

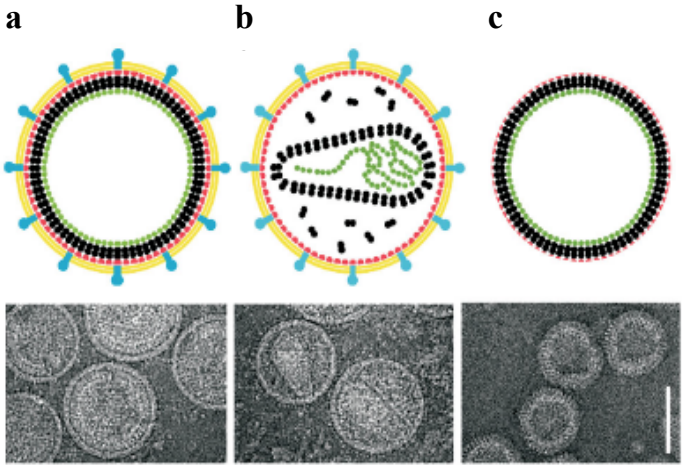


Figure 1.4 Cryo-electron micrographs and schematic representations of the structural organisation of immature (a) mature (b) and *in vitro* assembled Gag HIV-1 particles (c). **Blue:** viral envelope proteins **red:** MA domain of Gag protein, **black**: CA domain of Gag protein, **green:** NC domain of Gag protein and **yellow**: lipid bilayer. Scale bar 100nm Reprinted by permission from Macmillan Publishers Ltd: Nature Structural and Molecular Biology (Briggs et al., 2004), copyright 2004.

1.2.4.3 Matrix (MA)

The viral matrix protein (MA) is a 132-amino acid protein that has both a shuttling and a structural role within HIV infected cells. It exists in two forms: a cellular form (cMA) and a mature virion form (vMA). The former possesses three transport signals: one for nuclear localization, one for nuclear export and one for membrane localization which are thought to determine the localization of the Gag precursor intracellularly, as well as its targeting to the membrane for assembly and packaging. The vMA form on the other hand stabilizes the viral particles into an icosahedral form and contributes, upon infection, to the early post-entry events of reverse transcription, nuclear import, chromosome binding and integration (Bukrinskaya, 2007).

1.2.4.4 Capsid (CA)

The other main protein of the viral core is the capsid (CA). This consists of two independently folded domains separated by a flexible linker: an exposed N-terminal domain that forms the core surface of the mature virion and a C-terminal domain that is necessary for the multimerization and assembly of HIV particles. This C-terminal region also contains the major homology region (MHR), a domain that is highly conserved among retroviruses that is thought to play major roles in the Gag lattice formation, particle assembly and maturation (Ganser-Pornillos et al., 2008; Onyango et al., 2010).

1.2.4.5 Nucleocapsid (p8) and p6

The NC protein (p8) is a 44 amino-acid protein involved in the recognition and packaging of the viral RNA, the initiation of Gag multimerization as well as in the maintenance of the viral core (Gottlinger, 2001). It has a 67% sequence identity with its HIV-1 counterpart and folds into a similar structure that contains two zinc-finger motifs both of which are necessary for RNA binding (Matsui et al., 2009). On the other hand, the protein p6, initiates a late step in the budding process. This protein enables the dissociation of the newly formed virions from the host membrane by disrupting the tether that links them (Gottlinger, 2001).

1.2.5 Accessory Proteins

Three additional proteins exist in abundance inside the HIV-2 viral particles: Vpx, Vpu and Nef.

1.2.5.1 Vpx

Vpx is an accessory protein that is only found in members of the HIV-2/SIVsm family. It is incorporated in the HIV-2 virions by association with the Gag-p6 protein and its role is to facilitate the early post-entry events of the HIV-2 life cycle such as the reverse transcription, nuclear transport and localization of the pre-integration complexes (Fujita et al., 2008);(Fletcher et al., 1996). Even though the exact mechanisms through which Vpx exerts its various functions are not entirely clear, studies in dentritic cells (DCs) and macrophages suggest that the protein enhances the levels of reverse transcription, and thus infection of these two subsets, by targeting SAMHD1, a viral DNA

phosphohydrolase and host restriction factor, for proteosomal destruction (Laguette and Benkirane, 2012; Sharifi et al., 2012).

1.2.5.2 Vpr

The nuclear import of viral DNA in terminally differentiated macrophages, is also assisted also by the protein Vpr. However, this protein exerts additionally another function as well: it alters the cell cycle and proliferation status of the infected cells by inhibiting the progression from the G2 to the M phase of the cell cycle (Fletcher et al., 1996). This cell cycle arrest, which ensues once Vpr has associated with the CRL4 ubiquitin ligase complex through the adapter protein DCAF1, mimics a DNA damage response and stalls the cell at a phase that is advantageous for viral gene expression (Sharifi et al., 2012).

1.2.5.3 Nef

Whereas Vpx and Vpr counteract intracellular restriction factors to allow a productive infection, HIV-2 Nef enables the virus to persist once infection has been established. Nef increases the rate of CD4/CD8 internalization and CD4/CD8/MHC class I trafficking towards lysosomal compartments in infected cells, which down-modulates the levels of CD4, HLA-A and HLA-B on their surface. This decreased MHC class I expression allows the virus to evade a CTL-mediated immune response (DeGottardi et al., 2008; Laguette et al., 2010). In addition, HIV-2 Nef down-regulates the TCR and CD3 expression on the surface of T-cells which renders T- cells less responsive to a TCR-induced activation and thus more resistant to immune activation (Feldmann et al., 2009) and activation-induced cell death (Laguette et al., 2010).

1.2.5.4 Vif

Vif on the other hand, is an accessory protein that localizes to the nucleus and cytoplasm of infected cells. In HIV-1 infection, Vif acts to enhance the viral infectivity by counteracting the host protein APOBEC3G, a cytidine deaminase that destabilizes the nascent minus strand viral DNA during reverse transcription through a cytidine to uracil base editing (Ribeiro et al., 2005). However, the role of Vif in HIV-2 infection is less clear. Cell lines appear to be permissive to the virus when Vif is absent which suggests that APOBEC3G does not have the same effect on HIV-2 replication as it does for HIV-1 (Ribeiro et al., 2005; Wiegand et al., 2004). Nevertheless, it does suppress the virion incorporation of another member of the same restriction family, that of APOBEC3F, by causing it to sequester away from the budding virions (Wiegand et al., 2004).

1.3 Transmission and disease course

HIV-2 uses the same routes of transmission as HIV-1. It is transmitted through blood and blood products, sexual contact and vertically from mother to child during pregnancy and/or delivery. Earlier studies of HIV-2 had identified heterosexual contact and transfusion as the main routes of HIV-2 transmission (Poulsen et al., 1992). However, the introduction and implementation of blood screening for HIV-2 has led to a considerable decrease in the number of transfusion-related cases over the past decade (Carvalho et al., 2011).

Chronic HIV-2 infection increases the mortality rate of HIV-2 patients by two-fold compared to that of healthy, uninfected individuals (Poulsen et al., 1997; van der Loeff et al., 2010) but patients experience a slower rate of CD4 decline and lower

chronic levels of viraemia than HIV-1 patients (Drylewicz et al., 2008; Popper et al., 1999). In addition, its asymptomatic carriers disseminate the virus less readily than HIV-1 carriers, even in high-risk groups such as prostitutes (Kanki et al., 1994) and discordant couples (Kashyap et al., 2010). The incidence of HIV-2 acquisition per sexual act is five- to nine-fold lower than the one observed for HIV-1 (Marlink et al., 1994) which is most likely due to a lower level of viral shedding in the semen (Gottlieb et al., 2006) and female genital tract (Hawes et al., 2008). HIV-2 seropositivity is not associated with a higher risk for abortion or child death and children born to seropositive mothers do not appear to have a higher mortality rate than those born to seronegative mothers (Poulsen et al., 1992). In the absence of ART or HAART, the mother-to-child transmission rate of HIV-2 ranges between 0.6-4%. Mothers are more likely to pass on the virus to the neonates upon delivery or breastfeeding if they are immunocompromised and not adhering to therapy (Burgard et al., 2010; O'Donovan et al., 2000).

However, despite the low transmission efficiency of HIV-2 and more favourable prognosis for the majority of patients (Popper et al., 1999), infection with the virus ultimately leads to CD4 T-cell loss and AIDS. The same pathology is also observed in animals (baboons) that have been inoculated with either HIV-2_{UC2} or HIV-2_{UC3}. The acquisition of HIV-2 is followed by seroconversion and viral persistence that culminates in a disease that resembles HIV-1 AIDS in which the virus spreads in lymphoid tissues causing CD4 counts to drop profoundly (Barnett et al., 1994). Hence, even though HIV-2 viruses rank below HIV-1 group M viruses in terms of fitness (Arien et al., 2005) and some clades (e.g. B-E) show a lower replication potential of compared to others (e.g. clade A), HIV-2 is not merely an 'attenuated' retrovirus (Gao et al., 1994).

The poorest outcome is seen in patients with viral loads ≥ 10.000 copies/ ml (Berry et al., 2002). At such high loads, the transmission of the virus, such as in mother-to-child transmission, is no longer different to that of HIV-1 (O'Donovan et al., 2000). HIV-2 AIDS patients do not differ from HIV-1 AIDS patients in terms of clinical presentation either. The hallmark of HIV-2 AIDS is wasting whereas pulmonary tuberculosis (PTB) is the most frequent opportunistic infection. Other opportunistic infections include oesophageal candidiasis, cerebral toxoplasmosis, recurrent bacterial pneumonia, cryptosporidiosis, CMV disease and cryptococcosis (Matheron et al., 2003). CXCR4 HIV-2 isolates also infect human astrocytes and cause neurological disease with a mechanism similar to than employed by HIV-1 (Alvarez et al., 2008). However, HIV-2 AIDS patients are 12.4 times less likely to have Kaposi's sarcoma and vaginal candidiasis than HIV-1 infected patients (Ariyoshi et al., 1998; De Cock et al., 1993; Hawes et al., 2008; Martinez-Steele et al., 2007)

HIV-2 variants isolated from HIV-2 long-term non-progressors infect PBMCs *in vitro* with an entry efficiency that equals that of replication competent HIV-1 variants (Duvall et al., 2007). Moreover, CXCR4-tropic HIV-2 variants from progressors replicate in rates much higher than those recorded for some HIV-1 strains ($0.77\log_{10}$ pg/ml RT vs 0.32-0.58 $\log_{10}$ pg/ml respectively)(Blaak et al., 2006). In humans, the rate of progression appears to be faster in younger individuals (<50 years old) than old (Jaffar et al., 1997) and PBMC and plasma viral loads have been shown to contribute independently and be predictive of the rate of CD4+ T-cell decline (Gottlieb et al., 2002). Patients with CD4 T-cell counts <200 cells/µl have the same mortality rate and rate of CD4 decline as HIV-1 patients (Gottlieb et al., 2002; Martinez-Steele et al., 2007) as well as higher levels of proviral DNA (mean proviral load 663.3 copies/10^5 CD4 cells) compared to long-term non-progressors (Ariyoshi et al., 1996). In addition,

chronic and AIDS-stage patients have elevated LPS levels in their plasma, which is indicative of microbial translocation and a decreased responsiveness to innate stimuli such as TLR signalling (Nowroozalizadeh et al., 2010).

The expression of anti-viral cytokines such as IFN-α, IL-12 and MIP-1β is also compromised in progressive HIV-2 disease (Nowroozalizadeh et al., 2010). As CD4 counts drop, the sizes of naive and memory T repertoires become imbalanced and immune activation sets in. T-cells in HIV-2 infection upregulate a series of activation markers such as HLA-DR, CD38, CD69 and Fas and the frequency of cycling CD4+ T-cells (Ki67+) increases (Jaffar et al., 2005; Leligdowicz et al., 2010a; Sousa et al., 2002). Systemic markers of immune activation such as β2m-microglobulin, neopterin and suPAR also increase in the plasma of affected individuals (Jaffar et al., 2005).

1.4 Long-term non-progression in HIV-2 infection

Despite similar levels of proviral DNA in HIV-1 and HIV-2 infection however, chronic plasma viral load is approximately 30-fold lower in HIV-2 than HIV-1 infected patients. The majority of HIV-2 patients thus goes on to develop a long-term quiescent infection (Popper et al., 2000) with CD4 percentages ≥14%, low stable viral loads and no symptoms (eg. generalized lymphadenopathy, abdominal organomegaly or opportunistic infections) for at least 5 years after seroconversion (Ariyoshi et al., 1996; Berry et al., 2002; Marlink et al., 1994; Marlink et al., 1988; van der Loeff et al., 2010). A good predictor of outcome in HIV-1 infection is the viral load reached after the resolution of peak viremia (set point). Lower set points associate with a better prognosis and a longer incubation period (Mellors et al., 1996) and it has been shown that HIV-2 seroconverters reach a 28-fold lower set point than the one recorded for HIV-1 infection

(2500 versus 70.000 RNA copies/ml) (Andersson et al., 2000). The predicted mortality rate for those >60 years-old is similar to that of uninfected individuals and patients that live disease-free for 18-36 years are not uncommon (Utsumi et al., 2007; van der Loeff et al., 2010). Several explanations have been put forward to account for this phenomenon. It has been suggested that HIV-2 proviruses might be transcriptionally less active than HIV-1 proviruses, that some HIV-2 strains might be naturally less virulent or rendered less virulent through a mutational accumulation or that the adaptive immune response is better at clearing HIV-2 plasma viruses and controlling replication than in HIV-1 infection (Gottlieb et al., 2002; Onyango et al., 2010; Popper et al., 2000)

1.4.1 HIV-2 Replication Kinetics

The virulence of HIV strains depends on several genotypic elements. For HIV-2, some of these elements are regulatory and structural polymorphisms that lie within the LTR, env and gag regions of the virus (Grassly et al., 1998).

HIV-2 differs from HIV-1 in the number, as well as the identity of its regulatory elements (Popper et al., 2000). Its LTR regions are larger (Guyader et al., 1987) and contain only one kB site of transcriptional initiation as opposed to HIV-1 that has two. It also contains one more upstream site in its promoter, termed CD3R that is not found in HIV-1. The role of this element is to mediate the transcriptional response to TCR-activation but its action is much weaker than that of a kB site (Markovitz et al., 1990). Since the HIV-2 LTRs respond differently than HIV-1 to cellular stimuli such as, for instance, tumour necrosis factor alpha activation (Popper et al., 2000) it has been proposed that the low viral loads seen in HIV-2 infection might stem from differences in the transcriptional control of the two viruses especially as both HIV-1 and HIV-2

share a similar entry (Blaak et al., 2006) and proviral integration efficiency at transcriptionally active sites (MacNeil et al., 2006). This is further supported by HIV-2 replication studies that demonstrate the existence of lower levels of intermediate nucleic acids, such as mRNA, and reverse transcriptase accumulation per round of infectivity inside HIV-2 infected cells as opposed to cells infected with HIV-1 (MacNeil et al., 2007). Post-transcriptional events such as the rate of translation and genomic RNA packaging are also slower in HIV-2 infection (Soto-Rifo et al., 2011).

Another hypothesis that has been put forth as a likely explanation for the poor replication and low transmissibility of HIV-2 is the inability of the virus to fix mutations that that could increase its virulence (Barroso et al., 2011). Even though HIV-2 has a higher evolution rate than HIV-1 in advanced stages of disease (Skar et al., 2010), reaching up to 0.014 substitutions/site/year for envelope regions such as the C2 and C3, the quasi-species complexity in most patients is very low (Borrego et al., 2008) which suggests that occurring mutations are either synonymous or mutations that decrease viral fitness (Skar et al., 2010)

1.4.2 Host Restriction Factors

A number of host restriction factors have been identified in HIV-2 infection. These include TRIM factors as well as the restriction factors Lv2 and tetherin (Chakrabarti and Simon, 2010). TRIM5α is a host protein that belongs to the tripartite-motif family of proteins. It consists of a RING, a B-box, a coiled-coil, and a PRYSPRY (B30.2) domain that assemble into a trimer that mediates the restriction of the incoming viral capsids through its C-terminal SPRY motif (Reymond et al., 2001). This latter domain is also responsible for the species-specific retroviral function of TRIM5α and small

differences in its amino-acid sequence can affect the binding of the protein on the various retroviral capsids (Lukic and Campbell, 2012). HIV-2 isolates however do not show the same sensitivity to all restriction factors. For instance, HIV-2 isolates such as GH123 and UC12 are sensitive to the cynomolgus monkey (CM) TRIM5α and human TRIM5α whereas others are completely resistant (Chakrabarti and Simon, 2010).

One factor that determines TRIM5α resistance is the presence or absence of proline at the p26 capsid position 119, with its presence denoting TRIM5α sensitivity and its absence resistance. This residue retains its role as a determinant of TRIM5α sensitivity in other related viruses as well, such as for example SIVmac239 (Chakrabarti and Simon, 2010; Onyango et al., 2010) possibly by altering the level of capsid stability (Onyango et al., 2010).

Another host restriction factor that has been shown to limit HIV-2 replication of primary isolates *in vitro* such as CBL-23 is Lv2. Even though its exact mechanism of action has not been fully elucidated, Lv2 appears to interfere with the steps following viral reverse transcription. Restriction is dependent upon the route used for cytosolic entry and requires the entry of HIV-2 in endosomal compartments (Harrison and McKnight, 2011; Schmitz et al., 2004). However, as for TRIM5α, single amino-acid substitutions can also abolish an Lv2-mediated restriction. Such amino-acid substitutions have been mapped at envelope position 74 (G74E) and Gag position 207 (V207I) (Harrison and McKnight, 2011; Reuter et al., 2005).

Finally, tetherin (BST-2) is a type II transmembrane protein that immobilizes virions by infiltrating their lipid bilayers with its glycophosphatidylinositol (GPI) anchor and tethering them to the cell membrane of the host. However, as the protein HIV-2 Env usually counteracts tetherin, its role in HIV-2 infection is limited (Evans et al., 2010; Liberatore and Bieniasz, 2011).

1.4.3 HLA alleles and susceptibility genes

HLA class I alleles, and in particular the more diverse alleles of the B locus, have been long shown to influence the outcome of HIV disease (Kiepiela et al., 2004). The MHC class I molecules are proteins expressed on the surface of all nucleated cells and especially on cells of haematopoietic lineage with an antigen-presenting role (APCs) such as macrophages, dendritic cells, B-cells and CD4 T-cells (Murphy et al., 2008).

MHC class I molecules are composed of a heavy chain (HC) that is encoded by MHC genes and a copy of the protein β2-microglobulin. These fold together to generate a four domain structure, the role of which is to mediate the immune recognition of foreign as well as self- epitopes 8-10 amino-acids long by CD8+ T-cells (Murphy et al., 2008).

For antigen presentation to occur, HIV proteins must first be degraded to give rise to individual peptides. Two multi-domain complexes are responsible for the degradation of polyubiquitinylated proteins intracellularly: the proteasome and the immunoproteasome. The first consists of a polyprotein core (20S) and two attached protein complexes (19S) that specialize in the binding and unfolding of ubiquinated substrates (Kloetzel and Ossendorp, 2004). The second arises when IFN-γ activation triggers the replacement of three of the 20S subunits by the proteins LMP2, LMP7 and MECL-1 and induction of the proteasome activator complex (PAC, PA28), all of which act to increase the efficiency of antigen-processing and presentation (Kloetzel and Ossendorp, 2004). Poly-ubiquitinylated proteins such as those damaged by IFN-γ induced reactive oxygen species (Seifert et al., 2010) and defective ribosomal products (DRiPs), such as unfolded or misfolded proteins or proper length and sequence, proteins with sequence and prost-translational modification errors, prematurely

terminated proteins, proteins containing frame-shift errors and out-of-frame proteins, are all substrates for proteasome/immunoproteasome processing (Dolan et al., 2011). The resulting products associate with the peptide transporter TAP and translocate into the endoplasmic reticulum (ER) where they are further trimmed at their N-termini by proteases such as the ER-associated amino peptidases ERAAP1 and 2 to generate the epitopes that will finally be loaded onto the MHC class I molecules (Rock et al., 2010).

In the ER, the MHC class I chains fold partially and associate non-covalently with molecules of β2m. Most MHC class I chains in the ER are unstable in the absence of peptide and are thus stabilised by forming complexes with chaperone proteins such as calreticulin, ERp57, protein disulphide isomerase (PDI) and tapasin. These peptide-loading complexes (PLCs) not only stabilize the early conformations assumed by folded MHC I molecules but also ensure that the molecules are ultimately loaded with peptides of high affinity (Neefjes et al., 2011). Once peptide loading has occurred the MHC class I molecules are ready to leave the ER for presentation onto the cell surface. Inside the groove, the peptide is stabilized through a series of hydrogen bonds and ionic interactions that occur between the MHC class I cleft and its main anchor amino-acids and N- and C-termini (Murphy et al., 2008).

MHC class I members of the same or different HLA allotype can be differentiated with respect to their ability to control viral replication based on their individual peptide-binding groove amino-acid landscapes. For instance, the B*35 alleles B*3501 and B*3503 which differ by one amino acid at position 116 show different susceptibilities to disease progression and associate with different signatures of molecular evolution of at least one RT epitope in HIV-1 infection (Moore et al., 2002). Similar findings have been reported for the allelic pairs HLA-B*5702/B*5703 (Kloverpris et al., 2012) and HLA-B*4402/B*4403 (Macdonald et al., 2003). Likewise,

a three amino-acid difference between B*1510 and B*1503 translates into a differential association with viral control in individuals infected with HIV-2 (Yindom et al., 2010).

The strongest associations with effective viral control in HIV-1 infection have been noted for alleles, such as HLA-B*5701, B*5801 and B*63 (Gillespie et al., 2002) that have an inherent ability to accommodate peptides with a greater variety of residues, such as hydrophobic, negatively or positively charged and/or proline (Kiepiela et al., 2004). Other alleles, such as HLA-B*2705 owe their protective profile to their ability to preferentially present epitopes to CD8+ T-cells from structural viral proteins (eg. gag capsid proteins), the mutation of which bears a high fitness cost for the virus (Borghans et al., 2007). Protective alleles restrict immunodominant antigens and contribute more than non-protective alleles to the total CD8+ T-cell response in the early phases of HIV-1 infection (Altfeld et al., 2006). Finally, HLA allele heterozygosity is also thought to restrict viral replication by allowing for a greater variety of viral epitopes to be presented (Carrington et al., 1999).

Studies of HLA class I association with viral control in HIV-2 infection in patients of West African origin (Guinea-Bissauan) reveal similar allelic hierarchies. Despite being relatively infrequent in such populations, HLA-B*5701 and B*14 tend to correlate with higher CD4 counts and lower mean log viral loads. The reverse however is true for alleles such as B*1503 and B*0801 (Yindom et al., 2010).

Despite restricting qualitatively superior CD8+ T-cell responses (Almeida et al., 2007) however, protective alleles are not sufficient by themselves to provide a long-term restriction of viral replication (Migueles et al., 2000). SNPs located upstream or downstream of their genes are also important for disease susceptibility. One SNP (rs2395029) that has been shown to associate with a lower viral set point viral and long-

term non-progression in HIV-1 infection lies within the HERV-derived gene HCP5. This gene, whose function is still largely unknown, is in high linkage disequilibrium with the protective HLA-B*5701 allele and thought to contribute to its ability to limit viral replication (Fellay et al., 2007; Guergnon et al., 2012). The minor variant of this allele (G) is an independent predictor of disease progression (van Manen et al., 2009).

Another factor associated with a low infectivity and resistance to HIV-1 infection is the presence of a 32bp deletion within the CCR5 gene (Δ32). This mutation shifts the CCR5 ORF to introduce a premature stop codon that results in a truncated version of the CCR5 that the virus cannot bind. However, the absence of this deletion in African patients makes it less likely that it contributes to the viral control seen in HIV-2 infection (Rowland-Jones et al., 1998b).

On the other hand, SNPs, such as the intronic C>T CD4 SNP rs2255301 and the CD209 SNP rs8105483, are enriched in HIV-2 infected patients compared to uninfected controls. The first lies between the exons encoding the amino-terminal domain (D1) of the CD4 receptor and is thought to impact on the HIV-2 susceptibility to CD4 dependent viruses. The second, is located within the gene encoding for the C-type lectin DC-SIGN that is expressed on macrophages and dendritic cells and believed to play a role in the transport of HIV from the periphery to the lymph nodes. Patients with lower CD4 counts have a higher frequency of the G-A rs8105483 haplotype than patients who maintain higher CD4 counts (G-G) (Hennig et al., 2011).

1.5 The Immune Response to HIV-2

HIV-2 infection triggers both an innate and an adaptive immune response within its infected hosts. As with HIV-1 infection, they main targets of the virus are cells bearing the CD4-receptor such as dendritic cells (DCs), macrophages and CD4+ T-cells.

1.5.1 Dendritic cells

Dendritic cells belong to the innate arm of immunity and play a key role in the initiation of immune responses. Both plasmacytoid DCs (pDCs) and myeloid DCs (mDCs) present antigens to CD4+ T-cells and contribute to the immune response by producing antiviral factors such as IFN-α (pDCs) and IL-12 (mDCs) (Nowroozalizadeh et al., 2009). In the context of HIV-1 infection, DCs have been shown to contribute to the dissemination of the virus either by becoming infected or by transporting the virus in sites of CD4 T-cell activation (Duvall et al., 2007). They have also been shown to associate with the development of immune activation and induction of cellular apoptosis (Cavaleiro et al., 2009b).

DCs become activated when their intracellular pattern recognition receptors (PRRs) recognize a number of signature pathogen associated molecular patterns (PAMPs) on invading pathogens. For instance, Toll-like receptors (TLRs) such as TLR7/8 and TLR9 recognize single stranded RNA and unmethylated DNA respectively (Cavaleiro et al., 2009b; Nowroozalizadeh et al., 2009). HIV-1 env and capsid proteins have also the ability to activate DCs either through a bystander effect (Env) or through a cyclophilin-A (CYPA)- IRF3 (transcription factor interferon-regulatory factor 3) capsid-dependent mechanism (Bordon, 2010; Cavaleiro et al., 2009b).

The role of DCs in HIV-2 infection is less clear. Contrary to monocyte-derived macrophages (MDMs), which can be productively infected by HIV-2, immature and activated DCs in HIV-2 infection resist infection by primary and lab adapted strains and do not pass the virus onto CD4 T-cells (Duvall et al., 2007). When it occurs, infection is CD4-dependent and characterised by a much lower level of viral replication than the one seen in HIV-1 infection (Duvall et al., 2007). pDC depletion (Cavaleiro et al., 2009b) in HIV-2 infection can be observed in both aviraemic/ asymptomatic patients and progressors and thus is more likely virus-independent (Cavaleiro et al., 2009b). In terms of phenotype, pDCs display an activated profile (CD86+ PD-1L+) which suggests that they are either incompletely differentiated or tolerogenic (Cavaleiro et al., 2009a). The upregulation of CD40 after CpG-A stimulation is impaired (Cavaleiro et al., 2009b) and TLR9 responsiveness also wanes in advanced stages of HIV-2 disease (CD4 counts <200 cells/μl), (Cavaleiro et al., 2009b; Nowroozalizadeh et al., 2009). Myeloid DCs, on the other hand, appear to be better preserved, especially with regard to their resistance to Env (gp125)-mediated effects. There is no change in their morphology, expression of co-stimulatory markers, cytokine production or allostimulatory ability in the presence of HIV-2 envelope and it has been proposed that this might be one of the factors leading to the better outcomes observed in HIV-2 disease (Cavaleiro et al., 2009a).

1.5.2 Neutralising Antibodies

Chronic HIV-2 patients harbour primary HIV-2 variants that are sensitive to polyclonal and monoclonal neutralisation (de Silva et al., 2012; Kong et al., 2012; Ozkaya Sahin et al., 2012). Neutralisation is seen with longitudinal and contemporaneous plasma samples from HIV-2 infected patients as well as with plasma from HIV-1 infected

patients (intertype neutralisation) (Ozkaya Sahin et al., 2012). Such profiles of HIV-2 neutralisation have been shown to remain stable for up to 10 years (Ozkaya Sahin et al., 2012) and to reach reciprocal titers for some isolates as high as 10^6 (Ozkaya Sahin et al., 2012). Therefore, HIV-2 displays a high immunogenicity in natural infection (Kong et al., 2012). Even though the exact nature of these neutralising antibodies is not known at present, they are thought to share similar specificities to those of IgG antibodies that target regions such as the V3, V4 and CD4bs of HIV-1 (Kong et al., 2012).

Contrary to HIV-1 however, in HIV-2 infection there is no evidence of escape from neutralising antibodies (de Silva et al., 2012). Resistance seems to be an inherent characteristic of some envelopes, such as the envelope expressed by the clade A virus HIV-2_{ROD} but patients are able to raise potent neutralising antibodies even in the presence of such resistant variants (de Silva et al., 2012), most likely due to their longer viral incubation period, different conformations assumed by the HIV-2 envelope (eg more open and accessible V3 loop, differences in glycan shield density) (Ozkaya Sahin et al., 2012) and/or due to a higher degree of exposure of broadly cross-reactive epitopes on the surface of HIV-2 Env trimers. (Kong et al., 2012). Variants expressing resistant envelopes appear to circulate more frequently in patients with higher plasma viraemias and in patients with progressive HIV-2 disease (de Silva et al., 2012). Nevertheless, there is no association between the potency and breadth of neutralisation with viral load and progression in HIV-2 infection which has led to the conclusion that additional arms of immunity must be responsible for the dichotomy of disease outcomes seen in HIV-2 patients (Kong et al., 2012).

1.5.3 Natural Killer (NK) Cells

Even though NK cell percentages are lower in HIV-2 patients compared to healthy controls, at CD4 counts >500 cells/μl NK cytotoxicity is well preserved and cytokine producing NK $CD56^{bright}$ cells produce more IFN-γ, MIP-1β and RANTES than in HIV-1 infection. Hence, it has been postulated that NK cells might play an important role in early HIV-2 infection (Nuvor et al., 2006). The Killer Immunoglobulin-like receptors (KIRs) have also been implicated in the effectiveness of the HIV-2 specific immune response. Their role is to regulate the NK cell target recognition positively or negatively depending on their individual nature. Inhibitory KIRs such as KIR2DL and KIR3DL prevent NK function whereas activatory KIRs, such as KIR2DS and KIR3DS, provide activating signals to the NK cells. Both functions are necessary for host protection (Bashirova et al., 2011) and the recognition of HLA-B targets by KIRs is serologically defined. For instance, NK cells expressing the KIR3DS1 molecule specifically recognize Bw4 molecules with an isoleucine at position 80 (Bw4-80I) (Bashirova et al., 2011). Such activating KIRs may confer a protective advantage to populations harbouring higher frequencies of these alleles in HIV-2 infection (Yindom et al., 2010).

1.5.4 CD4 T-cells

During HIV-2 infection, the absolute numbers of naive CD4 and CD8 T-cells (CD45RA+CD62L+) in the peripheral blood of infected patients decrease (Sousa et al., 2002) and memory CD4 T-cells acquire an 'early differentiated' phenotype that is characterized by low levels of CD27 expression, CD45RO expression, lack of CD57 expression (Duvall et al., 2008) and an ability to produce IFN-γ and IL-2 (Alatrakchi et al., 2006). Terminally differentiated memory CD4 T-cells (CD57+CD45RO-) also develop in HIV-2 infected patients and these cells are characterised by higher levels of

CD4 polyfunctionality (Duvall et al., 2008) than their HIV-1 counterparts. This profile of higher IFN-γ, TNF-α, MIP-1β and IL-2 production and greater proliferative potential is especially true for Gag-specific CD4+ T-cells and CD4+ T-cells in asymptomatic patients with undetectable viral loads (Alatrakchi et al., 2006; Duvall et al., 2006; Duvall et al., 2008). However, as CD4+ CD45RA- T-cells are also enriched in CCR5+ expression, they are more likely to serve as HIV-2 infection targets (Soares et al., 2006). This coupled with the fact that their maintenance does not seem to translate into more polyfunctional CD8+ T-cell response or a better CD8 T-cell proliferative ability has led to the assumption that their preserved functionality might not fully account for the better outcomes observed in HIV-2 infection (Duvall et al., 2006).

1.5.5 CD8 T-cells

CD8+ cytotoxic T- lymphocytes (CTLs) are an equally important T-cell subset of antiviral immunity. Their appearance in primary HIV and SIV infection coincides with the decline of viral load at a time when HIV-specific antibodies are still undetectable (Borrow et al., 1994; Koup et al., 1994). *In vitro*, CTLs from humans and baboons inhibit the replication of HIV-1 and HIV-2 in autologous CD4+ T-lymphocytes (Blackbourn et al., 1997; Walker et al., 1986). *In vivo* these cells localise around the splenic white pulp where most infected CD4+ T-cells reside, and mediate their elimination (Cheynier et al., 1994). In addition, *in vivo* depletion of CD8+ T-cells in SIV-infected macaques leads to a rapid and dramatic increase in plasma viraemia, which further supports the importance of CTLs in setting viral replication under control (Jin et al., 1999; Schmitz et al., 1999). Viral epitopes targeted by CD8+ T-cells mutate rapidly, which indicates that CTLs exert considerable pressure on the virus in both HIV-1 and SIV infections (Goulder et al., 1997; Price et al., 2004).

CD8+ T-cells use T-cell receptors (TCR) to recognize target cells. The classic human TCR is a heterodimer composed of two chains; an α-chain and a β-chain. TCR β chains are encoded in the genome as variable (V), diversity (D), joining (J) and constant (C) gene segments whereas TCR α chains are encoded by variable (V), joining (J) and constant (C) gene segments only. There are approximately 42 different TRAV gene segments and 47 different TRBV segments dispersed in chromosomes 14 and 7 respectively (Arden et al., 1995). All V, D and J segments are flanked by recombination signal sequences (RSSs) that are seven and nine bases long and separated by 12-23 nucleotide spacers. These are recognized by RAG1 and RAG2, two recombination enzymes which act, along with high mobility group proteins, to assemble dissimilar segments separated by 12 and 23 bp spacers (12/23 rule) into synaptic complexes for recombination and non-homologous gene end-joining (Krangel, 2003). The random association of these V segments with D, J and C segments and the addition of non-germline nucleotides at the V (D) and J junctions (N diversity) produces each TCR chain (figure 1.5). This process also gives rise to three regions of hypervariability within the V segments, which are known as complementarity-determining regions (CDRs). The CDR1 and CDR2 regions are constant for each type of chain and contain germline-encoded residues whereas the CDR3 regions (loops) vary in their sequence and are involved in the recognition of the foreign peptides presented by MHC molecules on infected target cells (Arden et al., 1995; Garcia and Adams, 2005).

In addition, the αβ TCR is also non-covalently associated with a complex of CD3 protein subunits (γε, δε, ζζ), which carry ITAM motifs (Immunoreceptor tyrosine-based activation motifs) in their cytoplasmic portions. These are indispensable for the function of the TCR complex and the intracellular transduction and amplification of the αβ TCR ligation signals (Smith-Garvin et al., 2009)

Naive CD8+ T-cells develop in the thymus from bone marrow lymphoid progenitors that commit to a CD8 lineage before exiting to the periphery. They carry αβ TCRs that have a diminished potential to cause pathology upon tissue migration having been positively selected for their MHC class I restriction and negatively selected on

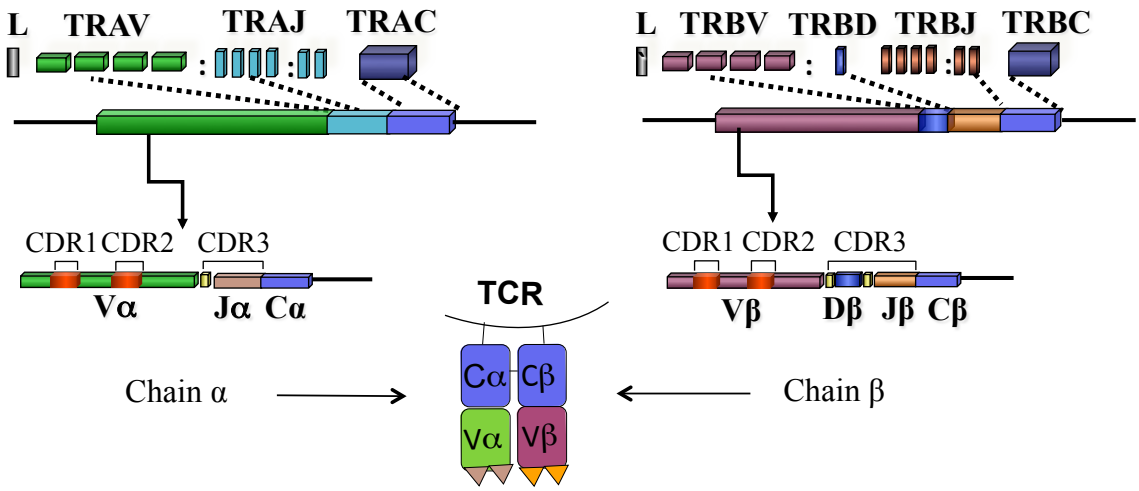


Figure 1.5 Gene rearrangement of the αβ TCR. L: leader sequence.

their ability for self-antigen recognition (Wang et al., 2009). In order to become activated, naive CD8+ T-cells that circulate between the blood and secondary lymphoid tissues must first encounter antigen and become 'instructed' by activated APCs. Three signals are necessary for their activation: a signal ensuing from the interaction of their TCR with the pMHC complex on the surface of APCs, a co-stimulatory signal that is also provided by co-stimulatory molecules on the APC (i.e. CD80, 4-1BB) and a third signal that comes from cytokines such as IL-12 and type I IFNs (Williams and Bevan, 2007).

Instruction of CD8+ T cells by CD4 T-cells is also necessary for increased CD8+ T-cell functionality. For instance, it has been shown that the CD4 T-cell mediated help favours a phenotype of enhanced IFN-γ and IL-2 responsiveness by CD8+ T-cells by promoting the histone acetylation of such loci during CD8+ T- cell differentiation (Northrop et al., 2006). However, the exact timing for this type of help is still debatable, as experimental evidence suggests both an involvement in the early phases of immunity during DC differentiation as well as in later stages of memory CD8+ T-cell development (Castellino and Germain, 2006).

Activated CD8+ T-cells utilise a variety of methods to kill infected cells and can undergo repeated cycles of antigen recognition and cytolysis during the acute and chronic phase of responses (figure 1.6) (Huse et al., 2008). The cytotoxic mechanisms that are triggered upon MHC recognition rely on the production of cytotoxic molecules such as perforin and granzyme as well as on cell-to-cell interactions between the CD8+ TNFR receptor Fas (Apo-1 or CD95) and its ligand. The first two factors (perforin/granzyme) cause the necrotic death of target cells by promoting the permeabilisation of the target membrane through the formation of pores, whereas Fas (CD95) triggers a program of necrotic death within infected cells (Kagi et al., 1996).

Target recognition also induces the concurrent production of an array of antiviral factors (IFN-γ) and chemokines (MIP-1α, RANTES) that are either secreted within the immunological synapse (IS) for cell killing and intercellular communication

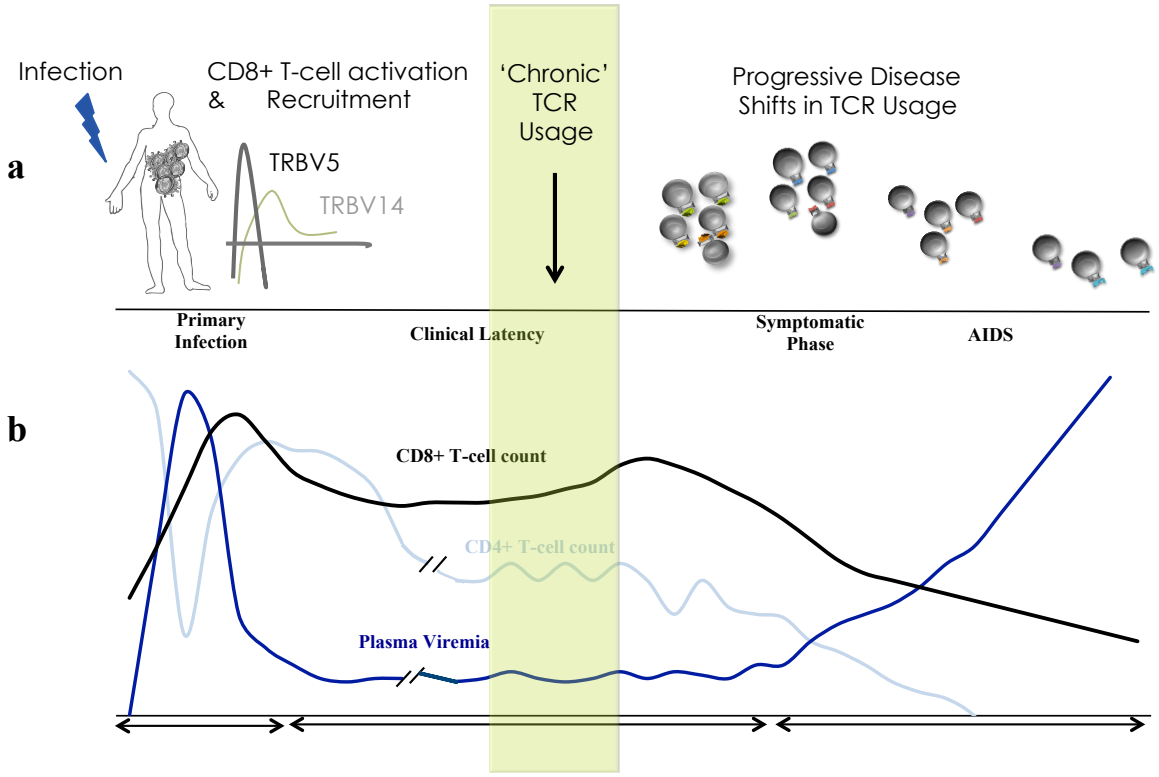


Figure 1.6 (a) CD8+ T-cell mobilisation over time (b) and course of infection. The acute phase of the HIV infection is characterised by high levels of viraemia and a profound drop in the numbers of CD4 helper T-cells. CD8+ T-cell mobilisation occurs approximately 1-2 weeks after infection and this initial wave of CTL recruitment coincides with the reduction of plasma virus levels and the restoration of CD4 T-cell counts. The chronic stage of the infection lasts between 2-10 years. During this time, CD4+ T-cell counts remain stable or fall slowly and viraemia fluctuates or is slowly rising. At the same time, CD8 TCR usage remains stable or fluctuates in response to bouts of viral replication but it is not until the symptomatic phase has began that major CD4 and CD8+ T-cell depletions and turbulences are seen (Poli et al., 1993; Sabin et al., 2000).

or produced systemically for bystander activation and the establishment of chemokine gradients (Huse et al., 2008).

HIV-2 patients have strong non-cytotoxic CD8+ T-cell antiviral activity. Cell-free culture supernatants suppress the replication of R5 and X4-tropic virus and it has been proposed that β-chemokines, which are produced with a higher magnitude in asymptomatic patients that maintain high CD4 counts, such as RANTES, MIP-1α and MIP-1β, may contribute to the containment of viral replication (Ahmed et al., 2005).

However, their role and contribution still remains debatable as it has also been shown that HIV-2 variants isolated from aviraemic patients resist inhibition by such chemokines (Blaak et al., 2008). In patients with CD4+ T-cell depletion the levels of plasma IL-7 are also elevated. However, this change in the IL-7 levels does not influence the homeostasis of differentiated CD8+ T-cells, as these progressively lose their IL-7Rα expression and thus their ability to respond to the cytokine. Hence, similarly to the β-chemokines, the role of these higher levels of IL-7 in HIV-2 infection remains unclear (Drylewicz et al., 2008).

The cytototoxic function of CD8+ T-cells in HIV-2 infection is better documented. HIV-2 infected patients have strong Gag-specific CD8+ T-cell IFN-γ responses that correlate well with the control of viraemia (Leligdowicz et al., 2007). However, studies involving Gambian patients have shown that at high CD4 percentages (>20%) there are no differences in the levels of immune responses or frequency of IFN-γ producing cells between HIV-1 and HIV-2 infection, despite the better control of viral replication observed in the latter infection (Jaye et al., 2004). In addition, Gag-specific CD8+ T-cells in HIV-2 are more polyfunctional but produce no IL-2 and show no differences in their proliferative capacity in comparison to their HIV-1 counterparts (Duvall et al., 2006). These cells are of a central memory or effector memory phenotype (Duvall et al., 2008) and are characterised by an intermediate phenotype (CD27+ perforin^{low} CD28+ variable) similar to that described in HIV-1 infection (Gillespie et al., 2005), as well as by an early differentiation phenotype (CD27+CD28+) (Leligdowicz et al., 2010b). The more polyfunctional population is the one that is terminally differentiated (CD57+) and degranulates more readily (Duvall et al., 2008).

The cross-recognition of variant epitopes has also been documented in HIV-2 infection where CD8+ T-cells have been shown to produce IFN-γ in response to not

only HIV-2 variant epitopes but to corresponding HIV-1 epitopes as well (Rowland-Jones et al., 1998a). In terms of CD8+TCR usage, a more polyclonal response has been proposed for HIV-2 infection, which was also put forward as a likely explanation for this enhanced capacity of CD8+ T-cell populations to tolerate substitutions (Lopes et al., 2003). However, the breadth of this response, in terms of number of TCR families mobilised, does not correlate with CD4 count or viral load and hence factors beyond this one must play a role in the control of viral replication (Lopes et al., 2003). Moreover, the overall magnitude of the responses does not predict the viral load (Jaye et al., 2004).

Hence, in HIV-2 infection the most robust T-cell responses identified to date are those directed towards the Gag protein. At higher CD4+ T-cell counts, Gag-specific T-cells are polyfunctional with the highest frequencies of IFN-γ production reported in individuals harboring lower viral loads (Leligdowicz et al., 2007). However, whether or not this association with viral load, which is thought to result from a CD8+ T- cell mediated response, mirrors a causal relationship between immune function and viral control remains debatable. This is because studies of acute HIV-2 infection are not currently available and most chronic infection studies published to date are cross-sectional and hence unable to offer a clear distinction between cause and effect. Nevertheless, the role of CD8+ T-cells in SIV infection and their documented contribution to the control of viral replication in macaques (Friedrich et al., 2007; Yamamoto et al., 2012) and the association of their function with viral suppression in HIV-1 infection (Borrow et al., 1994; Borrow et al., 1997; Koup et al., 1994; Saez-Cirion et al., 2007; Yang et al., 2012) as well as with viral clearance in infections such as CMV (Schmitt et al., 2011), render it plausible that this subset could potentially have an active role in the curtailment of viral replication in chronic HIV-2 infection. Thus,

the functional characteristics of HIV-2 specific CD8+ T-cells warrant further investigation especially as it has been previously proposed that successful immune responses to viruses depend on the quality, rather than quantity, of responding T-cells (Appay and Iglesias, 2011; Betts et al., 2006; Rowland-Jones et al., 2001). However, detailed, clonotypic studies in HIV-2 infection addressing the quality of CD8+ T-cells as well as their maintenance over time are currently lacking.

1.6 Aims of the thesis

The purpose of this thesis was to advance the current understanding on the function of HIV-2 specific Gag-restricted CD8+ T-cell immunity, as this has been shown to correlate with lower viral loads. More specifically, the aim was to investigate the quality of an HLA-B*3501 restricted HIV-2 specific Gag CD8+ T-cell response in terms of function and evolution and inform the rational design of follow-up studies.

The selection of the Gag epitope under study, namely of NPVPVGNIY (NY9), was guided by reports correlating its antigenic presentation with long-term non-progression (Leligdowicz et al., 2007; Rowland-Jones et al., 1995) as well as by the availability of reagents at the time of study initiation (i.e. HLA-B3501 expression constructs). In addition, the study was designed to look at qualitative aspects previously associated with HIV-1 control and with antiviral efficacy such as clonotypic constitution (Price et al., 2009b), polyfunctionality (Betts et al., 2006), functional avidity and sensitivity (Yang et al., 2003) and potential for cross-recognition (Rowland-Jones et al., 1998b). More specifically, this D.Phil. project aimed at answering the following questions:

- *Is there selection of public NY9-specific TCRs in HLA-B*3501 individuals or is the mobilised repertoire 'private' in its nature? (Chapter 3)*
- *Are there phenotypic differences between the different TRBV families that become mobilised in response to NY9 presentation? (Chapter 3)*
- *Is there HIV-1 cross-recognition by HIV-2 specific CD8+ T-cell clones (Chapter 3).*
- *Is there maintenance of TRBV usage over time in HIV-2 long-term non-progressors? (Chapter 4)*
- *Are there functional differences between different B*3501, NY9-restricted TRBV subsets in terms of cytotoxicity, avidity, sensitivity, cytokine production and cross-reactivity? (Chapter 4)*
- *What are the structural elements that define the observed readings of avidity and function (ie cross-recognition)? (Chapter 5)*

Hence, the ultimate aim was to determine, which qualitative attributes are present in asymptomatic chronic HIV-2 infection and which are not in an attempt to identify factors that a vaccine should be aiming to boost for prevention and/or therapy of HIV infection.

Chapter 2: Materials and Methods

2.1 Study Participants

Study participants were recruited from the Caio community-cohort in Guinea-Bissau. They were of the Manjako ethnic group, HIV-2 infected and ART-naive. Their selection was based on their HLA haplotype and expression of the HLA-B*3501 allele. In addition, some of them had previously shown a positive IFN-γ ELISpot response to the HIV-2 gag epitope NPVPGNIY. Ethical approval for the study was obtained from the Gambia Government/MRC Laboratories Joint Ethics Committee, the Ministry of health of Guinea-Bissau and the Oxford Tropical Research Ethics Committee. An additional sample obtained in 2005 from a B*3501 HIV-1 infected patient attending the John Warin Ward Clinic in Oxford was also used in the study. At the time of collection, the patient was on triple therapy with zidovudine, lopinavir and lamivudine. Ethical approval was obtained from the Oxford Research Ethics Committee.

2.2 Peripheral blood mononuclear cell (PBMC) isolation

Fiftheen milliliters of peripheral blood were collected by venipuncture into EDTA-containing vacutainer tubes (BD Biosciences). Plasma was separated by centrifugation at 1600rpm for 10min and then samples were diluted at a 1:1 ratio with H10 (RPMI 1640, 10% heat inactivated human serum) and transferred into 50ml Leucosep tubes (Greiner, Germany) preloaded with 15ml Ficoll-Hypaque. (Lymphoprep; Nycomed Pharma AS, Oslo, Norway). Tubes were centrifuged at 2000rpm for 20min with no brake and then PBMCs were collected from the plasma- Ficoll interface. The collected

cells were washed with H10 once and resuspended in 1ml freezing medium (FCS containing 10% DMSO) for cryopreservation.

2.3 CD4 Staining

For CD4 staining 500ul of fresh blood was mixed with 100ul Transfix and kept at 4^{o}C until analysis. CD4 T-cell percentages and absolute values were enumerated using the BD FACSCount system, as recommended by the manufacturer. Briefly, 50ul of whole blood, collected in EDTA, was mixed with the CD4 test kit reagents and incubated for 1 hr in the dark at room temperature. The samples were then fixed with 50ul fixative and ran on a FACs Count machine.

2.4 Generation of peptide- MHC class I tetrameric complexes

For protein expression, BL21(DE3)pLysS competent cells (Invitrogen,CA) were transformed with pGMT7 plasmids encoding either the HLA- B*3501 heavy chain or β2-microglobulin according to the manufacturer's instructions. The cells were subsequently streaked on LB agar/ampicillin plates and incubated at 37^{o}C for 14 hours. Liquid cultures were then set up and left to grow until an OD600 of 0,6 had been reached. The cells were induced with 1M IPTG. For protein recovery bacterial cells were sonicated, washed with 0.5% Triton X-100 thrice and resuspended in resuspension buffer (500mM Tris, 100mM NaCl, 1mM EDTA, 1mM DTT) and 4M Urea (Amersham Inc.).

Protein refolding was performed by adding 15mg of peptide (NPVPVGNIY) to 1lt of refolding buffer (100mM Tris, 400mM L-Arginine, 2mM EDTA, 5mM reduced and 0.5mM oxidised L-Glutathione) along with 25ug β2-microglobulin and 34ug B*35 heavy chain. Refolding was performed for 72 hours at 4^{o}C, after which time the protein

was concentrated down to a final volume of 5ml using a stirred cell ultrafiltration unit (Amicon). The concentrated protein was then purified by gel filtration and biotinylated overnight at room temperature using the enzyme BirA (Avidity, CO). Monomers were purified on an FPLC machine using a Superdex G75 column, concentrated and subjected to tetramerization. For tetramerization the biotinylated monomers were incubated at 4^{o}C with phycoerythrin (PE)-conjugated extravidin (Sigma) at a 4:1 molar ratio. Once ready, the tetramers were stored at 4^{o}C until use.

2.5 Establishment of EBV-transformed B-cell lines, short-term lines and CD8+ T-cell clones

Cryopreserved PBMCs (5 x 10^6 cells) were thawed and washed once with H10 (RPMI 1640, 10% Human AB Serum, 2mM L-Glutamine, 50U/ml penicillin/streptomycin). Subsequently, 50ul of 200ug/ml peptide were added to the PBMC pellet and the cells were incubated for 2.5hrs at 37^{o}C. Incubation was followed by the dilution of cells in 1ml H10, and transfer into wells of a 24-well plate. Three days after the initial seeding, IL-2 was added to the culture at a final concentration of 200U/ml and the short-term line was then maintained by restimulating the cells every 3 weeks with irradiated peptide-loaded EBV transformed B-cells.

For B-cell line production, 10^6 PBMCs were incubated with 200ul EBV supernatant for 3 hours at 37^{o}C / 5% $C0_2$. This initial incubation step was followed by the addition of 200ul R15 to the cells (R15: RPMI 1640, 15% FCS, 2mM L-glutamine, 50U/ml penicillin/streptomycin) containing a final concentration of 2ug/ml Cyclosporin A (SandImmune). The transformed cells were then seeded into two wells of a 96-well round bottom plate and incubated for 2 weeks, after which time cell media was replaced

by R10 (RPMI 1640, 10% FCS, 2mM L-glutamine, 50U/ml penicillin/streptomycin). Confluent cells were first re-seeded into the wells of a 24-well plate and then to a T25 flask where they were maintained in culture until cryopreservation.

Clones were generated from sorted CD8+ IFN-γ + CTL cells or CD8+ tetramer+ cells using the limiting dilution method. Where a tetramer was not available antigen specific CD8+ T-cells were sorted based on IFN-γ secretion. For this purpose 2 x 10^6 CTL cells at 37°C / 5% $C0_2$ for 3 hours with peptide-loaded autologous B-cells. The latter had been previously prepared by incubating the BCL cells for 1hr at 37°C / 5% $C0_2$ in the presence of peptide at a final concentration of 20ug/ml. Three hours later, CTL cells were washed once with cold PBS containing 1mM EDTA and labeled for IFN-γ and the marker CD8 using a PE-conjugated anti- IFN-γ antibody (MACs Cytokine secretion Assay) and 5ul of APC-conjugated anti-CD8 antibody (BD Biosciences). IFN-γ labeling was carried out according to the manufacturer's instructions (Miltenyi Biotec). Briefly, cells were first incubated with 20ul of Cytokine Catch Reagent (Miltenyi Biotec) for 5min on ice and then diluted with 1ml H5 (RPMI 1640, 5% Human AB serum, 2mM L-glutamine, 50U/ml penicillin/streptomycin). An additional 45min incubation at 37°C under slow continuous rotation followed, at the end of which time, cells were washed once with cold PBS/ EDTA buffer and stained with 20ul of PE- conjugated IFN-γ Detection Antibody (Miltenyi Biotec) for sorting. A different strategy was followed when tetramer was available. In those cases, thawed PBMCs were washed once in H10 and stained before sorting with 2ul PE-conjugated NY9 tetramer for 15min at 37°C, and then with 0.4ul CD8-APC (BD Bioscience, clone SK1) for 20 min at room temperature.

After sorting, all cells were seeded in a 96-well round bottom plate at a concentration of 1cell/well in the presence of 100ul cloning mix (feeders). The latter

consisted of $2x10^6$ mixed allogeneic irradiated PBMCs from three donors and 50ug/ml PHA. After two weeks, growing cells were plated out in 1ml of cloning mix, then maintained by weekly restimulations with feeders and IL-2 at a 200U/ml final concentration.

2.6 TCR Clonotyping

TCR clonotyping was performed on sorted CD8+ T-cells according to a protocol developed by the lab of Dr. Douek at the Vaccine Research Centre/NIH (MD, USA) in collaboration with Dr. Jorge Almeida and David Wolinsky. More specifically, PBMCs were thawed in 10ml cold FCS containing 10ul of DNAse I (Roche Applied Science), washed twice with H10 and stained with the live-dead stain ViViD (Molecular Probes) as well as with CD8-QD655, CD4-QD605, CD3-H7APC, CD14-PB, CD19-PB (BD Bioscience; Qdots from Invitrogen). Sorting was performed at the NIH Flow Cytometry facility and involved the selection of the CD3+CD4-CD8+Tet+ population. Between 350 and 6000 cells were sorted in 100ul RNAlater solution (QIAGEN). For TCR amplicon preparation, mRNA was isolated from the sorted cells using a uMACs mRNA isolation kit (Miltenyi Biotec) as per the manufacturer's instructions and subjected to reverse transcription and first round cDNA synthesis using the SMARTer cDNA synthesis kit (Clontech). Then a second PCR reaction was set up using the SMARTer primer 5' PCR Primer II A (5'-AAGCAGTGGTATCAACGCAGA GT-3') and either a TRAC-region primer or a TRBC-region primer (5'-GCTTCTGATGGGTCAAA CACAGCGACCTC-3'). The amplification conditions were as follows:

T (^{o}C)	Time	Number of Cycles
95^{o}C	30sec	1 cycle
95^{o}C 72^{o}C	5sec 2min	5 cycles
95^{o}C 70^{o}C 72^{o}C	5sec 10sec 1min	5 cycles
95^{o}C 68^{o}C 72^{o}C	95sec 10sec 1min	35 cycles

Following to the amplification, PCR products were visualized on a 1% agarose gel, purified using the Nucleospin Extract II kit (Macherey-Nagel) and cloned into pGEM-T Easy vectors (Promega) for bacterial transformation. The ligation for each TRAV and TRBV amplicon was carried out by setting up a 10.5ul reaction that contained a 5:1 cDNA to PGEM-T Easy vector ratio, and 1.5ul of T4 Ligase (Roche Applied Science). The ligated vectors were then used to transform 50ul of DH5a competent cells (Invitrogen) as per Invitrogen's instructions. The plates containing the transformed bacteria were incubated overnight at 37^{o}C and screened the following day for insert incorporation using colony PCR. This PCR was performed on a 96-well plate and for each colony a 25uL reaction was set up containing 2.5ul of 10x High-Fidelity buffer, 1ul of 50mM $MgSO_4$, 0.14ul High- Fidelity Taq Polymerase (Roche Applied Science), 0.5ul dNTPs (Invitrogen), 18.86uL water and 1ul of each of the primers M13F (5′-TTTTCCCAGTCACGAC-3′and M13R (5′-CAGGAAACAGCTATGAC-3′). The conditions for the PCR were as follows: denaturation at 94^{o}C for 5min, then 35 cycles of denaturation at 94^{o}C for 30sec, annealing at 57^{o}C for 30sec and extension at 68^{o}C for 2min followed by a final step of extension at 72^{o}C for 3min. Colonies confirmed as positive during this PCR screen were sent to Beckman Genomics in Massachusetts for Sanger sequencing. The returned TRAV and TRBV sequences were aligned and

analysed using the program Geneious v4.8 (Biomatters, Ltd). Incomplete, out of frame and other 'nonfunctional' sequences that could not be resolved by the individual chromatograms were excluded from the analysis. All results conform to the IMGT TCR nomenclature.

2.7 Tetramer Binding Assay

The avidity of the pMHC/TCR interaction was determined by staining each of the CD8+ T-cell clones with decreasing amounts of tetramer. For each stain, 10^5 cells were suspended in 100ul PBS and stained with fourteen 2-fold dilutions of PE-conjugated NY9 tetramer starting at a final concentration of 10ug/ml. Stains were performed for 15min at 37^{o}C after which time cells were washed with FACs buffer (PBS, 1% BSA, 0.1% NaN_3) and stained with 0.3ul CD8-APC. Background (non-specific) staining was controlled through the incubation of an irrelevant, NY9-non-specific CD8+ T-cell clone with 10ug/ml NY9 tetramer. An irrelevant tetramer (B*07 RPM) was also used in the NY9-specific stains as a guide for gating (unstained). After staining cells were washed once with FACs buffer and fixed using 2% paraformaldehyde. Events were acquired on a CyAn Flow Cytometer (Beckman-Coulter) using the program Summit. The analysis of the results was carried out with the program FlowJo (Treestar Inc.) and gating was based on the unstained sample (irrelevant tetramer B*07 RPM).

2.8 TCR sequencing

For CD8+ TCR sequencing 2-3x10^6 cells were harvested from each CTL clone culture and used for total mRNA isolation using the RNeasy Mini Kit (Qiagen) as per the

manufacturer's instructions. The isolated mRNA was then reverse-transcribed into cDNA using the SMARTer cDNA amplification kit (Clontech) as outlined in the manual. TRAV/TRBV amplicons were generated during the second strand PCR reaction by incorporating in the master mix either the primer TRAV: 5'- GTC CAT AGA CCT CAT GTC TAG -3' or TRBV: 5'-TCT GCT TCT GAT GGC TCA A-3' depending on the chain that required characterization. The conditions for this PCR were as follows:

Step	Temperature	Time	Cycles
Initial Den	95°C	1’	x10
Denaturation	95 °C	30’’	
Annealing	68 °C	30’’	
Extension	68 °C	2’	
Denaturation	95 °C	30’’	x20
Annealing	55 °C	30’’	
Extension	68 °C	2’	
Denaturation	95 °C	30’’	x 1 then at 4°C
Annealing	55 °C	30’’	
Extension	68 °C	7'	

The amplification products were then run on a 1.5% agarose gel and bands at 600 and 500kb respectively were excised from the gel and purified using the Nucleospin Gel and PCR Clean-Up kit (Macherey-Nagel). Purified DNA amplicons were cloned into TA TOPO vectors (Invitrogen) and used to transform TOP10 competent cells (Invitrogen). Transformed cells were plated on ampicillin containing plates (100ug/ml) and incubated overnight at 37°C. The next day, up to 14 different colonies were selected from each plate and used for small-scale culture preparation (10ml). Plasmids were isolated from the cultures at 14-16hrs post-inoculation using the Qiaprep Spin Mini Prep kit (Qiagen) and sent for sequencing. Sequencing was performed with the

primers T3 (5'-ATTAACC CTCACTAAAGGGA-3´) and T7 (5'-TAATACGACTCACTATAGGG-3') by John Frankland at the Weatherall Institute of Molecular Medicine (WIMM) sequencing facility using an automated ABI-377 DNA sequencer (Applied Biosciences Inc, USA). Sequences were analyzed using the program Geneious v4.8 (Biomatters, Ltd). CTL clones with two or more TRBV populations or with chromatograms that could not be fully resolved were characterized 'non-clonal' and were excluded from subsequent studies.

2.9 IFN-γ ELISpot Assay

ELISpot plates (MAIPN4550, Millipore, UK) were coated with 15ug/ml anti- human IFN-γ monoclonal antibody (1-DIK,MABTech, Sweden) and incubated for 2 hours at 37^{o}C. This incubation was followed by six PBS (Sigma) washing steps and blocking of the plate with 100ul/well warm R10 for 1h at room temperature. Plates were then seeded with CTL clones (500 cells/well) and HLA-matched peptide-laden BCL cells (10.000 cells/well) and incubated for 6hrs at 37^{o}C / 5% $C0_2$. Before adding to the wells, B-cells were pre-pulsed with peptide titrations or 10ug/ml peptide (add peptide) for 1h at 37^{o}C. Wells without peptide were also included in the assay as a control for peptide-dependence of interferon-γ secretion. Additional controls included wells containing RPMI 1640 media only (negative control) as well as PMA/Ionomycin (Sigma-Aldrich) stimulated clones (positive control: PMA; 0.05ug/ml, Ionomycin; 1ug/ml final concentration). At the end of the 6-hour incubation the cells were removed from the wells and plates were washed six times with PBS. A solution of 1 μg/ml of biotinylated anti-human IFN-γ monoclonal antibody was then added to the wells and the plates were incubated for an additional 2 hours at room temperature. After six further washes with PBS, a 1ug/ml of streptavidin-conjugated alkaline-phosphatase antibody (MABTech,

Sweden) was added to the plates, which were then incubated for one more hour at room temperature. At the end of this final incubation, plates were washed six times with PBS and developed with streptavidin-alkaline phosphatase and colorimetric substrate (Bio-Rad laboratories, CA). The number of spots was determined using an AID ELISpot reader system (Autoimmune Diagnostika, GmbH). A response was considered positive if R>3D and R-D>20 where R=SFU/5x10^2 clones and D=SFU media. Results were expressed as %maximal IFN-γ.

2.10 ^{51}Cr Release Assay

^{51}Cr release assays were performed in 96-well round bottom plates with HLA-matched BCLs labelled with 7.4MBq ^{51}Cr (PerkinElmer) for 1h at 37^{o}C and pulsed with individual peptides at 10ug/ml final concentration or with titrated peptides covering a range between 1.28x10^{-4}uM and 10uM. The following peptides were used:

	HIV-2	HIV-1
Index	NPVPVGNIY	PPIPVGEIY (B/D clade)
Variant	NPVPVG**S**IY	PPIPVG**D**IY (A/C clade)
	NP**I**PVGNIY	P**A**IPVGEIY
	NPVPV**R**NIY	PP**A**PVGEIY
	NP**I**PV**R**NIY	**N**PIPVGEIY
	SP**I**PVGNIY	PP**V**PVG**D**IY
		P**A**IPVG**D**IY
		NPIPVG**D**IY

B-cells were plated at 5000 cells per well and clones were added at a 5:1 effector-to-target ratio with the final incubation volume being adjusted to 100ul. Control wells were also included in the assay and comprised target cells in R10 for spontaneous background ^{51}Cr release as well as target cells in 5% Triton-X for maximal ^{51}Cr release. Plates were incubated at 37^{o}C / 5% $C0_2$ for 4 hours, at the end of which time, a total of 40ul of supernatant was removed from each well and transferred into a 96-well PET

microplate (PerkinElmer) along with 150ul of Supermix solution (PerkinElmer). Plates were read using a Beta counter (PerkinElmer).

All assays were performed in duplicate and background ^{51}Cr release was always less than 20%. The percent lysis was calculated from the formula (E-M/T-M) x 100, where E is the experimental release, M is the spontaneous release in the presence of R10 medium, and T is release in the presence of 5% Triton X.

2.11 Cytokine Bead Array Assay (Bio-Plex)

The levels of 27 cytokines, namely PDGF-b, IL-1b, IL-1a, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, Eotaxin, FGF-basic, G-CSF, GM-CSF, TNF-α, IFN-γ, IP10, MCP-1, MIP-1a, MIP-1b, RANTES, VEGF were determined in culture supernatants using a Bio-Plex Cytokine Assay (Bio-Rad laboratories,CA) according to the manufacturer's instructions. More specifically, CTL clones (effectors) and autologous EBV-transformed B- cells (targets) were plated at a 5: 1 effector to target ratio and cultured at 37^{o}C, 5% CO_2 for 24 hours either in the presence or in the absence of peptides. Control wells including B-cells with peptide and B-cells without peptide in R10 medium were also included in the assay to account for background B-cell cytokine production. Following the 24hour incubation, supernatants were removed from the assay wells and were centrifuged at 13.000rpm for 5 min to remove contaminating cells and stored at -80^{o}C.

On the day of the analysis standards, the working bead solution and detection antibodies for the assay were prepared and cytokine-specific beads were added to the wells of a 96-well filter plate (Bio-Rad laboratories) designated for the assay at a total volume of 50ul/well and vacuum washed twice. Next, samples and standards

(50ul/well) were added and incubated with the beads for 2 hours at room temperature. The incubation was followed by the addition of the detection antibody (50ul/well), a 30min rocking incubation at room temperature and addition of the streptavidin-PE antibody. Finally, a 10-minute incubation was performed and then the plate was washed three times with 100ul Bio-plex wash buffer in order to remove any unbound antibodies. For the analysis, the beads were resuspended in 125ul of Bio-Plex Assay buffer. Plate reading was performed at a CAL2/RP1 low setting and standard curves were constructed using the reference cytokine concentrations provided in the kit. Analysis of the results was performed using the Prism Software. (GraphPad, San Diego, CA).

2.12 Intracellular Staining

For intracellular staining, $2x10^6$ PBMCs were stimulated with either 2ug/ml peptide or 10ug/ml SEB (Sigma-Aldrich) for 1hr at 37^oC in the presence of CD107a-Alx680. Brefeldin A (Sigma-Aldrich) was then added at a final concentration of 1ug/ml and the cells were incubated for an additional 5 hours at 37^oC, 5% CO_2. At the end of the incubation, the cells were washed and stained with the live/dead dye aqua (Invitrogen) and an anti-TCRVb-FITC antibody (IoTest, Beckman Coulter) for 30min at 4^oC before being labelled with the surface antibodies anti-CD4-Cy55PE, CD8-QD705, CD27-Cy5PE (Beckman-Coulter) and CD45RO-TRPE (Beckman-Coulter) for 20min at room temperature. The CD8-QD705 antibody was conjugated in-house with CD8 purchased from BD Bioscience and QD fluorochome purchased from Invitrogen. The surface labelling was followed by the permeabilization of the cells at 4^oC for 30min. This step was carried out using the Cytofix/Cytoperm kit (BD Biosciences). Subsequently, the cells were labelled with CD3-H7APC (BD Biosciences), IFN-γ-PB (eBioscience),

TNFα-Cy7PE (BD), IL2-APC (BD) and MIP-1β-PE (BD) for 20min at room temperature, then washed twice and fixed with 1% formaldehyde. Cells were acquired on an LSRII flow cytometer and data were analysed using the software FlowJo version 8.7 (Treestar Inc).

For CTL clone intracellular cytokine staining, $0.5x10^5$ autologous B-cells were pulsed with seven 10-fold dilutions of the peptide NPVPVNGIY or 10ug/ml SEB (Sigma-Aldrich) for 1hr at 37^{o}C before being added to 10^5 NY9-specific CTL clone cells. For the peptide stimulated samples the starting concentration was 10ug/ml. An unstimulated (no peptide, no SEB) control was also included in the assay. One hour later, 1ul GolgiSTOP (Monensin; BD Biosciences), 1ul GolgiPLUG (Brefeldin A; BD Biosciences) and 3ul CD107a-APC (BD Biosciences) were added to the culture. Cells were then incubated for an additional 5hrs at 37^{o}C, 5% CO_2 following to which cells were washed and stained with anti-CD8-PB (BD Pharmingen) for 15min at room temperature. Then, cells were permeabilized using 250ul permeabilizing solution (Cytofix/Cytoperm; BD Biosciences) for 20min at 4^{o}C, washed and stained with the antibodies IFN-γ-FITC (BD Bioscience), TNFα-PECy7 (eBioscience) and IL-2 PE (BD Pharmingen) for 20min at room temperature. Cells were then washed twice and resuspended in 300ul 2% parafolmaldehyde. Events were acquired on a DAKO Cyan flow cytometer (Beckman Coulter) with the program Summit. Gating was based on CD8+ cells and at least 30.000 events were collected for each sample. The analysis of the results was carried out using the program Flow Jo (Treestar Inc).

2.13 Polychromatic Flow Cytometry

The panel used for the *ex-vivo* phenotypic analysis consisted of the antibodies PD-1 biotin /SA-PECy7 (R&D Systems, Invitrogen), anti-TCRVb-FITC (Beckman-Coulter),

CD3-H7APC (BD Biosciences), CD4-Cy55PE (Caltag), CD8-QD705, CD27Cy5PE, CD45RO-TRPE, 2B4-Cy5PE (Beckman-Coulter), CD160-Alx647 (Biolegend), CD57-QD565, CD127-PB (eBioscience). For TCR staining the monoclonal antibodies Vb5.1, Vb14, Vb13.1 and Vb17- FITC were used (IoTest, Beckman-Coulter). PBMCs ($2x10^6$) were initially stained with the live/dead fluorescent dye aqua (Invitrogen) for 3minutes and then with PD-1 biotin /SA and anti-TCRVb for 30min at 4^oC. Cells were then washed once with FACS buffer and stained with NY9 tetramer-PE for 20min at room temperature. This was followed by a second wash after which cells were stained with CD3/CD4/CD8/CD27/CD45RO/2B4/CD160/CD57/ CD127 for 20min at room temperature. After one last wash cells were fixed with 1% formaldehyde and acquired on an LSRII flow cytometer (BD Immunocytometry Systems). Forward scatter area versus forward scatter height was used to gate out cell aggregates. Dead cells were also removed from the analysis to reduce the background noise. Gating was performed on CD3+ CD8+ CD4- cells and 10^6 events were collected in total. The compensation was performed electronically with antibody capture beads (BD Biosciences) that were separately stained with each one of the monoclonal antibodies used in the stainings. The acquired data were analysed with the program FlowJo version 8.7 (Treestar Inc).

Chapter 3: *Ex-vivo* characterization of a conserved HIV-2 gag response: clonality, functionality and phenotype.

3.1 Introduction

Unlike viruses that are successfully cleared by the immune system of the host, such as Influenza, and viruses that are chronically controlled, such as EBV and CMV, HIV-1 and HIV-2 bring about a life-long infection that is characterised by immune evasion, continuous viral replication and immunological modulation. Among those infected with HIV only a small minority, the long-term non-progressors (LTNPs), will go on to control the virus for 8 years or more in the absence of anti-retroviral therapy (Grabar et al., 2009; Madec et al., 2009; Virgin et al., 2009).

LTNPs are HIV infected individuals that share a number of immunological and virological characteristics: they have CD4+ T-cell counts of 500 cells/μl or more, strong CD4+ and CD8+ T-cell responses, a higher CD4:CD8 ratio than progressors, lower levels of immune activation, an absence of spontaneous lymphocyte apoptosis, and low or even undetectable viral loads (Altfeld, 2003; Altfeld et al., 2003; Easterbrook, 1999; Madec et al., 2009). However, such individuals represent less than 2% of most cohorts and less than 5% of all HIV infected individuals (Grabar et al., 2009; Riva et al., 2011; Rowland-Jones, 1999; Rowland-Jones and Whittle, 2007) and their stratification is not always easy as multiple mechanisms of delayed disease progression and LTNP definitions have been proposed (figure 3.1) (Baker et al., 2009; Grabar et al., 2009).

Today, one community that represents a unique pool of HIV-infected long-term non-progressors is Caio. The village of Caio is situated in the northwestern part of Guinea-Bissau, approximately 100km from the capital Bissau and is a community-

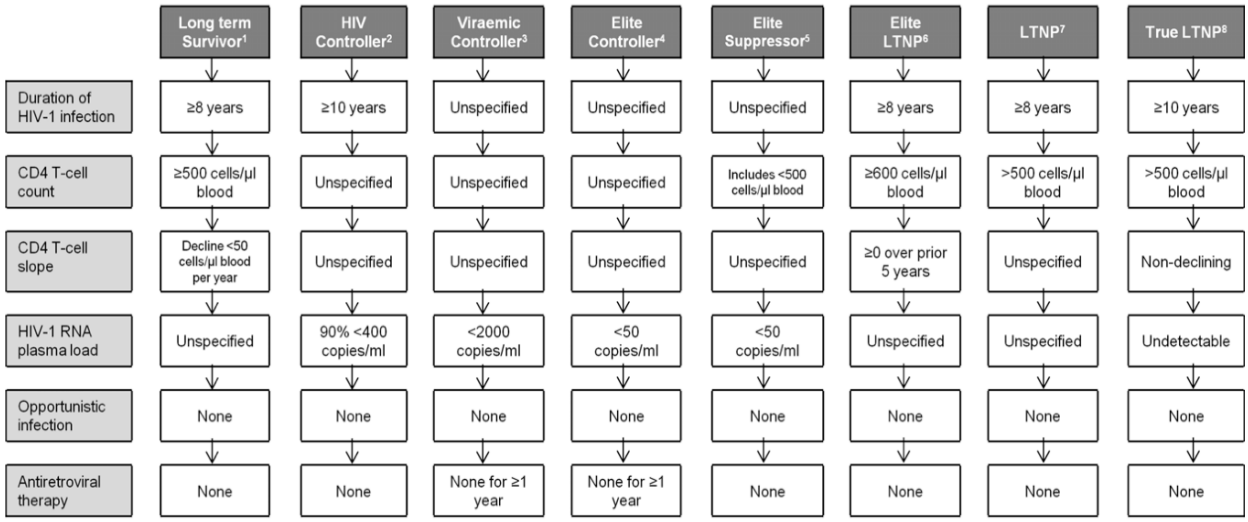


Figure 3.1 Slow progressor and LTNP classification currently used in the literature. Reproduced from (Mandalia et al., 2012) PloS ONE 7(2): e29844.doi:10.1371/journal.pone.0029844

based cohort with a high prevalence of HIV-2 that was established in 1989 by Dr. Dominique Ricard and followed ever since. The population is ethnically homogenous (Manjako) and dispersed among several zones that cover an area of approximately 9km. The HIV-2 prevalence among the inhabitants was 7.9% in 1989 but has since dropped to 4.7% (Tienen et al., 2010) and all patients are infected with clade A HIV-2 (Onyango et al., 2010). The high prevalence of the virus in the village has been attributed to an increased spread that took place during the war of independence (1963-74) (Poulsen et al., 2000).

Partial viral control after acute HIV-1 infection is often associated with persistent and strong $CD8^+$ T-cell responses directed against several HIV epitopes. However, the overall breadth of the CD8+ T-cell responses or the presence of a protective allele alone, such as HLA-B*5703 for example, cannot predict the chronic viral load (Kiepiela et al., 2007).

It is now well established that responses directed against the structural protein p24 Gag in HIV-1 infection correlate positively with markers of delayed progression

such as low viral load and a higher CD4 count in both chronic and elite HIV-1 controllers in the absence of therapy (Edwards et al., 2002; Kiepiela et al., 2007; Klein et al., 1995; Leligdowicz et al., 2007; Riviere et al., 1995). The correlation with the viral load is especially true when responses are mounted against three or more Gag epitopes (Kiepiela et al., 2007) and is independent of viral strain or clade (Boufassa et al., 2011; Dyer et al., 2008; Jia et al., 2012; Serwanga et al., 2009). Furthermore, when these responses are lost, an accelerated drop in CD4 counts and severe deterioration of T-cell function is normally observed (Klein et al., 1995).

HIV-2 infected patients also mount broad and strong homologous Gag-specific T-cell responses that are polyfunctional with over 60% of responding cells producing cytokines such as IFN-γ, TNF-α, MIP-1β and CD107α upon stimulation with the cognate antigen (Duvall et al., 2008). Among those responding to the Gag protein in the Caio cohort are individuals expressing the HLA-B*3501 allele (Leligdowicz et al., 2010b).

The HLA-B*35 molecules belong to the HLA-B7 supertype and comprise 8 main subtypes (B*3501-08), the most common of which is HLA-B*3501 which is also the most common class I molecule in much of West Africa (Allsopp et al., 1992; Hill et al., 1991). The HLA-B*35 alleles have been associated with a wide range of diseases including autoimmune conditions such as glomerulonephritis and thyroiditis (Amoli et al., 2002; Berthoux et al., 1983; Kramer et al., 2004; Mota et al., 1987). In HIV-1 infection, these alleles contribute to CD8+ T-cell responses to a level equivalent to that of protective alleles such as HLA-B*57 and HLA-B*27 in both infected individuals and vaccinated healthy seronegative volunteers (Friedrich et al., 2011; Zhai et al., 2008). In contrast, however, and despite their propensity to restrict immunodominant responses, their expression has been historically associated with a much faster disease

progression (Scorza-Smeraldi, 1988). Additional studies however revealed that only B*35 alleles that harbour Asn and either Phe or Tyr at heavy chain positions 114 and 116 contribute to an accelerated disease progression due to their peptide-binding profiles and their ability to down-modulate the function and proliferation of DCs and T-cells by binding the inhibitory monomyelocytic receptor ILT4 on their surfaces (Huang et al., 2009). Alleles such as B*3501 and B*3508 which preferentially bind peptides that contain P in position 2 and Tyrosine in position 9 (B*35-PY) have been shown to lack similar effects (Gao et al., 2001) and have been associated with a better clinical and immunological outcome (Jin et al., 2002; Rowland-Jones et al., 1998b).

Both gag and pol-derived peptides have been identified as B*3501-restricted in HIV-2 infection. Of particular interest among these epitopes is the Gag-derived epitope NPVPVGNIY ($Gag_{245-253}$) (Rowland-Jones et al., 1995), which is located in the structural helix (h7) that precedes the major homology region of HIV-2 (Price et al., 2009a). Previous HIV-2 studies involving the NPVPVGNIY epitope as well as the closely related HIV-1 peptide PPIPVGDIY (PY9; $Gag_{254-262}$) have established a correlation between the targeting of these peptides, a more broad IFN-γ production and the detection of cross-reactive responses (Rowland-Jones et al., 1999). More recently, Goepfert *et al*. proposed that the transmission in a discordant couple of a clade C virus with a B*35-restricted CTL-derived mutation in the PY9 epitope (TSNPPIPVG**D**IYKRW) from a B*35 individual to a non-B*35 partner may have contributed to the lower peak viral load observed in the recipient. Hence, conservation of this epitope might be important for viral fitness (Goepfert et al., 2008).

In the Caio cohort, studies involving this epitope have revealed an oligoclonal, and highly avid profile of IFN-γ production for NY9-specific CD8+ T-cells. In addition, these cells have been shown to share an early-differentiated phenotype

(Leligdowicz et al., 2010b). However, no previous study has addressed if the NY9 response is maintained over time. It is also unknown whether or not the *ex-vivo* polyfunctional signature that characterises Gag-specific responses in HIV-2 infection applies to this response as well and if the response is private or public in terms of its constituent clonotypes. Moreover, it is not clear at present to what extent the high functional avidity of IFN-γ production translates into an increased cytotoxicity and whether or not the various TRBV families mobilised in response to the NY9 epitope presentation differ between them in terms of phenotype. The purpose of this chapter was to address all these questions.

3.2 Results

3.2.1 The NY9-specific population persists in HLA-B*3501, HIV-2 infected patients for at least three years after their initial recruitment.

Eight HIV-2 infected patients, carriers of the HLA-B*3501 allele, were recruited for the purpose of this study. Of those, five (1,15,17, 21 and 30) had been previously shown to respond to the NY9 epitope whereas the remaining three (12, 26 and 27) were recruited as potential NY9 responders based on their HLA profile (figure 3.2). The classification of the patients as LTNPs followed that previously proposed by Leligdowicz *et al* (Leligdowicz et al., 2010b): all patients were infected with HIV-2 for an average of 13 years, were asymptomatic and had an undetectable viral load. The only patient that was found not to conform to this criterion was patient 26 who, at the time of the recruitment, had a CD4 count of 136 cells/μl.

Staining with NY9 tetramers confirmed the presence of NY9-specific CD8+ T-cells in 7/8 HIV-2 LTNPs. The sizes of the tetramer specific populations among circulating CD8+ T-cells ranged between 0.1-1.32%. A direct comparison between these frequencies and those detected in samples from a previous recruitment in 2006 was not possible however due to differences in the reagents (tetramers) and methods used.

(a)

EM_ID	Age	Sex	Duration of Infection	A1	A2	B1	B2	C1	C2	CD4%	[CD4]	VL†
EM_1	50.0	F	> 9 years (HIV-2)	23	30	B*07	B*3501	4	15	28.78	414	100
EM_12	65.3	F	> 13 years (HIV-2)	23	30	B*3501	B*5301	4	4	25.14	460	100
EM_15	78.3	M	> 17 years (HIV-2)	2	68	B*0801	B*3501	3	4	11.57	859	100
EM_17	75.4	F	> 17 years (HIV-2)	30	68	B*1503	B*3501	4	4	16.76	481	100
EM_21	61.3	M	> 9 years (HIV-2)	23	30	B*3501	B*4901	4	7	21.62	274	100
EM_26	56.2	M	>15 years (HIV-2)	23	23	B*3501	B*3501	4	4	n/d	134	666
EM_30	79.4	F	> 14 years (HIV-2)	3	33	B*3501	B*4201	4	17	37.87	705	100
J038	n/d	M	> 20 years (HIV-1)	3	68	B*3501	B*4401	4	7	n/d	840	3180

†Data from Caio database
nd:not determined

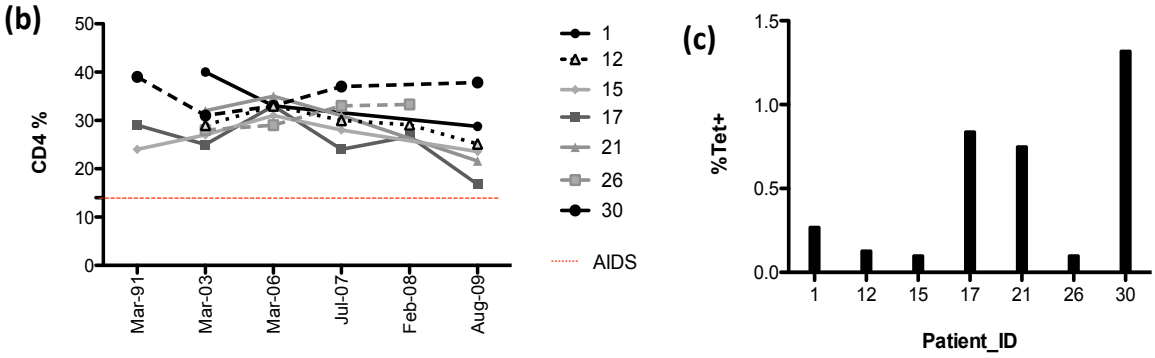


Figure 3.2 (a) Clinical characteristics of the recruited HIV-1/HIV-2 patients, (b) longitudinal CD4 data (%) and (c) Percentages of NY9+ T-cells among circulating CD8+ T-cells at the time of the study.

3.2.2 NY9-specific CD8+ T-cells produce an array of different cytokines.

CD8+ T-cell Gag-specific responses have previously been shown to carry a polyfunctional signature characterised by an increased production of CD107α, IFN-γ and MIP-1β (Duvall et al., 2008). To determine if the NY9-specific response was sharing this signature of polyfunctionality, PBMCs from four HIV-2 patients were thawed, stimulated with 2μM NY9-peptide and stained intracellularly for CD107α and an array of cytokines including IFN-γ, TNF-α, IL-2. and MIP-1β. As can be seen in figures 3.3b and 3.3c, NY9-specific CD8+ T-cells produced a variety of cytokines in

response to cognate antigen recognition. Subsets with four and three functions were detected and these made up an average of 13.45% of the NY9-specific response. In addition, and in line with what was previously proposed by Duvall *et al*, the most frequently produced cytokines were IFN-γ and MIP-1β (46.25% and 45.23% of the total NY9-response respectively), followed by TNF-α and IL-2 which were produced at much lower frequencies (8.64% and 2.84%). Degranulating CD8+ T-cells represented 58.01% of the total antigen-specific population and CD107α was detected in both polyfunctional and monofunctional populations.

When the individual profiles of the patients (figure 3.3c) were studied, some variations did however emerge. The two patients with the highest tetramer percentages (p21 and p30) appeared to have the most polyfunctional profiles. In particular, the pattern seen in patient 30 resembled the cytokine secretion profile obtained after SEB stimulation, which suggested a possible lack of functional impairment. On the other hand, patients 12 and 15, who had lower levels of NY9+ specific CD8+ T-cells, had a higher proportion of bi- and mono-functional CD8+ T-cells. Hence, NY9-specific CD8+ T-cells can produce an array of cytokines in response to their cognate antigen, most notably IFN-γ and MIP-1β. However, individuals mounting an NY9 response differ in their levels of polyfunctionality.

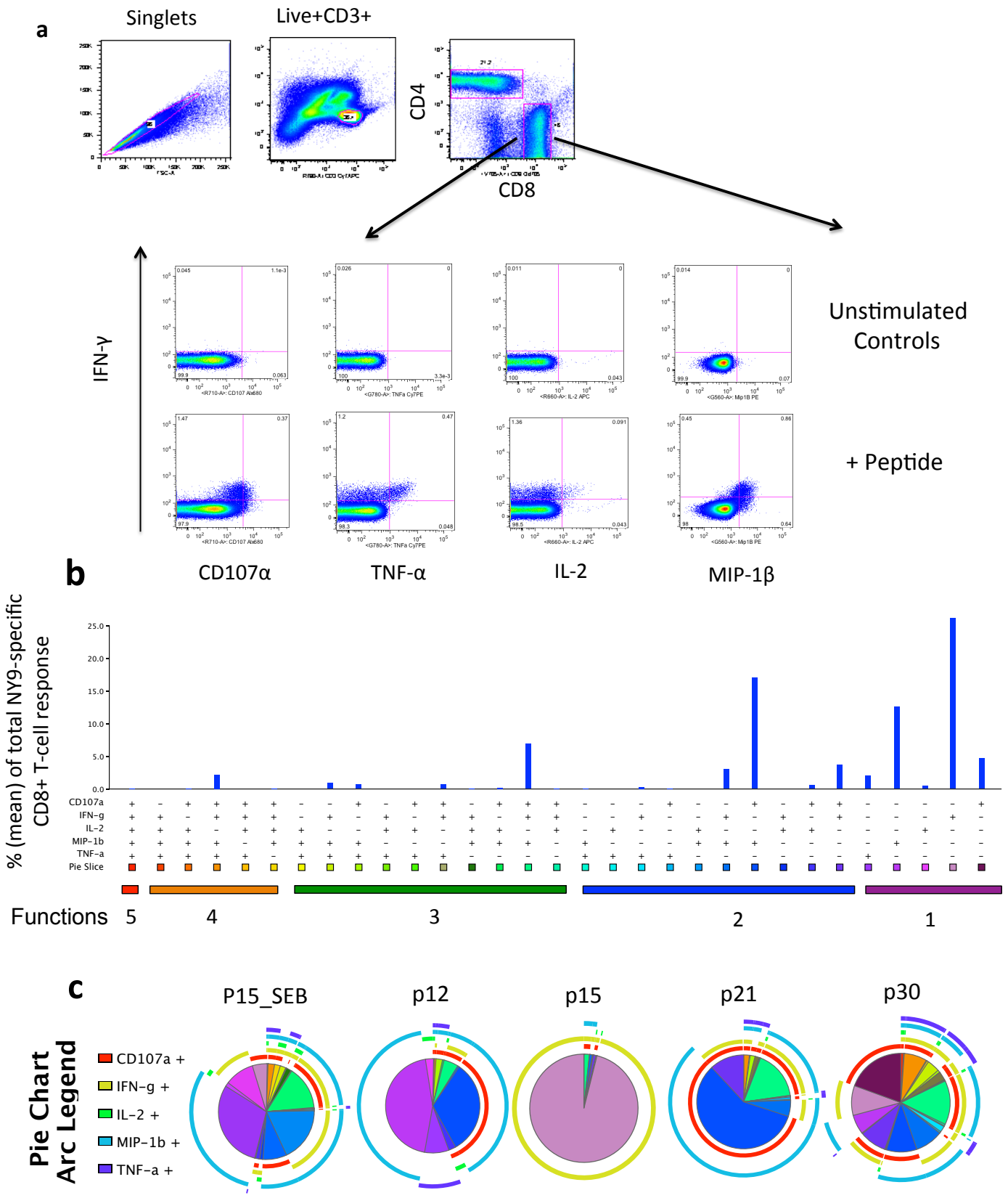


Figure 3.3 (a) The gating strategy used for the identification of CD8+cytokine+ subsets. (b) Boolean gating was used to create a total of 31 possible cytokine combinations for the cytokines under study. Graph bars represent the cytokine-producing subsets detected in the four patients under study (mean% of total NY9 response) whereas horizontal bars correspond to the overall number of functions/ (red=5, orange=4, green=3, blue=2 and purple=1). (c) These pie charts depict the different cytokine producing subsets detected in each patient (p12,p15, p21, p30). Slice colours follow those of individual cytokine combinations as given in b. Arcs denote individual functionalities .SEB: Staphylococcal Enterotoxin B. The program SPICE (NIH) was used for the analysis.

3.2.3 Clonotypes with public TCR features are rare among the NY9-specific CTLs despite the oligoclonal nature and chronic maintenance of the NY9 response.

To gain a better understanding on the nature of the individual clonotypes that contribute to the NY9-response, the TCR usage of NY9-specific populations was determined for seven of the patients under study (figure 3.4). The analysis confirmed the previously published oligoclonal profile of responses (Leligdowicz et al., 2010b) and extended these findings at a sequence level. It also revealed a diverse, 'private' TCR usage among NY9 responding populations. The NY9-response comprised a mean of 4-clonotypes/ patient. The only patient displaying a broader, more polyclonal usage was patient 26 who, at the time of sample collection, had started progressing towards disease. Nevertheless, public TCRs were not completely absent. Two out of seven patients (12 and 17) shared an identical TRAV sequence (TRAV12.3/TRAJ44-1) whereas patients 1 and 17 shared an identical TRBV (TRBV29.1/TRBJ1-1) sequence. These results suggest that public TCRs can be recruited in response to Gag epitopes such as NY9 in chronic HIV-2 infection but their frequency is low and their inter-patient sharing limited.

3.2.4 NY9-specific cells display an increased sensitivity for antigen, which appears to be several times higher than the sensitivity of HIV-1 CTL clones targeting the corresponding HIV-1 epitope.

Next, the ability of the individual NY9-specific clonotypes to lyse targets exogenously loaded with NY9 was assessed. For this purpose, tetramer-sorted PBMCs were used for the establishment of CD8+ T-cell clones using the limiting dilution method. Signs of

ID	TRAV	CDR3	TRAJ	Freq	ID	TRBV	CDR3	TRBJ	Freq
EM-1	12-2	CAVQLL	16-1	42	EM-1	5-1	CASSSLLGGSEAFF	1-1	54
	8-6	CAVSDA	37-1	18		29-1	CSVGYGNTEAFF	1-1	24
	6	CALDMMG	44-1	11		7-2	CASSPVGGGGADGYTF	1-2	1
	1-2	CAVD	44-1	3		19	CASSSQGNQPQHF	1-5	1
	12-2	CAAQLL	16-1	1		29-1	CSVEDAPGRADTQYF	2-3	3
		Seqs: 75					Seqs: 83		
EM-12	12-3	CAMD	44-1	23	EM-12	7-2	CASSLSPGWSEAFF	1-1	82
	4	CLVGEGL	29-1	20		6-1	CASSEDVPGNEQFF	2-1	4
	12-2	CAV	31-1	19		7-2	CASSLDGKSYEQYF	2-7	1
	26-1	CIVRVE	53-1	16		7-9	CASSSYQGAGTEAFF	1-1	1
	29	CAASS	57-1	2					
	4	CLVGEGR	29-1	1					
	13-2	CAER	39-1	1					
		Seqs: 82					Seqs: 88		
EM-15	29	CAASE	57-1	58	EM-15	5-1	CASTFEAGGPYNEQFF	2-1	47
	12-1	CVVNFE	9-01	13		7-9	CASSLYQGAGTEAFF	1-1	37
	12-3	CAMSVT	34-1	11		19	CASSRQEFAGNEQFF	2-1	2
	8-1	CAVNA	8-1	4		27	CASSLTYDAGNTIYF	1-3	2
	12-1	CVANFE	9-01	1					
		Seqs: 87					Seqs: 88		
EM-17	12-3	CAMD	44-1	74	EM-17	7-2	CASSLSPGWNEQFF	2-1	41
	8-6	CAVS	18-1	2		29-1	CSVAWGNTEAFF	1-1	34
	8-6	CAVS	37-1	3		29-1	CSVGYGNTEAFF	1-1	10
	13-2	CAEN	32-1	2		10-2	CASSWTSTYNEQFF	2-1	2
						7-2	CAGSLSPGWNEQFF	2-1	1
		Seqs: 81					Seqs: 88		
EM-21	13-2	CAVDCGD	9-1	58	EM-21	27	CASSPTIDSYEQYF	2-7	41
	12-3	CAMSPLY	43-1	11		11-2	CASSFDSHPNTEAFF	1-1	32
	10	CVVSAMSS	33-1	16		29-1	CSAAYGNTEAFF	1-1	10
						10-3	CAINEGAGDTQYF	2-3	1
						7-9	CASSLYQGAACEQYF	2-7	2
		Seqs: 85					Seqs: 86		
EM-26	26-2	CILREG	32-1	16	EM-26	6-5	CASSYMSSYEQYF	2-7	12
	29	CAALL	39-1	10		5-1	CASSLEGMFGGYNEQFF	2-1	11
	8-1	CAF	28-1	8		4-1	CASSQGTDTGGEKLFF	1-4	9
	30	CGTVG	6-1	8		4-1	CASSQAEGQGWGGEQ\	2-7	8
	38-2	CAYRS	24-2	5		19	CASSMGQGYNEQFF	2-1	7
	38-2	CAYRRD	44-1	5		16	CASSPGAAGMRYEQYF	2-7	6
	8-4	CAL	9-1	5		7-9	CASSPPRGGNNEQFF	2-1	6
	21	CAVRY	27-1	4		6-5	CASKSRGVNTEAFF	1-1	5
	13-1	CAAH	24-2	4		20-1	CSAMGPWGSMGRYQNI	2-1	4
	12-2	CAVNAL	16-1	4		6-5	CASSQGVFDEQYF	2-7	3
	14	CAMREGQ	17-1	2		20-1	CSAREGLYSNQPQHF	1-5	2
	39	CAVDR	12-1	2		29-1	CSVGDRGSGTEAFF	1-1	2
	25	CAG	17-1	1		11-2	CASSLGDRDNEKLFF	1-4	1
						4-2	CASSPQPAGGSNTGELF	2-2	1
						7-6	CASSISGPWDHDEQFF	2-1	1
		Seqs: 74					Seqs: 78		
EM-30	13-1	CAANRH	50-1	53	EM-30	19	CASSPQHGDTQYF	2-3	94
	10	CVVSAML	40-1	36					
		Seqs: 89					Seqs: 94		

Figure 3.4 TCR Vα and Vβ repertoire of tetramer positive NY9-specific CD8+ T-cells (CD3+CD8+Tet+) in 7 HIV-2 infected patients expressing the HLA-B*3501 allele. Public clonotypes (TRAV CDR3 or TRBV CDR3 identity) are shown in red.

growth were observed approximately two weeks after sorting, in five of the six plates seeded (1,15,17,21 and 30). Each plate represented each of the patients under study. The expanding CTL clones were then screened using tetramers and Vβ-specific antibodies for NY9-specificity and TCR usage. Where the identification of TCR usage

ID	TRAV	CDR3 sequence (aa)	TRAJ	TRBV	CDR3 sequence (aa)	TRBJ
1.3	8-6	**C A V S D A** G N T G K L I	37-1	29-1	C S V G Y G N T E A F F	1-1
15.1	29	**C A A S E** Q G G S E K L V F	57-1	7-9	**C A S S L Y Q G A G** T E A F	1-1
15.2	29	C A A S E Q G G S E K L V F	57-1	7-9	C A S S L Y Q G A G T E A F	1-1
15.4	29	C A A S E Q G G S E K L V F	57-1	7-9	C A S S L Y Q G A G T E A F	1-1
15.6	29	C A A S E Q G G S E K L V F	57-1	7-9	C A S S L Y Q G A G T E A F	1-1
15.28	29	C A A S E Q G G S E K L V F	57-1	7-9	C A S S L Y Q G A G T E A F	1-1
15.31	29	C A A S E Q G G S E K L V F	57-1	7-9	C A S S L Y Q G A G T E A F	1-1
15.33	29	C A A S E Q G G S E K L V F	57-1	7-9	C A S S L Y Q G A G T E A F	1-1
15.17	8-1	**C A V N A N A** G F Q K L V **F**	8-1	5-1	**C A S T F E A G G P** Y N E Q F F	2-1
15.22	8-1	C A V N A N A G F Q K L V F	8-1	5-1	C A S T F E A G G P Y N E Q F F	2-1
15.18	12-3	**C A M R A D** T G G F K T I F	9-1	5-1	**C A S S L D P T V R** D T Q Y F	2-3
15.26	12-3	C A M R A D T G G F K T I F	9-1	5-1	C A S S L D P T V R D T Q Y F	2-3
17.5	8-6	**C A V S** D R S T L G R L Y	18-1	29-1	**C S V A W G** N T E A F F	1-1
17.28	12-3	C A M D T G T A S K L T F	44-1	7-2	**C A S S L S P G W** N E Q F F	2-1
17.12	12-3	C A M D T G T A S K L T F	44-1	7-2	C A S S L S P G W N E Q F F	2-1
17.30	12-3	C A M D T G T A S K L T F	44-1	7-2	C A S S L S P G W N E Q F F	2-1
17.27	12-3	C A M D T G T A S K L T F	44-1	7-2	C A S S L S P G W N E Q F F	2-1
17.33	12-3	C A M D T G T A S K L T F	44-1	7-2	C A S S L S P G W N E Q F F	2-1
17.15	12-3	C A M D T G T A S K L T F	44-1	7-2	**C A S S L S P G R** N E Q F F	2-1
17.22	24	**C A S G** Y G Q N F V F	26-1	4-3	**C A S S E T G G Y** S P L H F	1-6
17.10	24	C A S G Y G Q N F V F	26-1	4-3	C A S S E T G G Y S P L H F	1-6
17.26	24	C A S G Y G Q N F V F	26-1	4-3	C A S S E T G G Y S P L H F	1-6
21.5	8-1	**C A V D** I K A A G N K L T	17-1	11-2	**C A S S F D S H P** N T E A F F	1-1
30.10	13-1 10	**C A A N R H** D K V I F **C V V S A M L** S G T Y K Y	50-1 40-1	19	**C A S S P Q H G** D T Q Y F	2-3
30.9	13.1	C A A N R H D K V I F	50-1	19	C A S S P Q H G D T Q Y F	2-3
30.11	13.1	C A A N R H D K V I F	50-1	19	C A S S P Q H G D T Q Y F	2-3
30.1	10	C V V S A M L S G T Y K Y	40-1	19	**C A S S P Q Q G** D T Q Y F	2-3
30.50	39	**C A V D F** G G T S Y G K L	52-1	20-1	**C S A S N Q G N G** G E L F F	2-2

Figure 3.5 αβ TCRs expressed by NY9-specific CTL clones. Public TCR sequences determined by clonotyping are shown in red. Dominant TCR families (eg TRAV29/TRBV7-9) were overrepresented among the established CTL clones

with Vβ-specific antibodies was not possible, a different approach was used, which involved the sequencing of individuals TCRs using a PCR method designed to amplify TCR chains in a non-selective way (chapter 4). The sequencing of the obtained TCR amplicons confirmed the overepresentation of dominant TCRs among the established CTL clones and established that the two shared chains (TRAV12.3 and TRBV29.1) were independent parts of two different TCRs (figure 3.5).

When the functional avidity of the NY9-clonotypes for their target antigens was determined using ^{51}Cr release assays, all patients studied were found to harbour CTLs that were cytotoxic and which possessed a high functional avidity (EC_{50}) for the cognate epitope. This avidity was ranking high among several functional avidities published to date and most importantly among a range of HIV-1 specific functional

avidities previously measured in our lab using the same method and E:T ratios (Dr. Tao Dong). In addition, when CTL clones established from an HIV-1 LTNP (patient J038), specific for the corresponding HIV-1 epitope PPIPVGEIY (PY9) were assessed for their strength of recognition, the recorded EC_{50} values were found to be much lower than those recorded for the HIV-2 specific CTL clones (figures 3.6 & 3.7). Therefore NY9-specific CD8+ T-cells are polyfunctional and able to lyse target cells with an increased sensitivity.

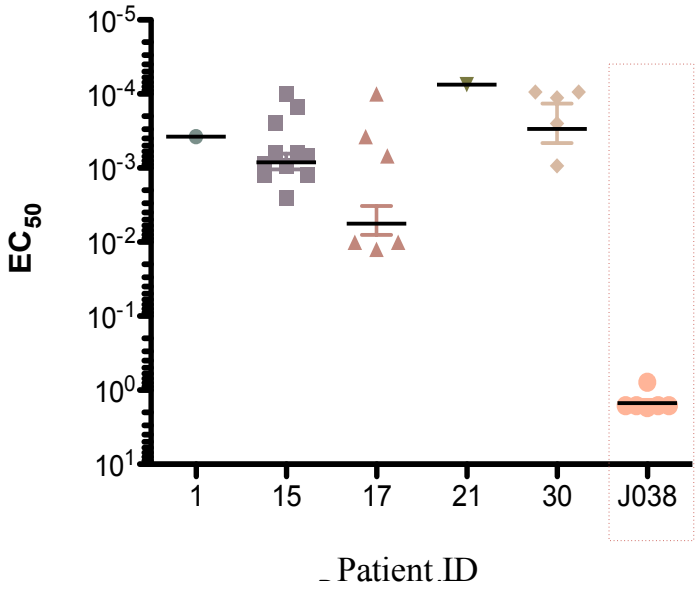


Figure 3.6 (a) Functional avidities (EC_{50}) of established CD8+ T-cell clones for the NY9 epitope in 5 HIV-2 patients (1,15,17,21 and 30) as determined by the ^{51}Cr release assay. The horizontal lines represent mean values and the functional avidities of PY9-specific CTL clones from an HIV-1 LTNP (J038) for the HIV-1 epitope PY9 are also given for comparison

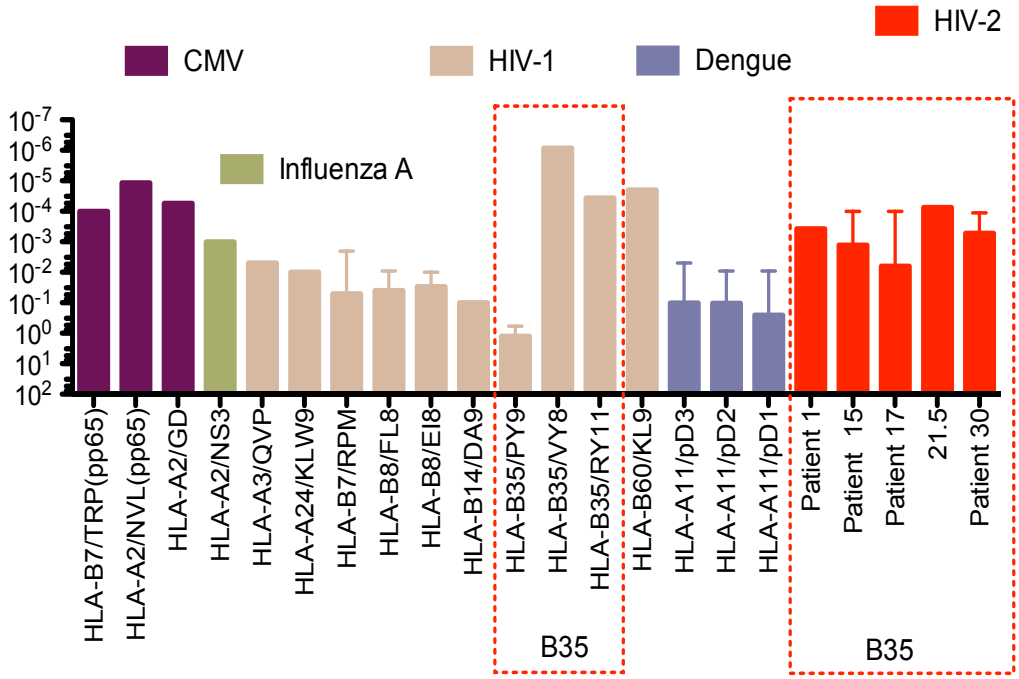
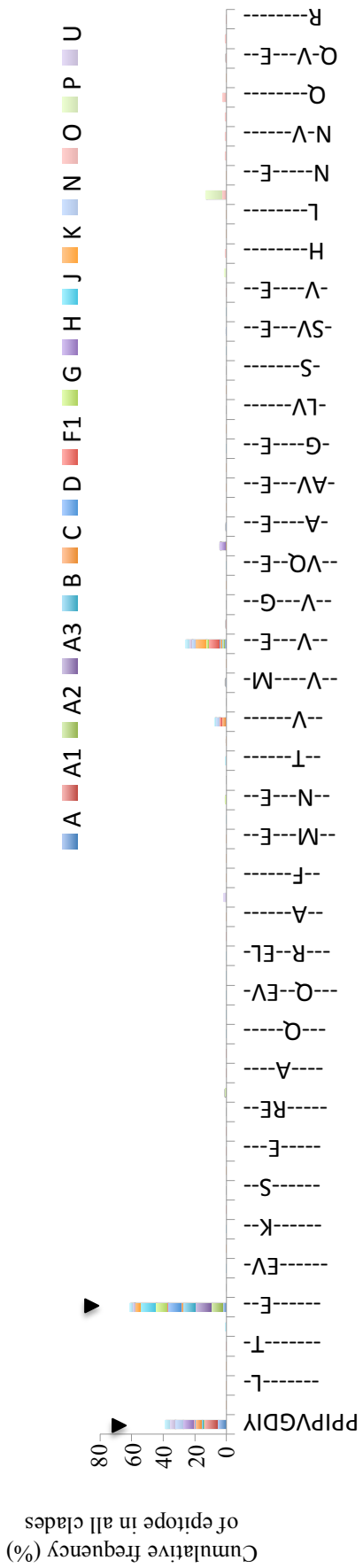


Figure 3.7 Comparison of published functional avidities (HIV-1 B*35) and of avidities previously measured in our lab (Influenza A/HIV-1/Dengue) with the functional avidities measured in the HIV-2 patients studied. Bars represent EC_{50} values (^{51}Cr release assays using CTL clones). The corresponding epitope and HLA restriction of each response is given on the X-axis. Bars within the red boxes represent functional avidities for HLA-B35-restricted epitopes. The HLA-B35/PY9 data denote the avidities of the PY9-specific HIV-1 CTL clones described earlier in this chapter whereas the data for the HLA-B35 VY9/RY11 restricted TCRs are from (Motozono et al., 2009). CMV-specific avidities are from (Day et al., 2007)

3.2.5 The HIV-1 epitope PY9 and its variants are effectively cross-recognised by the highly avid NY9-specific CTL clones.

Another aspect that characterises CD8+ T-cells responses in HIV-2 infection is cross-reactivity to HIV-1 antigens. To find out whether or not the NY9-specific CD8+ T-cells had the potential to cross-recognise the corresponding HIV-1 epitope (PY9) and its naturally occurring variants in a concentration dependent manner as previously suggested (Rowland-Jones et al., 1999), the established CTL clones were tested for their ability to produce IFN-γ and lyse B-cell targets in response to these epitopes (figure 3.8a). Even though cross-reactivity was seen in all patients for which clones

grew (3/3), clonotypes did not all share all the same cross-reactive potential, as each TCR recognized a different set of the variants tested. Overall, the clade B PY9 epitope was better recognized than the clade A/C epitope in both IFN-γ ELISPOT and ^{51}Cr release assays by both HIV-1 PY9 and HIV-2 NY9 specific CTLs (figure 3.8 b,c). These two epitopes differ in their respective amino-acids at position 7: in clade B/D variants this position is occupied by glutamic acid (E) whereas clade A/D variants have aspartic acid (D). However, as these residues form contacts with one of the MHC binding pockets (pocket E) (Schonbach et al., 1995), it is not clear if this particular difference in recognition mirrors differences in the TCR recognition or differences in MHC binding. The most cross-reactive TCR was found to be TRBV19, which showed a superior profile of cross-reactivity towards the clade B PY9 epitope independent of assay used (figure 3.8 b/c). However, when additional PY9 variants were tested, the emerging profiles were less consistent as different TCRs cross-recognised different variants with different efficiencies (figure 3.9). The most frequently recognised variant was PPVPVGEIY (3/3 HIV-2 specific TCRs) whereas PAVPVGEIY the least recognized (1/3 TCRs). Hence, the HIV-1 epitope PY9 and its naturally occurring variants can be effectively cross-recognised by NY9-specific CTL clones and at functional avidities that are comparable to those of HIV-1 specific CTLs. However, the nature of this cross-recognition heavily depends on the individual TCRs expressed.

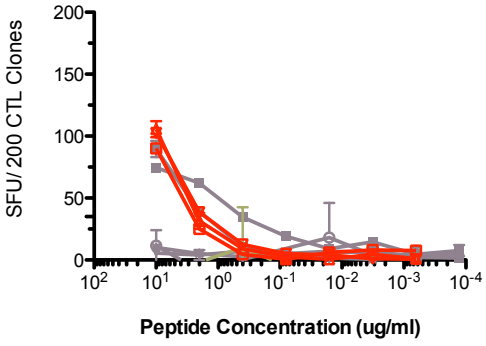


Cumulative frequency (%)
of epitope in all clades

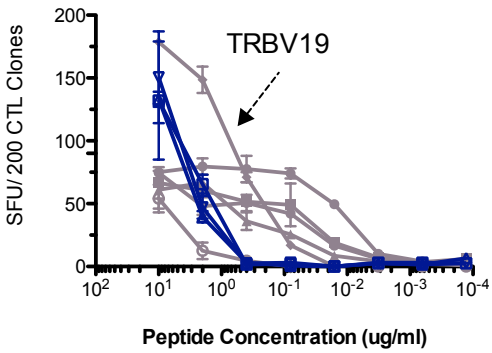
Figure 3.8 (a) Common variants of the PY9 epitope (X-axis) and their cumulative frequency among the different HIV-1 clades: A-U (%, Y-axis). Each bar represents the overall frequency of the epitope. Individual colours within each bar denote the different clades in which the epitope can be found. The two main (index) PY9 epitopes used in this study are marked by black arrows. The graph was compiled using information available in the Los Alamos database (http://www.hiv.lanl.gov/content/sequence/HIV/mainpage.html).

b **IFN-γ Secretion**

HIV-1 Epitope	EC_{50} Range (uM)	
	NY9 (HIV-2)specific CTLs	PY9 (HIV-1)-specific CTLs
P P I P V G **D** I Y	0.45	2.5-3.5
- - - - - - **E** - -	0.001- 4.5	3.9-4.4



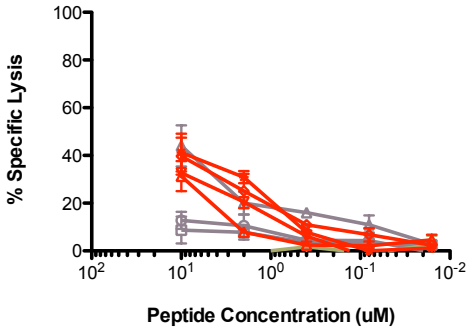
HIV- 1 (Clade A/C) : P P I P V G D I Y



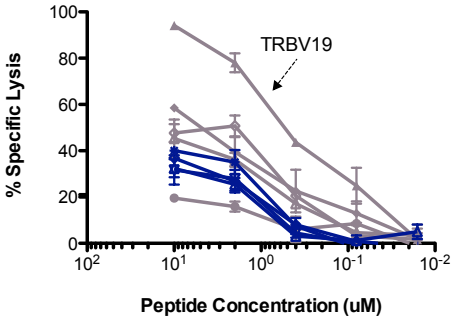
HIV- 1 (Clade B/D) : P P I P V G E I Y

C **Cytotoxicity**

HIV-1 Epitope	EC_{50} Range (uM)	
	NY9 (HIV-2)-specific CTLs	PY9 (HIV-1)-specific CTLs
P P I P V G **D** I Y	0.9-1.5	1-2
- - - - - - **E** - -	0.51-0.8	0.8-1



HIV- 1 (Clade A/C) : P P I P V G **D** I Y



HIV- 1 (Clade B/D) : P P I P V G **E** I Y

Figure 3.8 Heterologous cross-reactivity of HIV-2 clones (patients 15,17 and 30) for the HIV-1 epitope PY9 as measured by (b) ELISPOT and (c) ^{51}Cr release assay. The red and the blue lines on the graphs denote the autologous recognition of the epitopes by HIV-1, PY9-specific CTL clones. The TRBV family with the highest cross-reactivity observed (TRBV19) and the functional avidities (EC_{50}) for the autologous and heterologous recognitions are also given (black arrows and tables respectively).

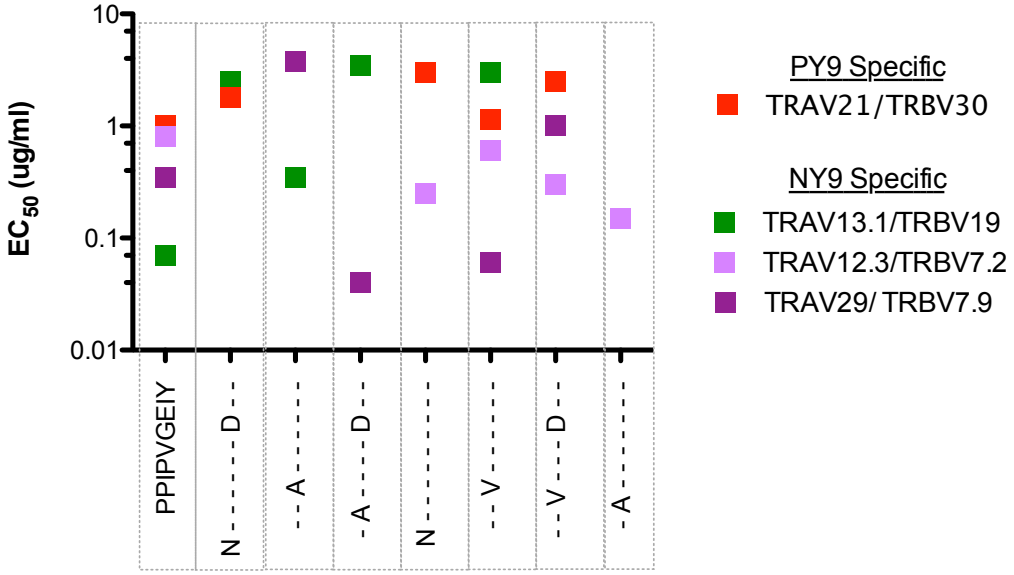


Figure 3.9 Cross-recognition of PY9 and of its naturally occurring variants by HIV-1 PY9-specific (red) and HIV-2 NY9-specific (green, pink, purple) CTL clones. Cytotoxicity was measured using 4hr ^{51}Cr release assays. For each peptide a titration was performed ($1.28x10^{-4}$ to 10ug/ml) and EC_{50} values were calculated by plotting the normalized maximal specific lysis against the concentration of the peptide (uM) and determining the concentration of each peptide required for half-maximal cytotoxicity

3.2.6 The expression of inhibitory molecules on the surface of CD8+ T-cells in HIV-2 infection is not associated with an impaired cytokine production, a decreased cytotoxicity or a lack of cross-reactivity.

One way to assess individual CTL clonotypes within a particular response with regard to their history of antigen encounter is by assessing their phenotype. Even though phenotype alone, and more specifically phenotypic aspects such as their status of differentiation do not always predict function (i.e. cytotoxic potential), it is thought that the expression and upregulation of molecules, such as the marker PD-1, denotes cells

EM_ID	TRBV family stained (clonotypic frequency)	Representation	Beckman Ab
EM_12	**TRBV6.1** (4/88)*	Subdominant	Vβ13.1
EM_15	**TRBV5.1** (47/88)	Dominant	Vβ5.1
EM_30	**TRBV19** (94/94)	Dominant	Vβ17

*Vβ mAb for dominant TRBV not available; staining denotes a subdominant population

Figure 3.10 Patients and TRBV families studied

that have previously contributed to an antiviral response (Appay et al., 2002; Petrovas et al., 2007). Previous studies involving the NY9-specific CD8+ T-cell population in HIV-2 infected patients suggested that NY9-specific cells share an overall early-differentiated phenotype (CD27+CD28+) that is additionally characterised by an increased expression of the marker PD-1 and the apoptotic marker CD95 (Leligdowicz et al., 2010b). To determine whether or not the dominance of particular clonotypes within the NY9-specific populations resulted in phenotypic characteristics that set them

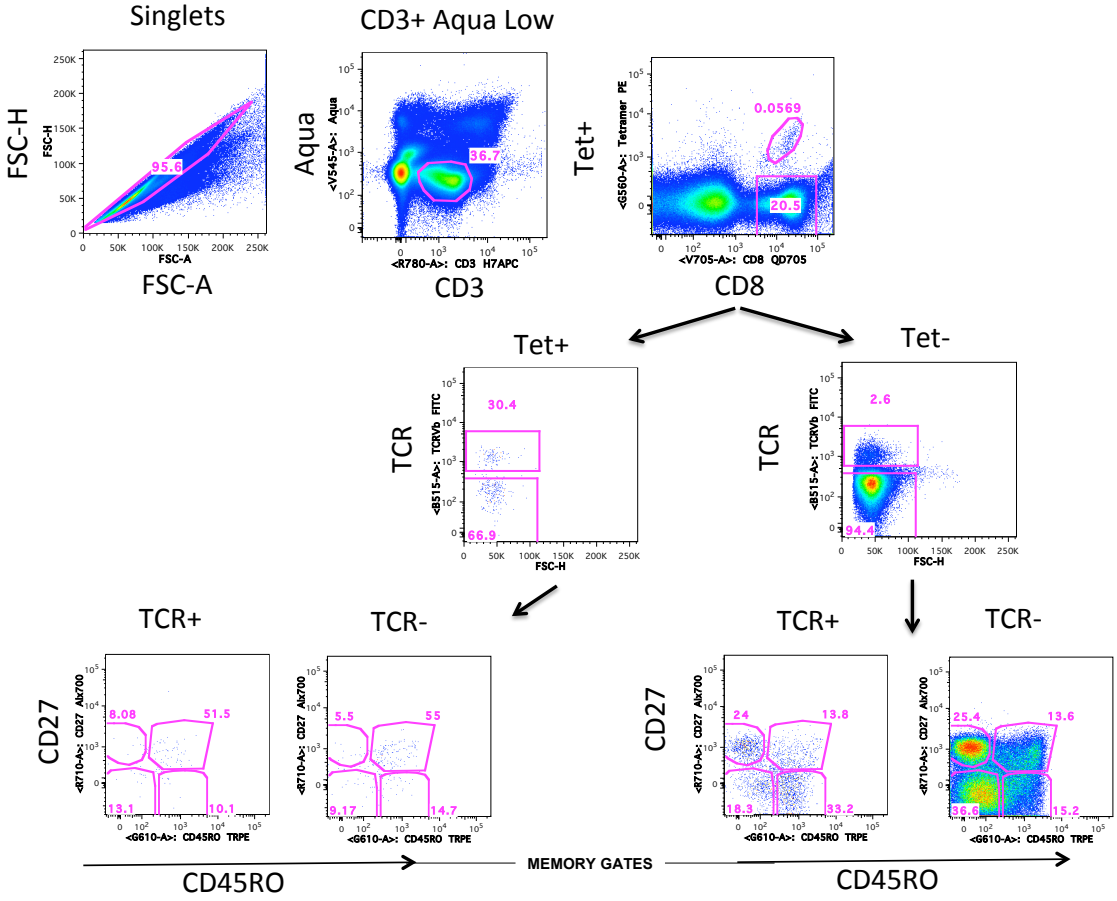


Figure 3.11 Gating strategy used for the identification of the different CD8+ T-cell memory populations.

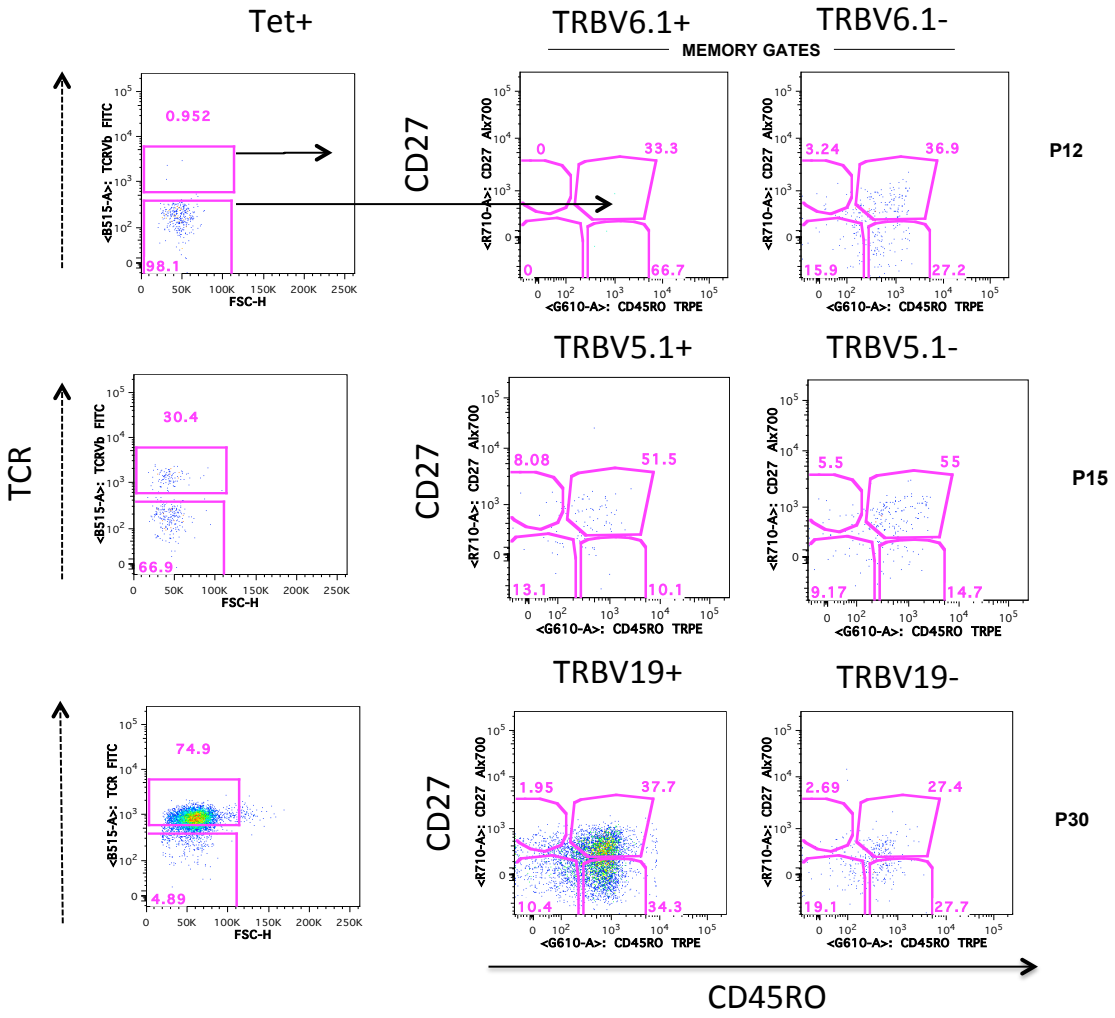


Figure 3.12 Differentiation stage of CD8+ NY9+ TCR+ and TCR- populations

apart from other NY9-specific T-cells, populations expressing dominant or subdominant TRBVs were stained using available TRBV-specific antibodies and a panel of surface antibodies specific for the differentiation markers CD27, CD45RO and inhibitory receptors PD-1, 2B4, CD160, CD127 (figures 3.10-11). Memory subsets were defined on the basis of CD27+ and CD45RO+ expression as these two markers have been shown previously to differentiate between naive and memory subsets with an increased accuracy (>90%) (De Rosa et al., 2001).

The analysis of the acquired data confirmed the previously proposed early-differentiated, 'immature' memory phenotype ($CD27^{high}CD45RO^{high}$) for NY9-specific

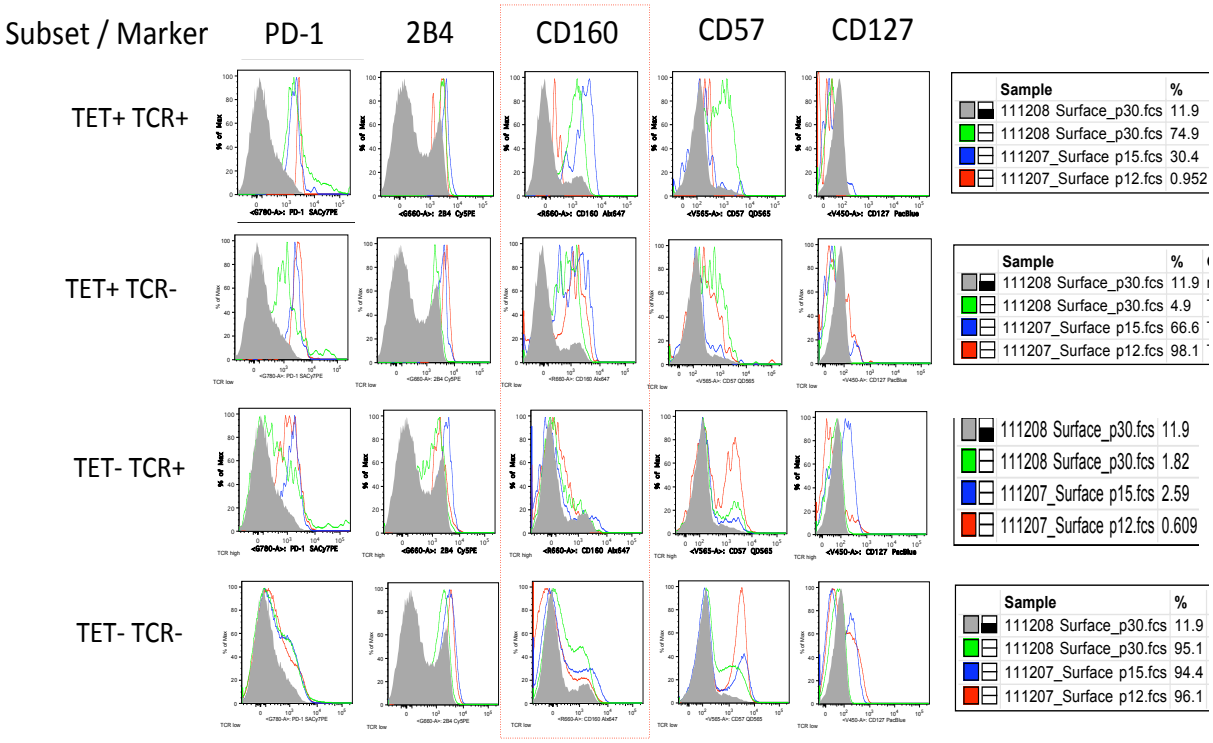


Figure 3.13 Histogram overlays depicting the expression levels of PD-1, 2B4, CD160, CD57 and CD127 on total memory NY9+ specific CD8+ T-cells and NY9- CD8+ populations. Colours represent the patients under study; gray: naïve, green: patient 30, blue: patient 15 and red: patient 12. The upregulation of the markers PD-1, 2B4 and CD160 on NY9-specific memory CD8+ subsets is independent of TCR usage.

CTLs. The proportion (average±SD) of $CD27^{high}CD45RO^{high}$ cells among tetramer-specific $TCR^{high}CD8+$ T-cells was 40.8±9.4% versus 7.8±6.9% for terminally differentiated CD8+ T-cells ($CD27^{low}CD45RO^{low}$). Similarly, among the NY9-specific TCR^{low} population, the proportions were 39.7±14% for $CD27^{high}CD45RO^{high}$ versus 14.7±5% for $CD27^{low}CD45RO^{low}$ T-cells (figure 3.12). In patient 30, the expression of CD57 was higher among TCR+ antigen-specific CD8+ T-cells than in TCR- cells and there was also a trend for a higher PD-1 expression among Tet+TCR+ CD8+ T-cells. In addition, the expression of CD160 was lower in subdominant clonotypes and the levels of CD127 were lower in Tet+ populations (figure 3.13). However, a statistical analysis was not possible due to sample size limitations. To gain a better understanding into the different combinations of inhibitory molecules expressed on NY9-specific

TCR+ and TCR$^-$ CD8+ T-cells, Boolean analysis was performed to create an array of 32 different phenotypic combinations of the markers under study. Overall, Tet+ populations were found to possess a higher frequency of cells expressing a simultaneous combination of 4 or 5 inhibitory molecules as well as a higher frequency of PD-1+CD127+ CD57+ cells. Moreover, memory NY9-specific CTLs expressing dominant TRBVs had a greater proportion of PD-1+CD57+ cells compared to those expressing subdominant TRBVs (TCR-) (25 vs 14%) and a higher frequency of 2B4+CD160+CD127+CD57+ T-cells that had downregulated the PD-1 marker (figure 3.14). Hence, specificity for a conserved Gag epitope in HIV-2 infection is associated with the upregulation of a series of inhibitory receptors. This phenotype appears to be shared by all NY9-specific cells, irrespective of dominance, and is not associated with an impaired cytokine secretion or a lack of cytotoxicity.

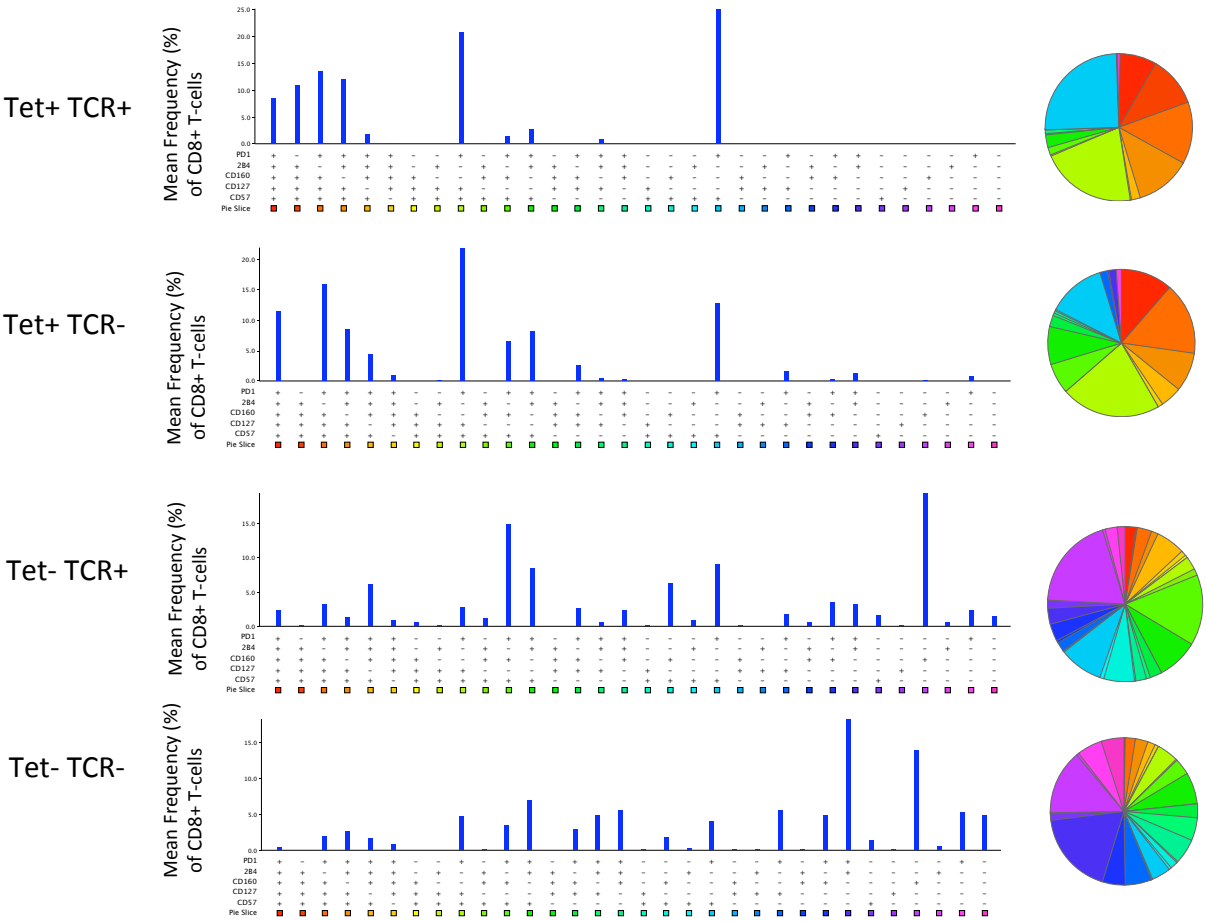


Figure 3.14 Graph representation of the frequency of expression (mean %) of PD-1, 2B4, CD160, CD57 and CD127 on bulk memory CD8+ T-cell populations in three patients (12, 15 & 30) subsequent to Boolean gating. The phenotypic markers studied are listed underneath the x-axis along with each of their respective combinations. Bars and pie chart slices represent the fraction of the total CD8+ T-cells expressing each of the 32 possible combinations.

3.3 Discussion

Conserved epitopes, such as the structural epitopes of Gag, have long been included in vaccination strategies for the induction of broader and more sustained CD8+ T-cell responses in both primate and human clinical trials (Dahirel et al., 2011; Goonetilleke et al., 2006; Knudsen et al., 2012; Niu et al., 2011; Rosario et al., 2010; Salmon-Ceron et al., 2009; Yang et al., 2011). The purpose of this chapter was to study in detail the natural response towards a relatively conserved HIV-2 Gag epitope and characterise attributes that could potentially contribute to viral control.

In line with previous findings (Pontesilli et al., 1998), in the absence of overt viral replication the NY9 response remained relatively stable over time in the patients under study. Research on the corresponding epitope in HIV-1 infection has established that immunoproteasomes within mature DCs and activated CD4+ T-cells have the potential to generate peptides containing an intact PPIPVGEIY (PY9) epitope (TNN**PPIPVGEIY**KRWIILG) at a high frequency. In addition, this epitope, has been shown to rank among the ten most frequently produced in proteosomal degradation experiments *in vitro,* along with peptides such as the HLA-B*27 restricted Gag peptide KK10 (Steers et al., 2011). Hence, it would not be unreasonable to hypothesise that the persistence of the NY9 response could potentially emerge from a similar profile of high frequency production within infected HLA-B*3501 patients. There are two possible explanations that could account for the lack of escape however: it could either be that the region is resistant to mutation due to its structural importance (Martinez-Picado et al., 2006), or that the mutations that arise are not selected (Bailey et al., 2006).

Even though the effective processing and presentation would enable the maintenance of the NY9 response, the generation and presentation of a Gag epitope

alone does not necessarily guarantee a better outcome. Studies in HIV-1 infection suggest that even though Gag p24 epitopes are targeted with a higher frequency in controllers than non-controllers, this targeting *per se* does not distinguish the two groups nor predicts outcome (Mothe et al., 2012). In agreement with these findings, strong Gag T-cell responses have previously been reported in viraemic HIV-2 patients (Jennes et al., 2008) and at least one of the patients under study (patient 26) showed signs of progression despite the presence of the NY9 response. Thus, it could be that in some B*3501 patients the NY9 response is qualitatively different and that the observed persistence mirrors the maintenance of an exceptionally active and long-lasting pool of CD8+ T-cells. Such a population could be maintained due to an ability to respond to minimal amounts of antigen and possibly escape variants as well.

The availability of TCR clonotypes in the periphery depends on a number of factors: on their production frequency (Quigley et al., 2010; Venturi et al., 2008), their thymic selection (Kosmrlj et al., 2010), the timing of their entry into the immune repertoire (time of first division) (Bousso et al., 1999), their avidity for particular peptide:MHC complexes (Alexander-Miller et al., 1996a), the initial antigen density triggering the activation (Bullock et al., 2003), as well as the nature of environmental antigens encountered previously by each individual (immunological history) (Lim et al., 2000). The initial response to HIV-1 infection is broad in terms of clonotypes (Schaubert et al., 2007) and during the chronic phase of infection the epitope-specific response becomes more focused (Day et al., 2007; Rudd et al., 2011). This focusing is not always reversible even when HAART is implemented (Giacoia-Gripp et al., 2005). The NY9-specific populations of the HIV-2 patients under study contained 4 different clonotypes on average. Hence, the results of this chapter on clonality parallel those of studies in chronic persistent infection such as EBV, CMV, HIV-1, HCV, LCMV and

SIV in that they demonstrate the development of a narrow, oligoclonal TCR repertoire over time (Berger et al., 2011; Leligdowicz et al., 2010b; Turnbull et al., 2006).

The TCR landscape of an oligoclonal response can be either 'flat' or 'hierarchical'. The former case differs from the latter in that all clonotypes are represented equally within the antigen-specific population as opposed to one or two clonotypes dominating the response. The NY9-restricted responses, presented in this chapter fall under the second category. Such hierarchical responses have often been associated with the presence of 'public' TCRs, namely TCRs shared by multiple individuals (Lim et al., 2000). Such 'public' TCRs have been shown to dominate several viral chronic responses towards immunodominant epitopes in a number of infections such as HIV-1, EBV, CMV and HSV-2 (Dong et al., 2010; Dong et al., 2004; Lim et al., 2000; Trautmann et al., 2005). However, the exact role of these 'public' TCRs is still at large unknown with some studies associating their presence with an increased control of viral replication (Price et al., 2009b) and others with less positive outcomes such as escape (Iglesias et al., 2011). Nevertheless, the determination of the clonotypic nature of a response is of paramount importance as it offers valuable insights into the principles that govern the recruitment of CD8+ T-cells over time and the efficacy of a given response. For instance, it has been suggested that for some epitopes, such as the D^b -restricted influenza A nucleoprotein epitope $NP_{366–374}$ in mice and the HLA-A*0201 matrix protein $M1_{58-66}$ in humans, the selection and development of a narrow, 'public' TCR Vβ response over time is associated with protection and viral clearance (Lawson et al., 2001; Zhong and Reinherz, 2004). However, a similar public narrowing is not always advantageous as in some cases, it can facilitates viral escape over time as in the case of the HLA-B*2705 restricted HIV p24 gag epitope KK10 (Iglesias et al., 2011). Understanding what type of response (ie.

public or private) lies behind a given clinical outcome, whether it is protection or progression, is necessary for rational vaccine design.

When the clonotypic constitution of NY9 response was examined, shared TCRs were not found to dominate, despite it being chronic and persistent. There was a very limited usage of public TCRs among the patients under study and most CD8+ T-cells expressed TCRs that were of a 'private' nature. Hence, if NY9-specific CD8+ T-cells play a role in the curtailment of viral replication in the patients under study then this is most likely achieved through a varied, 'private' response. On the other hand, the lack of dominant public clonotypes in this chronic infection could mean that either some CD8+ T-cells bearing public TCRs were recruited but became extinct over the years due to exhaustion and clonal turnover (Almeida et al., 2007) or that environmental factors promoted a differential modulation of individual repertoires in these West African patients in a way similar to that shown for monozygotic twins (Monteiro et al., 1995).

The structure of the epitope may also have contributed to the development and maintenance of a 'private' repertoire. Even though the structure of the NY9 epitope in complex with the HLA-B*3501 allele is not currently known, studies led by Turner *et al*, have demonstrated that the structural landscape of the epitope in complex with a particular HLA allele can play a significant role in the selection of public versus private TCRs. For instance, the presentation of a 'flat' H-2D^{b} influenza A epitope in mice triggers the development of an oligoclonal, 'public' TCR repertoire whereas the presentation of a protruding epitope such as the acid polymerase epitope PA224, favours the recruitment of TCRs with private specificities. The hypothesis put forward for this phenomenon is that a flat epitope would require more contacts with MHC α-helices for specificity and avidity. However, as most T-cells in the naive repertoire

with higher MHC affinities may have been negatively selected during thymic development only a few precursors would be able to meet this requirement for mobilisation (Turner et al., 2005). The high avidity of the NY9-specific CTL clones on the other hand was an avidity for antigen rather than MHC. In addition, the recorded avidity was much higher than that observed for HIV-1 clones targeting the corresponding HIV-1 epitope and also ranked high among many functional avidities reported to date by both our lab and those of others (Day et al., 2007; Motozono et al., 2009). In addition, in a recent study by Friedrich *et al*, B*35-restricted T-cells specific for the epitopes Env DL9 and Pol IL9 raised after vaccination with a tetravalent recombinant adenovirus vaccine (VRC-HIVADV014-VP) were also shown to possess a higher avidity compared to T-cells restricted by other alleles (Friedrich et al., 2011). It appears thus that when in complex with certain HIV epitopes, B*35 alleles may have the potential to restrict high avidity cytotoxic responses, much like protective alleles such as B*2701 (Almeida et al., 2007).

However, some caution is needed in the interpretation of this interesting finding. To begin with, only a small number of HIV-1 specific CTLs from a single HIV-1 patient was studied, and hence, there is a potential for bias arising from the restricted availability of HIV-1 samples. There is also a possibility that the observed differences between the HIV-2 CTL values recorded in this study and those published elsewhere could reflect methodological variations, such as for example differences in the E:T ratios, and not functional differences *per se*. Hence, it is not possible to conclude at present with confidence whether or not the finding of high functional avidity of B*3501 restricted, NY9-specific CD8+ T-cells can be more generally extrapolated to HIV-2 and HIV-1 specific CTLs.

The experimental approach followed in this chapter allowed a complete dissection of the profiles of functional avidities of cells bearing distinct TCRs, something that would have otherwise been impossible due to reagent (Vβ antibodies) and sample limitations. However, one potential argument against this *in vitro* approach is that functional avidities may have been modulated during culturing *in vitro.* Such an effect has previously been shown for HCV specific CD8+ T-cells (Yerly et al., 2008). However, as both the HIV-1 and HIV-2 CD8+ T-cell clones were expanded under the same conditions and for the same amount of time, it seems highly unlikely that their avidity differences are an artefact. No differences in the functional avidities were observed either after one, two or three rounds of *in vitro* expansion.

It is often said that highly avid CTLs might be better at limiting virus in the early stages of infection (Belyakov et al., 2006; Derby et al., 2001a). In latent HCMV infection, highly avid CD8+ T-cells have been shown to survive longer in long-term memory (Day et al., 2007) and in HCV infection such cells can be readily detected in patients clearing the pathogen (Yerly et al., 2008). Nevertheless, other studies point out that a high functional or structural avidity alone does not necessarily translate into a better outcome. For example, Mamu-A*01 macaques vaccinated with SIVmac239 Gag and challenged with SIVsmE660, fail to control viral replication *in vivo* despite a high frequency of highly avid GagCM9-specific CD8+ T-cells (Vojnov et al., 2011). Likewise, it has been proposed that highly avid TCR interactions might lead to a decreased functional responsiveness due to an up-regulation of inhibitory receptors (Ueno et al., 2004) and an increased rate of apoptosis (Alexander-Miller et al., 1996b; Lichterfeld et al., 2007). However, when functionality was assessed in the patients under study, no functional impairment was detected despite the high avidity. To the contrary, NY9-stimulated CD8+ T-cells were able to produce an array of cytokines in

response to 2μM of antigen. Hence, the results of this chapter reiterate that high avidity is an important element of an effective anti-viral response especially when accompanied by polyfunctionality, which has also been associated with HIV control (Betts et al., 2006).

Another attribute that has been associated with a better outcome in HIV-1 infection is cross-reactivity for homologous and heterologous antigens. In a recent study by Mothe *et al.* HIV-1 controllers were shown to raise Gag p24 responses that were characterised by an increased cross-reactivity for epitope variants compared to non-controllers (Mothe et al., 2012). Similarly, in rhesus macaques, immunisation with NYVAC and ALVAC HIV-1 constructs confers protection against HIV-2, which is thought to arise from the development of tissue-specific cross-reactive CTL responses in the vaccinated animals (Abimiku et al., 1995), and in HIV-2 infection, polyclonal CTL lines derived from patients expressing the HLA-B58 allele successfully recognize HIV-1 Gag epitopes such as the clade B $Gag_{240-249}$ epitope TW10 (Bertoletti et al., 1998).

However, not all HLA-alleles and HIV epitopes associate with a potential for cross-recognition. For instance, past studies have shown strong homologous cross-reactivity for variants of the HLA-B57-restricted KF11 epitope but not so much for the HLA-B57 restricted Gag p24 epitope ISPRTLNAW (Gillespie et al., 2002; Turnbull et al., 2006). The NY9 epitope has previously been implicated in cross-reactive responses in HIV-2 infection (Rowland-Jones et al., 1999) and it has been argued that such a cross-recognition might be associated with a broader and more diverse TCR usage (Lopes et al., 2003). However, these earlier studies did not assess TCR usage at a clonotypic level and those that did, did not address cytotoxicity. In this study, NY9-specific CTLs were found to possess an increased potential for heterologous (PY9)

variant cross-recognition and for some TCRs, such as the TRBV19 TCR, this recognition was functionally more avid than the autologous recognition by HIV-1 specific CTLs. As far as the TRBV19 TCR is concerned, it is plausible that this family is inherently more cross-reactive, as 'public' and 'private' TRBV19 (Vβ17) receptors have been implicated in homologous and heterologous cross-reactive responses in infections as diverse as EBV, HIV-1 and influenza A (Clute et al., 2010; Gillespie et al., 2006; Petrova et al., 2011). Hence, the results of this chapter suggest that cross-reactivity is not a function of a broader TCR usage alone but depends to a large extent - much as has been proposed for HIV-1 infection- on the individual TCRs used. Without the cross-reactive TCRs, a polyclonal response would not necessarily confer an adequate coverage, as each responding TCR would need to be recruited *de novo* from the naive subset (Turnbull et al., 2006). Pre-existing memory cross-reactive T-cells however, would be expected to expand faster than naive cells in the event of HIV-1 acquisition and hence, would have an advantage compared to newly primed CTLs. However whether or not this could prove ultimately beneficial remains unknown (Cornberg et al., 2006).

The fact that the lysis of the target cells by the NY9-specific CTLs occurred at low EC_{50} values, comparable or greater to those exhibited by HIV-1, PY9-specific CTLs, and in a concentration-dependent manner also hints towards a potentially physiological significance of this cross-reactivity. Furthermore, it establishes that this phenomenon was not an artefact of the use of high peptide concentrations. However, a previous study by Jennes *et al.*, indicated that the overall frequency of such cross-reactive responses *in vivo* is low and thus their true contribution to natural cross-protection might be lower than that estimated *in vitro* (Jennes et al., 2008). Even so, the detection of HIV-2-specific CD8+ T-cells bearing TCRs with a potential for cross-

recognition warrants further attention. Such clonotypes may have a greater potential to limit escape within HIV-2 hosts if present at adequate frequencies, and this latter attribute could potentially be modulated by vaccination.

Chronic antigenic stimulation enriches the expression of inhibitory molecules on the surface of CD8+ T-cells such as PD-1, 2B4, CD160 (Salisch et al., 2010). PD-1 is a member of the CD28 family of molecules that was first recognized in the context of HIV-1 infection as a negative regulator of T-cell function and survival (Day et al., 2006; Petrovas et al., 2006) and 2B4, which belongs to the SLAM/CD150 family of receptors (Mathew et al., 1993), has also been associated with a defective cytokine production by HIV-specific CTLs (Aldy et al., 2011). Since the upregulation of these receptors as well as of CD160 had been previously linked with immune 'exhaustion', a state characterised by a loss of effector functions and proliferative potential (Yamamoto et al., 2011), the question of whether or not differences in the expression of these markers could account for the observed segregation of NY9-specific T-cells into dominant and subdominant populations arose. The phenotypic analysis of NY9-specific CD8+ T-cell populations in three HIV-2 infected patients revealed an early, 'immature' phenotype and an overall up-regulated expression of PD-1, 2B4 and CD160 on NY9-specific populations. This was largely independent of TRBV family usage and dominance. However, memory NY9-restricted CTLs expressing dominant TRBVs had a greater proportion of CD57+PD-1+ cells and a trend for higher CD160 levels than subdominant TCRs, which potentially indicates the ability of these cells to respond more frequently to antigen. This is in line with previous studies suggesting an increased expression of PD-1+ and CD57+ in dominant HIV specific CD8+ T-cells (Conrad et al., 2011). However, contrary to these studies, the dominant TCRs in the HIV-2 infected patients did not exhibit a reduced potential for cross-recognition or low

CD127 expression. CD127 was upregulated in 66.7% of the total TCR+ NY9-specific T-cells, which suggests that these cells probably maintain a potential for increased homeostatic proliferation (Kaech et al., 2003) despite the upregulation of inhibitory markers such as PD-1 and 2B4.

In addition, the PD-1 upregulation was not associated with an impaired function. This finding supports a number of previously published observations on the expression of PD-1 within healthy individuals as well as HIV-1 infected elite controllers and asymptomatic HIV-2 patients that maintain undetectable viral loads (Duraiswamy et al., 2011; Salisch et al., 2010; Tendeiro et al., 2012). It is however at odds with reports proposing a role for PD-1 as a marker of exhaustion and disease progression (Day et al., 2006; Peretz et al., 2012; Trautmann et al., 2006). Indeed, PD-1 expression on CD8+ T-cells has long been associated with reduced levels of functionality in a number of chronic infection settings including HIV-1 (Day et al., 2006) and HCV (Golden-Mason et al., 2007). In addition, blockade of the PD-1 pathway has been shown to enhance the function of CD8+ T-cells in SIV-infected macaques (Velu et al., 2009). Hence, the observation that NY9-specific CD8+ T-cells retain functionality despite the observed PD-1 upregulation is noteworthy and warrants further investigation as the small number of patients studied for PD-1 expression in this thesis (n=3) makes it hard to deduce if the observed functionality of PD-1+ cells is a global characteristic of HIV-2 specific CD8+ T-cells or a characteristic of the studied NY9-specific subset alone. One possibility however is that the expression of this marker on NY9-specific CD8+ T-cells in asymptomatic HIV-2 infection denotes either their high avidity for antigen, as it has been previously suggested for HIV-1-specific CD8+ T-cells (Conrad et al., 2011) or an ongoing viral replication and a lack of escape from CD8+ T-cell recognition as it has been proposed for Gag-restricted, SIV-specific

CD8+ T-cells in Mamu-A*01 and A*02 rhesus macaques controlling an SIVmac infection (Salisch et al., 2010).

The upregulation of CD160 (BY55) in the dominant clonotypes is also an interesting finding. CD160 (BY55) is a GPI- anchored protein that shares a 44% homology with KIR receptors such as KIR2DL4. Its role is to mediate TCR signal transduction cascades associated with proliferation and cytotoxicity in NK cells, intestinal intraepithelial cells, γδ T-cells and circulating differentiated CD8+ T-cells of an intermediate (CD28- CD27+ CD28-) and late (CD27- CD28-) differentiation stage by engaging classical and non-classical MHC class I molecules on the surface of target cells (Agrawal et al., 1999; Anumanthan et al., 1998; Nikolova et al., 2002; Nikolova et al., 2005). Previous studies demonstrated that the number of CD8+CD160+ T-cells increases significantly during HIV-1 infection compared to healthy individuals, with up to 21.1% of the circulating CD3+CD8+ T-cells expressing the marker (Bensussan et al., 1993). In addition, the binding of CD160 to aggregated gag and pol loaded MHC tetramers has been shown to enhance the HIV-1 functional activity, granzyme B loading and cytotoxicity of CD8+ T-cell subsets (Agrawal et al., 1999; Nikolova et al., 2005). Due to its selective expression and association with proliferation cascades and cytotoxicity and its promiscuous MHC recognition, it has been hypothesised that CD160 expression might confer a functional advantage to those effector cells that express the molecule by allowing them to recognise virally infected cells even when HLA-A and B are downregulated (Nikolova et al., 2005). Hence, the higher expression of CD160 in dominant NY9-specific CTLs suggests that these cells might be representing CTLs with an enhanced antiviral efficacy.

All in all, the results of this chapter point out that in the context of HIV infection, viral containment might not necessarily result from a single correlate but

rather from a number of factors, both overlapping and redundant, that work in concert to bring about what is perceived as long-term non-progression. It is thus this interplay of factors that we need to understand better and to address in order to push the field of HIV vaccination forward.

Chapter 4: Functional characterization of CD8+ T-cell clones bearing distinct TCRs in HIV-2+ LTNPs.

4.1 Introduction

Over the past ten years, several studies have suggested that the 'quality' of a CD8+ T-cell response rather than its 'quantity' (i.e. magnitude and breadth) gives a more accurate prediction of antiviral efficacy (Addo et al., 2003). Most of the evidence regarding quality comes from the study of chronic infections in humans such as HIV-1 and EBV (Almeida et al., 2007; Betts et al., 2006; Ning et al., 2011), from the study of CD8+ T-cells in primary SIV infection such as the immunodominant Mamu-A*01 Gag CM9 response in macaques (Price et al., 2009b) as well as from the study of long-term non-progressors and controllers in HIV infection (Betts et al., 2006; Emu et al., 2005). Those studies show that patients or animals with a better prognosis harbour cytotoxic CD8+ T-cells that are more potent in their action than patients who fail to either clear or contain viral replication, depending on the nature of the virus under study.

The term 'quality' refers to a number of cellular attributes that characterize the *in vivo* and *in vitro* behaviour of CD8+ T-cells. Some of these qualities, such as the structural avidity for antigen and cross-reactivity for viral variants, depend more heavily on the characteristics of the individual TCRs (Buseyne and Riviere, 2001; Gillespie et al., 2006) whereas others, such as the functional avidity or sensitivity for antigen, the cytotoxic potential, the simultaneous secretion of several interleukins and chemokines (polyfunctionality) and the proliferative ability of the cells, are more of an amalgam of TCR usage and microenvironmental imprinting that CD8+ T-cells acquire upon activation (Appay and Iglesias, 2011; Price et al., 2009b; Youngblood et al., 2012).

It has been proposed that most of these qualities are strongly interconnected and hence their observed effect on viral control is one of an orchestrated action rather than the effect of a single qualitative attribute (Vojnov et al., 2010). Even so, the exact mechanisms that lead to such superior profiles of functionality, and quality, are still unclear (Iglesias et al., 2011).

In HIV-1 infection, some of the most recent studies have sought to identify correlates of protection at a clonotypic level (Iglesias et al., 2011; Price et al., 2009b). However, this topic has not yet been extensively addressed in HIV-2 infection. The only study currently available is a previous study published by Leligdowicz *et al*. in 2010 which examined TCR usage between two Vβ families, one dominant (TRBV19+) and a subdominant, in one HIV-2 long-term non-progressor. The published work demonstrated differences between the two families in terms of TCR avidity and IFN-γ production, as measured by IFN-γ ELISPOT, and suggested the selection of a superior quality NY9-restricted Vβ family in HIV-2 infection (TRBV19). However, it did not address whether or not this Vβ family persists in chronic infection and whether or not this 'superior' profile is restricted to the entire TRBV19 family, a single TRBV19 clonotype or a more generalized phenomenon of CD8+ T-cells in HIV-2 infection. In addition, even though IFN-γ production as measured by IFN-γ ELISPOT is a well-established method for the identification of Ag-specific responses, its use as a sole measure of quality has been debated (Valentine et al., 2008; Varadarajan et al., 2011)

The purpose of this chapter was to dissect the clonotypic constitution of the NY9 response in B*3501 patients in an attempt to identify qualitative features that might be associated with the control of viral replication. More specifically, this chapter aims at addressing the question of whether or not HIV-2 long-term non-progressors have persistence of particular clonotypes and to assess the qualitative features of such

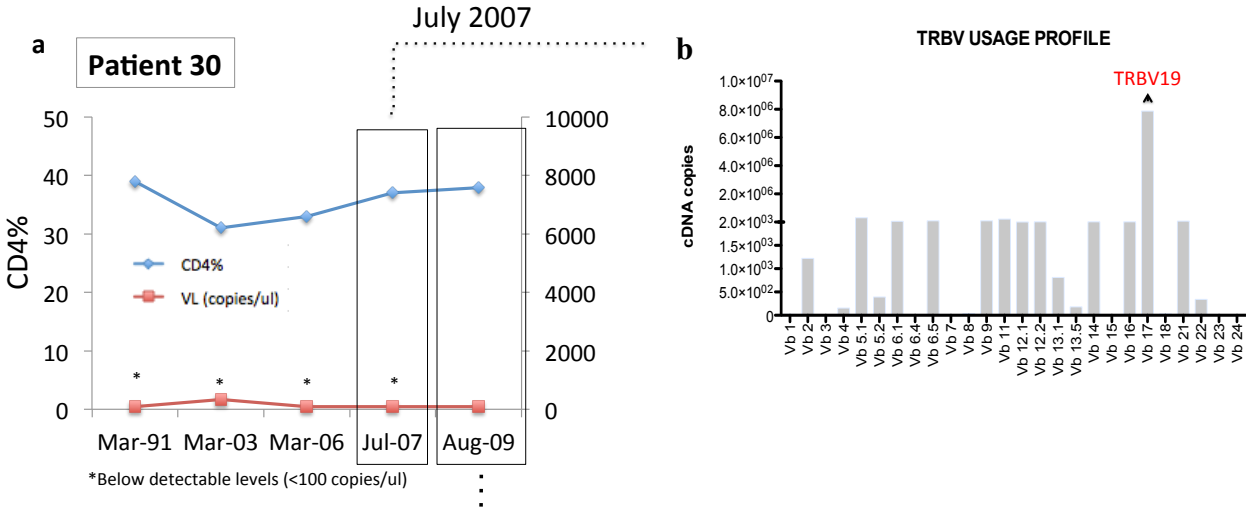
HIV-2 specific clonotypes *in vitro* using multiple methods. For this purpose, two different NY9- clonotypes - one dominant and one subdominant- that were successfully established from a single donor by CD8+ T-cell sorting and limiting dilution analysis were initially examined. The main focus was the question of how cells bearing a dominant receptor within a tetramer-specific population in HIV-2 infection compare to CD8+ T-cells bearing underrepresented TCRs (<1.5%) in terms of cytotoxicity, polyfunctionality, tetramer avidity, CD8 expression and cross-reactivity. The results were confirmed by studying the TCR usage of additional patients in our cohort.

Understanding how the clonal composition of a response affects the outcome in HIV-2 infection is of paramount importance as it could enable us to identify principles upon which HIV vaccination strategies could be based to achieve the mobilization of highly effective CTLs in a therapeutic or even prophylactic setting.

4.2 Results

4.2.1 Dominant TCR usage persists for two years in an HIV-2 LTNP despite an observed clonotyping narrowing.

Patient 30 is an HIV-2+ long-term non-progressor that has maintained an absolute CD4 count above 400 cells/µl and an undetectable viral load (<50 copies/ml) for more than 18 years. The patient's clinical data are summarised in chapter 3 and (figure 4.1). Previous work carried out in 2007 had identified a dominant NY9 ($Gag_{245-253}$) response in this patient and suggested the existence of a highly discernible Vβ family hierarchy within the NY9-specific CD8+ T-cell population (Leligdowicz et al., 2010b).



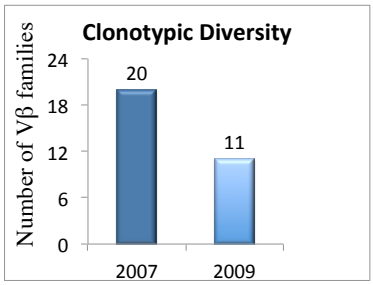
ID	TRAV	CDR3	TRAJ	Freq
EM-30	13-1	CAANRH	50-1	53
	10	CVVSAML	40-1	36
Seqs: 89				

ID	TRBV	CDR3	TRBJ	Freq
EM-30	19	CASSPQHGDTQYF	2-3	94
Seqs: 94				

Figure 4.1 (a) Clinical characteristics of patient 30 (b) TRBV usage profile as determined by quantitative qPCR in 2007 (Leligdowicz A. and TcLand-Expression Labs, France). The nomenclature used is that of Arden. (c) Dominant TCR usage as determined by clonotypic analysis of tetramer-sorted NY9+ CD3+ CD8+ T-cells in 2009. In this case, TRAV/TRBV families follow the IMGT nomenclature

EM_30	TRBV Family						
IMGT	TRBV9	TRBV20-1	TRBV28	TRBV29-1	TRBV5-1	TRBV5-4	TRBV7-3
Vβ	**Vb 1**	**Vβ 2**	**Vβ 3**	**Vβ 4**	**Vβ 5.1**	**Vβ 5.2**	**Vβ 6.1**
2009							

IMGT	TRBV7-9	TRBV7-2	TRBV4-1	TRBV12-3	TRBV3-1	TRBV25-1	TRBV10-3
Vβ	**Vβ 6.4**	**Vβ 6.5**	**Vβ 7**	**Vβ 8**	**Vβ 9**	**Vβ 11**	**Vβ 12.1**
2009							

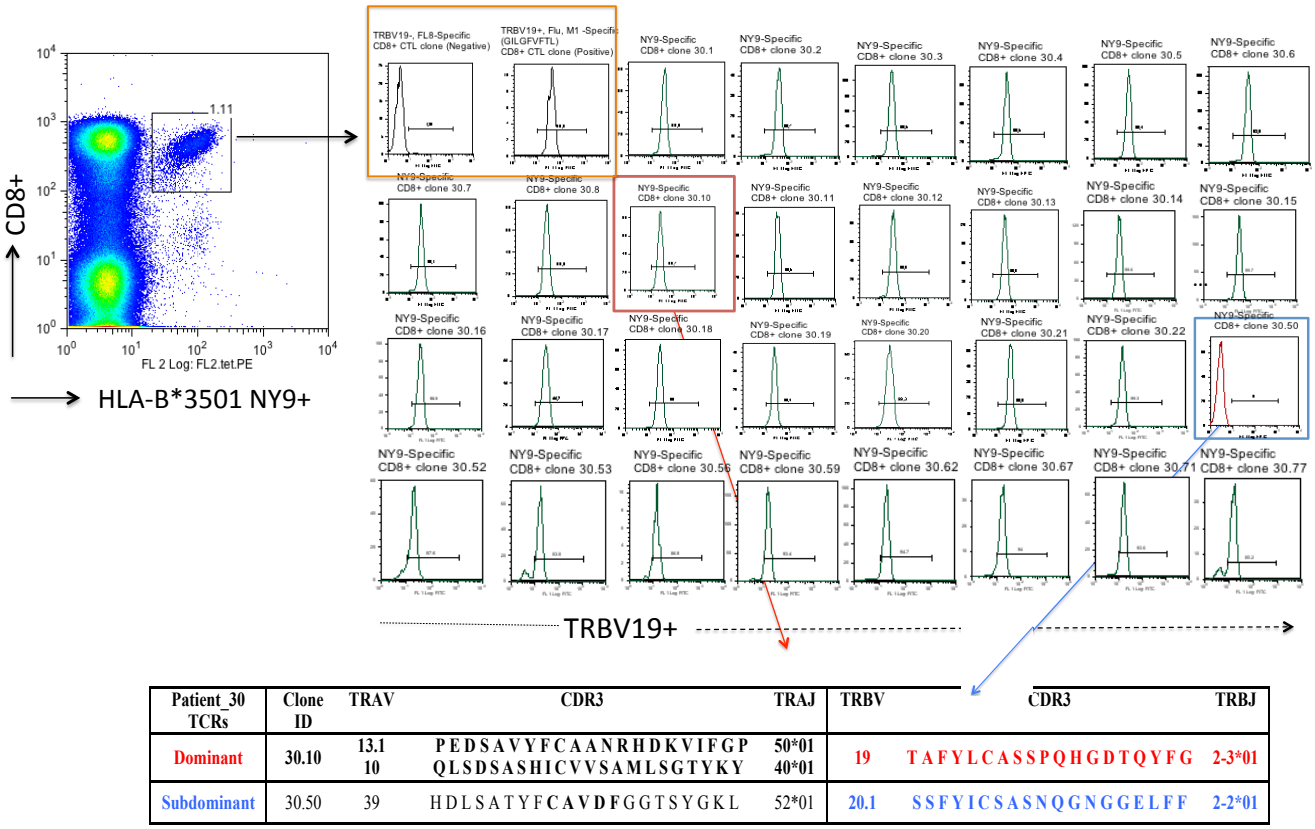


IMGT	TRBV10-1	TRBV6-1	TRBV6-4	TRBV27	TRBV24-1	TRBV14	TRBV19	TRBV18	TRBV11-1	TRBV2	TRBV13	TRBV15
Vβ	**Vβ 12.2**	**Vβ 13.1**	**Vβ 13.5**	**Vβ 14**	**Vβ 15**	**Vβ 16**	**Vβ 17**	**Vβ 18**	**Vβ 21**	**Vβ 22**	**Vβ 23**	**Vβ 24**
2009												

Figure 4.2 TRBV families detected in the NY9-specific, HLA-B*3501 restricted CD8+ T-cell response in patient 30 in 2009 (*light blue)*. Data for 2007 are from (Leligdowicz et al, 2010). IMGT: TCR family nomenclature based on IMGT. Vβ A combination of TCR clonotyping, Vβ-specific monoclonal antibody staining, TCR sequencing and spectratyping was used to ascertain TRBV usage.

To determine the nucleotide and amino-acid sequence of the clonotypes corresponding to the previously published TRBV19 family, archived pellets of flash-frozen CD8+ T-cell clones that were previously known to express the TRBV19 receptor were used for mRNA isolation and cDNA synthesis. Vβ-chains were then PCR-amplified from cDNA using a TRBV19-specific primer and a primer specific for the constant region of the beta chain (Cβ). Out of the three pellets screened only one pellet gave a PCR product. Sequencing of this product revealed a Vβ19 - Jβ2*3 pairing and a relatively short CDR3β loop bearing the amino acid sequence: CASSPQHG.

To determine if Vβ usage in this patient was still the same in 2009, cryopreserved PBMCs were thawed and stained with NY9 tetramer, anti-CD8 and a panel of Vβ-specific antibodies. The staining confirmed the presence of the NY9-specific response as well as the presence of the TRBV19 family (figure 4.2). CD8+NY9+ cells were then sorted and used for molecular TCR analyses (clonotyping, spectratyping) as well as for the establishment of CD8+ T-cell clones. The combination



Patient_30 TCRs	Clone ID	TRAV	CDR3	TRAJ	TRBV	CDR3	TRBJ
Dominant	30.10	13.1 10	PEDSAVYFCAANRHDKVIFGP QLSDSASHICVVSAMLSGTYKY	50*01 40*01	19	TAFYLCASSPQHGDTQYFG	2-3*01
Subdominant	30.50	39	HDLSATYFCAVDFGGTSYGKL	52*01	20.1	SSFYICSASNQGNGGELFF	2-2*01

Figure 4.3 Outline of the method used to generate the NY9-specific CD8+ T-cell clones from patient 30. CTL clones were established using the limiting dilution method from sorted CD8+NY9+ T-cells. 80 CTL clones grew all of which were screened for specificity using NY9 tetramers and for TCR usage using Vβ-specific mAbs. The histograms illustrate the TCR screen. *Orange box*: control cell lines (TRBV19+/TRBV19-) for gating, *red box*: TRBV19+ NY9-specific CTL clone, *blue box*: TRBV19- NY9-specific CTL clone. TCR usage and clonality were further confirmed by TCR sequencing.

of these four different methods for the analysis of TCR usage in 2009 was selected because the TCR landscape analysis that was performed in 2007 collaboratively with the TCLand Expression labs in France was no longer available, and full representation of circulating Vβ families would not have been achieved otherwise as different TCR usage methods offer varying degrees of Vβ coverage (Currier and Robinson, 2001; Dash et al., 2011; Douek et al., 2002; Genevee et al., 1992; Rufer, 2005; Sharrock et al., 1990) (table 4.1).

A total of 80 clones were established by limiting dilution all of which but one

Method	Description	Advantages	Disadvantages	Review
TCR Clonotyping	Amplification of expressed TCR sequences **using a single, 'global' set of TCR primers** from total mRNA isolated from tetramer sorted CD8+ T-cells. This is followed by bulk TA cloning of amplified sequences in bacterial vectors for sequence analysis of individual TCRs.	Unbiased method of TCR amplification. Excellent Vβ coverage (>98%) Good for unravelling patterns of TCR dominance and frequency within a given response.	Expensive Chance of identifying subdominant/rare Vβ families directly proportional to the number of bacterial colonies (or vectors) screened.	(Douek et al., 2002)
TCR Spectratyping	**CDR3-specific PCR amplifications** of TCR families using specific probes for the detection of CDR3 variation within each TCR family. The number of different CDR3 detected is a marker of TCR perplexity.	Semi-quantitative. Paired with sequencing it can provide information that covers not only the type of Vβ families expressed but also of the number of different clonotypes that are present (oligoclonal vs polyclonal response).	Can lead to biased representation of Vβ families depending on the annealing efficiencies of the CDR3-specific primers used. Can lead to biased representation of Vβ families due to primer cross-reactivity. Time consuming due to the number of PCR reactions involved.	(Currier and Robinson, 2001)
Gene-specific PCR amplification of TCRs	A **Vβ-specific primer panel** is used to set up a series of PCR amplifications the products of which reveal which Vβ families are expressed.		Can lead to biased representation of Vβ families depending on the annealing efficiencies of the Vβ-specific primers used. Time consuming due to the number of PCR reactions involved.	(Dash et al., 2011; Genevee et al., 1992)
Panel Vβ Antibody Staining	Screening of CD8+, Tetramer+ population with a panel of Vβ-specific monoclonal antibodies.	Fast and direct method for the quantification of the frequency of a given Vβ subset.	Coverage depends on the availability of Vβ antibodies No sequence information and hence, no distinction between different clonotypes/ family subgroups.	(Rufer, 2005)
Limiting Dilution TCR sequencing	Sorting of CD8+ Tet+ populations and plating using limiting dilution for the establishment of individual CD8 CTL clones.	The established CTL clones can be used for functional studies. Quantitative identification of dominant CTL clones	Difficult to grow and thus to identify subdominant CD8 T-cells/ T-cells with low frequency of representation (especially when responses are highly hierarchical and/or oligoclonal)	(Sharrock et al., 1990)

Table 4.1 : Comparison of methods available for TCR repertoire analysis.

were found to express the TRBV19 receptor. The remaining clone expressed a TRBV20-1 receptor (figure 4.3). Once the specificity of the clones was confirmed, their clonality was determined by PCR amplification. An unbiased TCR sequencing method was adopted for this purpose in which a 'global' 5'-end anchor primer (Clontech) was

used in combination with a constant domain primer (either alpha or beta) to amplify all TCR transcripts within each CTL clone culture. The advantage of this method is that it overrides the bias introduced by other 'traditional' TCR sequencing methods when it comes to the clonality of culture. Such 'traditional' methods selectively amplify TRAV/TRBV sequences by means of Vα and Vβ- specific primers and even though powerful enough to confirm Vβ-staining data and determine sequences they fail to properly address clonality, when a complete Vα/Vβ primer panel is not used, due to their selective nature of TCR amplification. The same method was also used to generate the cDNA needed for the molecular TCR analyses. The mRNA that was isolated from the uncloned portion of the sorted NY9+CD8+T-cells, was transcribed into cDNA and shipped to the lab of Dr. Brouard in Nantes, France for TCR spectratyping analysis. The PCR sequence analysis of the two CTL clones confirmed that both cultures were 100% clonal. It also revealed a dual TRAV usage for the TRBV19 CTL clone (figure 4.3). However, no TRAV10 surface expression was detected by Vα monoclonal antibody staining. The same sequencing analysis demonstrated a 100% match in nucleotide and amino-acid sequences for the TRBV19 TCRs that circulated in patient 30 in 2007 and 2009. This, in conjunction with the spectratyping, clonotyping and limiting dilution results, indicates that the TRBV19 receptor persists and dominates the NY9-specific response in this patient for at least two years despite an observed clonotypic narrowing (figure 4.2).

4.2.2 TRBV19+ is a 'private' receptor with a conserved, 'public' -Q-G motif

Since previous studies in our lab had revealed a selective expansion of the TRBV19 family in B*3501 patients that responded to the NY9 epitope (Leligdowicz et al., 2010b) the question of whether or not TRBV19 might be a shared, 'public' TCR arose. In keeping with the 2007 findings, the analysis of the 2009 samples by clonotyping and spectratyping revealed a higher representation of the TRBV19 family within the pool of NY9-specific CD8+ T-cells. However, an inter-patient comparison of TRBV19

Protein Level

TRBV19

Patient_ID	TRBV	CDR3	TRBJ
1	19	KNPTAFYL **C A S S S Q G** - NQPQH	1-5*01
	19	KNPTAFYL **C A S S L Q G L** - NTEAFF	1-1*01
15	19	KNPTAFYL **C A S S R Q E F A G** - NEQFF	2-1*01
21	19	KNPTAFYL **C A S S T Q V G** - DTQYF	2-3*01
26	19	KNPTAFYL **C A S S M G Q G** - NEQFF	2-1*01
30	19	KNPTAFYL **C A S S P Q H G** - DTQYF	2-3*01
Consensus Motif		**C A S S X Q X G**	

Nucleotide Level

ID	CDR3			
	TRBV	TRBD	N-VAR	TRBJ
1	TGT GCC AGT AGT TCA CAG GGT AAT CAG CCC CAG CAT TTT			
	TGT GCC AGT AGT TTA CAG GGC CTG AAC ACT GAA GCT TTC			
15	TGT GCC AGT AGT CGA CAG GAA TTT GCA GGC AAT GAG CAG TTC TTC			
21	TGT GCC AGT AGT ACC CAG GTT GGC GAT ACG CAG TAT TTT			
26	TGT GCC AGT AGT ATG GGA CAG GGG TAC AAT GAG CAG TTC TTC			
30	TGT GCC AGT AGT CCG CAA CAC GGA GAT ACG CAG TAT TTT			

Figure 4.4 The sequence of the dominant TRBV19+ receptor at the protein and nucleotide level. Short, germline encoded CDR3 regions (<6 nt long) and a higher degree of nucleotide triplet degeneracy are usually the hallmarks a higher production frequency.

sequences at the nucleotide and protein level revealed no public TCR usage, in terms of Vβ-Jβ pairing and/or CDR3β identity. Despite this observation, all TRBV19 receptors were found to possess a highly conserved -Q-G- amino acid motif (figure 4.4). The

encoding of the glutamine (Q) was both germ-line (TRDB encoded) and non-germ line (n-variation), whereas the conserved glycine (G) was made up exclusively of n-nucleotide additions. The TRBV20-1 TCRs were also compared for the presence of a motif. However, due to the limited representation of this family among the patients under study (n=2) no meaningful conclusions about motif usage could be drawn for this family.

4.2.3 Dominance of the TRBV19+ receptor cannot be explained by a higher production frequency.

Studies led previously by Quigley et al. and Venturi et al, have suggested that TCRs produced in mice with higher frequencies during VDJ recombination are overrepresented in the naive repertoire. Thus, such TCRs might be endowed with a kinetic advantage in the early stages of infection if they meet a minimum of antigen avidity for mobilization and might also have a greater potential for peripheral dominance in the chronic stages of infection. TCRs produced with higher frequencies are usually encoded by several different nucleotide triplets that 'converge' to the same amino-acids (Venturi et al., 2011). In addition, their CDR3β sequences contain a limited number of nucleotide additions (<3) (Quigley et al., 2010; Venturi et al., 2008). An alignment of all TRBV19 nucleotide sequences detected in patient 30 showed no evidence of VDJ convergence. There was only one nucleotide sequence encoding the observed TRBV19 and this was further confirmed by spectratyping analysis. Moreover, its CDR3β region contained a much higher number of nucleotide additions than the one anticipated for high frequency TCRs (8 vs 2) and included amino acids that were encoded by codons with a low degree of degeneracy (figure 4.5).

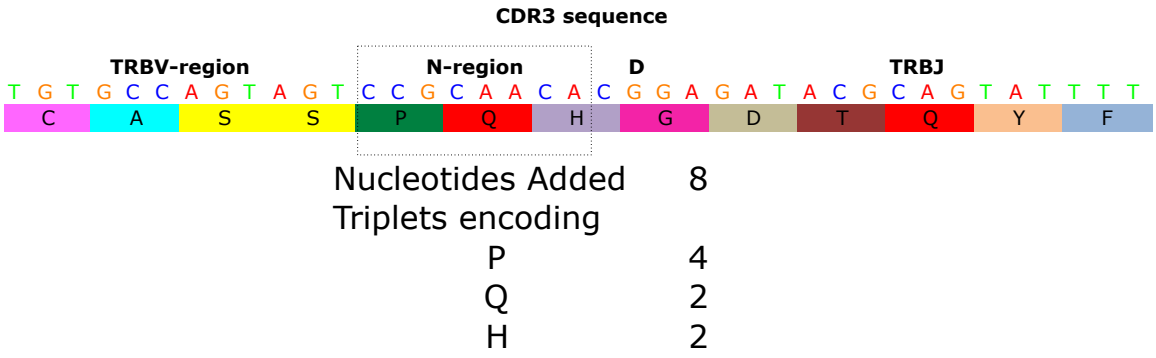


Figure 4.5 The sequence of the dominant TRBV19+ receptor. Short, germline encoded CDR3 regions (<6 nt long) and a higher degree of nucleotide triplet degeneracy are usually the hallmarks a higher production frequency.

Therefore, the dominance of this receptor in the NY9-specific CD8+ T-cell pool does not appear to stem from a higher production frequency.

4.2.4 The dominant TCR TRBV19 recognizes its cognate antigen with higher avidity than the subdominant TCR TRBV20-1.

The assessment of the structural avidity of a TCR:pMHC interaction can be carried out using a variety of methods. Some of these methods utilise single molecules synthesized *in vitro* to measure the thermodynamic characteristics of the TCR:pMHC interaction, such as the equilibrium constant K_D which describes the binding reaction AB <=> A+B that occurs in solution between the TCR and the pMHC whereas others rely on the technology of pMHC tetramers, to answer this question (Margulies, 2001). What is measured in the latter case is the affinity of the TCR for a multimeric pMHC complex. Even though this measurement does not offer but an approximation to the real affinity, it is considered a good and robust alternative to when single molecules are not readily

available (Davis et al., 2011; Margulies, 2001). Decreasing amounts of PE-Conjugated NY9 tetramer were used for the staining of the TRBV19 and TRBV20-1 CD8+ T-cell clones. Comparison of %tetramer and [MFI] values at different NY9 concentrations suggested that the dominant TCR has a higher avidity for the NY9: B*3501 complex than the subdominant TRBV20-1(figure 4.6). There was no difference in the levels of CD8 expression between the two CTL clones (figure 4.7).

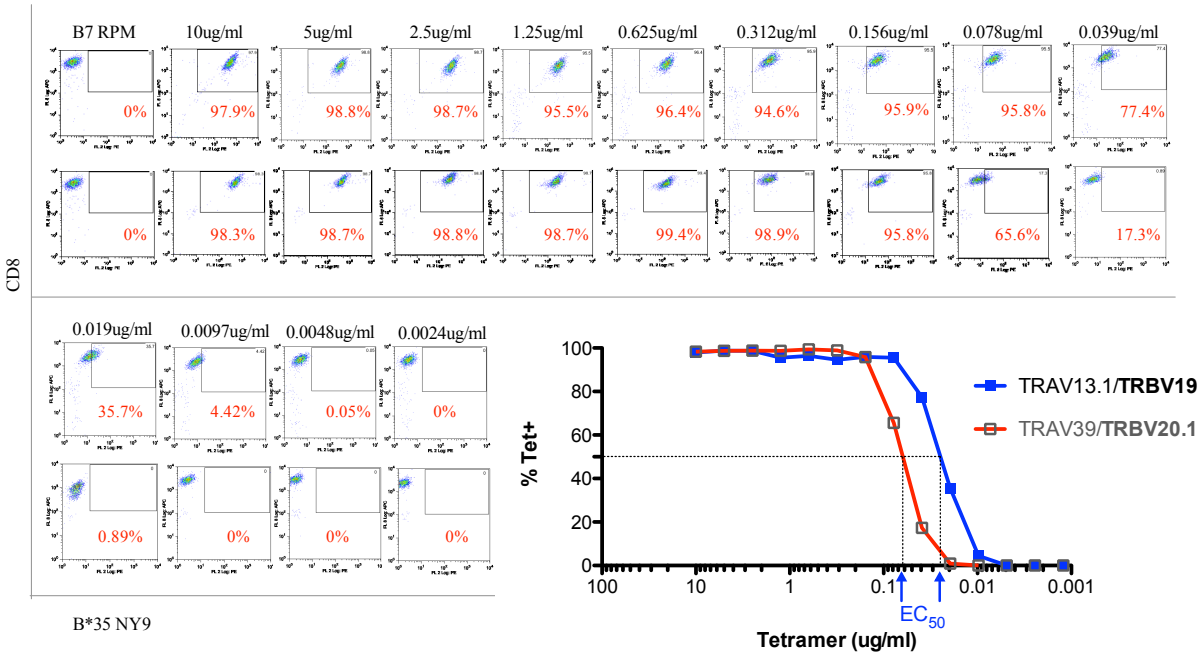


Figure 4.6 The TCR avidity of the CD8 CTL clones was determined by MHC class I (B*35 NY9) staining using decreasing amounts of tetramer. The dominant clonotype TRAV13.1/TRBV19 (upper row) displays a higher avidity than the subdominant clonotype TRAV39/TRBV20-1 (lower row). The gating was determined based on the irrelevant tetramer B7 RPM.

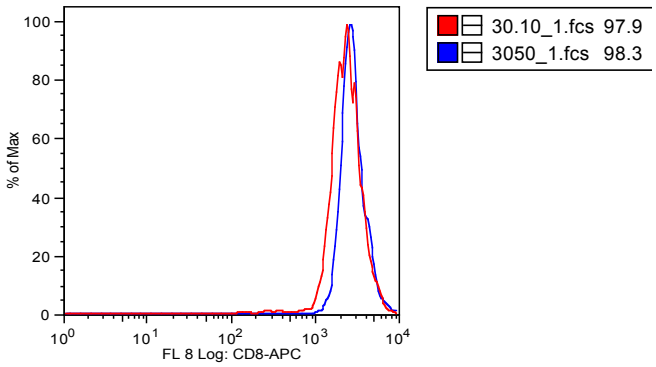


Figure 4.7 No difference in the levels of CD8 expression on the surface of TRBV19 (red) and TRBV20-1 (blue) NY9-specific CD8+ T- cell clones

4.2.5 CD8+ T-cell clones bearing the dominant TRBV19 TCR have a similar cytotoxic potential to TRBV20-1 clonotypes when antigen is in abundance but show a higher sensitivity at limiting Ag concentrations.

Next, the cytotoxic potentials and functional avidities of the two receptors were compared in a ^{51}Cr release assay using as targets autologous B-cell lines exogenously loaded with NY9 peptide. At higher antigen concentrations (10-10^{-3}uM) no difference was observed in the ability of the two CTL clones to lyse the B-cells. However, at lower antigen densities the functional avidity (EC_{50}) of the TRBV19 receptor was a log higher than that recorded for TRBV20-1 (figure 4.8)**.** To rule out any clone-specific effects with regard to functional avidity more than one established TRBV19 CTL clone were assessed for their ability to recognise the target cells. No differences were seen among different CTL clones expressing the same receptor.

Since ex-vivo studies involving PBMCs from patient 30 had previously suggested a polyfunctional profile for the NY9-specific clonotypes (chapter 3) the two CTL clones were then compared for their ability to degranulate and produce cytokines. Assessment of CD107α, IFN-γ, TNF-α and IL-2 production by intracellular staining revealed a profile of sensitivity that was similar to the one seen in ^{51}Cr release assays. At high antigen concentrations the two clones displayed an almost identical profile of polyfunctionality, which waned faster for clone TRBV20-1 as the concentration of Ag decreased (figure 4.9).

In addition, the TRBV19 clone displayed a more sustained profile of cytokine polyfunctionality and a greater magnitude of cytokine production. This was true for cytokines such as IFN-γ and TNF-α, but not so for IL-2, the production of which was greater in clone TRBV20-1 (figure 4.9c and appendix i).

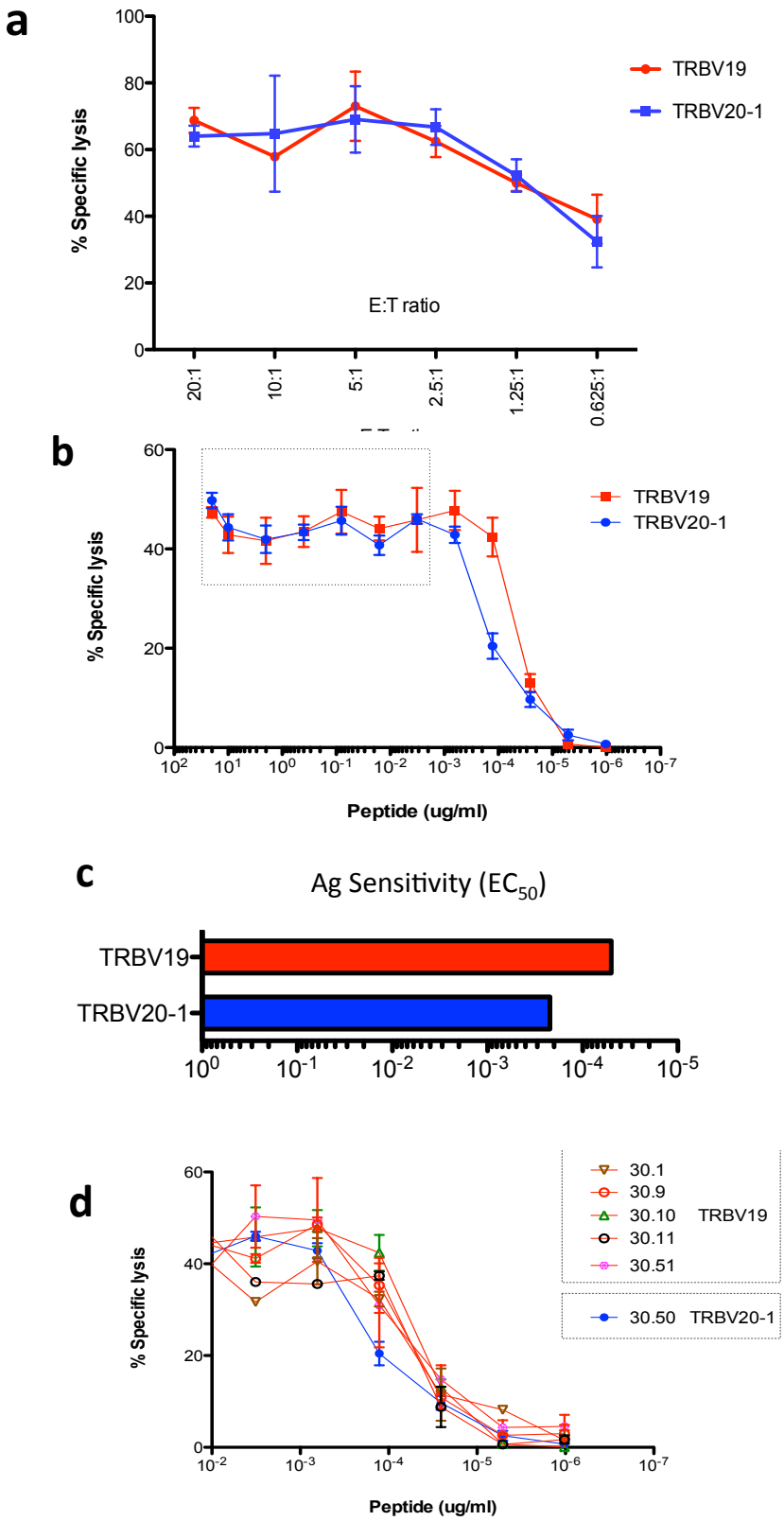


Figure 4.8 (a) Recognition of autologous BCLs exogenously loaded with 5uM NY9 peptide in a standard ^{51}Cr release assay. Both TCRs induce killing of B-cell targets at E:T ratios as low as 0.625:1. (b) TRBV19 and TRBV20-1 CTL clones show comparable levels of cytotoxicity for Ag concentrations between 10uM and 10^{-3} uM *(boxed area)*. Symbols represent means of duplicate wells while error bars represent SD. The E:T ratio was 5:1. (c) Functional sensitivity (EC_{50}) was calculated by normalizing the specific lysis so that the maximum value equated to 100% and then by assessing the concentration of peptide required for 50% maximum specific lysis. (d) Cytotoxicity and sensitivity were dependent on the TCR and not influenced by the CTL clone used.

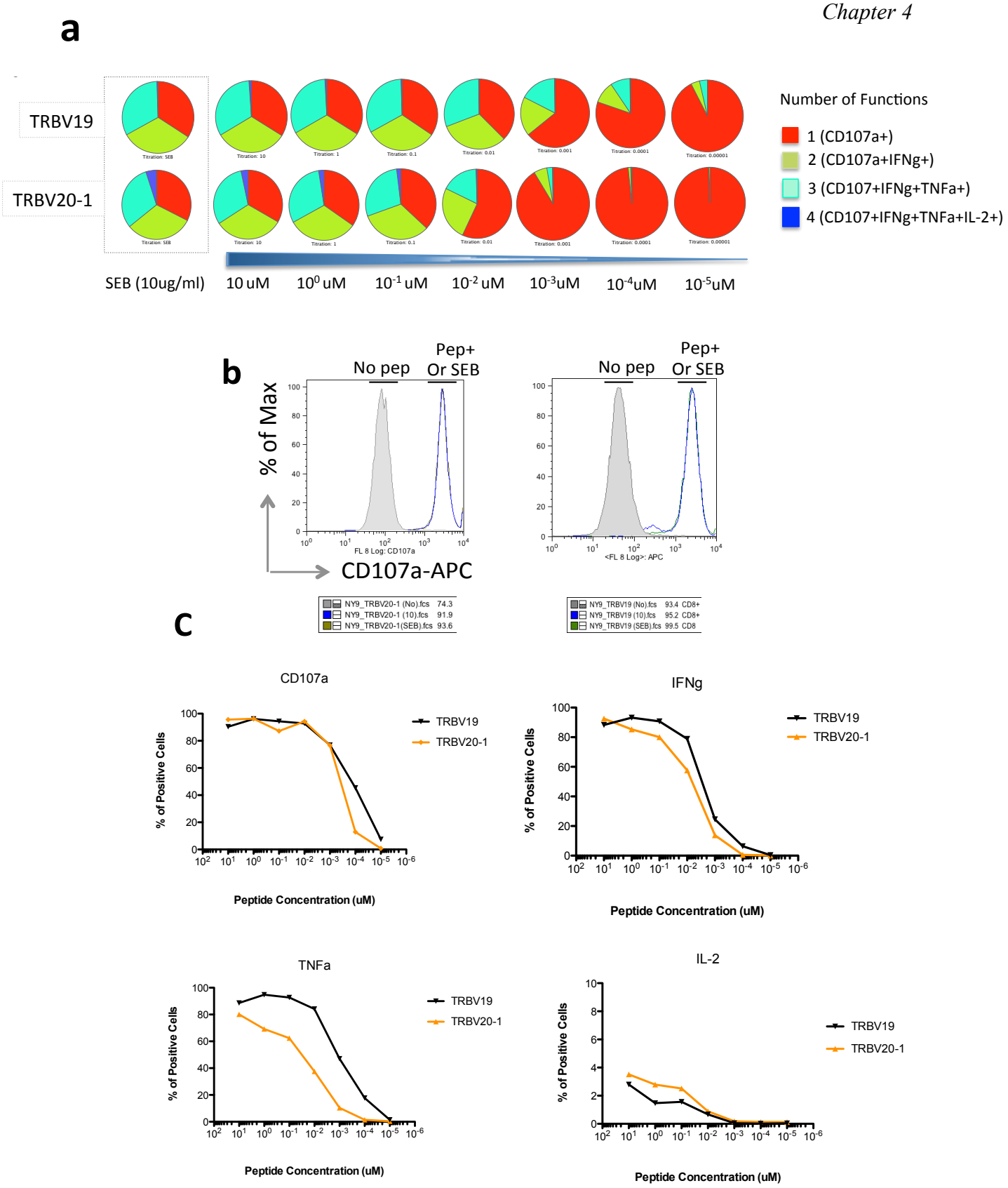


Figure 4.9 (a) Profiles of polyfunctionality of NY9-specific CTL clones expressing the TCRs TRBV19 and TRBV20-1 as determined by ICS . Each pie represents the background adjusted polyfunctional profile of the CTL clone at a given Ag concentration. SEB (10ug/ml) stimulation was also included in the assay to control for general CTL clone functionality. (b) Representative example of ICS production (here CD107α). (c) Sensitivities of CTL clones as assessed by CD107α, IFN-γ, TNF-α and IL-2 production (ICS).

Taken together these data demonstrate that the dominant TRBV19 clone has a higher sensitivity for antigen than the subdominant TRBV20-1 clone.

d

TCR	Sensitivity (EC_{50}) CD107a	IFNg (ELISPOT)
TRBV19	$1.8x10^{-4}$uM	$0.27x10^{-3}$uM
TRBV20-1	$5.4x10^{-4}$uM	$1.6x10^{-2}$ uM

Figure 4.9 (d) Functional sensitivity comparison for CTL clones 30.10 (TRBV19) and 30.50 (TRBV20-1) *[also appendix]*

4.2.6 Differences in magnitude but no overall differences in the types of cytokines produced by CD8+ T-cells bearing a TRBV19 versus a TRBV20-1 TCR.

To answer the question of whether or not the two CTL clones differed in their cytokine secretion beyond the production of 'standard' cytokines such as IFN-γ, TNF-α and IL-2, the production of 27 cytokines was assessed using a bead-array assay. The results confirmed previous observations in that there were no differences in the types of cytokines produced by the CD8+ T-cells bearing the two receptors (appendix II).

However, irrespective of Ag concentration, a greater magnitude of MIP-1β, IL-13, IL-6, GM-CSF and MCP-1 was secreted by the TRBV19 clone after 24hrs of incubation with autologous B-cell lines loaded with the NY9 epitope compared to the levels secreted by the TRBV20-1 clone (figure 4.10). This suggests that the TRBV19 expressing CTL clone is characterised by a superior profile of cytokine secretion when compared to the subdominant TCR TRBV20-1.

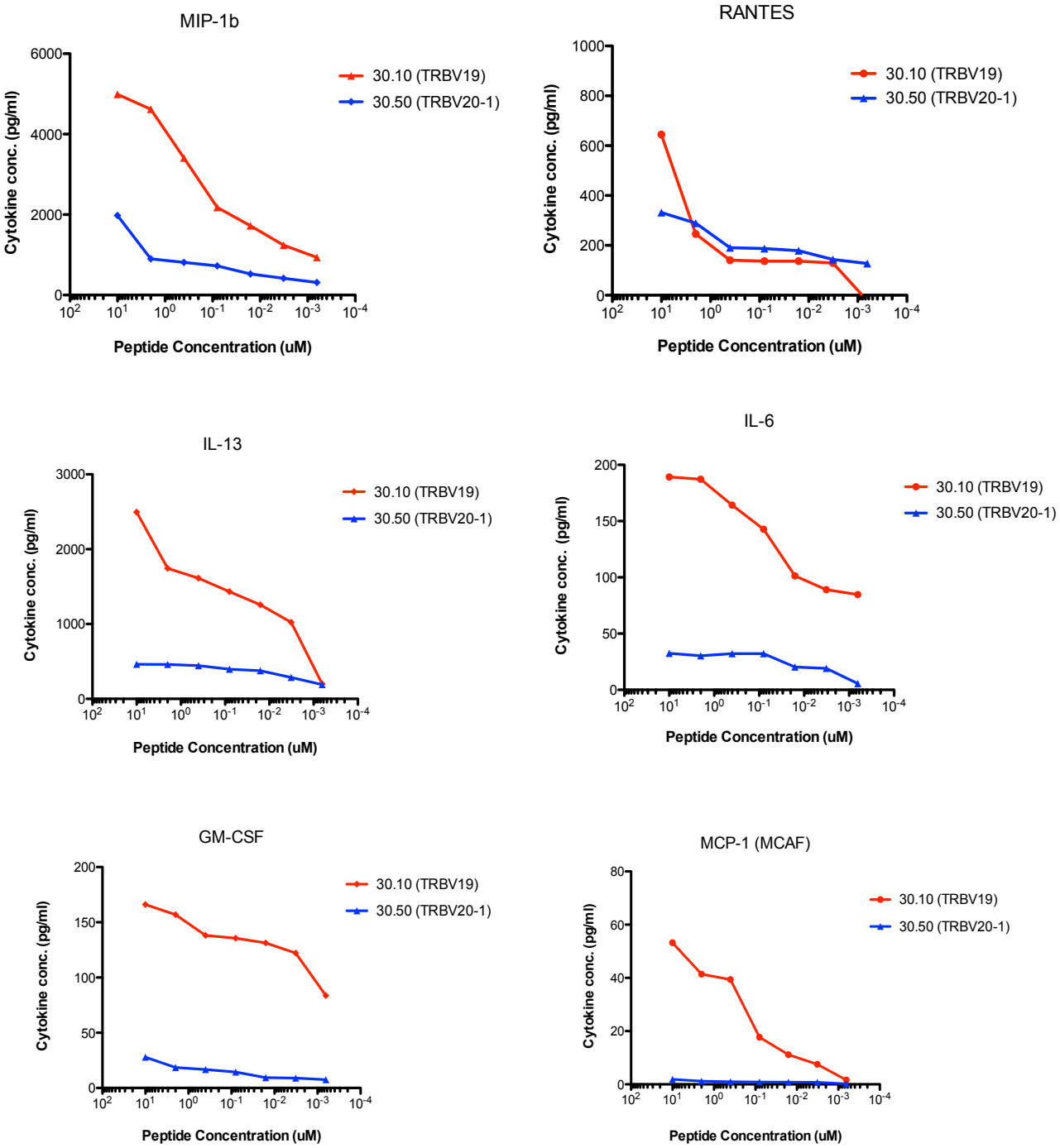


Figure 4.10 Background adjusted levels of MIP-1b, RANTES, IL-13, IL-6 and GM-CSF production by NY9-Specific CTL clones. Cytokine production was assessed following a 24hr- co-culture of the CTLs with autologous B-cells pulsed with decreasing concentrations of NY9 peptide (seven 5-fold dilutions starting at 10ug/ml). Cytokine production was measured using a bead array assay (Bio-plex).

4.2.7 The persisting TRBV19+ TCR is more resistant to variations of the NY9 epitope.

Even though the NY9 epitope is considered well conserved in ART-naive patients who control viral replication, variants of the sequence have been previously reported to emerge, especially in HLA-B*3501 patients who present with elevated viral loads (Leligdowicz et al., 2010b). Since escape from CD8+ T-cell responses is based on the emergence of viral variation and this is a major mechanism through which HIV-1 avoids CTL-mediated immune recognition (Goulder and Watkins, 2004), the question of whether or not the NY9-specific CD8+ T-cells under study could recognise the variants of the NY9 sequence seen in the Caio cohort and elsewhere arose. This question was also pertinent from a functional point of view since cross-reactive Gag-specific CD8+ T-cells have been previously shown to play a role in the containment of viruses with mutated epitope sequences (Johnson et al., 1991). However, up to date no study has provided data on the ability of HIV-2 specific CD8+ T-cells to cross-recognise epitope variants seen in HIV-2 patients, and hence the question of whether or

a

Epitope Variant	Source	MHC I Binding (strength)
NPVPVGNIY	Caio	Binder (+++)
- - - - - - S - -	Caio	Binder† (+++)
- - I - - - - - -	Caio	*nd*
- - - - - R - - -	Caio	*nd*
- - I - - R - - -	Caio	*nd*
S - I - - - - - -	7312A HIV-2 *	*nd*

** Los Alamos Database*

† Dr Tao Dong, unpublished observations.

Figure 4.11 (a) Table displaying the most commonly occurring variants of the NY9 epitope.

not the NY9-specific CD8+ T-cells possess this quality, and to what extend, remains unanswered. To answer this question, the two CTL clones were tested for their ability to cross-recognise target cells presenting common NY9 variants (figure 4.11). The TRBV19 CTL clone recognized all tested variants (5/5), albeit with different sensitivities. On the other hand, the TRBV20-1 CTL clone only recognized 2/5 variants tested. This shows that the TRBV19 receptor has a higher degree of flexibility when it comes to its antigen recognition than the subdominant TRBV20-1 receptor.

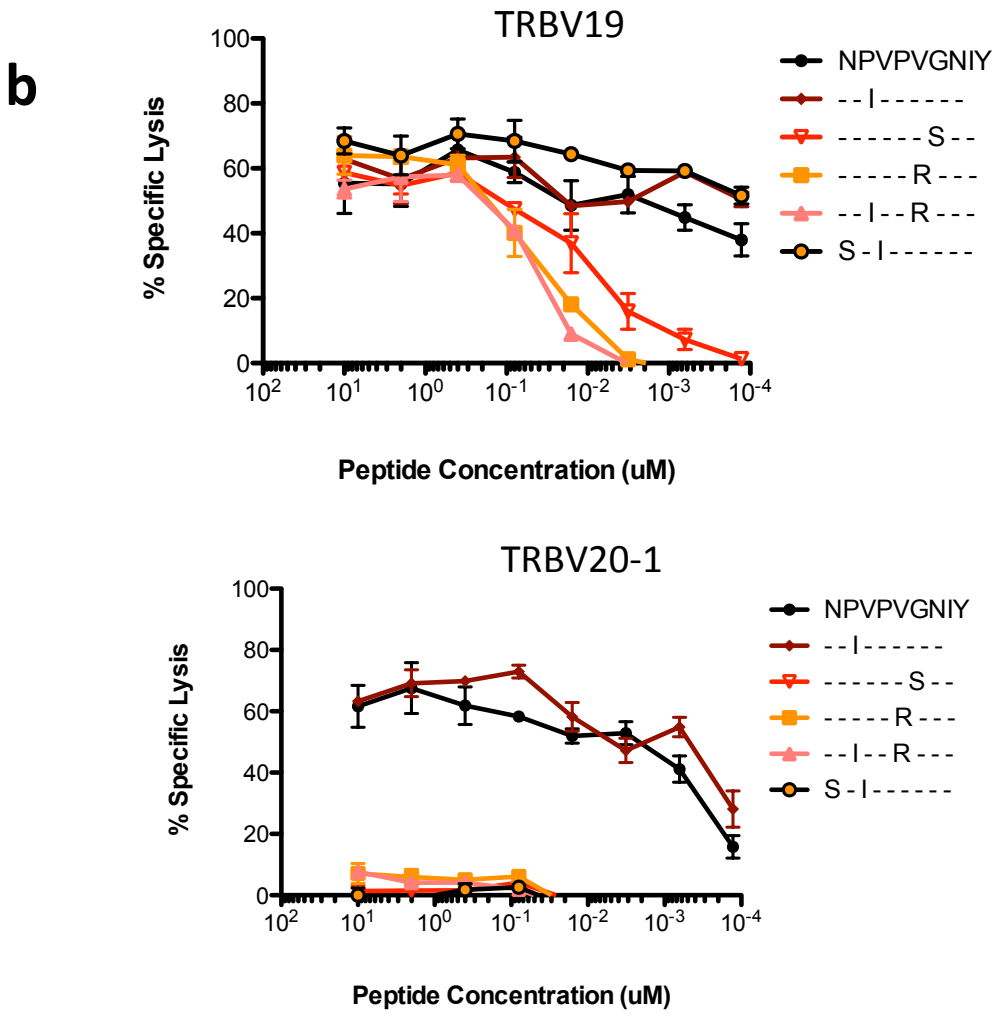


Figure 4.11 (b) The ability of TRBV19 and TRBV20-1 CTL clones to recognize targets (BCLs) exogenously loaded with decreasing amounts of NY9 variants was measured in standard 4hr ^{51}Cr release assays. The assays were performed at day 11 post restimulation. A 5:1 E:T ratio was used.

4.2.8 The phenotype of TRBV19+ CD8+ T-cells in patient 30 is characterised by the upregulation of multiple inhibitory markers.

To assess the phenotype of TRBV19 CD8+ T-cells in patient 30, cryopreserved PBMCs collected in 2009 were thawed and stained with monoclonal antibodies for CD57, PD-1, CD27, CD45RO, 2B4, CD160, and CD127 along with anti-TRBV19 (Vβ17), NY9-tetramer, anti-CD4, CD8 and CD3. Previous studies in the field have suggested a $PD1^{high}$, $CD127_{low}$ phenotype for dominant HIV-specific clonotypes (Almeida et al.,

2007; Conrad et al., 2011). In line with those results, the TRBV19 clone displayed a terminally differentiated, 'exhausted' phenotype compared to the general NY9 Tet-TRBV19+ population. This was characterised by a higher expression of CD57, PD-1 and CD160 (figures 4.12 &13). Unfortunately, due to the very low frequency of the TRBV20-1 family in the sample, a similar phenotypic analysis could not be performed for the TRBV20-1 population.

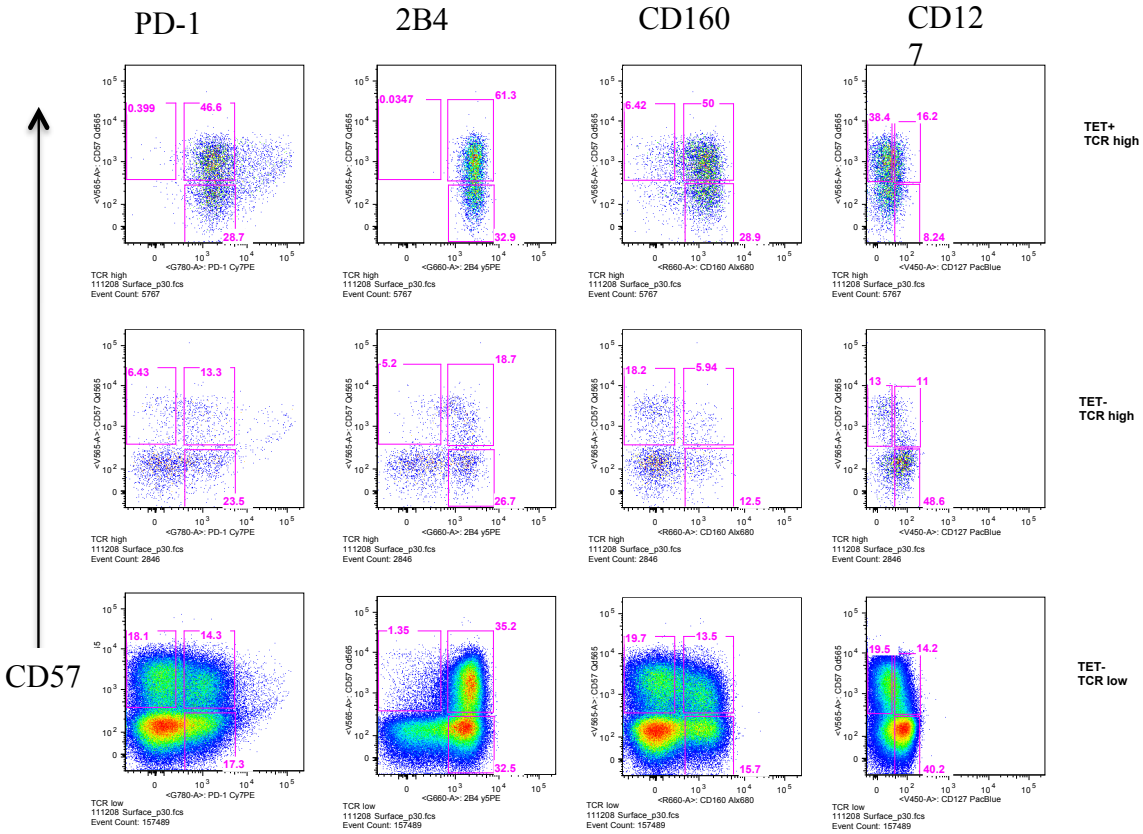


Figure 4.12 FACS plot showing the distribution of PD1, 2B4, CD160, CD127, and CD57 on bulk Tet+/TRBV19+, Tet -/TRBV19+, and Tet-/TRBV19- populations

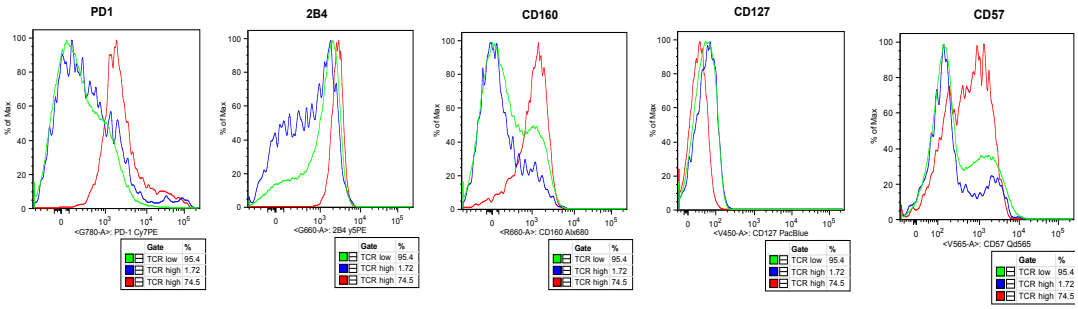


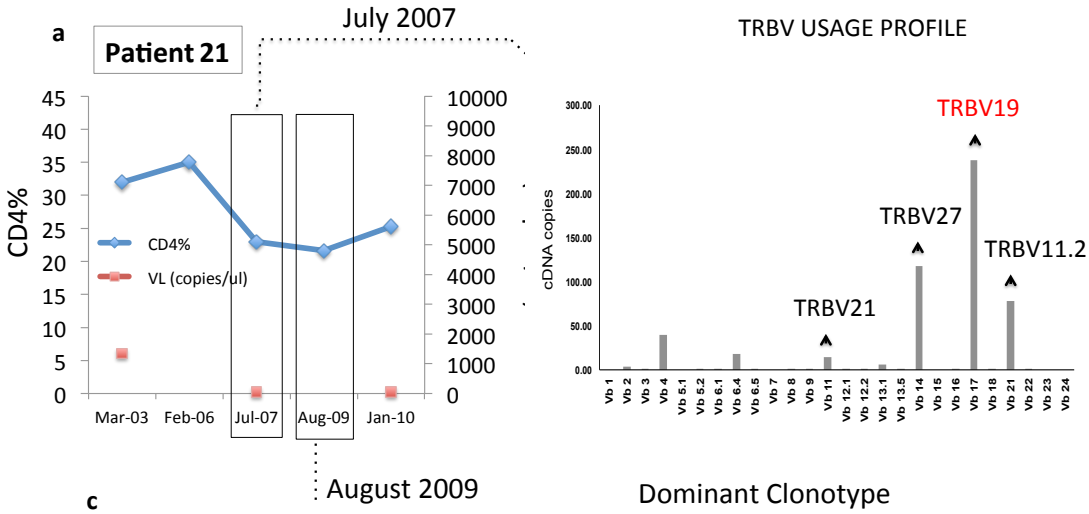
Figure 4.13 Total expression of PD1, 2B4, CD160, CD127, and CD57 on bulk Tet+/TRBV19+, Tet -/TRBV19+, and Tet-/TRBV19- populations. *red: Tet+/TRBV19+, blue: Tet-/TRBV19+, green: Tet-/TRBV19-*

4.2.9 A selective expansion of subdominant clonotypes ensues when TRBV19 clonal dominance is lost: the case of patient 21.

The analysis of two CTL clones with distinct TCR usage from patient 30 indicated that the dominant TRBV19 TCR possessed a higher avidity and sensitivity for Ag, a greater magnitude of cytokine production and an increased cross-reactivity for NY9 variants contributed to the observed dominance was not clear. To address this question, a similar analysis was performed in patient 21. This patient was also a long-term non-progressor and his clinical characteristics are given in chapter 3 and figure 4.14.

TCR clonotyping and spectratyping analyses in this patient using samples from 2009 revealed a narrowing in the clonotypic diversity comparable to that seen in patient 30 (figure 4.15). However, in this patient, in contrast to patient 30, there was no maintenance of the clonotypic hierarchy seen in 2007. The TRBV19 dominance had been lost and a selective expansion of previously subdominant TCRs had occurred**.** Unfortunately we were not able to generate clones from this patient for further clonotypic analyses. Instead, to determine what may have accounted for the observed

loss of dominance an archived TRBV19 CTL clone from 2007 that was available was used.



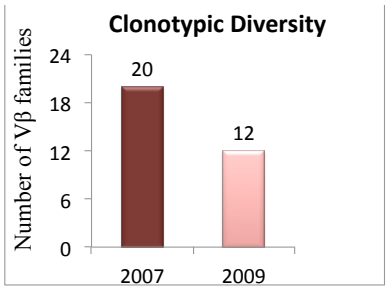
ID	TRAV	CDR3	TRAJ	Freq
EM-21	13-2	CAVDCGD	9-1	58
	12-3	CAMSPLY	43-1	11
	10	CVVSAMSS	33-1	16
		Seqs: 85		

ID	TRBV	CDR3	TRBJ	Freq
EM-21	27	CASSPTIDSYEQYF	2-7	41
	11-2	CASSFDSHPNTEAFF	1-1	32
	29-1	CSAAYGNTEAFF	1-1	10
	10-3	CAINEGAGDTQYF	2-3	1
	7-9	CASSLYQGAACEQYF	2-7	2
		Seqs: 86		

Figure 4.14 (a) Clinical characteristics of patient 21. (b) TRBV usage profile as determined by quantitative qPCR in 2007. Arden nomenclature used. (Leligdowicz A and TcLand-expression Labs, France). (c) TCR profile as determined by clonotypic analysis of tetramer- sorted (NY9+) CD3+CD8+ T-cells in 2009. TRAV/TRBV families follow the IMGT nomenclature.

EM_21	TRBV Family						
IMGT	TRBV9	TRBV20-1	TRBV28	TRBV29-1	TRBV5-1	TRBV5-4	TRBV7-3
Vβ	**Vβ 1**	**Vβ 2**	**Vβ 3**	**Vβ 4**	**Vβ 5.1**	**Vβ 5.2**	**Vβ 6.1**
2009							

IMGT	TRBV7-9	TRBV7-2	TRBV4-1	TRBV12-3	TRBV3-1	TRBV25-1	TRBV10-3
Vβ	**Vβ 6.4**	**Vβ 6.5**	**Vβ 7**	**Vβ 8**	**Vβ 9**	**Vβ 11**	**Vβ 12.1**
2009							



IMGT	TRBV10-1	TRBV6-1	TRBV6-4	TRBV27	TRBV24-1	TRBV14	TRBV19	TRBV18	TRBV11-1	TRBV2	TRBV13	TRBV15
Vβ	**Vβ 12.2**	**Vβ 13.1**	**Vβ 13.5**	**Vβ 14**	**Vβ 15**	**Vβ 16**	**Vβ 17**	**Vβ 18**	**Vβ 21**	**Vβ 22**	**Vβ 23**	**Vβ 24**
2009												

Figure 4.15 TRBV families detected in the NY9 -specific, HLA-B*3501 restricted CD8+ T-cell response in patient 21 in 2009 (*pink)* and overall clonotypic diversity in 2007 (brown) (Leligdowicz et al, 2010) and 2009 (pink). IMGT: IMGT database nomenclature. Vβ: Arden nomenclature. TCR usage in 2009 was ascertained using a combination of Vβ-antibody staining, TCR clonotyping and TCR spectratyping.

4.2.10 TRBV19 clonotypes from patients 21 and 30 share an identical profile of avidity, sensitivity and cytotoxicity.

Comparison of the two TRBV19 CTL clones revealed an identical profile of avidity, sensitivity and cytotoxicity. However, when the magnitude of IFN-γ production was assessed, some differences did emerge. There was no difference in the magnitude or sensitivity of TNF-α, MIP-1β or IL-2 production. Nevertheless, the dominant TRBV19 CTL clone produced more IFN-γ than the clone that became ultimately replaced (figure 4.16).

4.2.11 TRBV19_21 and TRBV19_30 display dissimilar profiles of sensitivity to variants of the NY9 epitope

The ability of the older TRBV19 CTL clone to recognize variants of the NY9 epitope was also examined. As it was true for the dominant TRBV19 clone from patient 30, this clone also recognized all variants tested (5/5). However, the sensitivity of the two CTL clones for the different variants differed. More specifically, the 2007 clone recognized three of the most common variants with a much lower sensitivity than the persisting TRBV19 CTL clone (figure 4.17).

a

CTL_ID	TRAV	CDR3a	TRAJ	TRBV	CDR3b	TRBJ
21.8	10	L S D S A S Y I **C V V S A M S S** S N Y Q L	33*01	19	T A F Y L **C A S S T Q V G** D T Q Y F G	2-3*01
30.10	13.1	P E D S A V Y F **C A A N R H** D K V I F G P	50*01	19	T A F Y L **C A S S P Q H G** D T Q Y F G	2-3*01

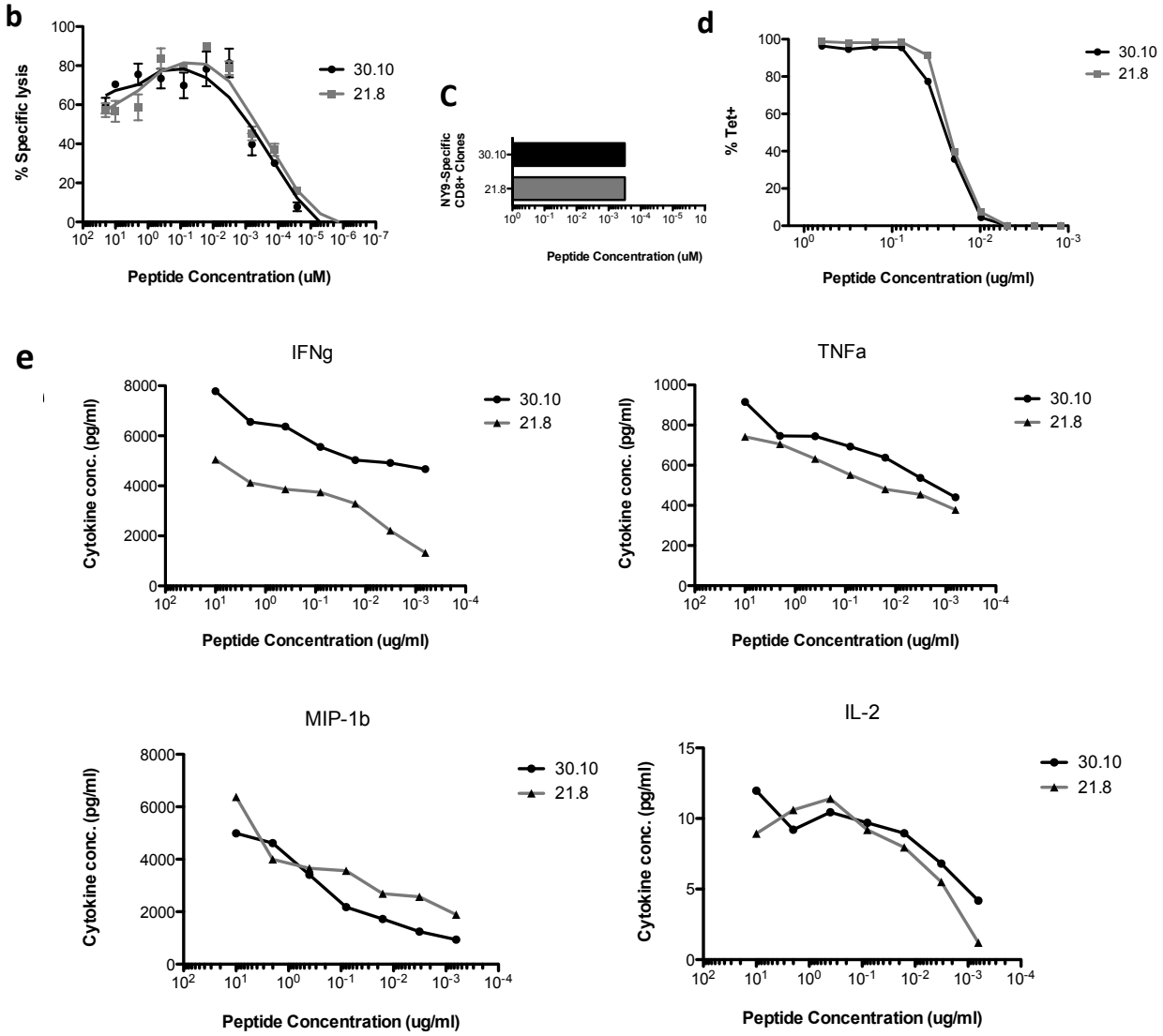
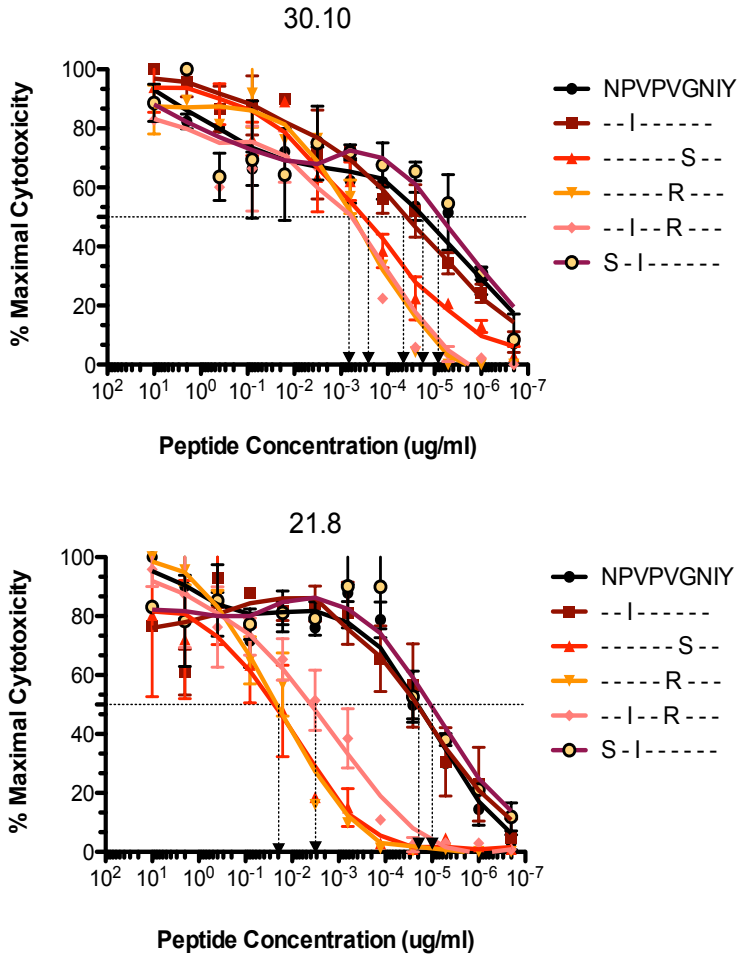


Figure 4.16 (a) TCR sequences of TRBV19 clones in patients 21 and 30 (b) The ability of the clones to recognize infected cells was measured in a ^{51}Cr release assay using B-cells lines pulsed with decreasing concentrations of NY9 peptide. (c) The functional avidity of the clones was determined by normalizing the specific lysis to 100% and by assessing the concentration of peptide required for 50% maximum specific lysis. (d) The TCR avidity of the two TRBV19 clones was determined by tetramer staining in the presence of decreasing tetramer concentrations. (e) Levels of IFN-γ, TNF-α, MIP-1β and IL-2 production by TRBV19 CTL clones in culture supernatants following a 24hr co-culture with peptide (NY9) laden B-cells. A 5:1 E:T ratio was used.

a



b

EC_{50} (Antigen Conc. ug/ml)

30.10	Variant	21.8	D
0.00002	**NPVPVGNIY**	0.00001	+
0.0000068	**--I------**	0.0032	+++
0.00024	**------S--**	0.02	+++
0.0008	**-----R---**	0.02	+++
0.0008	**--I--R---**	0.00002	++
0.00001	**S-I------**	0.00002	+

Figure 4.17 (a) Recognition of the most common NY9 variants by NY9-specific TRBV19+ CTL clones in a standard ^{51}Cr release assay. The functional avidities (EC_{50}) for each clone and each variant are summarized in table b. D; magnitude of difference.

4.2.12 Irrespective of dominance, all NY9-specific B*3501 restricted TRBV families harbour conserved CDR3β motifs

Additional dominant and subdominant CTL clones established from other B*3501 HIV-2 patients mounting a NY9-specific CD8+ T-cell response (patient 17 and patient 15) were also examined for their qualitative features.

Analysis of a dominant TCR from patient 17 and of a subdominant TCR from patient 15, both of which were long-term non-progressors, revealed motifs that invariably contained a conserved glycine (G) at amino acid position 8 of the CDR3β sequence (figure 4.18) as did the persisting and replaced TRBV19 receptors.

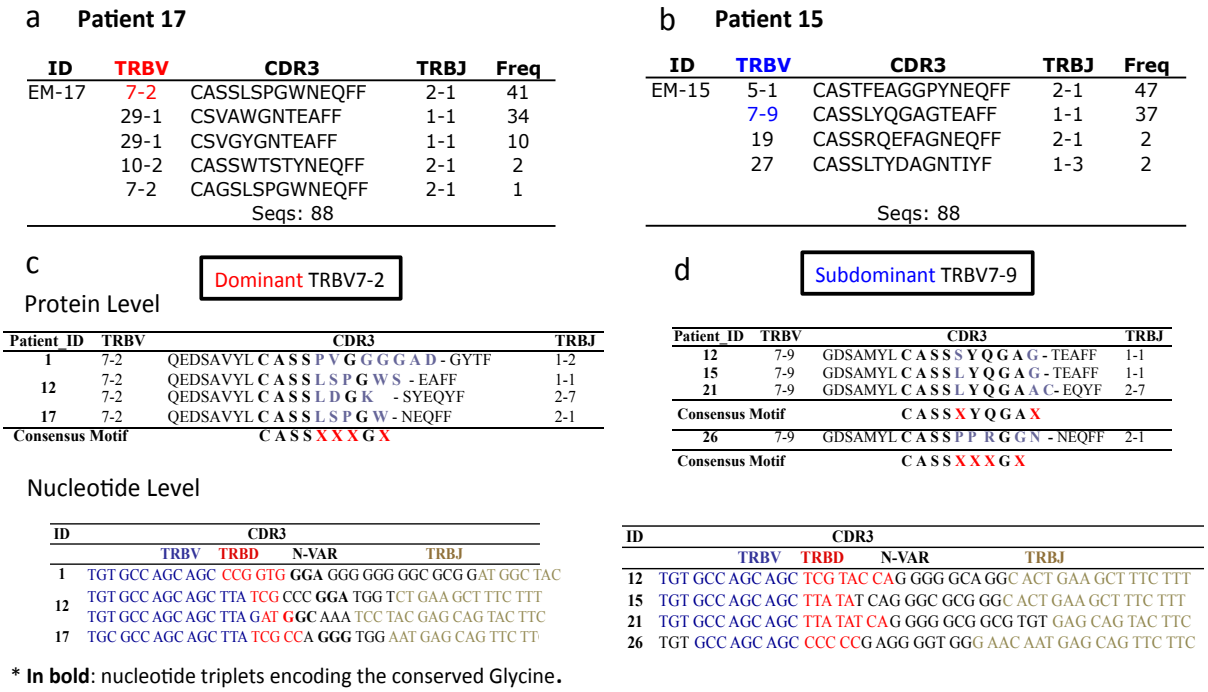


Figure 4.18 Clonotypic architecture of the NY9-specific, B*3501 restricted CD8+ T-cell response in (a) patient 17 and (b) patient 15. (c), (d) Interpatient comparison of the TRBV7-2 and TRBV7-9 sequences at the protein and DNA level.

4.2.13 NY9-specific dominant clonotypes have a higher avidity and a greater sensitivity for antigen than subdominant clonotypes.

The TRBV7-9 and TRBV7-2 CTL clones were also tested for their respective avidities for antigen, their sensitivities and polyfunctionality. Once again, to ascertain that the observed profiles of antigen avidity were associated with TCR usage rather than the individual clone itself, multiple CTL clones bearing the same receptor were tested wherever this was possible.

No avidity differences were found by tetramer staining for clones expressing the same TCR. The dominant TRBV7-2 clone however displayed a greater avidity and sensitivity for Ag than the subdominant TRBV7-9 (figure 4.19), which was consistent with the pattern observed for the TRBV19 and TRBV20-1 CTL clones. Hence, avidity and sensitivity for Ag appear to distinguish dominant from subdominant NY9-specific TCRs in HIV-2 infection.

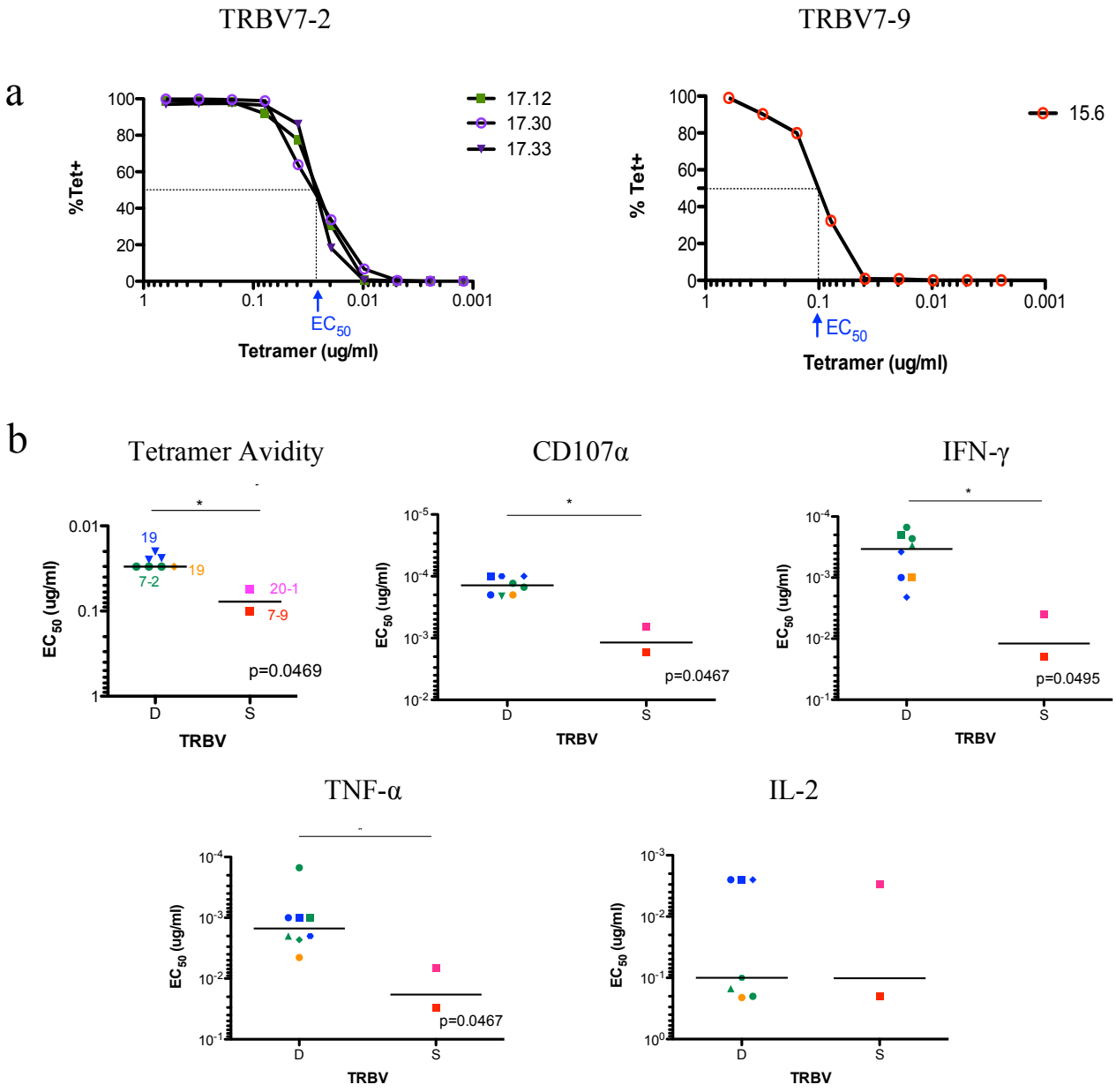


Figure 4.19 (a) TCR avidity measurement of CTL clones bearing the dominant TRBV7-2 and subdominant TRBV7-9 receptors. (b) Plots of Ag sensitivity as measured by tetramer titration assays and ICS (CD107α, IFN-γ, TNF-α and IL-2) after 6hrs of stimulation. The EC_{50} value on the y-axis represents the concentration of NY9 Ag in which half-maximal recognition occured. Each symbol stands for a single clone and each colour for a single TCR (*blue and orange represent TCRB19 TCRs from patients 30 and 21 respectively, green: TRBV7-2, pink: TRBV20-1 and red: TRBV7-9*). Horizontal lines depict group medians. Comparisons between groups were performed with the Mann-Whitney test. P values <0.05 were considered significant. TCR statuses (*D;* dominant TCR *or S:* subdominant) are plotted on the x-axis.

4.2.14 NY9-specific CD8+ T-cells with the TRBV7-2 or the persisting TRBV19 TCR display a higher Ag sensitivity for the common N>S NY9 variant *NPVPVGNSIY*.

The TRBV7-2 and TRBV7-9 CTL clones were also assessed for their ability to cross-recognize NY9 variants. Both clones displayed a considerable amount of flexibility recognizing all five variants tested. However, variants were recognized with different sensitivities by the two TCRs. Most importantly, a comparison of all TCRs under study revealed a high variability with regard to the recognition of the common N>S variant. TCRs such as the persisting TRBV19 and the dominant TRBV7-2 displayed a much higher avidity for this variant than subdominant or replaced TCRs (figures 4.20-21). It is therefore plausible that antigenic shifts plays a role in the clonal dominance observed in HIV-2 infection similar to that seen in mice infected with influenza A viruses (Haanen et al., 1999).

EC50 Ag Conc. (ug/ml)					
	Dominant		Subdominant/Replaced		
	30.1	17.33	21.8	30.5	15.6
Epitope Variant	**TRBV19(D)**	**TRBV7-2**	**TRBV19(R)**	**TRBV20-1**	**TRBV7-9**
NPVPVGNIY	0.00002	0.0025	0.00001	0.00076	0.0029
- - I - - - - - -	0.0000068	0.0025	0.0032	0.0009	0.0029
- - - - - - S - -	0.00024	0.0098	0.02	nr	0.2
- - - - - R - - -	0.0008	0.4	0.02	nr	0.025
- - I - - R - - -	0.0008	0.5	0.00002	nr	0.08
S - I - - - - - -	0.00001	0.00054	0.00002	nr	0.00065

nr=not recognized

Figure 4.20 Functional avidities of the TCRs under study for different NY9 variants. The EC_{50} values represent the amount of Ag needed for half-maximal target lysis. D: Dominant, R: Replaced

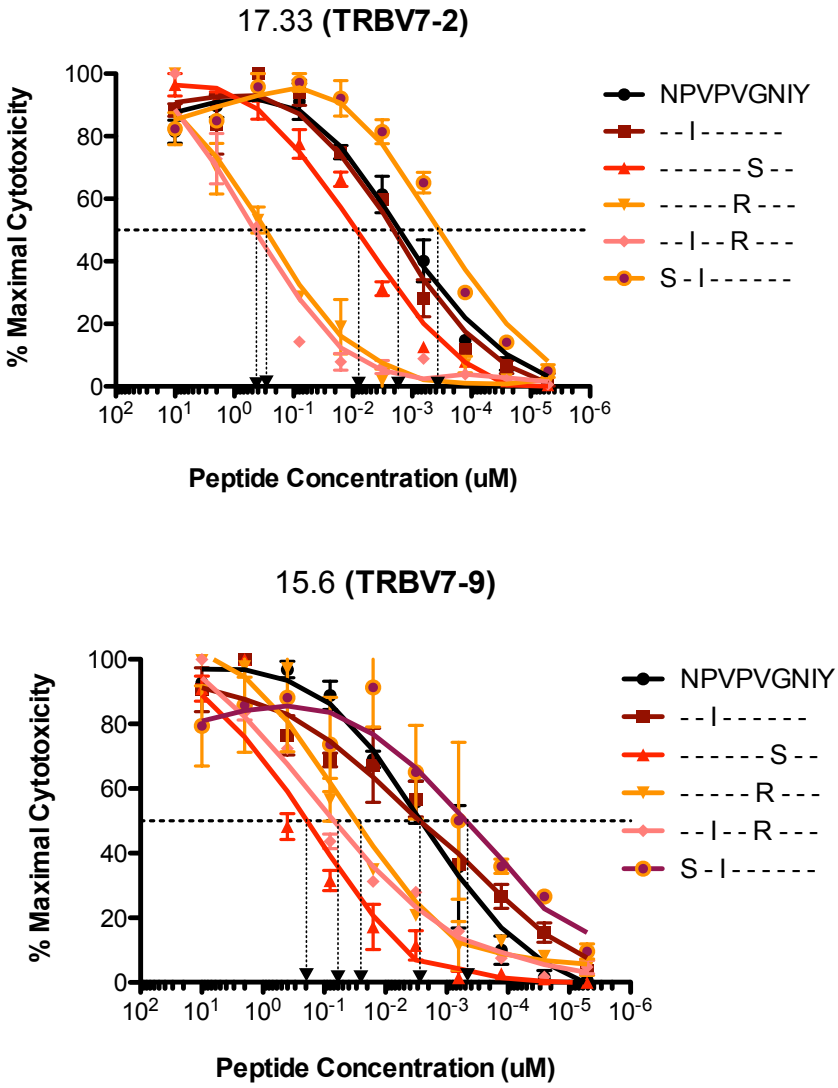


Figure 4.21 TRBV7-2 and TRBV7-9-specific NY9 variant recognition hierarchy as measured in ^{51}Cr release assays. B-cell lines were exogenously loaded with titrations of the most commonly occurring NY9 variants and used as targets at a 5:1 E:T ratio.

4.3 Discussion

The main focus of this chapter was the clonotypic architecture of the NY9-specific B*3501-restricted CD8+ T-cell population in one HIV-2 long-term non-progressor that has maintained the LTNP status for 18 years in the absence of anti-retroviral therapy. Previous research involving this patient had established a clear profile of clonotypic dominance within the NY9-specific CD8+ T-cell pool (Leligdowicz et al., 2010b). As previous studies in HIV-1 infection have linked the dominance of a single clonotype to the control of viral replication in long-term non-progressors (Almeida et al., 2007; Dong et al., 2004; Gillespie et al., 2006), it was hypothesised that viral control in HIV-2 infection might also be associated with the dominance of TCR clonotypes that possess superior qualities. CD8+ T-cell clones were established by limiting dilution and studied *in vitro* for their ability to lyse targets, their avidity and sensitivity for antigen, their polyfunctionality, their levels of CD8 expression and lastly for their ability to cross-recognize variants of the NY9 epitope.

The study of HIV-1 long-term non-progression proposes that during chronic HIV infection, and in the absence of high-level viral replication, the antigen-specific TCR repertoire displays a relative stability in terms of clonotypic constitution (van Bockel et al., 2011). This is also true for other persisting chronic viral infections, such as EBV, where it has been shown that CTL responses mounted towards two immunodominant latent EBV epitopes remain clonotypically unchanged for up to 18 years (Miles et al., 2005). Changes and perturbations are most commonly seen in patients who progress to disease (Gorochov et al., 1998) or experience a rebound in viral replication due to escape (Meyer-Olson et al., 2006), during the transition from acute to chronic infection (Pantaleo et al., 1997) or during the first few months following HAART initiation (Conrad et al., 2012; Soudeyns et al., 2000). However, a

longitudinal analysis of the NY9-specific clonotypes in two HIV-2 long-term non-progressors suggested a contraction of several NY9-specific TRBV families over time as some of the families detected in 2007 could no longer be detected in the 2009 sample despite using a variety of methods (Vβ monoclonal antibody staining, TCR clonotyping, TCR spectratyping and limiting dilution). This finding is at odds with a previous study in HIV-2 infection which suggested a broad TCR usage in HIV-2 infection (Lopes et al., 2003) but reiterates observations from chronic viral infections such HCMV, where in the face of low-level replication, TCRs with very low functional avidity contract below the limits of detection (Day et al., 2007). It has also been suggested that the loss of certain CTL clonotypes during the chronic phase of HIV-1 infection could be due to the clonal turn-over of exhausted clonotypes (Almeida et al., 2007). Indeed, when the phenotype of the dominant TRBV19 clonotype was studied, a picture of exhaustion, characterized by an increased expression of PD-1, 2B4, CD160 and CD57, emerged. Another possible explanation of course is that this change simply mirrors the advanced age of the patients under study (median age= 66.5years). Proof for such an age-related effect comes from a recent study in neonatal, adult and old mice that demonstrated an age-driven evolution of Ag-specific responses in HSV-1 infection. TCR repertoires in older mice were found to possess a more pronounced clonal hierarchy and were characterized by a gradual focusing that became more pronounced as age advanced (Rudd et al., 2011).

Despite this oligoclonal narrowing, at least one dominant HIV-2-specific clonotype (TRBV19) was found to persist in the peripheral NY9-specific CD8+ T-cell pool for two years. It is possible that this persisting TCR represents a clonotype selected during the early, acute stages of infection. Early mobilized, 'acute phase' TCR clonotypes that persist throughout the course of infection, even in the context of rapid

progression, have been previously described in HIV-1 (Islam et al., 2001), HCMV (Day et al., 2007), HCV infection (Miles et al., 2011) as well as in the context of vaccination (Price et al., 2009b). However, this is the first time that such a clonotypic persistence is described for HIV-2 infection. Since several studies in the past exploring the clonotypic constitution of EBV, HIV and SIV-specific CD8+ T-cell repertoires have linked TCR persistence to shared, public TCRs (Argaet et al., 1994; Dong et al., 2004; Lim et al., 2000; Price et al., 2009b) the TRBV19 sequence was also analysed for the presence of public features. Nevertheless, the persisting TRBV19 receptor was not of such 'public' nature.

While the persistence of a single clonotype might be associated with long-term non-progression, a narrow response is not always advantageous. The study of HIV-1 patients that carry the B*2705 allele has shown that even though these patients are able to mount a protective response against the immunodominant gag epitope KK10 which is characterised by a high functional avidity and sensitivity, the narrow nature of this response does not allow them to contain viral variants with even minimal changes such as the single KK10L_{268}M mutation (Iglesias et al., 2011). Indeed, while the persisting TRBV19 clonotype in patient 30 displays a superior quality and a high degree of functionality, this is not true for the TRBV20-1 CTL clone. The latter has a lower avidity and sensitivity for the NY9 antigen than the dominant TRBV19 clonotype and it does not demonstrate the same flexibility in the recognition of NY9 variants that the dominant TCR displays. Therefore, it is plausible that this subdominant TRBV20-1 CTL clone and its progeny would have been less effective at containing the virus and/or at limiting escape in the absence of the TRBV19 clonotype. This suggests that single clonotypes can be associated with the control of viral replication in HIV-2 infection.

The results of this chapter also point out that all dominant clonotypes in HIV-2 infection are characterised by a higher antigen avidity and sensitivity than subdominant clonotypes as well as an increased polyfunctionality at limiting Ag concentrations. The terms 'avidity', 'sensitivity' and 'polyfunctionality' were used throughout this chapter to describe the functional profiles associated with each CTL clone. The term 'avidity' for instance was employed to describe the intrinsic affinity of a particular TCR for a given pMHC complex as measured by tetramer staining whereas the term 'sensitivity' was used as previously described by Purbhoo *et al*. (Purbhoo et al., 2004) to characterise the minimum number of peptide laden MHC complexes that a CTL needs to encounter for half-maximal function. Finally, the concurrent production of cytokines at a given antigen concentration was described using the term 'polyfunctionality' as previously used by Duvall *et al*. (Duvall et al., 2008). A number of methods were utilized to measure the sensitivity or 'functional avidity' of each TCR for the NY9 epitope: ELISPOT and ICS were carried out to measure sensitivity in terms of IFN-γ and TNF-α secretion, whereas the cytotoxic potential of each CTL clone at various antigen concentrations was measured by CD107α staining and ^{51}Cr release. It was noted that irrespectively of the method used, dominant TCRs displayed a better 'quality' than subdominant clonotypes especially at limiting antigen concentrations. It has been previously suggested that such a profile may confer a kinetic advantage in the early stages of infection when viral replication is still low but not when sufficient stimulus is present. (Price et al., 2005)

Whereas avidity and sensitivity could confer an advantage to dominant clonotypes in the presence of low-antigen loads, this is less likely for polyfunctionality. Earlier studies on the quality of CD8 T-cells in HIV-1 infection suggested that while polyfunctionality is a correlate of quality, it does not distinguish controllers from non-

controllers or dominant clonotypes from co-dominant (Chakrabarti and Simon, 2010; Janbazian et al., 2012). In the same line, the TRBV20-1 TCR did not dominate the NY9-specific response in patient 30 despite a presumed increased potential for proliferation associated with an increased ability to secrete IL-2 (Akinsiku et al., 2011). Differences were also observed in the magnitude of IFN-γ production between the two TRBV19 CTL clones, despite an almost identical TRBV usage, avidity and cytotoxicity. However, even though avidity and sensitivity best described the dominant clonotypes in this study the loss of the TRBV19 dominance in patient 21 suggests that additional qualities besides high avidity and sensitivity are needed for this dominance to be sustained. Indeed, several studies point out that the functional avidity alone does not always explain the relative dominance of certain clonotypes within an antigen specific response. For instance, dominant and subdominant clonotypes in latent HCMV and EBV infection show no difference in their functional avidities (Day et al., 2007; Janbazian et al., 2012). Probably, the most consistent finding associated with dominance and persistence in this study is the ability of the different TCRs to cross-recognize common variants of the NY9 epitope with high sensitivity. This ability was not found to be dependent on the presence of individual TCR motifs as previously proposed (Price et al., 2004). Instead, all TCR clonotypes studied, irrespective of dominance and/or persistence, presented the conserved motif CASSXXXGX. Therefore, it is more likely that this motif represents a structural requirement for the NY9: B*3501 interaction rather than a characteristic associated with TCR dominance as it has been previously postulated for similar motifs in HCV, HCMV and SIV infection (Day et al., 2007; Miles et al., 2011; Venturi et al., 2008). The hypothesis that cross-recognition may be the driving force behind clonotypic persistence however is not new. A previous study led by Van Bockel *et al*, in HIV-1 infection demonstrated that

dominant, persistent KK10-specific T-cell clonotypes are inherently more promiscuous for variants of the KK10 epitope (van Bockel et al., 2011). In addition, the dominance of a few cross-reactive TCRs could also explain the clonotypic narrowing of the NY9-response over time as it has been proposed in studies of LCMV-infection (Cornberg et al., 2006). Hence, it is tempting to speculate that either the appearance of a viral variant or some localized bouts of viral replication may have sustained the persistence of the dominant clonotypes over time in the HIV-2 patients under study. Likewise, similar mechanisms, namely escape or exhaustion due to low-level viral replication, may have also accounted for the loss of the TRBV19 clonotype in patient 21. The lack however of viral sequences from these patients does not allow any further or definite conclusions to be drawn. Still, it is possible that despite the undetectable viral loads in the patients under study some basal HIV-2 replication that could give rise to viral variants may have been taking place in peripheral sites as previously proposed for patients with undetectable viral loads due to ART treatment (Soares et al., 2011). This hypothesis is further supported by a recent study demonstrating, by means of ultra-deep sequencing (pyrosequencing), that epitope conservation does not necessarily translate into a lack of viral variation and that viral variation in SIV infected macaques is dynamic, frequent and diverse even for epitopes previously considered as stable (Bimber et al., 2009).

To summarize, this chapter suggests that for the virus to remain under control the persistence of TCR clonotypes within the NY9-specific CD8+ T-cell pool is necessary. This could be achieved either by the dominance and persistence of a single, cross-reactive TCR like we see in patient 30 or through the relative co-dominance of a broader pool of clonotypes as the one emerging in patient 21 after the contraction of the dominant TRBV19 CTL clone. In this way, small changes in the clonotypic dominance or antigenic variations are less likely to affect progression.

Chapter 5: Cloning and expression of highly avid, cross-reactive HLA-B*3501 restricted NY9- specific TCRs.

5.1 Introduction

The first step in the sequence of events that lead to CD8+ T-cell activation or function is the recognition of a peptide-laden MHC class I molecule on the surface of an APC. As already mentioned in the introduction, this recognition is mediated by heterodimeric αβ T-cell receptors (TCRs) on the surface of T-cells. Each TCR is a constituent unit of a larger multiprotein complex (TCR/CD3) that involves several transmembrane proteins with a potential for intracellular signal transduction.

The TCR signal begins with the activation of src kinases (Lck and Fyn) and phosphorylation of ITAMs (Immunoreceptor Tyrosine-based Activation Motifs) in the intracellular portion of the TCR CD3 and ζ molecules. This event is followed by the recruitment and activation of the ZAP-70 protein through phosphorylation, and the subsequent activation of adaptor molecules such as LAT (Linker Activator for T-Cells) and TRIM (T-Cell Receptor Interacting Molecule) by PTKs (Protein Tyrosine Kinases). These initiate then multiple downstream pathways through successive protein tyrosine phosphorylation cascades such as the ERK (Extracellular Signal Regulated Kinase), JNK (c-Jun N-terminal Kinase), NF-KappaB (Nuclear Factor-KappaB) and NFAT (Nuclear Factor of Activated T-Cells) pathways which ultimately induce the transcription of genes, the products of which are associated with CD8+ T-cell function (Cantrell, 1996).

Several factors determine the strength of pMHC: TCR interaction, which determines then in turn, even if in part, the ensuing functional result: the nature of the bound MHC epitope (agonist/antagonist) (Lyons et al., 1996), the stability of the pMHC

complex (Busch and Pamer, 1998), the thermodynamic affinity and kinetics of the monomeric pMHC:TCR engagement itself (Krogsgaard et al., 2003; Ueno et al., 2004), as well as the density of MHC and TCR molecules on the surface of the T-cell (Gonzalez et al., 2005; Kim et al., 1996). Additionally, accessory molecules such as integrins (LFA-1) and co-receptors (CD8, CD2 and CD28) also contribute to the stabilisation of this interaction (Margulies, 2001). It has been shown that for each TCR, the dwell-time of pMHC:TCR interaction must fall within a certain restricted range in order for CD8+ T-cell activation to occur and that both accelerated and prolonged half-lives in terms of dissociation (K_D,$t_{1/2}$) can have a negative effect on the ensuing signalling cascade (Kalergis et al., 2001). Within a primary response, the strength of the TCR: pMHC engagement can directly affect the magnitude of expansion and CD8+ T-cell contraction, as well as the T-cell migration kinetics from tissues to periphery (Zehn et al., 2009) and levels of intracellular Ca2+ release and influx (Chen et al., 2010). In anamnestic responses, those activated memory CD8+ T-cells that bear TCRs that recognize their cognate antigens with high avidities (~$5x10^{-4}$uM) lyse infected cells earlier in the course of infection and more rapidly than low-avidity CTLs (Derby et al., 2001a). Furthermore, the strength of TCR engagement and co-stimulation have been also shown to modulate aspects of T-cell immunity such as the sensitivity for Ag and/or IL-2 production post-transcriptionally, through a mechanism that most likely relies on the proportionate induction intracellularly of regulatory miRNA molecules (Curtale et al., 2010; Haasch et al., 2002; Li et al., 2007). Hence, in the periphery, much like during thymic development, the quality of the pMHC: TCR recognition can determine whether or not activation will occur and if so, what the functional outcome will be.

Over the past few years several studies have pointed out that higher TCR avidities correlate with higher T-cell functional capacities (Almeida et al., 2007; Derby

et al., 2001a; Vingert et al., 2010). For instance, increases in the multivalent TCR affinity have been shown to increase the pMHC:TCR multimerization and LAT/p-ERK1/2 mediated intracellular signalling (Schmid et al., 2010). However, this functional enhancement is not infinite (Schmid et al., 2010) and supraphysiologic avidities are not necessarily advantageous as they entail a risk of non-specific recognition and can also trigger CD8+ T-cell apoptosis (Alexander-Miller et al., 1996b). Moreover, even though functional avidity strongly correlates with structural avidity, this correlation is not always true (al-Ramadi et al., 1995; Derby et al., 2001b). For instance, it has been shown that functional avidity can also be modulated during the course of infection, by signals arising from multiple co-stimulatory modalities such as CTLA-4 and/or GM-CSF signals, a phenomenon that resembles in principle a process of 'avidity maturation' (Hodge et al., 2005). One question that therefore arose, following the identification of a series of NY9-specific TCRs with a naturally high functional avidity during the studies described in chapters 3 and 4, was, to what extent the high functional avidity observed in HIV-2 infection can be attributed to the biophysical properties of the pMHC: TCR interaction itself or to a combination of structural avidity and 'avidity maturation'.

Furthermore, the work of the previous two chapters also demonstrated that HIV-2 CTL clones possess an increased potential for homologous cognate antigen (NY9) recognition and heterologous antigen (PY9) cross-recognition. It has been previously postulated that such a characteristic is a necessary attribute of the TCR (Mason, 1998), yet the molecular principles of cross-reactivity remain unclear despite it being a desirable attribute of a protective T-cell response for broader coverage, as only a handful of structures addressing this mechanism have been solved so far (figure 5.1).

Hence, the purpose of the work for this chapter was to clone, express and refold these highly avid, cross-reactive and non-cross-reactive TCRs for comparative structural studies, in an attempt to understand better the principles that lie behind CD8 TCR selection and mobilization in the initial phases as well as chronic phases of antigen exposure.

PMID	Title	Reference
12244309	A Functional and Structural Basis for TCR Cross-Reactivity in Multiple Sclerosis	(Lang et al., 2002)
15583017	T Cell Cross-Reactivity and Conformational Changes During TCR Engagement.	(Lee et al., 2004)
15837811	Structural and Kinetic Basis for Heightened Immunogenicity of T Cell Vaccines	(Chen et al., 2005)
16951352	Strong TCR Conservation and Altered T Cell Cross-Reactivity Characterize a B*57-Restricted Immune Response in HIV-1 Infection.	(Gillespie et al., 2006)
18941216	Distinct CDR3 Conformations in TCRs Determine the Level of Cross-Reactivity for Diverse Antigens, but Not the Docking Orientation	(Jones et al., 2008)
20064447	T cell receptor cross-reactivity directed by antigen-dependent tuning of peptide-MHC molecular flexibility.	(Borbulevych et al., 2009)
20711359	Protective efficacy of cross-reactive CD8+ T cells recognising mutant viral epitopes depends on peptide-MHC-I structural interactions and T cell activation threshold.	(Valkenburg et al., 2010)
20616031	Cross-reactive CD8+ T-cell immunity between the pandemic H1N1-2009 and H1N1-1918 influenza A viruses.	(Gras et al., 2010)
21086445	Loss of recognition by cross-reactive T cells and its relation to a C-terminus-induced conformational reorientation of an HLA-B*2705-bound peptide	(Loll et al., 2011)
21937447	Loss of T Cell Antigen Recognition Arising from Changes in Peptide and Major Histocompatibility Complex Protein Flexibility: Implications for vaccine design	(Insaidoo et al., 2011)
22019736	Disparate degrees of hypervariable loop flexibility control T-cell receptor cross-reactivity, specificity, and binding mechanism.	(Scott et al., 2011)

Figure 5.1 Summary of studies addressing CTL cross-reactivity at the structural level.

5.2 Results

5.2.1 Amplification and cloning of TCRs into expression vectors

PID	CID	fAV	PY9 Cx	TRAV	CDR3	TRAJ	TRBV	CDR3	TRBJ
1	1.3	0.000375	N/D	8.6	C A V S D A	37-1	29.1	C S V G Y G	1-1*01
17	17.5	0.0001	N/D	8.6	C A V S	18-1	29.1	C S V A W G	1-1*01
	17.15	0.000375	0.8	12.3	C A M D	44-1	7.2	C A S S L S P G R	2-1*01
21	21.8*	0.0000625	N/D	13.2	C A V C G D	9-1	19	C A S S T Q V G	2-3*01
30	30.50	0.0002	1	39	C A V D F	52-1	20.1	C S A S N Q G N G	2-2*01
	30.10	0.000094	0.07	13.1	C A A N R H	50-1	19	C A S S P Q H G	2-3*01
J038	C2	1.616	Index	21	C A V P G G I	50-1	30	C A W R G T A L L Q	1-2*01

Figure 5.2 Functional characteristics of TCRs selected for expression. fAV: Functional avidity (EC_{50}) as measured by 51Cr release assay. Green: high functional avidity, Blue: medium functional avidity, Red: low functional avidity. PY9 Cx: Level of heterologous PY9 cross-reactivity (PPIPVG**E**IY). Cross-reactivity values represent EC_{50} in ^{51}Cr release assays using titrated amounts of the epitope. N/D: Not determined. * Archived TRBV19 TCR from 2007 (Chapter 4).

Six HIV-2 NY9-specific TCRs with different abilities for cognate (NY9) and heterologous (PY9) peptide recognition as well as one HIV-1 PY9-specific TCR (J038 C2) (Figure 5.2) were selected for expression. mRNA was isolated from ~10^6 CTL clone cells and reverse transcribed into cDNA before being subjected to a three-step PCR for the production of TCR inserts. The TCR primers were engineered according to the method described by Boulter *et al.* incorporating cysteine codons (C*) at positions threonine 48 (TRAC) and serine 57 (TRBC) respectively to allow the formation of a non-native αβ interchain disulphide bridge during refolding (Boulter et al., 2003). They were also designed to carry codons optimised for expression in *E.coli*, as well as the NdeI and XhoI restriction sites at the 5' and 3' end respectively for directional cloning into the pET22b+ vector.

Each chain was amplified using three sets of primers: for the TCR alpha-chain these were the sets αTCR/1AR, 1AF/Aend and αTCR/Aend, whereas for TCR β chains these were amplified using the sets βTCR/1BR, 1BF/Bend and βTCR/Bend (Figure 5.3). The sequences of the designed oligonucleotides were as follows:

TCR_ID	Oligo_ID	Sequence (5'->3')
1.3†	L_001**a**8.6	GAGACATATGCAGTCTGTTACCCAGCTTGACAGCCAAGTCCCTG
17.5†	L_001**b**29	GAGACATATGGCGGTTATCTCTCAAAAGCCAAGCAGGGATAT
17.15	L_005**a**12-3	GATACATATGCAGCAGAAAGAAGTGGAGCAGGATCCTGGACCACTCAGTGTTCC
	L_005**b**7-2	GAGACATATGGGTGTTTCTCAGTCTCCCAGTAACAAGGTCACAGAG
21.8†	L_004**a**13.1/2	GATACATATGGAAAACGTTGAACAGCATCCTTCTACCCTGAGTGTCC
30.10†	L_004**b**19	GATACATATGGGCATCACCCAGTCCCCAAAGTACCTGTTCAGAAAGGAAGG
30.50	L_002**a**39	GAGACATATGGAACTGAAAGTTGAACAAAACCCTCTGTTCCTGAGC
	L_002**b**20-1	GATACATATGGGTTCTGGTCTTGGTGCTGTCGTCTCTCAACATCCG
C2	L_003**a**21	GATACATATGAAACAAGAGGTTACGCAGATTCCTGCAGCTCTGAGTGTCCCAG
	L_003**b**30	GATACATATGCAGACCATCCACCAATGGCCAGCGACCCTGGT

†Same TRAV/TRBV family used NdeI

The primers 1AR, 1BR, Aend and Bend were primers used previously in the lab (Dr. G. Stewart-Jones). All amplifications were carried out in 50µl reactions containing 5µl 10x buffer, 10µM dNTPs, 3.3U High Fidelity Polymerase (Roche), 2µl cDNA, 39µl H_20 and 10pmol of each of the primers whereas the conditions for the amplifications consisted of a 2min initial denaturation step at 94°C followed by 35 cycles of denaturation at 94°C for 30sec, annealing at 60°C (α-chains) or 65°C (β-chains) for 30sec, and 1min elongation at 72°C. A final round of elongation at 72°C for 8min was also performed.

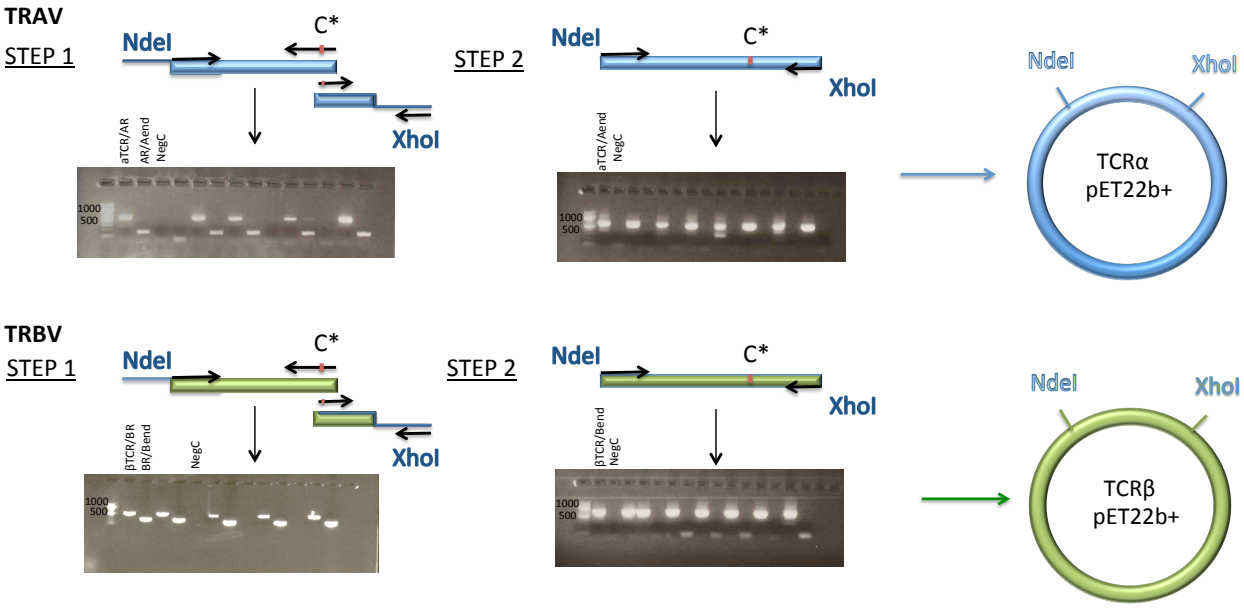
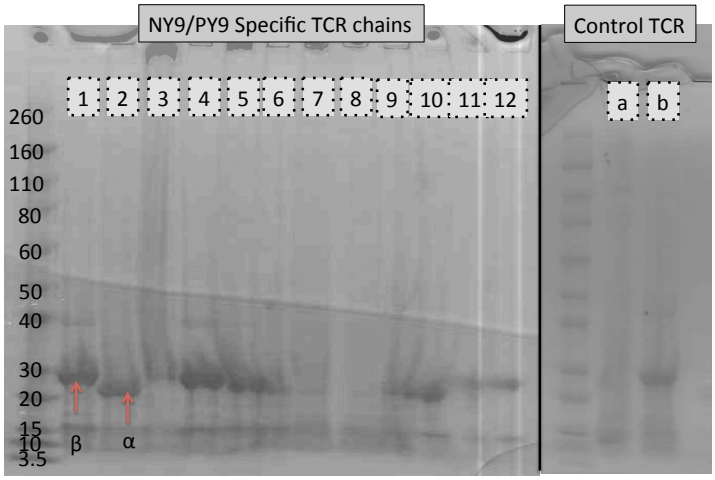


Figure 5.3 Overlapping PCR strategy used for the generation of expression constructs. Primers αTCR/1AR, 1AF/Aend and βTCR/1BR, 1BF/Bend were used in step 1 whereas αTCR/Aend and βTCR/Bend in step 2. NegC: Negative control.

Next, the TCR inserts were cloned into pET22b+ expression vectors (Novagen). The cloning was successful for 5/7 TCRs (17.15, 21.8, 30.10, 30.50 and C2) and all constructs were verified by sequencing which was performed at the WIMM sequencing facility by John Frankland. Then, the constructs were used for BLR (DE3) Competent cell (Novagen) transformation for protein expression as inclusion bodies (IBs). Expression was induced with 0.5M IPTG at OD_{600}=0.9 and after a 6hr incubation at 21^{o}C the protein was purified from IB pellets by washing three times in Triton X-100 (Sigma-Aldrich) and resolubilised in 8M Guanidine-HCl, 15mM DTT and 100mM Tris pH 8.0 (Figure 5.4). The chains of HLA-B*3501 molecules and β2 microglobulin were expressed as described in the Materials and Methods *(2.4 Generation of peptide MHC class I tetramer complexes)*. A TCR previously expressed in the lab (Dr. G. Stewart-Jones) (Figure 5.4) was also purified from inclusion bodies and used as a control during the studies. This was the KF11 (KAFSPEVIPMF) specific, B*5701-restricted AGA-1 TCR (TRAV5/TRBV19).



The lowest yields were observed for the TCR 30.50.

Figure 5.4 Coomassie stained SDS-PAGE gel of IB purified TCR proteins. Red arrows denote the bands for the a-chains (~23kDa) and β-chains (~28kDa).
Lane 1: TRBV19 (TCR 21.8)
Lane 2: TRAV21 (TCR C2)
Lane 3: TRBV30 (TCR C2)
Lane 4: TRBV19 (TCR 30.10)
Lane 5: TRAV12.3 (TCR 17.15)
Lane 6: TRAV10 (TCR 30.10)
Lane 7: TRAV39 (TCR 30.50)
Lane 8: TRBV20-1 (TCR 30.50)
Lane 9: TRAV10 (TCR 21.8)
Lane 10: TRAV13.2 (TCR 21.8)
Lane 11: TRAV13.1 (TCR 30.10)
Lane 12: TRBV7.2 (TCR 17.15)

5.2.2 TCR & MHC class I refolding and anion-exchange chromatography

For TCR refolding, paired soluble TCR chain proteins (30mg/each) were injected into 1litre of refolding buffer (5M Urea, 200mM L-arginine, 50mM Tris pH8.0, 1mM EDTA, 1.5mM reduced glutathione and 0.15mM oxidized glutathione) and left stirring

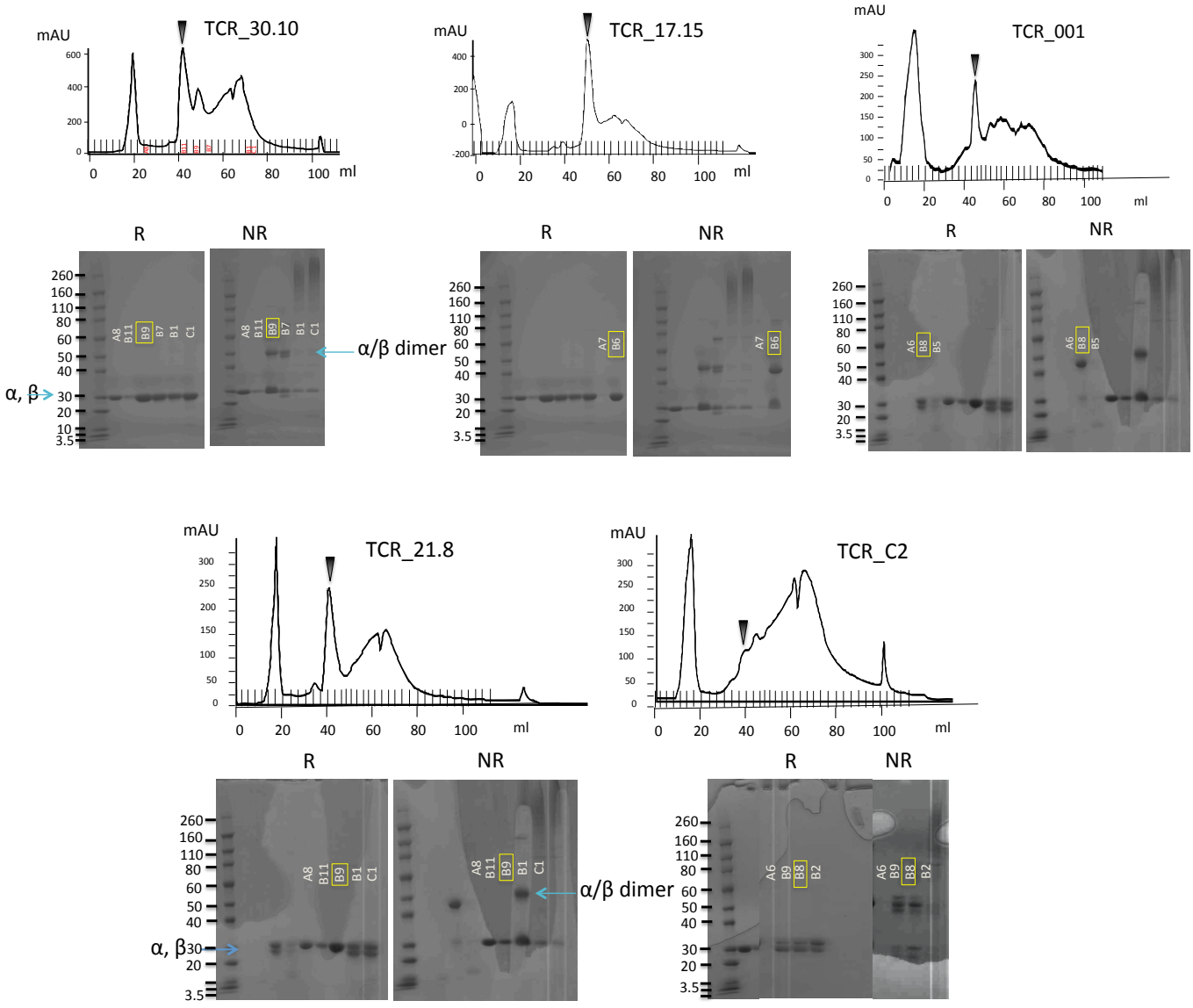


Figure 5.5 Purification of the different TCRs under study on anion-exchange and coomassie stained SDS-PAGE. TCR001 is the control TCR. The x-axis on the graphs represents elution volumes (ml). R: reducing, NR: non-reducing conditions. Black arrows and yellow boxes indicate the fractions selected for further purification.

at 4°C for 48hrs.The refolded mixtures were then dialysed for 2 weeks against 16lt of 6.25mM Tris pH 8.0 at 4°C. The HLA-B*3501 molecules were also refolded with peptides NY9 and PY9 as described in the materials and methods. At the end of the dialysis period, the refolded protein was harvested, filtered through a 0.22μM filter (Stericup, PVDF, Millipore), loaded for purification onto 5ml HiTrap HQP anion exchange columns (GE Healthcare) and eluted with 18% 1M NaCl, 10mM Tris pH 8.0. TCR-containing fractions were then pooled together and analysed by coomassie stained SDS-PAGE under reducing and non-reducing conditions to confirm the formation of disulphide bonds and TCR dimers (Figure 5.5). Refolding was achieved for 4/5 TCRs, namely for the HIV-2 NY9-specific TCRs 30.10, 17.15, 21.8 and the control TCR AGA-1 and SDS-PAGE analysis revealed both TCR dimers as well as unbound α and β-chain monomers in the refolds. However, due to time and sample volume limitations further αβ chain linkage confirmation by mass-spectrometry was not performed.

A similar approach was adopted for the purification of p:B*3501 complexes (Figure 5.6). The refolded proteins were purified by anion-exchange chromatography using 1ml HiTrap HQP columns and eluted with a constant NaCl gradient and 10mM Tris pH 8.0.

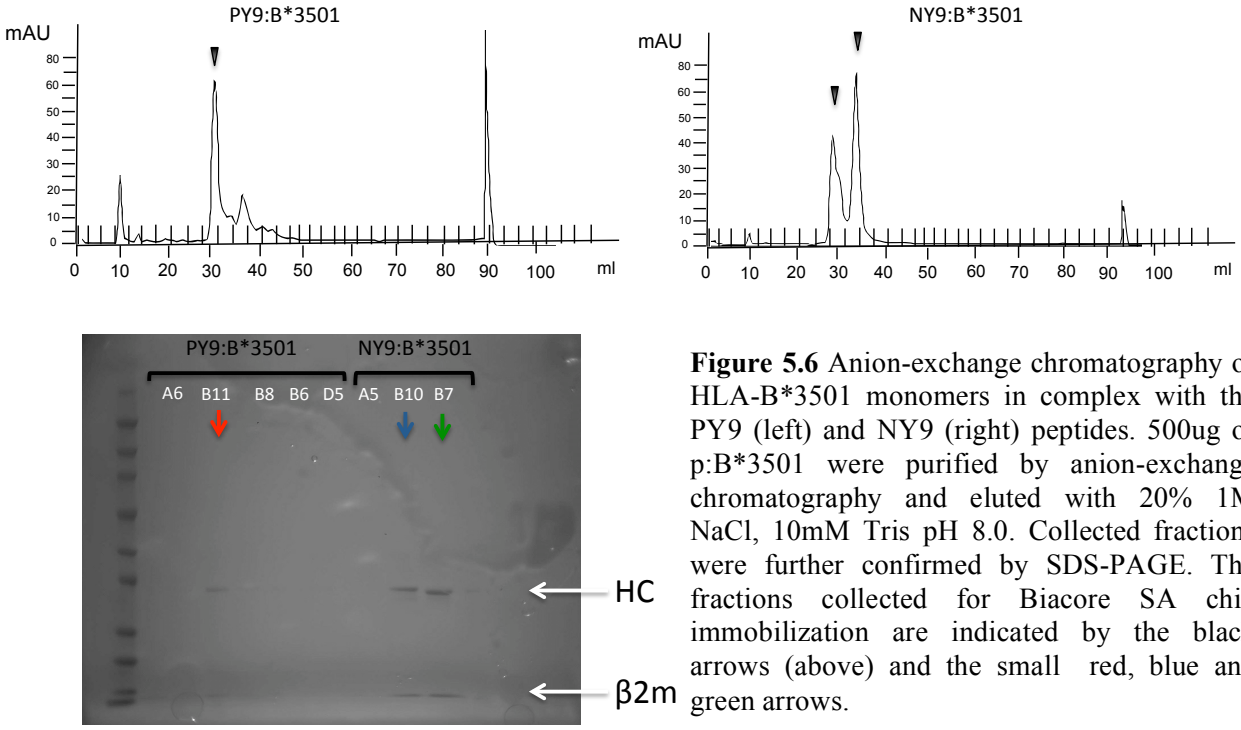


Figure 5.6 Anion-exchange chromatography of HLA-B*3501 monomers in complex with the PY9 (left) and NY9 (right) peptides. 500ug of p:B*3501 were purified by anion-exchange chromatography and eluted with 20% 1M NaCl, 10mM Tris pH 8.0. Collected fractions were further confirmed by SDS-PAGE. The fractions collected for Biacore SA chip immobilization are indicated by the black arrows (above) and the small red, blue and green arrows.

5.2.3 Ni+ Affinity and Size Exclusion Chromatography

The collected fractions from the anion-exchange chromatography that contained the TCR were then subjected to two additional rounds of purification including Ni+ affinity and Superdex 200 (10/300 GL) size-exclusion chromatography (GE Healthcare) in order to obtain a homogenous sample. After the final elution (10mM HEPES pH 7.0, 100mM NaCl) the fractions containing the main peak were pooled together and analysed on a Coomassie stained SDS-PAGE gel under reducing and non-reducing conditions (Figure 5.7) to confirm the presence of the α/β heterodimer and then were concentrated for surface plasmon reasonance (BIAcore) analysis.

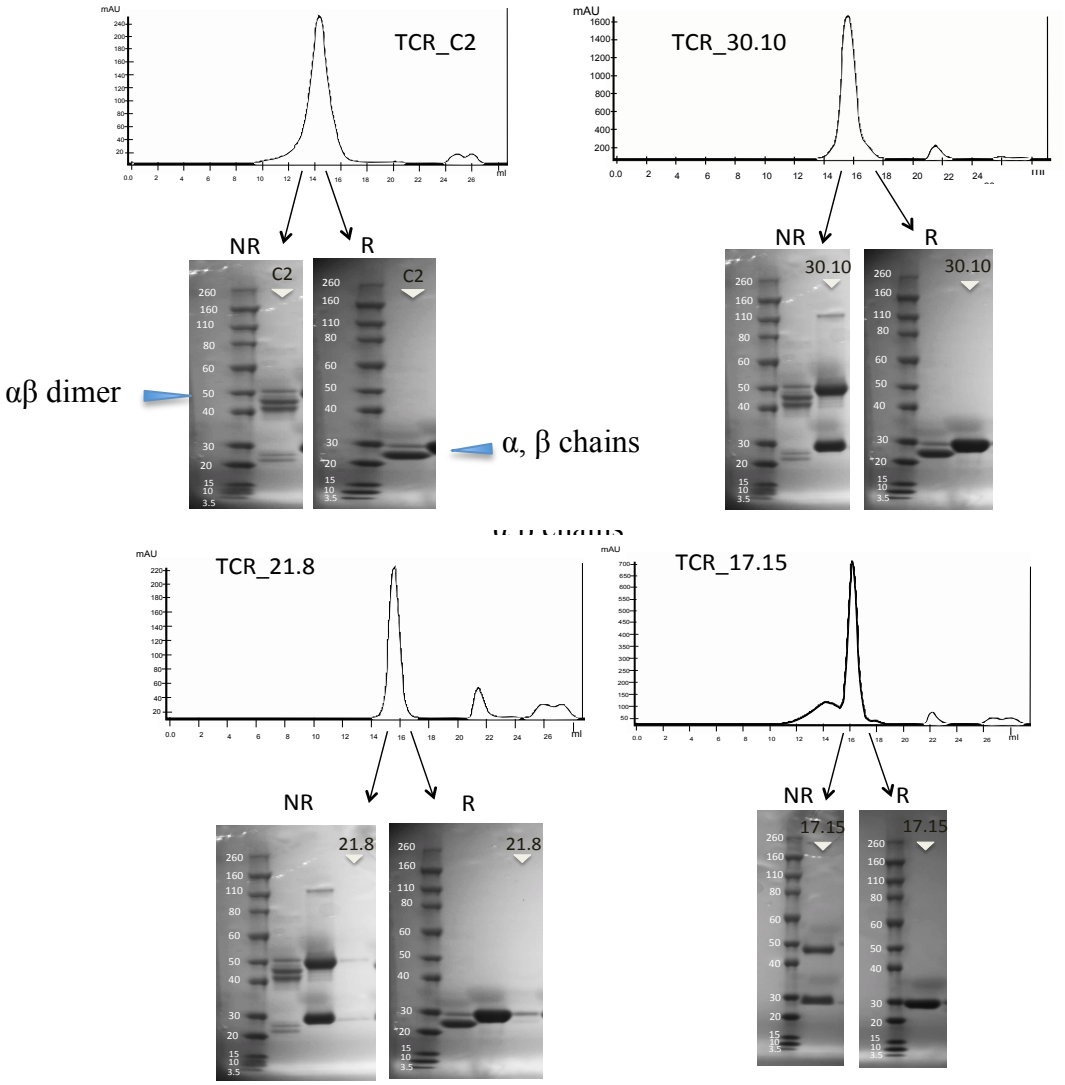


Figure 5.7 SD200 gel filtration of refolded TCRs and analysis of selected fractions on SDS-PAGE (R:reduced/NR:non-reduced) White arrows indicate the bands corresponding to the refolded TCRs.

5.2.4 Surface Plasmon Resonance (SPR) analysis

The binding of the expressed TCRs to their ligands was assessed using SPR analysis. For this purpose, 1000RU of each biotinylated p:HLA-B*3501 complex were immobilised onto adjacent flow cells (IFC1-3) on a Streptavidin (SA)-coated sensor chip (GE Healthcare) whereas a fourth flow cell (IFC4) was left uncoupled and used as a negative (no binding) control. The KF11: HLA-B*5701 monomer was also immobilised on a second SA chip for the kinetic analysis of the binding of the AGA-1 TCR. This TCR whose binding expression, refolding and binding kinetics had been previously confirmed (Dr. G.Gillespie and G.Stewart-Jones) was included in the analysis as a positive (binding) control, to rule out methodological errors that may have occurred during the steps of expression, refolding and/or purification of the TCRs as well as an internal control for the Biacore analysis itself.

Following to the p:MHC immobilisation, each TCR was injected over the corresponding chip (TCRs C2, 30.10 and 21.8 over the p:B*3501 SA chip and the AGA-1 TCR over the KF11:B*5701) and kinetic data were collected for further analysis. The SPR studies were carried out on a BIAcore 3000 machine (GE Healthcare) and the analysis was performed using the BIAcore software. The results of the injection of each TCR over the different immobilised p:MHCs are shown in figure 5.8. As it can be seen in the figures 5.8b-e the response for each TCR, which was calculated by subtracting the response of the negative control flow cell (pink line) from that recorded for the p:MHC containing flow cells (blue, green and red lines), turned out to be negative. This indicated that the four NY9=specific TCRs did not bind to their target p:MHCs antigens contrary to the positive control AGA-1 TCR which gave a binding response, as expected (figure 5.8, f).

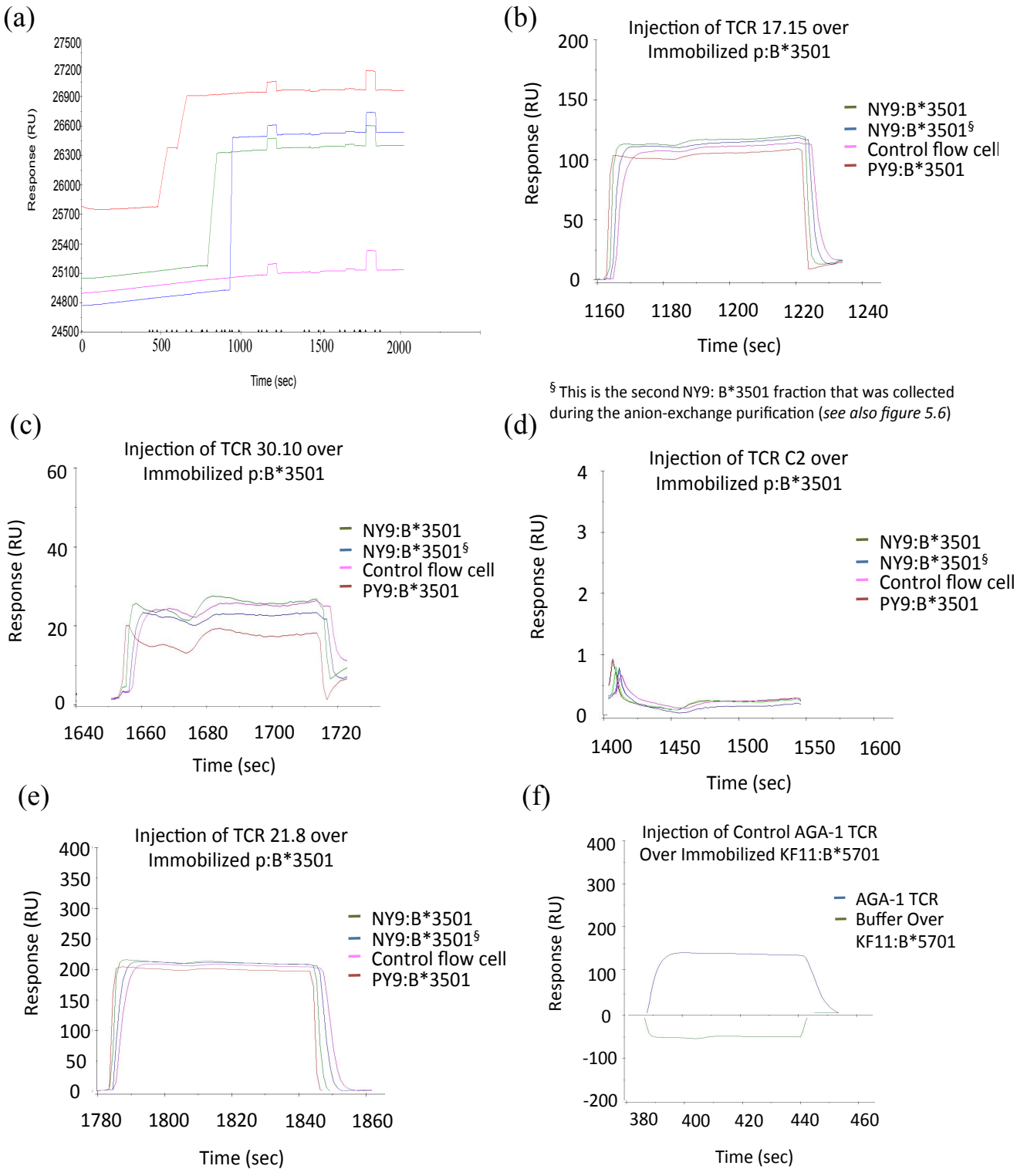


Figure 5.8 Sensorgrams depicting the injection of the NY9/PY9-specific TCRs over the different immobilised p:MHCs (red, blue, green lines) and their observed responses (a) Summary of all injections. (b) Injection of 17.15 TCR over the three immobilised p:MHCs and the negative control flow cell (in pink) (c) Injection of C2 TCR over the three immobilised p:MHCs and the negative control flow cell (in pink) (d) Injection of 30.10 TCR over the three immobilised p:MHCs and the negative control flow cell (in pink) (e) Injection of 21.8 TCR over the three immobilised p:MHCs and the negative control flow cell (in pink) and (f) Injection of mock AGA-1 TCR over immobilised KF11: HLA-B*5701. As it can be seen from the sensorgrams (b-e) the background response seen with each TCR flowing over an empty flow cell (pink line) is no different from the responses recorded when each respective TCR was injected over the different p:MHCs.. The only exception was the AGA-1 TCR for which the response recorded was different from the response seen for the no-binding, negative control (green line: 5.8f).

5.3 Discussion

The purpose of this chapter was to clone and express a number of highly avid, cross-reactive NY9/PY9-specific TCRs for biophysical studies and crystallization. Seven TCRs were selected for the study, four of which were successfully cloned, expressed and refolded for SPR analysis.

SPR is a method that has been extensively used for the study of pMHC: TCR interactions along with isothermal titration calorimetry (ITC) and fluorescence anisotropy (Piepenbrink et al., 2009). It measures how the refractive index of a surface (sensor) that contains an immobilised protein changes, as a second protein-ligand is injected over this surface. The recorded changes are then analysed to determine whether or not the two proteins, immobilised and injected, have interacted with each other (Margulies et al., 1996). Compared to alternative methods, SPR is advantageous in that it requires relatively low amounts of each protein and does not require labelling of the molecules under study, minimizing thus the danger of structural interference. In addition, it allows the determination of a number of biophysical and thermodynamic parameters of the TCR: pMHC interaction such as the binding kinetics (Kon/Koff), affinity (K_D), entropic (ΔS) and enthalpic (ΔH) profiles as well as heat capacity changes (ΔCp) (Krogsgaard et al., 2003; Margulies et al., 1996; Piepenbrink et al., 2009; van der Merwe and Barclay, 1996). The engagement of the TCR is normally characterized by a low affinity (~0.1-100uM), a slow on-rate and a fast-off rate (Boniface and Davis, 1995) and crystallization studies have revealed that most TCRs position over pMHC complexes in a semi-diagonal mode, with the Vα and Vβ domains aligning over the N- and C-terminal halves of the peptide respectively (Garcia and Adams, 2005). This interaction relies on an a number of favourable enthalpic changes that stabilize the TCR: pMHC complex by overcoming an entropic penalty (Boniface et al., 1999). It has

thus been suggested that the particulars of these biophysical and structural profiles, such as the docking topology (Adams et al., 2011; Stewart-Jones et al., 2003), the TCR chain establishing the specificity (Ishizuka et al., 2008), the CDR3 loop length, flexibility and its amino-acid landscape (Borg et al., 2005; Ding et al., 1998; Dong et al., 2004) may account, at least in some systems, for the immunodominant selection, functional profile or cross-reactive potential of certain TCRs. Each TCR possesses a binding 'hotspot' where most of the residues that play a significant role in the stabilisation and specificity of the recognition are located. For the immunodominant HLA-A2 restricted Flu-specific JM22 TCR the majority of stabilising interactions in the 'binding hotspot' are contributed almost entirely by the Vβ CDR1 and CDR2 loops and some CDR3 amino-acids (Ishizuka et al., 2008). Other studies however, such as those exploring the HLA-B8 restricted EBV (FLR)-specific LC13 TCR place those 'hotspots' within the CDR3 loops (Borg et al., 2005).

The structure of the bound epitope can also have a profound impact on the selected TCR repertoire with bulged epitopes, such as the HLA-B*5703 restricted epitope KF11 (KAFSPEVIPMF), being associated with antigenic immunodominance and HIV-1 non-progression (Stewart-Jones et al., 2005) and more flat 'featureless' epitopes favouring the mobilization of a narrower, highly hierarchical antigen-specific TCR repertoire during the course of infection (Stewart-Jones et al., 2003; Turner et al., 2005).

However, when the four refolded NY9/PY9-specific TCRs under study were assayed for their ability to recognise their respective ligands, no binding was detected. A number of factors could account for this lack of interaction: it could be that the assumed conformations of the refolded TCRs were not physiological; that the refolded pMHC complexes did not contain the desired peptides or optimal epitopes or that the

constructs had incorporated mutations in the early steps of plasmid amplification that led to alterations in the previously characterised TCRs CDR3 amino-acid profiles. Indeed, the pMHC: TCR interaction is extremely sensitive to single amino-acid changes. Variations within the CDR3 regions of the TCR have been shown previously to alter or abrogate binding at the molecular level and hence to affect the response to a given peptide either by reducing or by eliminating the production of certain cytokines and/or the potential for recognition (Goyarts et al., 1998; Ishizuka et al., 2008; Kalergis et al., 2001; Robbins et al., 2008). An analogous mechanism that relies on the selection of mutated epitopes that abrogate CTL recognition either by destabilising the p:MHC interaction or by altering the residues at TCR contact sites is also one of the many strategies implemented by HIV for immune escape (Cale et al., 2011; Jones et al., 2004). However, as the sequence of all constructs was verified following plasmid amplification, and mass-spectrometry analysis of MHC class I bound epitopes revealed no diversifications (appendix III), the lack of binding most likely resulted from the fact that the refolded TCRs might have been misidentified, mispaired αα or ββ homodimers. The formation of such homodimers in solution, and especially of ββ homodimers during refolding, has been previously described (Li et al., 1997; Zhou et al., 2011) and is proposed to account for up to 80% of the refolded proteins (van Boxel et al., 2009). Nevertheless, a definite conclusion regarding the presence of αβ homodimers in the refolded protein solutions was not possible in this study, as the molecular weights of the different dimers are very similar (αβ TCR ~53kDa vs ~57 for a ββ TCR) and SDS-PAGE does not provide a means for accurate, definite distinction. Future studies of these receptors however, will need to incorporate additional steps of quality control involving mass spectrometry as proposed by Van Boxel *et al*. to confirm the presence

of the desired αβ heterodimers as well as the integrity of the α and β chains (van Boxel et al., 2009).

Even though binding was not detected between the expressed TCRs and their cognate p: B*3501 complexes, these receptors and the corresponding NY9/PY9 complexes, still warrant further study at a structural level due to their contribution in unique profiles of avidity and cross-recognition. Especially with regard to the latter, it should be noted that most studies up to date have been focusing on either cross-reactive recognition of closely related ligands by CTL lines (Valkenburg et al., 2010) or the recognition of different MHC alleles (Burrows et al., 1997) but studies of multiple cross-reactive TCRs targeting the same pMHC allele are limited. Nevertheless, the few studies available to date support the idea that each TCR possesses a unique profile of cross-reactivity. For instance, the study of two TCRs specific for the Tax/HLA-A2 complex in HTLV-1 infection revealed a differential functional response among the two receptors to a peptide substitution designed to remove a prominent interfacial hydrogen bond. The difference stemmed from electrostatic differences in the two recognitions and could not be predicted by the profile of response towards the index epitope as both receptors has similar binding affinities and kinetics for the latter (Davis-Harrison et al., 2005).

Hence biophysical and crystallographic studies, as well as those involving TCR gene transfers in *in vitro* systems (i.e. TCR transfer in Jurkat cells), are advantageous in that they allow an evaluation of different TCR mediated interactions and, depending on the method, downstream signalling pathways without the high level of noise and complexity that normally characterises physiological *in vitro* cellular assays (Iancu et al., 2011; Irving et al., 2012; Thomas et al., 2010). The findings can then be applied to predict or increase the immunogenicity of epitopes (Cole et al., 2010; Dash et al., 2007)

and the avidities of TCRs through peptide anchor residue optimization or TCR engineering by either site-directed mutagenesis (Varela-Rohena et al., 2008), phage display (Li et al., 2005) or CDR3 swapping (Bennett et al., 2010) as well as for the prediction of TCR mobilisation (Roomp and Domingues, 2011) which is necessary for rational vaccine design (Apostolopoulos et al., 2000; Dash et al., 2007). It will thus be interesting to see in the future whether or not the selected NY9/PY9-specific TCRs possess any unusual characteristics at an atomic level and how these characteristics modulate the observed findings of high avidity and cross-reactivity in HIV infection.

Chapter 6: Discussion and future directions

Over the past 30 years HIV-1 infection has evolved from a disease with an unfavourable prognosis into a manageable chronic disease. A lot of advances have contributed towards this end: the development of new drug classes and drugs, such as the CCR5 antagonist Maraviroc and the integrase inhibitor Raltegravir that were introduced into the market in 2007 (Arts and Hazuda, 2012; Hughes et al., 2009); the emergence of testing methods that permit the identification of HIV-seropositive individuals earlier post-acquisition by combining antibody testing with antigen (p24) detection (Bischof et al., 2011); the expansion of the access to ART in resource-limited settings (WHO/UNAIDS/UNICEF, 2011); the development of more sensitive methods for the detection of resistance (Gega and Kozal, 2011); as well as the emergence of a new culture of adherence to antiretroviral regimens (Mann et al., 2012). However, despite these advances and despite a number of well-orchestrated research efforts spanning more than 3 decades, an HIV vaccine is still missing.

In 2007, the much-promising rAd5 Merck STEP vaccine trial triggered fervent debates over the need for more basic research after it failed to confer to the vaccinated volunteers the same level of protection previously demonstrated in animal models (Cohen, 2007). It also prompted discussions over the suitability of certain methods such as the IFN-γ ELISPOT as a major determinant of immunogenicity in trials and on whether or not a single correlate such as the breadth and/or magnitude of a response can predict a post-vaccination outcome with sufficient power (Fuchs et al., 2010; McElrath et al., 2008). What was then realised was that for an accurate characterisation of vaccine-induced HIV-specific CD8+ T-cell immune responses, a more holistic approach was necessary that would incorporate the characterisation of more than one

parameters, such as the potential for viral suppression (Saez-Cirion et al., 2010) or the production of multiple cytokines (Horton et al., 2007) alongside breadth and magnitude of the overall T-cell response.

The Merck trial also exemplified the complexity of the system that we are currently dealing with: not only is HIV a virus with great plasticity that triggers a multi-layered response involving multiple interconnected host pathways (Brass et al., 2008) but also our access to the true sites of infection such as the mucosa and lymph nodes remains extremely limited. Despite this, the advances on the immunological front have been encouraging and researchers are now, assisted by methodological advances of the past decade, such as 18-colour flow cytometry, beginning to re-evaluate the current knowledge on epitope specific responses by placing more emphasis on the fine architecture of each response (Seder et al., 2008). Systems biology is also emerging as a new way of looking into vaccine-induced immunity and is expected to play a bigger role in the evaluation of such responses in the years to come (Andersen-Nissen et al., 2012; Rappuoli and Aderem, 2011).

HIV-2 is a virus that affects approximately 1-2 million people around the world and patients progressing to disease share a clinical picture that is indistinguishable from that caused by HIV-1 (Gottlieb et al., 2008). However, contrary to the progress made in HIV-1 infection regarding the treatment of seropositive patients, the management of HIV-2 progressors as well as dual HIV-1/2 patients still remains a challenge as the virus is naturally resistant to drug classes such as the non-nucleoside reverse transcriptase inhibitors (NNRTIs), fusion inhibitors such as enfurvirtide (T-20) and some protease inhibitors (i.e. nelfinavir). There are also lower thresholds for the acquisition of resistance to nucleoside reverse transcriptase inhibitors (NRTIs) with as few as two mutations conferring full resistance to essential antiretroviral drugs such as

zidovudine and lamivudine (Gilleece et al., 2010; Gottlieb et al., 2008). In addition, HIV-2 patients undergoing triple therapy (triple nucleoside analogues or protease inhibitors) have been shown to have a poor CD4 recovery one year after the initiation of treatment, with a median increase of 41 cells/μl despite strong virological responses (median viral load decrease1.1$\log_{10}$ copies/ml) (Matheron et al., 2006). Hence, HIV-2 infected individuals have fewer options than HIV-1 patients when it comes to therapy which explains why developed countries affected by the virus like France and Portugal have started placing HIV-2 more prominently in their research agendas over the past years.

Much as in HIV-1 infection, HIV-2 patients with a better prognosis tend to target more frequently conserved epitopes such as epitopes of the protein gag (Leligdowicz et al., 2007). The aim of this thesis was thus to examine in depth such a response, and more specifically the HLA-B*3501-restricted NY9 response in an attempt to identify factors that could potentially be associated with viral control and which could potentially guide vaccine design in the future. For this purpose, PBMCs were collected from a number of HIV-2 infected ART-naive patients from the village of Caio, in Guinea-Bissau and used for *ex vivo* analyses and for the establishment of CTL clones.

The initial characterisation of the response revealed that NY9-specific CD8+ T-cells possessed a number of superior attributes: they were able to produce an array of cytokines, more notably IFN-γ and MIP-1β, had a greater avidity and sensitivity for antigen and an ability to cross-recognise the heterologous HIV-1 antigen PY9 as well as some of its variants. Most strikingly however, the increased polyfunctionality exhibited by the NY9-specific CTLs was not hampered by the expression of inhibitory markers on their surface. These findings suggest that protection might not necessarily be elusive

even in the face of alleles that have historically been associated with progression (Dinges et al., 2010). They also indicate that in HIV-2 infection CD8+ T-cells contribute actively to viral control. A similar profile of avidity and polyfunctionality however, has not been yet described for the corresponding HIV-1 epitope PY9. It will be thus interesting to address in the future the reasons behind this disparity in terms of epitope processing and presentation as such studies could guide the selection of epitopes in the future. The latter is one of the main considerations of HIV vaccination and so far various strategies have been used in an attempt to achieve immunogenicity, broad CD8+ T-cell coverage and persistence. These strategies include poly-epitope constructs with optimal CTL epitopes or with whole natural viral proteins or conserved HIV regions assembled together, constructs with polyvalent mosaic protein strings that have been optimised *in silico* for T-cell recognition, consensus or ancestral protein sequences of circulating variants or overlapping peptide sets scrambled together (Bazhan et al., 2010; Burgers et al., 2006; Fischer et al., 2007; Kesturu et al., 2006; Korber et al., 2009; Letourneau et al., 2007; Nickle et al., 2003; Thomson et al., 2005). The data presented herein demonstrate that the presentation of the NY9 epitope within patients expressing the HLA-B*3501 allele leads to the recruitment of several highly avid, polyfunctional cross-reactive CD8+ T-cells. One question therefore that vaccine strategies will need to address in the future is whether or not HIV-2 epitopes could be used in constructs as alternatives to corresponding HIV-1 epitopes for which a potent response cannot be achieved.

The results of chapter 4 on the other hand offer a more mechanistic basis for the better outcomes associated with the NY9-specific responses. In line with the findings of Buseyne *et al.* from HIV-1 infection (Buseyne and Riviere, 2001), it was observed that within HIV-2 LTNPs two mechanisms exist that potentially limit viral replication; one

is the dominance of a single cross-reactive TCR and the other the maintenance of several clonotypes specific for a single pMHC complex. As outlined in the chapter's discussion however, each option carries its own benefits and risks and future research will need to address which of the two types of responses might ultimately prove more beneficial to the host.

A further finding of chapter 4 was that dominant clonotypes exhibited a greater sensitivity and functional avidity for antigen as well as a superior profile of cross-reactivity towards one of the most common NY9 variants. How these superior clonotypes survive long-term (>13 years) however is not clear at present, especially in the setting of very low viral load. It has been proposed that antigen dose might play a role in the survival of CD8+ T-cell clones since higher antigen densities have been shown to carry an increased risk for apoptosis especially for the more avid T-cells (Alexander-Miller et al., 1996b). Therefore, in order to untangle how antigen density modulates CTL responses during the acute and chronic phases of disease further research is deemed necessary. Such studies are bound to benefit from methodological advancements such as the development of microarray platforms, which have already been successfully used to analyse gene-expression patterns in CD8+ T-cells (Li et al., 2009).

Apart from the antigen dose however, the amount of antigenic variation maintained within aviraemic HIV-2 infected LTNPs is also of paramount importance as it could hold the key to a better understanding of the principles governing clonotypic selection. The use of new technologies such as next-generation sequencing and pyrosequencing has already suggested that aviremic patients might be harbouring a much greater antigenic variation than previously thought (Bimber et al., 2009). It will

thus be interesting to see whether or not escape variants emerge within aviraemic HIV-2 patients and how these affect CTL recognition and progression.

Finally, this thesis also set the basis for future molecular studies of the identified NY9-specific TCRs by generating a series of expression constructs. It is anticipated that these constructs will enable a further characterisation of the principles governing avidity and cross-reactivity of HIV-2 specific CD8+ T-cells in the future.

To conclude, much like in HIV-1 infection, long-term non-progression in HIV-2 infection arises from a number of different intertwining factors. It is exactly those patterns of interaction that we need to understand better in order to produce an effective HIV vaccine, a goal that appears, after the RV144 vaccine trial in Thailand reported an encouraging efficacy of 31.2% in the modified intention-to-treat analysis (Rerks-Ngarm et al., 2009), no longer an impossibility.

References

Abimiku, A.G., Franchini, G., Tartaglia, J., Aldrich, K., Myagkikh, M., Markham, P.D., Chong, P., Klein, M., Kieny, M.P., Paoletti, E., and et al. (1995). HIV-1 recombinant poxvirus vaccine induces cross-protection against HIV-2 challenge in rhesus macaques. Nat Med *1*, 321-329.

Adams, J.J., Narayanan, S., Liu, B., Birnbaum, M.E., Kruse, A.C., Bowerman, N.A., Chen, W., Levin, A.M., Connolly, J.M., Zhu, C.*, et al.* (2011). T cell receptor signaling is limited by docking geometry to peptide-major histocompatibility complex. Immunity *35*, 681-693.

Addo, M.M., Yu, X.G., Rathod, A., Cohen, D., Eldridge, R.L., Strick, D., Johnston, M.N., Corcoran, C., Wurcel, A.G., Fitzpatrick, C.A.*, et al.* (2003). Comprehensive epitope analysis of human immunodeficiency virus type 1 (HIV-1)-specific T-cell responses directed against the entire expressed HIV-1 genome demonstrate broadly directed responses, but no correlation to viral load. J Virol *77*, 2081-2092.

Agrawal, S., Marquet, J., Freeman, G.J., Tawab, A., Bouteiller, P.L., Roth, P., Bolton, W., Ogg, G., Boumsell, L., and Bensussan, A. (1999). Cutting edge: MHC class I triggering by a novel cell surface ligand costimulates proliferation of activated human T cells. J Immunol *162*, 1223-1226.

Ahmed, R.K., Norrgren, H., da Silva, Z., Blaxhult, A., Fredriksson, E.L., Biberfeld, G., Andersson, S., and Thorstensson, R. (2005). Antigen-specific beta-chemokine production and CD8 T-cell noncytotoxic antiviral activity in HIV-2-infected individuals. Scand J Immunol *61*, 63-71.

Akinsiku, O.T., Bansal, A., Sabbaj, S., Heath, S.L., and Goepfert, P.A. (2011). Interleukin-2 production by polyfunctional HIV-1-specific CD8 T cells is associated with enhanced viral suppression. J Acquir Immune Defic Syndr *58*, 132-140.

al-Ramadi, B.K., Jelonek, M.T., Boyd, L.F., Margulies, D.H., and Bothwell, A.L. (1995). Lack of strict correlation of functional sensitization with the apparent affinity of MHC/peptide complexes for the TCR. J Immunol *155*, 662-673.

Alatrakchi, N., Damond, F., Matheron, S., Beretta-Tempelhoff, S., Campa, P., Carcelain, G., Brun-Vezinet, F., and Autran, B. (2006). Proliferative, IFNgamma and IL-2-producing T-cell responses to HIV-2 in untreated HIV-2 infection. AIDS *20*, 29-34.

Aldy, K.N., Horton, N.C., Mathew, P.A., and Mathew, S.O. (2011). 2B4+ CD8+ T cells play an inhibitory role against constrained HIV epitopes. Biochem Biophys Res Commun *405*, 503-507.

Alexander-Miller, M.A., Leggatt, G.R., and Berzofsky, J.A. (1996a). Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. Proc Natl Acad Sci U S A *93*, 4102-4107.

Alexander-Miller, M.A., Leggatt, G.R., Sarin, A., and Berzofsky, J.A. (1996b). Role of antigen, CD8, and cytotoxic T lymphocyte (CTL) avidity in high dose antigen induction of apoptosis of effector CTL. J Exp Med *184*, 485-492.

Allsopp, C.E., Harding, R.M., Taylor, C., Bunce, M., Kwiatkowski, D., Anstey, N., Brewster, D., McMichael, A.J., Greenwood, B.M., and Hill, A.V. (1992). Interethnic genetic differentiation in Africa: HLA class I antigens in The Gambia. Am J Hum Genet *50*, 411-421.

Almaghrabi, R.S., Ali, B., Al-Thawadi, S., Halim, M.A., and Alrajhi, A.A. (2011). Human immunodeficiency virus type 2 in two Saudi families. Ann Saudi Med *31*, 417-420.

Almeida, J.R., Price, D.A., Papagno, L., Arkoub, Z.A., Sauce, D., Bornstein, E., Asher, T.E., Samri, A., Schnuriger, A., Theodorou, I.*, et al.* (2007). Superior control of HIV-1 replication by CD8+ T cells is reflected by their avidity, polyfunctionality, and clonal turnover. J Exp Med *204*, 2473-2485.

Altfeld, M. (2003). Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection. AIDS *17*, 2581-2591.

Altfeld, M., Addo, M.M., Rosenberg, E.S., Hecht, F.M., Lee, P.K., Vogel, M., Yu, X.G., Draenert, R., Johnston, M.N., Strick, D.*, et al.* (2003). Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection. AIDS *17*, 2581-2591.

Altfeld, M., Kalife, E.T., Qi, Y., Streeck, H., Lichterfeld, M., Johnston, M.N., Burgett, N., Swartz, M.E., Yang, A., Alter, G.*, et al.* (2006). HLA Alleles Associated with Delayed Progression to AIDS Contribute Strongly to the Initial CD8(+) T Cell Response against HIV-1. PLoS Med *3*, e403.

Alvarez, S., Blanco, A., Kern, F., Fresno, M., and Munoz-Fernandez, M.A. (2008). HIV-2 induces NF-kappaB activation and cyclooxygenase-2 expression in human astroglial cells. Virology *380*, 144-151.

Amoli, M.M., Thomson, W., Hajeer, A.H., Calvino, M.C., Garcia-Porrua, C., Ollier, W.E., and Gonzalez-Gay, M.A. (2002). HLA-B35 association with nephritis in Henoch-Schonlein purpura. J Rheumatol *29*, 948-949.

Andersen-Nissen, E., Heit, A., and McElrath, M.J. (2012). Profiling immunity to HIV vaccines with systems biology. Curr Opin HIV AIDS *7*, 32-37.

Andersson, S., Norrgren, H., da Silva, Z., Biague, A., Bamba, S., Kwok, S., Christopherson, C., Biberfeld, G., and Albert, J. (2000). Plasma viral load in HIV-1 and HIV-2 singly and dually infected individuals in Guinea-Bissau, West Africa: significantly lower plasma virus set point in HIV-2 infection than in HIV-1 infection. Arch Intern Med *160*, 3286-3293.

Anumanthan, A., Bensussan, A., Boumsell, L., Christ, A.D., Blumberg, R.S., Voss, S.D., Patel, A.T., Robertson, M.J., Nadler, L.M., and Freeman, G.J. (1998). Cloning of BY55, a novel Ig superfamily member expressed on NK cells, CTL, and intestinal intraepithelial lymphocytes. J Immunol *161*, 2780-2790.

Apostolopoulos, V., Yu, M., McKenzie, I.F., and Wilson, I.A. (2000). Structural implications for the design of molecular vaccines. Curr Opin Mol Ther *2*, 29-36.

Appay, V., Dunbar, P.R., Callan, M., Klenerman, P., Gillespie, G.M., Papagno, L., Ogg, G.S., King, A., Lechner, F., Spina, C.A.*, et al.* (2002). Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nat Med *8*, 379-385.

Appay, V., and Iglesias, M.C. (2011). Antigen sensitivity and T-cell receptor avidity as critical determinants of HIV control. Curr Opin HIV AIDS *6*, 157-162.

Arden, B., Clark, S.P., Kabelitz, D., and Mak, T.W. (1995). Human T-cell receptor variable gene segment families. Immunogenetics *42*, 455-500.

Argaet, V.P., Schmidt, C.W., Burrows, S.R., Silins, S.L., Kurilla, M.G., Doolan, D.L., Suhrbier, A., Moss, D.J., Kieff, E., Sculley, T.B., and Misko, I.S. (1994). Dominant selection of an invariant T cell antigen receptor in response to persistent infection by Epstein-Barr virus. J Exp Med *180*, 2335-2340.

Arhel, N. (2010). Revisiting HIV-1 uncoating. Retrovirology *7*, 96.

Arien, K.K., Abraha, A., Quinones-Mateu, M.E., Kestens, L., Vanham, G., and Arts, E.J. (2005). The replicative fitness of primary human immunodeficiency virus type 1 (HIV-1) group M, HIV-1 group O, and HIV-2 isolates. J Virol *79*, 8979-8990.

Ariyoshi, K., Berry, N., Wilkins, A., Ricard, D., Aaby, P., Naucler, A., Ngom, P.T., Jobe, O., Jaffar, S., Dias, F.*, et al.* (1996). A community-based study of human immunodeficiency virus type 2 provirus load in rural village in West Africa. J Infect Dis *173*, 245-248.

Ariyoshi, K., Schim van der Loeff, M., Cook, P., Whitby, D., Corrah, T., Jaffar, S., Cham, F., Sabally, S., O'Donovan, D., Weiss, R.A.*, et al.* (1998). Kaposi's sarcoma in the Gambia, West Africa is less frequent in human immunodeficiency virus type 2 than in human immunodeficiency virus type 1 infection despite a high prevalence of human herpesvirus 8. J Hum Virol *1*, 193-199.

Arts, E.J., and Hazuda, D.J. (2012). HIV-1 Antiretroviral Drug Therapy. Cold Spring Harb Perspect Med *2*, a007161.

Bailey, J.R., Williams, T.M., Siliciano, R.F., and Blankson, J.N. (2006). Maintenance of viral suppression in HIV-1-infected HLA-B*57+ elite suppressors despite CTL escape mutations. J Exp Med *203*, 1357-1369.

Baker, B.M., Block, B.L., Rothchild, A.C., and Walker, B.D. (2009). Elite control of HIV infection: implications for vaccine design. Expert Opin Biol Ther *9*, 55-69.

Barin, F., Cazein, F., Lot, F., Pillonel, J., Brunet, S., Thierry, D., Damond, F., Brun-Vezinet, F., Desenclos, J.C., and Semaille, C. (2007). Prevalence of HIV-2 and HIV-1 group O infections among new HIV diagnoses in France: 2003-2006. AIDS *21*, 2351-2353.

Barin, F., M'Boup, S., Denis, F., Kanki, P., Allan, J.S., Lee, T.H., and Essex, M. (1985). Serological evidence for virus related to simian T-lymphotropic retrovirus III in residents of west Africa. Lancet *2*, 1387-1389.

Barnett, S.W., Murthy, K.K., Herndier, B.G., and Levy, J.A. (1994). An AIDS-like condition induced in baboons by HIV-2. Science *266*, 642-646.

Barroso, H., Borrego, P., Bartolo, I., Marcelino, J.M., Familia, C., Quintas, A., and Taveira, N. (2011). Evolutionary and structural features of the C2, V3 and C3 envelope regions underlying the differences in HIV-1 and HIV-2 biology and infection. PLoS One *6*, e14548.

Bashirova, A.A., Thomas, R., and Carrington, M. (2011). HLA/KIR restraint of HIV: surviving the fittest. Annu Rev Immunol *29*, 295-317.

Bazhan, S.I., Karpenko, L.I., Ilyicheva, T.N., Belavin, P.A., Seregin, S.V., Danilyuk, N.K., Antonets, D.V., and Ilyichev, A.A. (2010). Rational design based synthetic polyepitope DNA vaccine for eliciting HIV-specific CD8+ T cell responses. Mol Immunol *47*, 1507-1515.

Belyakov, I.M., Kuznetsov, V.A., Kelsall, B., Klinman, D., Moniuszko, M., Lemon, M., Markham, P.D., Pal, R., Clements, J.D., Lewis, M.G.*, et al.* (2006). Impact of vaccine-induced mucosal high-avidity CD8+ CTLs in delay of AIDS viral dissemination from mucosa. Blood *107*, 3258-3264.

Bennett, M.S., Joseph, A., Ng, H.L., Goldstein, H., and Yang, O.O. (2010). Fine-tuning of T-cell receptor avidity to increase HIV epitope variant recognition by cytotoxic T lymphocytes. AIDS *24*, 2619-2628.

Bensussan, A., Rabian, C., Schiavon, V., Bengoufa, D., Leca, G., and Boumsell, L. (1993). Significant enlargement of a specific subset of CD3+CD8+ peripheral

blood leukocytes mediating cytotoxic T-lymphocyte activity during human immunodeficiency virus infection. Proc Natl Acad Sci U S A *90*, 9427-9430.

Berger, C.T., Frahm, N., Price, D.A., Mothe, B., Ghebremichael, M., Hartman, K.L., Henry, L.M., Brenchley, J.M., Ruff, L.E., Venturi, V.*, et al.* (2011). High-Functional-Avidity Cytotoxic T Lymphocyte Responses to HLA-B-Restricted Gag-Derived Epitopes Associated with Relative HIV Control. J Virol *85*, 9334-9345.

Berry, N., Jaffar, S., Schim van der Loeff, M., Ariyoshi, K., Harding, E., N'Gom, P.T., Dias, F., Wilkins, A., Ricard, D., Aaby, P.*, et al.* (2002). Low level viremia and high CD4% predict normal survival in a cohort of HIV type-2-infected villagers. AIDS Res Hum Retroviruses *18*, 1167-1173.

Berthoux, F.C., Genin, C., Le Petit, J.C., and Laurent, B. (1983). [Immunogenetics of intercapillary glomerulonephritis due to IgA deposits]. Nephrologie *4*, 283-287.

Bertoletti, A., Cham, F., McAdam, S., Rostron, T., Rowland-Jones, S., Sabally, S., Corrah, T., Ariyoshi, K., and Whittle, H. (1998). Cytotoxic T cells from human immunodeficiency virus type 2-infected patients frequently cross-react with different human immunodeficiency virus type 1 clades. J Virol *72*, 2439-2448.

Betts, M.R., Nason, M.C., West, S.M., De Rosa, S.C., Migueles, S.A., Abraham, J., Lederman, M.M., Benito, J.M., Goepfert, P.A., Connors, M.*, et al.* (2006). HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. Blood *107*, 4781-4789.

Bimber, B.N., Burwitz, B.J., O'Connor, S., Detmer, A., Gostick, E., Lank, S.M., Price, D.A., Hughes, A., and O'Connor, D. (2009). Ultradeep pyrosequencing detects complex patterns of CD8+ T-lymphocyte escape in simian immunodeficiency virus-infected macaques. J Virol *83*, 8247-8253.

Bischof, J.J., Kuruc, J.D., Embry, J.A., Hatch, J.E., Ashton, F.A., Schmitz, J.L., Miller, W.C., Leone, P.A., and Gay, C.L. (2011). Prospective study of the ARCHITECTHIV Ag/Ab Combo fourth generation assay to detect HIV infection in sexually transmitted infection clinics. AIDS *25*, 1927-1929.

Blaak, H., Boers, P.H., van der Ende, M.E., Schuitemaker, H., and Osterhaus, A.D. (2008). CCR5-restricted HIV type 2 variants from long-term aviremic individuals are less sensitive to inhibition by beta-chemokines than low pathogenic HIV type 1 variants. AIDS Res Hum Retroviruses *24*, 473-484.

Blaak, H., van der Ende, M.E., Boers, P.H., Schuitemaker, H., and Osterhaus, A.D. (2006). In vitro replication capacity of HIV-2 variants from long-term aviremic individuals. Virology *353*, 144-154.

Blackbourn, D.J., Locher, C.P., Ramachandran, B., Barnett, S.W., Murthy, K.K., Carey, K.D., Brasky, K.M., and Levy, J.A. (1997). CD8+ cells from HIV-2-infected baboons control HIV replication. AIDS *11*, 737-746.

Boniface, J.J., and Davis, M.M. (1995). T-cell recognition of antigen. A process controlled by transient intermolecular interactions. Ann N Y Acad Sci *766*, 62-69.

Boniface, J.J., Reich, Z., Lyons, D.S., and Davis, M.M. (1999). Thermodynamics of T cell receptor binding to peptide-MHC: evidence for a general mechanism of molecular scanning. Proc Natl Acad Sci U S A *96*, 11446-11451.

Borbulevych, O.Y., Piepenbrink, K.H., Gloor, B.E., Scott, D.R., Sommese, R.F., Cole, D.K., Sewell, A.K., and Baker, B.M. (2009). T cell receptor cross-reactivity directed by antigen-dependent tuning of peptide-MHC molecular flexibility. Immunity *31*, 885-896.

Bordon, Y. (2010). Dendritic cells: An inner eye for HIV. Nat Rev Immunol *10*, 678.

Borg, N.A., Ely, L.K., Beddoe, T., Macdonald, W.A., Reid, H.H., Clements, C.S., Purcell, A.W., Kjer-Nielsen, L., Miles, J.J., Burrows, S.R., *et al.* (2005). The CDR3 regions of an immunodominant T cell receptor dictate the 'energetic landscape' of peptide-MHC recognition. Nat Immunol *6*, 171-180.

Borghans, J.A., Molgaard, A., de Boer, R.J., and Kesmir, C. (2007). HLA alleles associated with slow progression to AIDS truly prefer to present HIV-1 p24. PLoS One *2*, e920.

Borrego, P., Marcelino, J.M., Rocha, C., Doroana, M., Antunes, F., Maltez, F., Gomes, P., Novo, C., Barroso, H., and Taveira, N. (2008). The role of the humoral immune response in the molecular evolution of the envelope C2, V3 and C3 regions in chronically HIV-2 infected patients. Retrovirology *5*, 78.

Borrow, P., Lewicki, H., Hahn, B.H., Shaw, G.M., and Oldstone, M.B. (1994). Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol *68*, 6103-6110.

Borrow, P., Lewicki, H., Wei, X., Horwitz, M.S., Peffer, N., Meyers, H., Nelson, J.A., Gairin, J.E., Hahn, B.H., Oldstone, M.B., and 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. Nat Med *3*, 205-211.

Boufassa, F., Saez-Cirion, A., Lechenadec, J., Zucman, D., Avettand-Fenoel, V., Venet, A., Rouzioux, C., Delfraissy, J.F., Lambotte, O., and Meyer, L. (2011). CD4 dynamics over a 15 year-period among HIV controllers enrolled in the ANRS French observatory. PLoS One *6*, e18726.

Boulter, J.M., Glick, M., Todorov, P.T., Baston, E., Sami, M., Rizkallah, P., and Jakobsen, B.K. (2003). Stable, soluble T-cell receptor molecules for crystallization and therapeutics. Protein Eng *16*, 707-711.

Bousso, P., Levraud, J.P., Kourilsky, P., and Abastado, J.P. (1999). The composition of a primary T cell response is largely determined by the timing of recruitment of individual T cell clones. J Exp Med *189*, 1591-1600.

Brass, A.L., Dykxhoorn, D.M., Benita, Y., Yan, N., Engelman, A., Xavier, R.J., Lieberman, J., and Elledge, S.J. (2008). Identification of host proteins required for HIV infection through a functional genomic screen. Science *319*, 921-926.

Briggs, J.A., Simon, M.N., Gross, I., Krausslich, H.G., Fuller, S.D., Vogt, V.M., and Johnson, M.C. (2004). The stoichiometry of Gag protein in HIV-1. Nat Struct Mol Biol *11*, 672-675.

Broutet, N., de Queiroz Sousa, A., Basilio, F.P., Sa, H.L., Simon, F., and Dabis, F. (1996). Prevalence of HIV-1, HIV-2 and HTLV antibody, in Fortaleza, Ceara, Brazil, 1993-1994. Int J STD AIDS *7*, 365-369.

Bukrinskaya, A. (2007). HIV-1 matrix protein: a mysterious regulator of the viral life cycle. Virus Res *124*, 1-11.

Bullock, T.N., Mullins, D.W., and Engelhard, V.H. (2003). Antigen density presented by dendritic cells in vivo differentially affects the number and avidity of primary, memory, and recall CD8+ T cells. J Immunol *170*, 1822-1829.

Burgard, M., Jasseron, C., Matheron, S., Damond, F., Hamrene, K., Blanche, S., Faye, A., Rouzioux, C., Warszawski, J., and Mandelbro, L. (2010). Mother-to-child transmission of HIV-2 infection from 1986 to 2007 in the ANRS French Perinatal Cohort EPF-CO1. Clin Infect Dis *51*, 833-843.

Burgers, W.A., van Harmelen, J.H., Shephard, E., Adams, C., Mgwebi, T., Bourn, W., Hanke, T., Williamson, A.L., and Williamson, C. (2006). Design and preclinical

evaluation of a multigene human immunodeficiency virus type 1 subtype C DNA vaccine for clinical trial. J Gen Virol *87*, 399-410.

Burrows, S.R., Silins, S.L., Khanna, R., Burrows, J.M., Rischmueller, M., McCluskey, J., and Moss, D.J. (1997). Cross-reactive memory T cells for Epstein-Barr virus augment the alloresponse to common human leukocyte antigens: degenerate recognition of major histocompatibility complex-bound peptide by T cells and its role in alloreactivity. Eur J Immunol *27*, 1726-1736.

Busch, D.H., and Pamer, E.G. (1998). MHC class I/peptide stability: implications for immunodominance, in vitro proliferation, and diversity of responding CTL. J Immunol *160*, 4441-4448.

Buseyne, F., and Riviere, Y. (2001). The flexibility of the TCR allows recognition of a large set of naturally occurring epitope variants by HIV-specific cytotoxic T lymphocytes. Int Immunol *13*, 941-950.

Calado, M., Matoso, P., Santos-Costa, Q., Espirito-Santo, M., Machado, J., Rosado, L., Antunes, F., Mansinho, K., Lopes, M.M., Maltez, F*., et al.* (2010). Coreceptor usage by HIV-1 and HIV-2 primary isolates: the relevance of CCR8 chemokine receptor as an alternative coreceptor. Virology *408*, 174-182.

Cale, E.M., Bazick, H.S., Rianprakaisang, T.A., Alam, S.M., and Letvin, N.L. (2011). Mutations in a dominant Nef epitope of simian immunodeficiency virus diminish TCR:epitope peptide affinity but not epitope peptide:MHC class I binding. J Immunol *187*, 3300-3313.

Camacho, R.J. (2012). Special Aspects of the Treatment of HIV-2-Infected Patients. Intervirology *55*, 179-183.

Cantrell, D.A. (1996). T cell antigen receptor signal transduction pathways. Cancer Surv *27*, 165-175.

Carrington, M., Nelson, G.W., Martin, M.P., Kissner, T., Vlahov, D., Goedert, J.J., Kaslow, R., Buchbinder, S., Hoots, K., and O'Brien, S.J. (1999). HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. Science *283*, 1748-1752.

Carvalho, A., Valadas, E., Franca, L., Carvalho, C., Aleixo, M., Mendez, J., Marques, R., Sarmento, A., Doroana, M., Antunes, F*., et al.* (2011). Population mobility and the changing epidemics of HIV-2 in Portugal. HIV Med.

Castellino, F., and Germain, R.N. (2006). Cooperation between CD4+ and CD8+ T cells: when, where, and how. Annu Rev Immunol *24*, 519-540.

Cavaleiro, R., Baptista, A.P., Foxall, R.B., Victorino, R.M., and Sousa, A.E. (2009a). Dendritic cell differentiation and maturation in the presence of HIV type 2 envelope. AIDS Res Hum Retroviruses *25*, 425-431.

Cavaleiro, R., Baptista, A.P., Soares, R.S., Tendeiro, R., Foxall, R.B., Gomes, P., Victorino, R.M., and Sousa, A.E. (2009b). Major depletion of plasmacytoid dendritic cells in HIV-2 infection, an attenuated form of HIV disease. PLoS Pathog *5*, e1000667.

CDC, M. (2011). HIV-2 Infection Surveillance, United States, 1987-2009. JAMA: The Journal of the American Medical Association *306*, 1079-1081.

Chakrabarti, L.A., and Simon, V. (2010). Immune mechanisms of HIV control. Curr Opin Immunol *22*, 488-496.

Chen, J.L., Morgan, A.J., Stewart-Jones, G., Shepherd, D., Bossi, G., Wooldridge, L., Hutchinson, S.L., Sewell, A.K., Griffiths, G.M., van der Merwe, P.A*., et al.* (2010). Ca2+ release from the endoplasmic reticulum of NY-ESO-1-specific T cells is modulated by the affinity of TCR and by the use of the CD8 coreceptor. J Immunol *184*, 1829-1839.

Chen, J.L., Stewart-Jones, G., Bossi, G., Lissin, N.M., Wooldridge, L., Choi, E.M., Held, G., Dunbar, P.R., Esnouf, R.M., Sami, M.*, et al.* (2005). Structural and kinetic basis for heightened immunogenicity of T cell vaccines. J Exp Med *201*, 1243-1255.

Chen, Z., Luckay, A., Sodora, D.L., Telfer, P., Reed, P., Gettie, A., Kanu, J.M., Sadek, R.F., Yee, J., Ho, D.D.*, et al.* (1997). Human immunodeficiency virus type 2 (HIV-2) seroprevalence and characterization of a distinct HIV-2 genetic subtype from the natural range of simian immunodeficiency virus-infected sooty mangabeys. J Virol *71*, 3953-3960.

Cheynier, R., Henrichwark, S., Hadida, F., Pelletier, E., Oksenhendler, E., Autran, B., and Wain-Hobson, S. (1994). HIV and T cell expansion in splenic white pulps is accompanied by infiltration of HIV-specific cytotoxic T lymphocytes. Cell *78*, 373-387.

Ciccozzi, M., Babakir-Mina, M., Cella, E., Bertoli, A., Lo Presti, A., Maniar, J.K., Perno, C.F., and Ciotti, M. (2011). A case of Italian HIV type 2 infection: a genetic analysis. AIDS Res Hum Retroviruses *27*, 1333-1335.

Clute, S.C., Naumov, Y.N., Watkin, L.B., Aslan, N., Sullivan, J.L., Thorley-Lawson, D.A., Luzuriaga, K., Welsh, R.M., Puzone, R., Celada, F., and Selin, L.K. (2010). Broad cross-reactive TCR repertoires recognizing dissimilar Epstein-Barr and influenza A virus epitopes. J Immunol *185*, 6753-6764.

Cochrane, A.W., Chen, C.H., and Rosen, C.A. (1990). Specific interaction of the human immunodeficiency virus Rev protein with a structured region in the env mRNA. Proc Natl Acad Sci U S A *87*, 1198-1202.

Cohen, J. (2007). AIDS research. Promising AIDS vaccine's failure leaves field reeling. Science *318*, 28-29.

Cole, D.K., Edwards, E.S., Wynn, K.K., Clement, M., Miles, J.J., Ladell, K., Ekeruche, J., Gostick, E., Adams, K.J., Skowera, A.*, et al.* (2010). Modification of MHC anchor residues generates heteroclitic peptides that alter TCR binding and T cell recognition. J Immunol *185*, 2600-2610.

Conrad, J.A., Ramalingam, R.K., Duncan, C.B., Smith, R.M., Wei, J., Barnett, L., Simons, B.C., Lorey, S.L., and Kalams, S.A. (2012). Anti-retroviral therapy reduces the magnitude and T cell receptor repertoire diversity of HIV-specific T cell responses without changing T cell clonotype dominance. J Virol.

Conrad, J.A., Ramalingam, R.K., Smith, R.M., Barnett, L., Lorey, S.L., Wei, J., Simons, B.C., Sadagopal, S., Meyer-Olson, D., and Kalams, S.A. (2011). Dominant Clonotypes within HIV-Specific T Cell Responses Are Programmed Death-1high and CD127low and Display Reduced Variant Cross-Reactivity. The Journal of Immunology *186*, 6871-6885.

Cornberg, M., Chen, A.T., Wilkinson, L.A., Brehm, M.A., Kim, S.K., Calcagno, C., Ghersi, D., Puzone, R., Celada, F., Welsh, R.M., and Selin, L.K. (2006). Narrowed TCR repertoire and viral escape as a consequence of heterologous immunity. J Clin Invest *116*, 1443-1456.

Currier, J.R., and Robinson, M.A. (2001). Spectratype/immunoscope analysis of the expressed TCR repertoire. Curr Protoc Immunol *Chapter 10*, Unit 10 28.

Curtale, G., Citarella, F., Carissimi, C., Goldoni, M., Carucci, N., Fulci, V., Franceschini, D., Meloni, F., Barnaba, V., and Macino, G. (2010). An emerging player in the adaptive immune response: microRNA-146a is a modulator of IL-2 expression and activation-induced cell death in T lymphocytes. Blood *115*, 265-273.

Dahirel, V., Shekhar, K., Pereyra, F., Miura, T., Artyomov, M., Talsania, S., Allen, T.M., Altfeld, M., Carrington, M., Irvine, D.J.*, et al.* (2011). Coordinate

linkage of HIV evolution reveals regions of immunological vulnerability. Proc Natl Acad Sci U S A *108*, 11530-11535.

Dash, B.P., Mukherjee, S., Suhas, V.L., and Chandra, N. (2007). Interaction profiling of T-cell epitopes with MHC-class I molecules. Protein Pept Lett *14*, 557-564.

Dash, P., McClaren, J.L., Oguin, T.H., 3rd, Rothwell, W., Todd, B., Morris, M.Y., Becksfort, J., Reynolds, C., Brown, S.A., Doherty, P.C., and Thomas, P.G. (2011). Paired analysis of TCRalpha and TCRbeta chains at the single-cell level in mice. J Clin Invest *121*, 288-295.

Davis, M.M., Altman, J.D., and Newell, E.W. (2011). Interrogating the repertoire: broadening the scope of peptide-MHC multimer analysis. Nat Rev Immunol *11*, 551-558.

Davis-Harrison, R.L., Armstrong, K.M., and Baker, B.M. (2005). Two different T cell receptors use different thermodynamic strategies to recognize the same peptide/MHC ligand. J Mol Biol *346*, 533-550.

Day, C.L., Kaufmann, D.E., Kiepiela, P., Brown, J.A., Moodley, E.S., Reddy, S., Mackey, E.W., Miller, J.D., Leslie, A.J., DePierres, C*., et al.* (2006). PD-1 expression on HIV-specific T cells is associated with T-cell exhaustion and disease progression. Nature *443*, 350-354.

Day, E.K., Carmichael, A.J., ten Berge, I.J.M., Waller, E.C.P., Sissons, J.G.P., and Wills, M.R. (2007). Rapid CD8+ T Cell Repertoire Focusing and Selection of High-Affinity Clones into Memory Following Primary Infection with a Persistent Human Virus: Human Cytomegalovirus. The Journal of Immunology *179*, 3203-3213.

De Cock, K.M., Adjorlolo, G., Ekpini, E., Sibailly, T., Kouadio, J., Maran, M., Brattegaard, K., Vetter, K.M., Doorly, R., and Gayle, H.D. (1993). Epidemiology and transmission of HIV-2. Why there is no HIV-2 pandemic. JAMA *270*, 2083-2086.

De Rosa, S.C., Herzenberg, L.A., and Roederer, M. (2001). 11-color, 13-parameter flow cytometry: identification of human naive T cells by phenotype, function, and T-cell receptor diversity. Nat Med *7*, 245-248.

de Silva, T.I., Aasa-Chapman, M., Cotten, M., Hue, S., Robinson, J., Bibollet-Ruche, F., Sarge-Njie, R., Berry, N., Jaye, A., Aaby, P*., et al.* (2012). Potent autologous and heterologous neutralizing antibody responses occur in HIV-2 infection across a broad range of infection outcomes. J Virol *86*, 930-946.

DeGottardi, M.Q., Specht, A., Metcalf, B., Kaur, A., Kirchhoff, F., and Evans, D.T. (2008). Selective downregulation of rhesus macaque and sooty mangabey major histocompatibility complex class I molecules by Nef alleles of simian immunodeficiency virus and human immunodeficiency virus type 2. J Virol *82*, 3139-3146.

Derby, M., Alexander-Miller, M., Tse, R., and Berzofsky, J. (2001a). High-avidity CTL exploit two complementary mechanisms to provide better protection against viral infection than low-avidity CTL. J Immunol *166*, 1690-1697.

Derby, M.A., Wang, J., Margulies, D.H., and Berzofsky, J.A. (2001b). Two intermediate-avidity cytotoxic T lymphocyte clones with a disparity between functional avidity and MHC tetramer staining. Int Immunol *13*, 817-824.

Ding, Y.H., Smith, K.J., Garboczi, D.N., Utz, U., Biddison, W.E., and Wiley, D.C. (1998). Two human T cell receptors bind in a similar diagonal mode to the HLA-A2/Tax peptide complex using different TCR amino acids. Immunity *8*, 403-411.

Dinges, W.L., Richardt, J., Friedrich, D., Jalbert, E., Liu, Y., Stevens, C.E., Maenza, J., Collier, A.C., Geraghty, D.E., Smith, J.*, et al.* (2010). Virus-specific CD8+ T-cell responses better define HIV disease progression than HLA genotype. J Virol *84*, 4461-4468.

Dolan, B.P., Bennink, J.R., and Yewdell, J.W. (2011). Translating DRiPs: progress in understanding viral and cellular sources of MHC class I peptide ligands. Cell Mol Life Sci *68*, 1481-1489.

Doms, R.W., Earl, P.L., Chakrabarti, S., and Moss, B. (1990). Human immunodeficiency virus types 1 and 2 and simian immunodeficiency virus env proteins possess a functionally conserved assembly domain. J Virol *64*, 3537-3540.

Doms, R.W., and Moore, J.P. (2000). HIV-1 membrane fusion: targets of opportunity. J Cell Biol *151*, F9-14.

Dong, L., Li, P., Oenema, T., McClurkan, C.L., and Koelle, D.M. (2010). Public TCR use by herpes simplex virus-2-specific human CD8 CTLs. J Immunol *184*, 3063-3071.

Dong, T., Stewart-Jones, G., Chen, N., Easterbrook, P., Xu, X., Papagno, L., Appay, V., Weekes, M., Conlon, C., Spina, C.*, et al.* (2004). HIV-specific cytotoxic T cells from long-term survivors select a unique T cell receptor. J Exp Med *200*, 1547-1557.

Douek, D.C., Betts, M.R., Brenchley, J.M., Hill, B.J., Ambrozak, D.R., Ngai, K.L., Karandikar, N.J., Casazza, J.P., and Koup, R.A. (2002). A novel approach to the analysis of specificity, clonality, and frequency of HIV-specific T cell responses reveals a potential mechanism for control of viral escape. J Immunol *168*, 3099-3104.

Dougan, S., Patel, B., Tosswill, J.H., and Sinka, K. (2005). Diagnoses of HIV-1 and HIV-2 in England, Wales, and Northern Ireland associated with west Africa. Sex Transm Infect *81*, 338-341.

Drylewicz, J., Matheron, S., Lazaro, E., Damond, F., Bonnet, F., Simon, F., Dabis, F., Brun-Vezinet, F., Chene, G., and Thiebaut, R. (2008). Comparison of viro-immunological marker changes between HIV-1 and HIV-2-infected patients in France. AIDS *22*, 457-468.

Duraiswamy, J., Ibegbu, C.C., Masopust, D., Miller, J.D., Araki, K., Doho, G.H., Tata, P., Gupta, S., Zilliox, M.J., Nakaya, H.I.*, et al.* (2011). Phenotype, function, and gene expression profiles of programmed death-1(hi) CD8 T cells in healthy human adults. J Immunol *186*, 4200-4212.

Duvall, M.G., Jaye, A., Dong, T., Brenchley, J.M., Alabi, A.S., Jeffries, D.J., van der Sande, M., Togun, T.O., McConkey, S.J., Douek, D.C.*, et al.* (2006). Maintenance of HIV-specific CD4+ T cell help distinguishes HIV-2 from HIV-1 infection. J Immunol *176*, 6973-6981.

Duvall, M.G., Lore, K., Blaak, H., Ambrozak, D.A., Adams, W.C., Santos, K., Geldmacher, C., Mascola, J.R., McMichael, A.J., Jaye, A.*, et al.* (2007). Dendritic cells are less susceptible to human immunodeficiency virus type 2 (HIV-2) infection than to HIV-1 infection. J Virol *81*, 13486-13498.

Duvall, M.G., Precopio, M.L., Ambrozak, D.A., Jaye, A., McMichael, A.J., Whittle, H.C., Roederer, M., Rowland-Jones, S.L., and Koup, R.A. (2008). Polyfunctional T cell responses are a hallmark of HIV-2 infection. Eur J Immunol *38*, 350-363.

Dyer, W.B., Zaunders, J.J., Yuan, F.F., Wang, B., Learmont, J.C., Geczy, A.F., Saksena, N.K., McPhee, D.A., Gorry, P.R., and Sullivan, J.S. (2008).

Mechanisms of HIV non-progression; robust and sustained CD4+ T-cell proliferative responses to p24 antigen correlate with control of viraemia and lack of disease progression after long-term transfusion-acquired HIV-1 infection. Retrovirology *5*, 112.

Easterbrook, P. (1999). Long-term non-progression in HIV infection: definitions and epidemiological issues. Journal of Infection *38*, 71-73.

Edinger, A.L., Clements, J.E., and Doms, R.W. (1999). Chemokine and orphan receptors in HIV-2 and SIV tropism and pathogenesis. Virology *260*, 211-221.

Edwards, B.H., Bansal, A., Sabbaj, S., Bakari, J., Mulligan, M.J., and Goepfert, P.A. (2002). Magnitude of functional CD8+ T-cell responses to the gag protein of human immunodeficiency virus type 1 correlates inversely with viral load in plasma. J Virol *76*, 2298-2305.

Emu, B., Sinclair, E., Favre, D., Moretto, W.J., Hsue, P., Hoh, R., Martin, J.N., Nixon, D.F., McCune, J.M., and Deeks, S.G. (2005). Phenotypic, functional, and kinetic parameters associated with apparent T-cell control of human immunodeficiency virus replication in individuals with and without antiretroviral treatment. J Virol *79*, 14169-14178.

Evans, D.T., Serra-Moreno, R., Singh, R.K., and Guatelli, J.C. (2010). BST-2/tetherin: a new component of the innate immune response to enveloped viruses. Trends Microbiol *18*, 388-396.

Fan, N., Rank, K.B., Leone, J.W., Heinrikson, R.L., Bannow, C.A., Smith, C.W., Evans, D.B., Poppe, S.M., Tarpley, W.G., Rothrock, D.J., and et al. (1995). The differential processing of homodimers of reverse transcriptases from human immunodeficiency viruses type 1 and 2 is a consequence of the distinct specificities of the viral proteases. J Biol Chem *270*, 13573-13579.

Faria, N.R., Hodges-Mameletzis, I., Silva, J.C., Rodes, B., Erasmus, S., Paolucci, S., Ruelle, J., Pieniazek, D., Taveira, N., Trevino, A.*, et al.* (2012). The phylogeographic footprint of colonial history in the global dispersal of HIV-2 group A. J Gen Virol.

Feldmann, J., Leligdowicz, A., Jaye, A., Dong, T., Whittle, H., and Rowland-Jones, S.L. (2009). Downregulation of the T-cell receptor by human immunodeficiency virus type 2 Nef does not protect against disease progression. J Virol *83*, 12968-12972.

Fellay, J., Shianna, K.V., Ge, D., Colombo, S., Ledergerber, B., Weale, M., Zhang, K., Gumbs, C., Castagna, A., Cossarizza, A.*, et al.* (2007). A whole-genome association study of major determinants for host control of HIV-1. Science *317*, 944-947.

Fischer, W., Perkins, S., Theiler, J., Bhattacharya, T., Yusim, K., Funkhouser, R., Kuiken, C., Haynes, B., Letvin, N.L., Walker, B.D.*, et al.* (2007). Polyvalent vaccines for optimal coverage of potential T-cell epitopes in global HIV-1 variants. Nat Med *13*, 100-106.

Fletcher, T.M., 3rd, Brichacek, B., Sharova, N., Newman, M.A., Stivahtis, G., Sharp, P.M., Emerman, M., Hahn, B.H., and Stevenson, M. (1996). Nuclear import and cell cycle arrest functions of the HIV-1 Vpr protein are encoded by two separate genes in HIV-2/SIV(SM). EMBO J *15*, 6155-6165.

Friedrich, D., Jalbert, E., Dinges, W.L., Sidney, J., Sette, A., Huang, Y., McElrath, M.J., and Horton, H. (2011). Vaccine-induced HIV-specific CD8+ T cells utilize preferential HLA alleles and target-specific regions of HIV-1. J Acquir Immune Defic Syndr *58*, 248-252.

Friedrich, T.C., Valentine, L.E., Yant, L.J., Rakasz, E.G., Piaskowski, S.M., Furlott, J.R., Weisgrau, K.L., Burwitz, B., May, G.E., Leon, E.J., *et al.* (2007). Subdominant CD8+ T-cell responses are involved in durable control of AIDS virus replication. J Virol *81*, 3465-3476.

Fuchs, J.D., Sobieszczyk, M.E., Hammer, S.M., and Buchbinder, S.P. (2010). Lessons drawn from recent HIV vaccine efficacy trials. J Acquir Immune Defic Syndr *55 Suppl 2*, S128-131.

Fujita, M., Otsuka, M., Miyoshi, M., Khamsri, B., Nomaguchi, M., and Adachi, A. (2008). Vpx is critical for reverse transcription of the human immunodeficiency virus type 2 genome in macrophages. Journal of Virology *82*, 7752-7756.

Gallo, S.A., Reeves, J.D., Garg, H., Foley, B., Doms, R.W., and Blumenthal, R. (2006). Kinetic studies of HIV-1 and HIV-2 envelope glycoprotein-mediated fusion. Retrovirology *3*, 90.

Ganser-Pornillos, B.K., Yeager, M., and Sundquist, W.I. (2008). The structural biology of HIV assembly. Curr Opin Struct Biol *18*, 203-217.

Gao, F., Yue, L., Robertson, D.L., Hill, S.C., Hui, H., Biggar, R.J., Neequaye, A.E., Whelan, T.M., Ho, D.D., Shaw, G.M., and et al. (1994). Genetic diversity of human immunodeficiency virus type 2: evidence for distinct sequence subtypes with differences in virus biology. J Virol *68*, 7433-7447.

Gao, X., Nelson, G.W., Karacki, P., Martin, M.P., Phair, J., Kaslow, R., Goedert, J.J., Buchbinder, S., Hoots, K., Vlahov, D., *et al.* (2001). Effect of a single amino acid change in MHC class I molecules on the rate of progression to AIDS. N Engl J Med *344*, 1668-1675.

Garcia, K.C., and Adams, E.J. (2005). How the T cell receptor sees antigen--a structural view. Cell *122*, 333-336.

Gega, A., and Kozal, M.J. (2011). New technology to detect low-level drug-resistant HIV variants. Future Virol *6*, 17-26.

Genevee, C., Diu, A., Nierat, J., Caignard, A., Dietrich, P.Y., Ferradini, L., Roman-Roman, S., Triebel, F., and Hercend, T. (1992). An experimentally validated panel of subfamily-specific oligonucleotide primers (V alpha 1-w29/V beta 1-w24) for the study of human T cell receptor variable V gene segment usage by polymerase chain reaction. Eur J Immunol *22*, 1261-1269.

Giacoia-Gripp, C.B., Neves, I., Jr., Galhardo, M.C., and Morgado, M.G. (2005). Flow cytometry evaluation of the T-cell receptor Vbeta repertoire among HIV-1 infected individuals before and after antiretroviral therapy. J Clin Immunol *25*, 116-126.

Gilleece, Y., Chadwick, D.R., Breuer, J., Hawkins, D., Smit, E., McCrae, L.X., Pillay, D., Smith, N., and Anderson, J. (2010). British HIV Association guidelines for antiretroviral treatment of HIV-2-positive individuals 2010. HIV Med *11*, 611-619.

Gillespie, G.M., Kaul, R., Dong, T., Yang, H.B., Rostron, T., Bwayo, J.J., Kiama, P., Peto, T., Plummer, F.A., McMichael, A.J., and Rowland-Jones, S.L. (2002). Cross-reactive cytotoxic T lymphocytes against a HIV-1 p24 epitope in slow progressors with B*57. AIDS *16*, 961-972.

Gillespie, G.M., Pinheiro, S., Sayeid-Al-Jamee, M., Alabi, A., Kaye, S., Sabally, S., Sarge-Njie, R., Njai, H., Joof, K., Jaye, A., *et al.* (2005). CD8+ T cell responses to human immunodeficiency viruses type 2 (HIV-2) and type 1 (HIV-1) gag proteins are distinguishable by magnitude and breadth but not cellular phenotype. Eur J Immunol *35*, 1445-1453.

Gillespie, G.M., Stewart-Jones, G., Rengasamy, J., Beattie, T., Bwayo, J.J., Plummer, F.A., Kaul, R., McMichael, A.J., Easterbrook, P., Dong, T.*, et al.* (2006). Strong TCR conservation and altered T cell cross-reactivity characterize a B*57-restricted immune response in HIV-1 infection. J Immunol *177*, 3893-3902.

Goepfert, P.A., Lumm, W., Farmer, P., Matthews, P., Prendergast, A., Carlson, J.M., Derdeyn, C.A., Tang, J., Kaslow, R.A., Bansal, A.*, et al.* (2008). Transmission of HIV-1 Gag immune escape mutations is associated with reduced viral load in linked recipients. J Exp Med *205*, 1009-1017.

Golden-Mason, L., Palmer, B., Klarquist, J., Mengshol, J.A., Castelblanco, N., and Rosen, H.R. (2007). Upregulation of PD-1 expression on circulating and intrahepatic hepatitis C virus-specific CD8+ T cells associated with reversible immune dysfunction. J Virol *81*, 9249-9258.

Gonzalez, P.A., Carreno, L.J., Coombs, D., Mora, J.E., Palmieri, E., Goldstein, B., Nathenson, S.G., and Kalergis, A.M. (2005). T cell receptor binding kinetics required for T cell activation depend on the density of cognate ligand on the antigen-presenting cell. Proc Natl Acad Sci U S A *102*, 4824-4829.

Goonetilleke, N., Moore, S., Dally, L., Winstone, N., Cebere, I., Mahmoud, A., Pinheiro, S., Gillespie, G., Brown, D., Loach, V.*, et al.* (2006). Induction of multifunctional human immunodeficiency virus type 1 (HIV-1)-specific T cells capable of proliferation in healthy subjects by using a prime-boost regimen of DNA- and modified vaccinia virus Ankara-vectored vaccines expressing HIV-1 Gag coupled to CD8+ T-cell epitopes. J Virol *80*, 4717-4728.

Gorochov, G., Neumann, A.U., Kereveur, A., Parizot, C., Li, T., Katlama, C., Karmochkine, M., Raguin, G., Autran, B., and Debre, P. (1998). Perturbation of CD4+ and CD8+ T-cell repertoires during progression to AIDS and regulation of the CD4+ repertoire during antiviral therapy. Nat Med *4*, 215-221.

Gottlieb, G.S., Eholie, S.P., Nkengasong, J.N., Jallow, S., Rowland-Jones, S., Whittle, H.C., and Sow, P.S. (2008). A call for randomized controlled trials of antiretroviral therapy for HIV-2 infection in West Africa. AIDS *22*, 2069-2072; discussion 2073-2064.

Gottlieb, G.S., Hawes, S.E., Agne, H.D., Stern, J.E., Critchlow, C.W., Kiviat, N.B., and Sow, P.S. (2006). Lower levels of HIV RNA in semen in HIV-2 compared with HIV-1 infection: implications for differences in transmission. AIDS *20*, 895-900.

Gottlieb, G.S., Sow, P.S., Hawes, S.E., Ndoye, I., Redman, M., Coll-Seck, A.M., Faye-Niang, M.A., Diop, A., Kuypers, J.M., Critchlow, C.W.*, et al.* (2002). Equal plasma viral loads predict a similar rate of CD4+ T cell decline in human immunodeficiency virus (HIV) type 1- and HIV-2-infected individuals from Senegal, West Africa. J Infect Dis *185*, 905-914.

Gottlinger, H.G. (2001). The HIV-1 assembly machine. AIDS *15 Suppl 5*, S13-20.

Goulder, P.J., Phillips, R.E., Colbert, R.A., McAdam, S., Ogg, G., Nowak, M.A., Giangrande, P., Luzzi, G., Morgan, B., Edwards, A.*, et al.* (1997). Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nat Med *3*, 212-217.

Goulder, P.J., and Watkins, D.I. (2004). HIV and SIV CTL escape: implications for vaccine design. Nat Rev Immunol *4*, 630-640.

Goyarts, E.C., Vegh, Z., Kalergis, A.M., Horig, H., Papadopoulos, N.J., Young, A.C., Thomson, C.T., Chang, H.C., Joyce, S., and Nathenson, S.G. (1998). Point mutations in the beta chain CDR3 can alter the T cell receptor recognition

pattern on an MHC class I/peptide complex over a broad interface area. Mol Immunol *35*, 593-607.

Grabar, S., Selinger-Leneman, H., Abgrall, S., Pialoux, G., Weiss, L., and Costagliola, D. (2009). Prevalence and comparative characteristics of long-term nonprogressors and HIV controller patients in the French Hospital Database on HIV. AIDS *23*, 1163-1169.

Gras, S., Kedzierski, L., Valkenburg, S.A., Laurie, K., Liu, Y.C., Denholm, J.T., Richards, M.J., Rimmelzwaan, G.F., Kelso, A., Doherty, P.C.*, et al.* (2010). Cross-reactive CD8+ T-cell immunity between the pandemic H1N1-2009 and H1N1-1918 influenza A viruses. Proc Natl Acad Sci U S A *107*, 12599-12604.

Grassly, N.C., Xiang, Z., Ariyoshi, K., Aaby, P., Jensen, H., Schim van der Loeff, M., Dias, F., Whittle, H., and Breuer, J. (1998). Mortality among human immunodeficiency virus type 2-positive villagers in rural Guinea-Bissau is correlated with viral genotype. J Virol *72*, 7895-7899.

Groom, H.C., Anderson, E.C., and Lever, A.M. (2009). Rev: beyond nuclear export. J Gen Virol *90*, 1303-1318.

Guergnon, J., Dalmasso, C., Broet, P., Meyer, L., Westrop, S.J., Imami, N., Vicenzi, E., Morsica, G., Tinelli, M., Zanone Poma, B.*, et al.* (2012). Single-Nucleotide Polymorphism-Defined Class I and Class III Major Histocompatibility Complex Genetic Subregions Contribute to Natural Long-term Nonprogression in HIV Infection. J Infect Dis *205*, 718-724.

Guyader, M., Emerman, M., Sonigo, P., Clavel, F., Montagnier, L., and Alizon, M. (1987). Genome organization and transactivation of the human immunodeficiency virus type 2. Nature *326*, 662-669.

Haanen, J.B., Wolkers, M.C., Kruisbeek, A.M., and Schumacher, T.N. (1999). Selective expansion of cross-reactive CD8(+) memory T cells by viral variants. J Exp Med *190*, 1319-1328.

Haasch, D., Chen, Y.W., Reilly, R.M., Chiou, X.G., Koterski, S., Smith, M.L., Kroeger, P., McWeeny, K., Halbert, D.N., Mollison, K.W.*, et al.* (2002). T cell activation induces a noncoding RNA transcript sensitive to inhibition by immunosuppressant drugs and encoded by the proto-oncogene, BIC. Cell Immunol *217*, 78-86.

Harrich, D., and Hooker, B. (2002). Mechanistic aspects of HIV-1 reverse transcription initiation. Rev Med Virol *12*, 31-45.

Harrison, I.P., and McKnight, A. (2011). Cellular entry via an actin and clathrin-dependent route is required for Lv2 restriction of HIV-2. Virology *415*, 47-55.

Hauser, H., Lopez, L.A., Yang, S.J., Oldenburg, J.E., Exline, C.M., Guatelli, J.C., and Cannon, P.M. (2010). HIV-1 Vpu and HIV-2 Env counteract BST-2/tetherin by sequestration in a perinuclear compartment. Retrovirology *7*, 51.

Hawes, S.E., Sow, P.S., Stern, J.E., Critchlow, C.W., Gottlieb, G.S., and Kiviat, N.B. (2008). Lower levels of HIV-2 than HIV-1 in the female genital tract: correlates and longitudinal assessment of viral shedding. AIDS *22*, 2517-2525.

Hennig, B.J., Velez-Edwards, D.R., Schim van der Loeff, M.F., Bisseye, C., Edwards, T.L., Tacconelli, A., Novelli, G., Aaby, P., Kaye, S., Scott, W.K.*, et al.* (2011). CD4 intragenic SNPs associate with HIV-2 plasma viral load and CD4 count in a community-based study from Guinea-Bissau, West Africa. J Acquir Immune Defic Syndr *56*, 1-8.

Hill, A.V., Allsopp, C.E., Kwiatkowski, D., Anstey, N.M., Twumasi, P., Rowe, P.A., Bennett, S., Brewster, D., McMichael, A.J., and Greenwood, B.M. (1991).

Common west African HLA antigens are associated with protection from severe malaria. Nature *352*, 595-600.

Hodge, J.W., Chakraborty, M., Kudo-Saito, C., Garnett, C.T., and Schlom, J. (2005). Multiple costimulatory modalities enhance CTL avidity. J Immunol *174*, 5994-6004.

Horton, H., Thomas, E.P., Stucky, J.A., Frank, I., Moodie, Z., Huang, Y., Chiu, Y.L., McElrath, M.J., and De Rosa, S.C. (2007). Optimization and validation of an 8-color intracellular cytokine staining (ICS) assay to quantify antigen-specific T cells induced by vaccination. J Immunol Methods *323*, 39-54.

Huang, J., Goedert, J.J., Sundberg, E.J., Cung, T.D., Burke, P.S., Martin, M.P., Preiss, L., Lifson, J., Lichterfeld, M., Carrington, M., and Yu, X.G. (2009). HLA-B*35-Px-mediated acceleration of HIV-1 infection by increased inhibitory immunoregulatory impulses. J Exp Med *206*, 2959-2966.

Hughes, C.A., Robinson, L., Tseng, A., and MacArthur, R.D. (2009). New antiretroviral drugs: a review of the efficacy, safety, pharmacokinetics, and resistance profile of tipranavir, darunavir, etravirine, rilpivirine, maraviroc, and raltegravir. Expert Opin Pharmacother *10*, 2445-2466.

Huse, M., Quann, E.J., and Davis, M.M. (2008). Shouts, whispers and the kiss of death: directional secretion in T cells. Nat Immunol *9*, 1105-1111.

Iancu, E.M., Baumgaertner, P., Wieckowski, S., Speiser, D.E., and Rufer, N. (2011). Profile of a serial killer: cellular and molecular approaches to study individual cytotoxic T-cells following therapeutic vaccination. J Biomed Biotechnol *2011*, 452606.

Iglesias, M.C., Almeida, J.R., Fastenackels, S., van Bockel, D.J., Hashimoto, M., Venturi, V., Gostick, E., Urrutia, A., Wooldridge, L., Clement, M.*, et al.* (2011). Escape from highly effective public CD8+ T-cell clonotypes by HIV. Blood *118*, 2138-2149.

Insaidoo, F.K., Borbulevych, O.Y., Hossain, M., Santhanagopolan, S.M., Baxter, T.K., and Baker, B.M. (2011). Loss of T cell antigen recognition arising from changes in peptide and major histocompatibility complex protein flexibility: implications for vaccine design. J Biol Chem *286*, 40163-40173.

Irving, M., Zoete, V., Hebeisen, M., Schmid, D., Baumgartner, P., Guillaume, P., Romero, P., Speiser, D., Luescher, I., Rufer, N., and Michielin, O. (2012). Interplay between T cell receptor binding kinetics and the level of cognate peptide presented by major histocompatibility complexes governs CD8+ T cell responsiveness. J Biol Chem.

Ishizuka, J., Stewart-Jones, G.B., van der Merwe, A., Bell, J.I., McMichael, A.J., and Jones, E.Y. (2008). The structural dynamics and energetics of an immunodominant T cell receptor are programmed by its Vbeta domain. Immunity *28*, 171-182.

Islam, S.A., Hay, C.M., Hartman, K.E., He, S., Shea, A.K., Trocha, A.K., Dynan, M.J., Reshamwala, N., Buchbinder, S.P., Basgoz, N.O., and Kalams, S.A. (2001). Persistence of human immunodeficiency virus type 1-specific cytotoxic T-lymphocyte clones in a subject with rapid disease progression. J Virol *75*, 4907-4911.

Jaffar, S., Van der Loeff, M.S., Eugen-Olsen, J., Vincent, T., Sarje-Njie, R., Ngom, P., Meyer, A.M., Berry, N., Aaby, P., and Whittle, H. (2005). Immunological predictors of survival in HIV type 2-infected rural villagers in Guinea-Bissau. AIDS Res Hum Retroviruses *21*, 560-564.

Jaffar, S., Wilkins, A., Ngom, P.T., Sabally, S., Corrah, T., Bangali, J.E., Rolfe, M., and Whittle, H.C. (1997). Rate of decline of percentage CD4+ cells is faster in HIV-1 than in HIV-2 infection. J Acquir Immune Defic Syndr Hum Retrovirol *16*, 327-332.

Janbazian, L., Price, D.A., Canderan, G., Filali-Mouhim, A., Asher, T.E., Ambrozak, D.R., Scheinberg, P., Boulassel, M.R., Routy, J.P., Koup, R.A.*, et al.* (2012). Clonotype and repertoire changes drive the functional improvement of HIV-specific CD8 T cell populations under conditions of limited antigenic stimulation. J Immunol *188*, 1156-1167.

Jaye, A., Sarge-Njie, R., Schim van der Loeff, M., Todd, J., Alabi, A., Sabally, S., Corrah, T., and Whittle, H. (2004). No differences in cellular immune responses between asymptomatic HIV type 1- and type 2-infected Gambian patients. J Infect Dis *189*, 498-505.

Jennes, W., Camara, M., Dieye, T., Mboup, S., and Kestens, L. (2008). Higher homologous and lower cross-reactive Gag-specific T-cell responses in human immunodeficiency virus type 2 (HIV-2) than in HIV-1 infection. J Virol *82*, 8619-8628.

Jia, M., Hong, K., Chen, J., Ruan, Y., Wang, Z., Su, B., Ren, G., Zhang, X., Liu, Z., Zhao, Q.*, et al.* (2012). Preferential CTL targeting of Gag is associated with relative viral control in long-term surviving HIV-1 infected former plasma donors from China. Cell Res.

Jin, X., Bauer, D.E., Tuttleton, S.E., Lewin, S., Gettie, A., Blanchard, J., Irwin, C.E., Safrit, J.T., Mittler, J., Weinberger, L.*, et al.* (1999). Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. J Exp Med *189*, 991-998.

Jin, X., Gao, X., Ramanathan, M., Jr., Deschenes, G.R., Nelson, G.W., O'Brien, S.J., Goedert, J.J., Ho, D.D., O'Brien, T.R., and Carrington, M. (2002). Human immunodeficiency virus type 1 (HIV-1)-specific CD8+-T-cell responses for groups of HIV-1-infected individuals with different HLA-B*35 genotypes. J Virol *76*, 12603-12610.

Johnson, R.P., Trocha, A., Yang, L., Mazzara, G.P., Panicali, D.L., Buchanan, T.M., and Walker, B.D. (1991). HIV-1 gag-specific cytotoxic T lymphocytes recognize multiple highly conserved epitopes. Fine specificity of the gag-specific response defined by using unstimulated peripheral blood mononuclear cells and cloned effector cells. J Immunol *147*, 1512-1521.

Jones, L.L., Colf, L.A., Stone, J.D., Garcia, K.C., and Kranz, D.M. (2008). Distinct CDR3 conformations in TCRs determine the level of cross-reactivity for diverse antigens, but not the docking orientation. J Immunol *181*, 6255-6264.

Jones, N.A., Wei, X., Flower, D.R., Wong, M., Michor, F., Saag, M.S., Hahn, B.H., Nowak, M.A., Shaw, G.M., and Borrow, P. (2004). Determinants of human immunodeficiency virus type 1 escape from the primary CD8+ cytotoxic T lymphocyte response. J Exp Med *200*, 1243-1256.

Kaech, S.M., Tan, J.T., Wherry, E.J., Konieczny, B.T., Surh, C.D., and Ahmed, R. (2003). Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat Immunol *4*, 1191-1198.

Kagi, D., Ledermann, B., Burki, K., Zinkernagel, R.M., and Hengartner, H. (1996). Molecular mechanisms of lymphocyte-mediated cytotoxicity and their role in immunological protection and pathogenesis in vivo. Annu Rev Immunol *14*, 207-232.

Kalergis, A.M., Boucheron, N., Doucey, M.A., Palmieri, E., Goyarts, E.C., Vegh, Z., Luescher, I.F., and Nathenson, S.G. (2001). Efficient T cell activation requires an optimal dwell-time of interaction between the TCR and the pMHC complex. Nat Immunol *2*, 229-234.

Kanki, P.J., Travers, K.U., S, M.B., Hsieh, C.C., Marlink, R.G., Gueye, N.A., Siby, T., Thior, I., Hernandez-Avila, M., Sankale, J.L., and et al. (1994). Slower heterosexual spread of HIV-2 than HIV-1. Lancet *343*, 943-946.

Kannangai, R., Nair, S.C., Sridharan, G., Prasannakumar, S., and Daniel, D. (2010). Frequency of HIV type 2 infections among blood donor population from India: a 10-year experience. Indian J Med Microbiol *28*, 111-113.

Kashyap, B., Gautam, H., Chadha, S., and Bhalla, P. (2010). Delayed progression and inefficient transmission of HIV-2. Southeast Asian J Trop Med Public Health *41*, 570-573.

Kati, W.M., Johnson, K.A., Jerva, L.F., and Anderson, K.S. (1992). Mechanism and fidelity of HIV reverse transcriptase. J Biol Chem *267*, 25988-25997.

Kesturu, G.S., Colleton, B.A., Liu, Y., Heath, L., Shaikh, O.S., Rinaldo, C.R., Jr., and Shankarappa, R. (2006). Minimization of genetic distances by the consensus, ancestral, and center-of-tree (COT) sequences for HIV-1 variants within an infected individual and the design of reagents to test immune reactivity. Virology *348*, 437-448.

Kiepiela, P., Leslie, A.J., Honeyborne, I., Ramduth, D., Thobakgale, C., Chetty, S., Rathnavalu, P., Moore, C., Pfafferott, K.J., Hilton, L.*, et al.* (2004). Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. Nature *432*, 769-775.

Kiepiela, P., Ngumbela, K., Thobakgale, C., Ramduth, D., Honeyborne, I., Moodley, E., Reddy, S., de Pierres, C., Mncube, Z., Mkhwanazi, N.*, et al.* (2007). CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. Nat Med *13*, 46-53.

Kim, D.T., Rothbard, J.B., Bloom, D.D., and Fathman, C.G. (1996). Quantitative analysis of T cell activation: role of TCR/ligand density and TCR affinity. J Immunol *156*, 2737-2742.

Kim, S.S., Kim, E.Y., Park, K.Y., Suh, S.D., Park, H.K., Shin, Y.O., Bae, M., and Lee, J.S. (2000). Introduction of human immunodeficiency virus 2 infection into South Korea. Acta Virol *44*, 15-22.

Klein, M.R., van Baalen, C.A., Holwerda, A.M., Kerkhof Garde, S.R., Bende, R.J., Keet, I.P., Eeftinck-Schattenkerk, J.K., Osterhaus, A.D., Schuitemaker, H., and Miedema, F. (1995). Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. J Exp Med *181*, 1365-1372.

Kloetzel, P.M., and Ossendorp, F. (2004). Proteasome and peptidase function in MHC-class-I-mediated antigen presentation. Curr Opin Immunol *16*, 76-81.

Kloverpris, H.N., Stryhn, A., Harndahl, M., van der Stok, M., Payne, R.P., Matthews, P.C., Chen, F., Riddell, L., Walker, B.D., Ndung'u, T.*, et al.* (2012). HLA-B*57 Micropolymorphism shapes HLA allele-specific epitope immunogenicity, selection pressure, and HIV immune control. J Virol *86*, 919-929.

Knudsen, M.L., Mbewe-Mvula, A., Rosario, M., Johansson, D.X., Kakoulidou, M., Bridgeman, A., Reyes-Sandoval, A., Nicosia, A., Ljungberg, K., Hanke, T., and Liljestrom, P. (2012). Superior Induction of T Cell Responses to Conserved HIV-1 Regions by Electroporated Alphavirus Replicon DNA Compared to That with Conventional Plasmid DNA Vaccine. J Virol *86*, 4082-4090.

Kong, R., Li, H., Bibollet-Ruche, F., Decker, J.M., Zheng, N.N., Gottlieb, G.S., Kiviat, N.B., Sow, P.S., Georgiev, I., Hahn, B.H., *et al.* (2012). Broad and potent neutralizing antibody responses elicited in natural HIV-2 infection. J Virol *86*, 947-960.

Korber, B.T., Letvin, N.L., and Haynes, B.F. (2009). T-cell vaccine strategies for human immunodeficiency virus, the virus with a thousand faces. J Virol *83*, 8300-8314.

Kosmrlj, A., Read, E.L., Qi, Y., Allen, T.M., Altfeld, M., Deeks, S.G., Pereyra, F., Carrington, M., Walker, B.D., and Chakraborty, A.K. (2010). Effects of thymic selection of the T-cell repertoire on HLA class I-associated control of HIV infection. Nature *465*, 350-354.

Koup, R.A., Safrit, J.T., Cao, Y., Andrews, C.A., McLeod, G., Borkowsky, W., Farthing, C., and Ho, D.D. (1994). Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol *68*, 4650-4655.

Kramer, A.B., Roozendaal, C., and Dullaart, R.P. (2004). Familial occurrence of subacute thyroiditis associated with human leukocyte antigen-B35. Thyroid *14*, 544-547.

Krangel, M.S. (2003). Gene segment selection in V(D)J recombination: accessibility and beyond. Nat Immunol *4*, 624-630.

Krogsgaard, M., Prado, N., Adams, E.J., He, X.L., Chow, D.C., Wilson, D.B., Garcia, K.C., and Davis, M.M. (2003). Evidence that structural rearrangements and/or flexibility during TCR binding can contribute to T cell activation. Mol Cell *12*, 1367-1378.

Kwong, P.D., Wyatt, R., Robinson, J., Sweet, R.W., Sodroski, J., and Hendrickson, W.A. (1998). Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature *393*, 648-659.

Laguette, N., and Benkirane, M. (2012). How SAMHD1 changes our view of viral restriction. Trends Immunol *33*, 26-33.

Laguette, N., Bregnard, C., Benichou, S., and Basmaciogullari, S. (2010). Human immunodeficiency virus (HIV) type-1, HIV-2 and simian immunodeficiency virus Nef proteins. Mol Aspects Med *31*, 418-433.

Laguette, N., Sobhian, B., Casartelli, N., Ringeard, M., Chable-Bessia, C., Segeral, E., Yatim, A., Emiliani, S., Schwartz, O., and Benkirane, M. (2011). SAMHD1 is the dendritic- and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. Nature *474*, 654-657.

Lang, H.L., Jacobsen, H., Ikemizu, S., Andersson, C., Harlos, K., Madsen, L., Hjorth, P., Sondergaard, L., Svejgaard, A., Wucherpfennig, K., *et al.* (2002). A functional and structural basis for TCR cross-reactivity in multiple sclerosis. Nat Immunol *3*, 940-943.

Lawson, T.M., Man, S., Williams, S., Boon, A.C., Zambon, M., and Borysiewicz, L.K. (2001). Influenza A antigen exposure selects dominant Vbeta17+ TCR in human CD8+ cytotoxic T cell responses. Int Immunol *13*, 1373-1381.

Le Tortorec, A., and Neil, S.J. (2009). Antagonism to and intracellular sequestration of human tetherin by the human immunodeficiency virus type 2 envelope glycoprotein. J Virol *83*, 11966-11978.

Lee, J.K., Stewart-Jones, G., Dong, T., Harlos, K., Di Gleria, K., Dorrell, L., Douek, D.C., van der Merwe, P.A., Jones, E.Y., and McMichael, A.J. (2004). T cell cross-reactivity and conformational changes during TCR engagement. J Exp Med *200*, 1455-1466.

Leligdowicz, A., Feldmann, J., Jaye, A., Cotten, M., Dong, T., McMichael, A., Whittle, H., and Rowland-Jones, S. (2010a). Direct relationship between virus load and systemic immune activation in HIV-2 infection. J Infect Dis *201*, 114-122.

Leligdowicz, A., Onyango, C., Yindom, L.M., Peng, Y., Cotten, M., Jaye, A., McMichael, A., Whittle, H., Dong, T., and Rowland-Jones, S. (2010b). Highly avid, oligoclonal, early-differentiated antigen-specific CD8+ T cells in chronic HIV-2 infection. Eur J Immunol *40*, 1963-1972.

Leligdowicz, A., Yindom, L.M., Onyango, C., Sarge-Njie, R., Alabi, A., Cotten, M., Vincent, T., da Costa, C., Aaby, P., Jaye, A.*, et al.* (2007). Robust Gag-specific T cell responses characterize viremia control in HIV-2 infection. J Clin Invest *117*, 3067-3074.

Letourneau, S., Im, E.J., Mashishi, T., Brereton, C., Bridgeman, A., Yang, H., Dorrell, L., Dong, T., Korber, B., McMichael, A.J., and Hanke, T. (2007). Design and pre-clinical evaluation of a universal HIV-1 vaccine. PLoS One *2*, e984.

Li, H., Lebedeva, M.I., Ward, E.S., and Mariuzza, R.A. (1997). Dual conformations of a T cell receptor V alpha homodimer: implications for variability in V alpha V beta domain association. J Mol Biol *269*, 385-394.

Li, Q., Smith, A.J., Schacker, T.W., Carlis, J.V., Duan, L., Reilly, C.S., and Haase, A.T. (2009). Microarray analysis of lymphatic tissue reveals stage-specific, gene expression signatures in HIV-1 infection. J Immunol *183*, 1975-1982.

Li, Q.J., Chau, J., Ebert, P.J., Sylvester, G., Min, H., Liu, G., Braich, R., Manoharan, M., Soutschek, J., Skare, P.*, et al.* (2007). miR-181a is an intrinsic modulator of T cell sensitivity and selection. Cell *129*, 147-161.

Li, Y., Moysey, R., Molloy, P.E., Vuidepot, A.L., Mahon, T., Baston, E., Dunn, S., Liddy, N., Jacob, J., Jakobsen, B.K., and Boulter, J.M. (2005). Directed evolution of human T-cell receptors with picomolar affinities by phage display. Nat Biotechnol *23*, 349-354.

Liberatore, R.A., and Bieniasz, P.D. (2011). Tetherin is a key effector of the antiretroviral activity of type I interferon in vitro and in vivo. Proc Natl Acad Sci U S A *108*, 18097-18101.

Lichterfeld, M., Yu, X.G., Mui, S.K., Williams, K.L., Trocha, A., Brockman, M.A., Allgaier, R.L., Waring, M.T., Koibuchi, T., Johnston, M.N.*, et al.* (2007). Selective depletion of high-avidity human immunodeficiency virus type 1 (HIV-1)-specific CD8+ T cells after early HIV-1 infection. J Virol *81*, 4199-4214.

Lim, A., Trautmann, L., Peyrat, M.A., Couedel, C., Davodeau, F., Romagne, F., Kourilsky, P., and Bonneville, M. (2000). Frequent contribution of T cell clonotypes with public TCR features to the chronic response against a dominant EBV-derived epitope: application to direct detection of their molecular imprint on the human peripheral T cell repertoire. J Immunol *165*, 2001-2011.

Liu, H.Y., Soda, Y., Shimizu, N., Haraguchi, Y., Jinno, A., Takeuchi, Y., and Hoshino, H. (2000). CD4-Dependent and CD4-independent utilization of coreceptors by human immunodeficiency viruses type 2 and simian immunodeficiency viruses. Virology *278*, 276-288.

Loll, B., Ruckert, C., Hee, C.S., Saenger, W., Uchanska-Ziegler, B., and Ziegler, A. (2011). Loss of recognition by cross-reactive T cells and its relation to a C-terminus-induced conformational reorientation of an HLA-B*2705-bound peptide. Protein Sci *20*, 278-290.

Lopes, A.R., Jaye, A., Dorrell, L., Sabally, S., Alabi, A., Jones, N.A., Flower, D.R., De Groot, A., Newton, P., Lascar, R.M.*, et al.* (2003). Greater CD8+ TCR

heterogeneity and functional flexibility in HIV-2 compared to HIV-1 infection. J Immunol *171*, 307-316.

Lukic, Z., and Campbell, E.M. (2012). The Cell Biology of TRIM5alpha. Curr HIV/AIDS Rep *9*, 73-80.

Lyons, D.S., Lieberman, S.A., Hampl, J., Boniface, J.J., Chien, Y., Berg, L.J., and Davis, M.M. (1996). A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. Immunity *5*, 53-61.

Macdonald, W.A., Purcell, A.W., Mifsud, N.A., Ely, L.K., Williams, D.S., Chang, L., Gorman, J.J., Clements, C.S., Kjer-Nielsen, L., Koelle, D.M*., et al.* (2003). A naturally selected dimorphism within the HLA-B44 supertype alters class I structure, peptide repertoire, and T cell recognition. J Exp Med *198*, 679-691.

MacNeil, A., Sankale, J.L., Meloni, S.T., Sarr, A.D., Mboup, S., and Kanki, P. (2006). Genomic sites of human immunodeficiency virus type 2 (HIV-2) integration: similarities to HIV-1 in vitro and possible differences in vivo. J Virol *80*, 7316-7321.

MacNeil, A., Sarr, A.D., Sankale, J.L., Meloni, S.T., Mboup, S., and Kanki, P. (2007). Direct evidence of lower viral replication rates in vivo in human immunodeficiency virus type 2 (HIV-2) infection than in HIV-1 infection. J Virol *81*, 5325-5330.

Madec, Y., Boufassa, F., Avettand-Fenoel, V., Hendou, S., Melard, A., Boucherit, S., Surzyn, J., Meyer, L., and Rouzioux, C. (2009). Early control of HIV-1 infection in long-term nonprogressors followed since diagnosis in the ANRS SEROCO/HEMOCO cohort. J Acquir Immune Defic Syndr *50*, 19-26.

Mandalia, S., Westrop, S.J., Beck, E.J., Nelson, M., Gazzard, B.G., and Imami, N. (2012). Are Long-Term Non-Progressors Very Slow Progressors? Insights from the Chelsea and Westminster HIV Cohort, 1988-2010. PLoS One *7*, e29844.

Mann, B., Milloy, M.J., Kerr, T., Zhang, R., Montaner, J., and Wood, E. (2012). Improved adherence to modern antiretroviral therapy among HIV-infected injecting drug users. HIV Med.

Mansson, F., Biague, A., da Silva, Z.J., Dias, F., Nilsson, L.A., Andersson, S., Fenyo, E.M., and Norrgren, H. (2009). Prevalence and incidence of HIV-1 and HIV-2 before, during and after a civil war in an occupational cohort in Guinea-Bissau, West Africa. AIDS *23*, 1575-1582.

Margulies, D.H. (2001). TCR avidity: it's not how strong you make it, it's how you make it strong. Nat Immunol *2*, 669-670.

Margulies, D.H., Plaksin, D., Khilko, S.N., and Jelonek, M.T. (1996). Studying interactions involving the T-cell antigen receptor by surface plasmon resonance. Curr Opin Immunol *8*, 262-270.

Markovitz, D.M., Hannibal, M., Perez, V.L., Gauntt, C., Folks, T.M., and Nabel, G.J. (1990). Differential regulation of human immunodeficiency viruses (HIVs): a specific regulatory element in HIV-2 responds to stimulation of the T-cell antigen receptor. Proc Natl Acad Sci U S A *87*, 9098-9102.

Marlink, R., Kanki, P., Thior, I., Travers, K., Eisen, G., Siby, T., Traore, I., Hsieh, C.C., Dia, M.C., Gueye, E.H., and et al. (1994). Reduced rate of disease development after HIV-2 infection as compared to HIV-1. Science *265*, 1587-1590.

Marlink, R.G., Ricard, D., M'Boup, S., Kanki, P.J., Romet-Lemonne, J.L., N'Doye, I., Diop, K., Simpson, M.A., Greco, F., Chou, M.J., and et al. (1988). Clinical, hematologic, and immunologic cross-sectional evaluation of individuals exposed to human immunodeficiency virus type-2 (HIV-2). AIDS Res Hum Retroviruses *4*, 137-148.

Martinez-Picado, J., Prado, J.G., Fry, E.E., Pfafferott, K., Leslie, A., Chetty, S., Thobakgale, C., Honeyborne, I., Crawford, H., Matthews, P.*, et al.* (2006). Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. J Virol *80*, 3617-3623.

Martinez-Steele, E., Awasana, A.A., Corrah, T., Sabally, S., van der Sande, M., Jaye, A., Togun, T., Sarge-Njie, R., McConkey, S.J., Whittle, H., and Schim van der Loeff, M.F. (2007). Is HIV-2- induced AIDS different from HIV-1-associated AIDS? Data from a West African clinic. AIDS *21*, 317-324.

Mason, D. (1998). A very high level of crossreactivity is an essential feature of the T-cell receptor. Immunol Today *19*, 395-404.

Matheron, S., Damond, F., Benard, A., Taieb, A., Campa, P., Peytavin, G., Pueyo, S., Brun-Vezinet, F., and Chene, G. (2006). CD4 cell recovery in treated HIV-2-infected adults is lower than expected: results from the French ANRS CO5 HIV-2 cohort. AIDS *20*, 459-462.

Matheron, S., Pueyo, S., Damond, F., Simon, F., Lepretre, A., Campa, P., Salamon, R., Chene, G., and Brun-Vezinet, F. (2003). Factors associated with clinical progression in HIV-2 infected-patients: the French ANRS cohort. AIDS *17*, 2593-2601.

Mathew, P.A., Garni-Wagner, B.A., Land, K., Takashima, A., Stoneman, E., Bennett, M., and Kumar, V. (1993). Cloning and characterization of the 2B4 gene encoding a molecule associated with non-MHC-restricted killing mediated by activated natural killer cells and T cells. J Immunol *151*, 5328-5337.

Matsui, T., Tanaka, T., Endoh, H., Sato, K., Tanaka, H., Miyauchi, E., Kawashima, Y., Nagai-Makabe, M., Komatsu, H., Kohno, T.*, et al.* (2009). The RNA recognition mechanism of human immunodeficiency virus (HIV) type 2 NCp8 is different from that of HIV-1 NCp7. Biochemistry *48*, 4314-4323.

Maueia, C., Costa, D., Meggi, B., Ismael, N., Walle, C., Curvo, R., Abreu, C., Bhatt, N., Tanuri, A., Jani, I., and Ferreira, O. (2011). Frequency of human immunodeficiency virus type-2 in hiv infected patients in Maputo City, Mozambique. Virology Journal *8*, 408.

McElrath, M.J., De Rosa, S.C., Moodie, Z., Dubey, S., Kierstead, L., Janes, H., Defawe, O.D., Carter, D.K., Hural, J., Akondy, R.*, et al.* (2008). HIV-1 vaccine-induced immunity in the test-of-concept Step Study: a case-cohort analysis. Lancet *372*, 1894-1905.

Mellors, J.W., Rinaldo, C.R., Jr., Gupta, P., White, R.M., Todd, J.A., and Kingsley, L.A. (1996). Prognosis in HIV-1 infection predicted by the quantity of virus in plasma. Science *272*, 1167-1170.

Meyer-Olson, D., Brady, K.W., Bartman, M.T., O'Sullivan, K.M., Simons, B.C., Conrad, J.A., Duncan, C.B., Lorey, S., Siddique, A., Draenert, R.*, et al.* (2006). Fluctuations of functionally distinct CD8+ T-cell clonotypes demonstrate flexibility of the HIV-specific TCR repertoire. Blood *107*, 2373-2383.

Migueles, S.A., Sabbaghian, M.S., Shupert, W.L., Bettinotti, M.P., Marincola, F.M., Martino, L., Hallahan, C.W., Selig, S.M., Schwartz, D., Sullivan, J., and Connors, M. (2000). HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. Proc Natl Acad Sci U S A *97*, 2709-2714.

Miles, J.J., Silins, S.L., Brooks, A.G., Davis, J.E., Misko, I., and Burrows, S.R. (2005). T-cell grit: large clonal expansions of virus-specific CD8+ T cells can dominate in the peripheral circulation for at least 18 years. Blood *106*, 4412-4413.

Miles, J.J., Thammanichanond, D., Moneer, S., Nivarthi, U.K., Kjer-Nielsen, L., Tracy, S.L., Aitken, C.K., Brennan, R.M., Zeng, W., Marquart, L.*, et al.* (2011). Antigen-driven patterns of TCR bias are shared across diverse outcomes of human hepatitis C virus infection. J Immunol *186*, 901-912.

Monteiro, J., Hingorani, R., Choi, I.H., Silver, J., Pergolizzi, R., and Gregersen, P.K. (1995). Oligoclonality in the human CD8+ T cell repertoire in normal subjects and monozygotic twins: implications for studies of infectious and autoimmune diseases. Mol Med *1*, 614-624.

Moore, C.B., John, M., James, I.R., Christiansen, F.T., Witt, C.S., and Mallal, S.A. (2002). Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. Science *296*, 1439-1443.

Mota, A.H., Fainboim, H., Terg, R., and Fainboim, L. (1987). Association of chronic active hepatitis and HLA B35 in patients with hepatitis B virus. Tissue Antigens *30*, 238-240.

Mothe, B., Llano, A., Ibarrondo, J., Zamarreno, J., Schiaulini, M., Miranda, C., Ruiz-Riol, M., Berger, C.T., Herrero, M.J., Palou, E.*, et al.* (2012). CTL responses of high functional avidity and broad variant cross-reactivity are associated with HIV control. PLoS One *7*, e29717.

Motozono, C., Yanaka, S., Tsumoto, K., Takiguchi, M., and Ueno, T. (2009). Impact of intrinsic cooperative thermodynamics of peptide-MHC complexes on antiviral activity of HIV-specific CTL. J Immunol *182*, 5528-5536.

Murphy, K.E., Travers, P., and Walport, M. (2008). Janeway's Immunobiology. D. Schanck, ed. (New York: Garland Science, Francis & Taylor group), pp. 126-132.

Neefjes, J., Jongsma, M.L., Paul, P., and Bakke, O. (2011). Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nat Rev Immunol *11*, 823-836.

Nickle, D.C., Jensen, M.A., Gottlieb, G.S., Shriner, D., Learn, G.H., Rodrigo, A.G., and Mullins, J.I. (2003). Consensus and ancestral state HIV vaccines. Science *299*, 1515-1518; author reply 1515-1518.

Nikolova, M., Marie-Cardine, A., Boumsell, L., and Bensussan, A. (2002). BY55/CD160 acts as a co-receptor in TCR signal transduction of a human circulating cytotoxic effector T lymphocyte subset lacking CD28 expression. Int Immunol *14*, 445-451.

Nikolova, M.H., Muhtarova, M.N., Taskov, H.B., Kostov, K., Vezenkov, L., Mihova, A., Boumsell, L., and Bensussan, A. (2005). The CD160+ CD8high cytotoxic T cell subset correlates with response to HAART in HIV-1+ patients. Cell Immunol *237*, 96-105.

Ning, R.J., Xu, X.Q., Chan, K.H., and Chiang, A.K. (2011). Long-term carriers generate Epstein-Barr virus (EBV)-specific CD4(+) and CD8(+) polyfunctional T-cell responses which show immunodominance hierarchies of EBV proteins. Immunology *134*, 161-171.

Niu, L., Termini, J.M., Kanagavelu, S.K., Gupta, S., Rolland, M.M., Kulkarni, V., Pavlakis, G.N., Felber, B.K., Mullins, J.I., Fischl, M.A., and Stone, G.W. (2011). Preclinical evaluation of HIV-1 therapeutic ex vivo dendritic cell vaccines expressing consensus Gag antigens and conserved Gag epitopes. Vaccine *29*, 2110-2119.

Northrop, J.K., Thomas, R.M., Wells, A.D., and Shen, H. (2006). Epigenetic remodeling of the IL-2 and IFN-gamma loci in memory CD8 T cells is influenced by CD4 T cells. J Immunol *177*, 1062-1069.

Nowroozalizadeh, S., Mansson, F., da Silva, Z., Repits, J., Dabo, B., Pereira, C., Biague, A., Albert, J., Nielsen, J., Aaby, P., *et al.* (2009). Studies on toll-like receptor stimuli responsiveness in HIV-1 and HIV-2 infections. Cytokine *46*, 325-331.

Nowroozalizadeh, S., Mansson, F., da Silva, Z., Repits, J., Dabo, B., Pereira, C., Biague, A., Albert, J., Nielsen, J., Aaby, P., *et al.* (2010). Microbial translocation correlates with the severity of both HIV-1 and HIV-2 infections. J Infect Dis *201*, 1150-1154.

Nuvor, S.V., van der Sande, M., Rowland-Jones, S., Whittle, H., and Jaye, A. (2006). Natural killer cell function is well preserved in asymptomatic human immunodeficiency virus type 2 (HIV-2) infection but similar to that of HIV-1 infection when CD4 T-cell counts fall. J Virol *80*, 2529-2538.

O'Donovan, D., Ariyoshi, K., Milligan, P., Ota, M., Yamuah, L., Sarge-Njie, R., and Whittle, H. (2000). Maternal plasma viral RNA levels determine marked differences in mother-to-child transmission rates of HIV-1 and HIV-2 in The Gambia. MRC/Gambia Government/University College London Medical School working group on mother-child transmission of HIV. AIDS *14*, 441-448.

Onyango, C.O., Leligdowicz, A., Yokoyama, M., Sato, H., Song, H., Nakayama, E.E., Shioda, T., de Silva, T., Townend, J., Jaye, A., *et al.* (2010). HIV-2 capsids distinguish high and low virus load patients in a West African community cohort. Vaccine *28 Suppl 2*, B60-67.

Ozkaya Sahin, G., Holmgren, B., da Silva, Z., Nielsen, J., Nowroozalizadeh, S., Esbjornsson, J., Mansson, F., Andersson, S., Norrgren, H., Aaby, P., *et al.* (2012). Potent intratype neutralizing activity distinguishes human immunodeficiency virus type 2 (HIV-2) from HIV-1. J Virol *86*, 961-971.

Pantaleo, G., Soudeyns, H., Demarest, J.F., Vaccarezza, M., Graziosi, C., Paolucci, S., Daucher, M., Cohen, O.J., Denis, F., Biddison, W.E., *et al.* (1997). Evidence for rapid disappearance of initially expanded HIV-specific CD8+ T cell clones during primary HIV infection. Proc Natl Acad Sci U S A *94*, 9848-9853.

Pepin, J., Plamondon, M., Alves, A.C., Beaudet, M., and Labbe, A.C. (2006). Parenteral transmission during excision and treatment of tuberculosis and trypanosomiasis may be responsible for the HIV-2 epidemic in Guinea-Bissau. AIDS *20*, 1303-1311.

Peretz, Y., He, Z., Shi, Y., Yassine-Diab, B., Goulet, J.P., Bordi, R., Filali-Mouhim, A., Loubert, J.B., El-Far, M., Dupuy, F.P., *et al.* (2012). CD160 and PD-1 Co-Expression on HIV-Specific CD8 T Cells Defines a Subset with Advanced Dysfunction. PLoS Pathog *8*, e1002840.

Petrova, G.V., Naumova, E.N., and Gorski, J. (2011). The polyclonal CD8 T cell response to influenza M158-66 generates a fully connected network of cross-reactive clonotypes to structurally related peptides: a paradigm for memory repertoire coverage of novel epitopes or escape mutants. J Immunol *186*, 6390-6397.

Petrovas, C., Casazza, J.P., Brenchley, J.M., Price, D.A., Gostick, E., Adams, W.C., Precopio, M.L., Schacker, T., Roederer, M., Douek, D.C., and Koup, R.A. (2006). PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. J Exp Med *203*, 2281-2292.

Petrovas, C., Price, D.A., Mattapallil, J., Ambrozak, D.R., Geldmacher, C., Cecchinato, V., Vaccari, M., Tryniszewska, E., Gostick, E., Roederer, M., *et al.* (2007). SIV-specific CD8+ T cells express high levels of PD1 and cytokines but have

impaired proliferative capacity in acute and chronic SIVmac251 infection. Blood *110*, 928-936.

Piepenbrink, K.H., Gloor, B.E., Armstrong, K.M., and Baker, B.M. (2009). Methods for quantifying T cell receptor binding affinities and thermodynamics. Methods Enzymol *466*, 359-381.

Poli, G., Pantaleo, G., and Fauci, A.S. (1993). Immunopathogenesis of human immunodeficiency virus infection. Clin Infect Dis *17 Suppl 1*, S224-229.

Pontesilli, O., Klein, M.R., Kerkhof-Garde, S.R., Pakker, N.G., de Wolf, F., Schuitemaker, H., and Miedema, F. (1998). Longitudinal analysis of human immunodeficiency virus type 1-specific cytotoxic T lymphocyte responses: a predominant gag-specific response is associated with nonprogressive infection. J Infect Dis *178*, 1008-1018.

Popper, S.J., Sarr, A.D., Gueye-Ndiaye, A., Mboup, S., Essex, M.E., and Kanki, P.J. (2000). Low plasma human immunodeficiency virus type 2 viral load is independent of proviral load: low virus production in vivo. J Virol *74*, 1554-1557.

Popper, S.J., Sarr, A.D., Travers, K.U., Gueye-Ndiaye, A., Mboup, S., Essex, M.E., and Kanki, P.J. (1999). Lower human immunodeficiency virus (HIV) type 2 viral load reflects the difference in pathogenicity of HIV-1 and HIV-2. J Infect Dis *180*, 1116-1121.

Poulsen, A.G., Aaby, P., Jensen, H., and Dias, F. (2000). Risk factors for HIV-2 seropositivity among older people in Guinea-Bissau. A search for the early history of HIV-2 infection. Scand J Infect Dis *32*, 169-175.

Poulsen, A.G., Aaby, P., Larsen, O., Jensen, H., Naucler, A., Lisse, I.M., Christiansen, C.B., Dias, F., and Melbye, M. (1997). 9-year HIV-2-associated mortality in an urban community in Bissau, west Africa. Lancet *349*, 911-914.

Poulsen, A.G., Kvinesdal, B.B., Aaby, P., Lisse, I.M., Gottschau, A., Molbak, K., Dias, F., and Lauritzen, E. (1992). Lack of evidence of vertical transmission of human immunodeficiency virus type 2 in a sample of the general population in Bissau. J Acquir Immune Defic Syndr *5*, 25-30.

Price, A.J., Marzetta, F., Lammers, M., Ylinen, L.M., Schaller, T., Wilson, S.J., Towers, G.J., and James, L.C. (2009a). Active site remodeling switches HIV specificity of antiretroviral TRIMCyp. Nat Struct Mol Biol *16*, 1036-1042.

Price, D.A., Asher, T.E., Wilson, N.A., Nason, M.C., Brenchley, J.M., Metzler, I.S., Venturi, V., Gostick, E., Chattopadhyay, P.K., Roederer, M.*, et al.* (2009b). Public clonotype usage identifies protective Gag-specific CD8+ T cell responses in SIV infection. J Exp Med *206*, 923-936.

Price, D.A., Brenchley, J.M., Ruff, L.E., Betts, M.R., Hill, B.J., Roederer, M., Koup, R.A., Migueles, S.A., Gostick, E., Wooldridge, L.*, et al.* (2005). Avidity for antigen shapes clonal dominance in CD8+ T cell populations specific for persistent DNA viruses. J Exp Med *202*, 1349-1361.

Price, D.A., West, S.M., Betts, M.R., Ruff, L.E., Brenchley, J.M., Ambrozak, D.R., Edghill-Smith, Y., Kuroda, M.J., Bogdan, D., Kunstman, K.*, et al.* (2004). T cell receptor recognition motifs govern immune escape patterns in acute SIV infection. Immunity *21*, 793-803.

Purbhoo, M.A., Irvine, D.J., Huppa, J.B., and Davis, M.M. (2004). T cell killing does not require the formation of a stable mature immunological synapse. Nat Immunol *5*, 524-530.

Quigley, M.i.F., Greenaway, H.Y., Venturi, V., Lindsay, R., Quinn, K.M., Seder, R.A., Douek, D.C., Davenport, M.P., and Price, D.A. (2010). Convergent

recombination shapes the clonotypic landscape of the na√ Øve T-cell repertoire. Proceedings of the National Academy of Sciences *107*, 19414-19419.

Rambaut, A., Posada, D., Crandall, K.A., and Holmes, E.C. (2004). The causes and consequences of HIV evolution. Nat Rev Genet *5*, 52-61.

Rappuoli, R., and Aderem, A. (2011). A 2020 vision for vaccines against HIV, tuberculosis and malaria. Nature *473*, 463-469.

Reeves, J.D., Hibbitts, S., Simmons, G., McKnight, A., Azevedo-Pereira, J.M., Moniz-Pereira, J., and Clapham, P.R. (1999). Primary human immunodeficiency virus type 2 (HIV-2) isolates infect CD4-negative cells via CCR5 and CXCR4: comparison with HIV-1 and simian immunodeficiency virus and relevance to cell tropism in vivo. J Virol *73*, 7795-7804.

Rerks-Ngarm, S., Pitisuttithum, P., Nitayaphan, S., Kaewkungwal, J., Chiu, J., Paris, R., Premsri, N., Namwat, C., de Souza, M., Adams, E.*, et al.* (2009). Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. N Engl J Med *361*, 2209-2220.

Reuter, S., Kaumanns, P., Buschhorn, S.B., and Dittmar, M.T. (2005). Role of HIV-2 envelope in Lv2-mediated restriction. Virology *332*, 347-358.

Reymond, A., Meroni, G., Fantozzi, A., Merla, G., Cairo, S., Luzi, L., Riganelli, D., Zanaria, E., Messali, S., Cainarca, S.*, et al.* (2001). The tripartite motif family identifies cell compartments. EMBO J *20*, 2140-2151.

Ribeiro, A.C., Maia e Silva, A., Santa-Marta, M., Pombo, A., Moniz-Pereira, J., Goncalves, J., and Barahona, I. (2005). Functional analysis of Vif protein shows less restriction of human immunodeficiency virus type 2 by APOBEC3G. J Virol *79*, 823-833.

Riva, A., Vicenzi, E., Galli, M., and Poli, G. (2011). Strenuous resistance to natural HIV-1 disease progression: viral controllers and long-term nonprogressors. Future Virol *6*, 521-533.

Riviere, Y., McChesney, M.B., Porrot, F., Tanneau-Salvadori, F., Sansonetti, P., Lopez, O., Pialoux, G., Feuillie, V., Mollereau, M., Chamaret, S., and et al. (1995). Gag-specific cytotoxic responses to HIV type 1 are associated with a decreased risk of progression to AIDS-related complex or AIDS. AIDS Res Hum Retroviruses *11*, 903-907.

Robbins, P.F., Li, Y.F., El-Gamil, M., Zhao, Y., Wargo, J.A., Zheng, Z., Xu, H., Morgan, R.A., Feldman, S.A., Johnson, L.A.*, et al.* (2008). Single and dual amino acid substitutions in TCR CDRs can enhance antigen-specific T cell functions. J Immunol *180*, 6116-6131.

Rock, K.L., Farfan-Arribas, D.J., and Shen, L. (2010). Proteases in MHC class I presentation and cross-presentation. J Immunol *184*, 9-15.

Roomp, K., and Domingues, F.S. (2011). Predicting interactions between T cell receptors and MHC-peptide complexes. Mol Immunol *48*, 553-562.

Rosario, M., Bridgeman, A., Quakkelaar, E.D., Quigley, M.F., Hill, B.J., Knudsen, M.L., Ammendola, V., Ljungberg, K., Borthwick, N., Im, E.J.*, et al.* (2010). Long peptides induce polyfunctional T cells against conserved regions of HIV-1 with superior breadth to single-gene vaccines in macaques. Eur J Immunol *40*, 1973-1984.

Rowland-Jones, S. (1999). Long-term non-progression in HIV infection: clinico pathological issues. Journal of Infection *38*, 67-70

.

Rowland-Jones, S., Colbert, R.A., Dong, T., McAdam, S., Brown, M., Ariyoshi, K., Sabally, S., Whittle, H., and McMichael, A. (1998a). Distinct recognition of

closely-related HIV-1 and HIV-2 cytotoxic T-cell epitopes presented by HLA-B*2703 and B*2705. AIDS *12*, 1391-1393.

Rowland-Jones, S., Sutton, J., Ariyoshi, K., Dong, T., Gotch, F., McAdam, S., Whitby, D., Sabally, S., Gallimore, A., Corrah, T., and et al. (1995). HIV-specific cytotoxic T-cells in HIV-exposed but uninfected Gambian women. Nat Med *1*, 59-64.

Rowland-Jones, S.L., Dong, T., Dorrell, L., Ogg, G., Hansasuta, P., Krausa, P., Kimani, J., Sabally, S., Ariyoshi, K., Oyugi, J.*, et al.* (1999). Broadly cross-reactive HIV-specific cytotoxic T-lymphocytes in highly-exposed persistently seronegative donors. Immunol Lett *66*, 9-14.

Rowland-Jones, S.L., Dong, T., Fowke, K.R., Kimani, J., Krausa, P., Newell, H., Blanchard, T., Ariyoshi, K., Oyugi, J., Ngugi, E.*, et al.* (1998b). Cytotoxic T cell responses to multiple conserved HIV epitopes in HIV-resistant prostitutes in Nairobi. J Clin Invest *102*, 1758-1765.

Rowland-Jones, S.L., Pinheiro, S., Kaul, R., Hansasuta, P., Gillespie, G., Dong, T., Plummer, F.A., Bwayo, J.B., Fidler, S., Weber, J.*, et al.* (2001). How important is the 'quality' of the cytotoxic T lymphocyte (CTL) response in protection against HIV infection? Immunol Lett *79*, 15-20.

Rowland-Jones, S.L., and Whittle, H.C. (2007). Out of Africa: what can we learn from HIV-2 about protective immunity to HIV-1? Nat Immunol *8*, 329-331.

Rudd, B.D., Venturi, V., Davenport, M.P., and Nikolich-Zugich, J. (2011). Evolution of the antigen-specific CD8+ TCR repertoire across the life span: evidence for clonal homogenization of the old TCR repertoire. J Immunol *186*, 2056-2064.

Rufer, N. (2005). Molecular tracking of antigen-specific T-cell clones during immune responses. Curr Opin Immunol *17*, 441-447.

Sabin, C.A., Devereux, H., Phillips, A.N., Hill, A., Janossy, G., Lee, C.A., and Loveday, C. (2000). Course of viral load throughout HIV-1 infection. J Acquir Immune Defic Syndr *23*, 172-177.

Saez-Cirion, A., Lacabaratz, C., Lambotte, O., Versmisse, P., Urrutia, A., Boufassa, F., Barre-Sinoussi, F., Delfraissy, J.F., Sinet, M., Pancino, G., and Venet, A. (2007). HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection ex vivo and peculiar cytotoxic T lymphocyte activation phenotype. Proc Natl Acad Sci U S A *104*, 6776-6781.

Saez-Cirion, A., Shin, S.Y., Versmisse, P., Barre-Sinoussi, F., and Pancino, G. (2010). Ex vivo T cell-based HIV suppression assay to evaluate HIV-specific CD8+ T-cell responses. Nat Protoc *5*, 1033-1041.

Salisch, N.C., Kaufmann, D.E., Awad, A.S., Reeves, R.K., Tighe, D.P., Li, Y., Piatak, M., Jr., Lifson, J.D., Evans, D.T., Pereyra, F.*, et al.* (2010). Inhibitory TCR coreceptor PD-1 is a sensitive indicator of low-level replication of SIV and HIV-1. J Immunol *184*, 476-487.

Salmon-Ceron, D., Durier, C., Desaint, C., Cuzin, Surenaud, M., Henin, Y., Lelievre, J., Bonnet, B., Pialoux, G., Poizot-Martin, I.*, et al.* (2009). OA04-01. Safety and immunogenicity of LIPO-5, a HIV-1 lipopeptide vaccine: results of ANRS VAC18, a phase 2, randomized, double-blind, placebo-controlled trial. Retrovirology *6*, O25.

Schaubert, K.L., Price, D.A., Frahm, N., Li, J., Ng, H.L., Joseph, A., Paul, E., Majumder, B., Ayyavoo, V., Gostick, E.*, et al.* (2007). Availability of a Diversely Avid CD8+ T Cell Repertoire Specific for the Subdominant HLA-A2-Restricted HIV-1 Gag p2419¬ñ27 Epitope. The Journal of Immunology *178*, 7756-7766.

Schmid, D.A., Irving, M.B., Posevitz, V., Hebeisen, M., Posevitz-Fejfar, A., Sarria, J.C., Gomez-Eerland, R., Thome, M., Schumacher, T.N., Romero, P.*, et al.* (2010). Evidence for a TCR affinity threshold delimiting maximal CD8 T cell function. J Immunol *184*, 4936-4946.

Schmitt, A., Tonn, T., Busch, D.H., Grigoleit, G.U., Einsele, H., Odendahl, M., Germeroth, L., Ringhoffer, M., Ringhoffer, S., Wiesneth, M.*, et al.* (2011). Adoptive transfer and selective reconstitution of streptamer-selected cytomegalovirus-specific CD8+ T cells leads to virus clearance in patients after allogeneic peripheral blood stem cell transplantation. Transfusion *51*, 591-599.

Schmitz, C., Marchant, D., Neil, S.J., Aubin, K., Reuter, S., Dittmar, M.T., and McKnight, A. (2004). Lv2, a novel postentry restriction, is mediated by both capsid and envelope. J Virol *78*, 2006-2016.

Schmitz, J.E., Kuroda, M.J., Santra, S., Sasseville, V.G., Simon, M.A., Lifton, M.A., Racz, P., Tenner-Racz, K., Dalesandro, M., Scallon, B.J.*, et al.* (1999). Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science *283*, 857-860.

Schonbach, C., Ibe, M., Shiga, H., Takamiya, Y., Miwa, K., Nokihara, K., and Takiguchi, M. (1995). Fine tuning of peptide binding to HLA-B*3501 molecules by nonanchor residues. J Immunol *154*, 5951-5958.

Schramm, B., Penn, M.L., Palacios, E.H., Grant, R.M., Kirchhoff, F., and Goldsmith, M.A. (2000). Cytopathicity of human immunodeficiency virus type 2 (HIV-2) in human lymphoid tissue is coreceptor dependent and comparable to that of HIV-1. J Virol *74*, 9594-9600.

Scorza-Smeraldi, R., Fabio, G., Lazzarin, A., Eisera, N., Uberti Foppa, C., Moroni, M., Zanussi, C. (1988). HLA-Associated susceptibility to AIDS: HLA B35 is a major risk factor for Italian HIV-infected intravenous drug addicts. Human Immunology *22*, 73-79.

Scott, D.R., Borbulevych, O.Y., Piepenbrink, K.H., Corcelli, S.A., and Baker, B.M. (2011). Disparate degrees of hypervariable loop flexibility control T-cell receptor cross-reactivity, specificity, and binding mechanism. J Mol Biol *414*, 385-400.

Seder, R.A., Darrah, P.A., and Roederer, M. (2008). T-cell quality in memory and protection: implications for vaccine design. Nat Rev Immunol *8*, 247-258.

Seifert, U., Bialy, L.P., Ebstein, F., Bech-Otschir, D., Voigt, A., Schroter, F., Prozorovski, T., Lange, N., Steffen, J., Rieger, M.*, et al.* (2010). Immunoproteasomes preserve protein homeostasis upon interferon-induced oxidative stress. Cell *142*, 613-624.

Serwanga, J., Shafer, L.A., Pimego, E., Auma, B., Watera, C., Rowland, S., Yirrell, D., Pala, P., Grosskurth, H., Whitworth, J.*, et al.* (2009). Host HLA B*allele-associated multi-clade Gag T-cell recognition correlates with slow HIV-1 disease progression in antiretroviral therapy-naive Ugandans. PLoS One *4*, e4188.

Sharifi, H.J., Furuya, A.M., and de Noronha, C.M. (2012). The role of HIV-1 Vpr in promoting the infection of nondividing cells and in cell cycle arrest. Curr Opin HIV AIDS *7*, 187-194.

Sharrock, C.E., Kaminski, E., and Man, S. (1990). Limiting dilution analysis of human T cells: a useful clinical tool. Immunol Today *11*, 281-286.

Sierra, S., Kupfer, B., and Kaiser, R. (2005). Basics of the virology of HIV-1 and its replication. J Clin Virol *34*, 233-244.

Skar, H., Borrego, P., Wallstrom, T.C., Mild, M., Marcelino, J.M., Barroso, H., Taveira, N., Leitner, T., and Albert, J. (2010). HIV-2 genetic evolution in patients with advanced disease is faster than that in matched HIV-1 patients. J Virol *84*, 7412-7415.

Smith-Garvin, J.E., Koretzky, G.A., and Jordan, M.S. (2009). T cell activation. Annu Rev Immunol *27*, 591-619.

Soares, R., Foxall, R., Albuquerque, A., Cortesao, C., Garcia, M., Victorino, R.M., and Sousa, A.E. (2006). Increased frequency of circulating CCR5+ CD4+ T cells in human immunodeficiency virus type 2 infection. J Virol *80*, 12425-12429.

Soares, R.S., Tendeiro, R., Foxall, R.B., Baptista, A.P., Cavaleiro, R., Gomes, P., Camacho, R., Valadas, E., Doroana, M., Lucas, M*., et al.* (2011). Cell-associated viral burden provides evidence of ongoing viral replication in aviremic HIV-2-infected patients. J Virol *85*, 2429-2438.

Soto-Rifo, R., Limousin, T., Rubilar, P.S., Ricci, E.P., Decimo, D., Moncorge, O., Trabaud, M.A., Andre, P., Cimarelli, A., and Ohlmann, T. (2011). Different effects of the TAR structure on HIV-1 and HIV-2 genomic RNA translation. Nucleic Acids Res.

Soudeyns, H., Campi, G., Rizzardi, G.P., Lenge, C., Demarest, J.F., Tambussi, G., Lazzarin, A., Kaufmann, D., Casorati, G., Corey, L., and Pantaleo, G. (2000). Initiation of antiretroviral therapy during primary HIV-1 infection induces rapid stabilization of the T-cell receptor beta chain repertoire and reduces the level of T-cell oligoclonality. Blood *95*, 1743-1751.

Sousa, A.E., Carneiro, J., Meier-Schellersheim, M., Grossman, Z., and Victorino, R.M. (2002). CD4 T cell depletion is linked directly to immune activation in the pathogenesis of HIV-1 and HIV-2 but only indirectly to the viral load. J Immunol *169*, 3400-3406.

Steers, N.J., Currier, J.R., Kijak, G.H., di Targiani, R.C., Saxena, A., Marovich, M.A., Kim, J.H., Michael, N.L., Alving, C.R., and Rao, M. (2011). Cell type-specific proteasomal processing of HIV-1 Gag-p24 results in an altered epitope repertoire. J Virol *85*, 1541-1553.

Stewart-Jones, G.B., Gillespie, G., Overton, I.M., Kaul, R., Roche, P., McMichael, A.J., Rowland-Jones, S., and Jones, E.Y. (2005). Structures of three HIV-1 HLA-B*5703-peptide complexes and identification of related HLAs potentially associated with long-term nonprogression. J Immunol *175*, 2459-2468.

Stewart-Jones, G.B., McMichael, A.J., Bell, J.I., Stuart, D.I., and Jones, E.Y. (2003). A structural basis for immunodominant human T cell receptor recognition. Nat Immunol *4*, 657-663.

Svarovskaia, E.S., Cheslock, S.R., Zhang, W.H., Hu, W.S., and Pathak, V.K. (2003). Retroviral mutation rates and reverse transcriptase fidelity. Front Biosci *8*, d117-134.

Tendeiro, R., Foxall, R.B., Baptista, A.P., Pinto, F., Soares, R.S., Cavaleiro, R., Valadas, E., Gomes, P., Victorino, R.M., and Sousa, A.E. (2012). PD-1 and its ligand PD-L1 are progressively up-regulated on CD4 and CD8 T-cells in HIV-2 infection irrespective of the presence of viremia. AIDS *26*, 1065-1071.

Thomas, S., Stauss, H.J., and Morris, E.C. (2010). Molecular immunology lessons from therapeutic T-cell receptor gene transfer. Immunology *129*, 170-177.

Thomson, S.A., Jaramillo, A.B., Shoobridge, M., Dunstan, K.J., Everett, B., Ranasinghe, C., Kent, S.J., Gao, K., Medveckzy, J., Ffrench, R.A., and Ramshaw, I.A. (2005). Development of a synthetic consensus sequence

scrambled antigen HIV-1 vaccine designed for global use. Vaccine *23*, 4647-4657.

Tienen, C., van der Loeff, M.S., Zaman, S.M., Vincent, T., Sarge-Njie, R., Peterson, I., Leligdowicz, A., Jaye, A., Rowland-Jones, S., Aaby, P., and Whittle, H. (2010). Two distinct epidemics: the rise of HIV-1 and decline of HIV-2 infection between 1990 and 2007 in rural Guinea-Bissau. J Acquir Immune Defic Syndr *53*, 640-647.

Trautmann, L., Janbazian, L., Chomont, N., Said, E.A., Gimmig, S., Bessette, B., Boulassel, M.R., Delwart, E., Sepulveda, H., Balderas, R.S.*, et al.* (2006). Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. Nat Med *12*, 1198-1202.

Trautmann, L., Rimbert, M., Echasserieau, K., Saulquin, X., Neveu, B., Dechanet, J., Cerundolo, V., and Bonneville, M. (2005). Selection of T cell clones expressing high-affinity public TCRs within Human cytomegalovirus-specific CD8 T cell responses. J Immunol *175*, 6123-6132.

Turnbull, E.L., Lopes, A.R., Jones, N.A., Cornforth, D., Newton, P., Aldam, D., Pellegrino, P., Turner, J., Williams, I., Wilson, C.M.*, et al.* (2006). HIV-1 epitope-specific CD8+ T cell responses strongly associated with delayed disease progression cross-recognize epitope variants efficiently. J Immunol *176*, 6130-6146.

Turner, S.J., Kedzierska, K., Komodromou, H., La Gruta, N.L., Dunstone, M.A., Webb, A.I., Webby, R., Walden, H., Xie, W., McCluskey, J.*, et al.* (2005). Lack of prominent peptide-major histocompatibility complex features limits repertoire diversity in virus-specific CD8+ T cell populations. Nat Immunol *6*, 382-389.

Ueno, T., Tomiyama, H., Fujiwara, M., Oka, S., and Takiguchi, M. (2004). Functionally impaired HIV-specific CD8 T cells show high affinity TCR-ligand interactions. J Immunol *173*, 5451-5457.

UNAIDS (2010). Report on the global AIDS epidemic 2010.

Utsumi, T., Nagakawa, H., Uenishi, R., Kusakawa, S., and Takebe, Y. (2007). An HIV-2-infected Japanese man who was a long-term nonprogressor for 36 years. AIDS *21*, 1834-1835.

Valadas, E., Franca, L., Sousa, S., and Antunes, F. (2009). 20 years of HIV-2 infection in Portugal: trends and changes in epidemiology. Clin Infect Dis *48*, 1166-1167.

Valentine, L.E., Piaskowski, S.M., Rakasz, E.G., Henry, N.L., Wilson, N.A., and Watkins, D.I. (2008). Recognition of Escape Variants in ELISPOT Does Not Always Predict CD8+ T-Cell Recognition of Simian Immunodeficiency Virus-Infected Cells Expressing the Same Variant Sequences. Journal of Virology *82*, 575-581.

Valkenburg, S.A., Gras, S., Guillonneau, C., La Gruta, N.L., Thomas, P.G., Purcell, A.W., Rossjohn, J., Doherty, P.C., Turner, S.J., and Kedzierska, K. (2010). Protective efficacy of cross-reactive CD8+ T cells recognising mutant viral epitopes depends on peptide-MHC-I structural interactions and T cell activation threshold. PLoS Pathog *6*, e1001039.

van Bockel, D.J., Price, D.A., Munier, M.L., Venturi, V., Asher, T.E., Ladell, K., Greenaway, H.Y., Zaunders, J., Douek, D.C., Cooper, D.A.*, et al.* (2011). Persistent survival of prevalent clonotypes within an immunodominant HIV gag-specific CD8+ T cell response. J Immunol *186*, 359-371.

van Boxel, G.I., Stewart-Jones, G., Holmes, S., Sainsbury, S., Shepherd, D., Gillespie, G.M., Harlos, K., Stuart, D.I., Owens, R., and Jones, E.Y. (2009). Some lessons

from the systematic production and structural analysis of soluble (alpha)(beta) T-cell receptors. J Immunol Methods *350*, 14-21.

van der Loeff, M.F., Larke, N., Kaye, S., Berry, N., Ariyoshi, K., Alabi, A., van Tienen, C., Leligdowicz, A., Sarge-Njie, R., da Silva, Z.*, et al.* (2010). Undetectable plasma viral load predicts normal survival in HIV-2-infected people in a West African village. Retrovirology *7*, 46.

van der Merwe, P.A., and Barclay, A.N. (1996). Analysis of cell-adhesion molecule interactions using surface plasmon resonance. Curr Opin Immunol *8*, 257-261.

van Manen, D., Kootstra, N.A., Boeser-Nunnink, B., Handulle, M.A., van't Wout, A.B., and Schuitemaker, H. (2009). Association of HLA-C and HCP5 gene regions with the clinical course of HIV-1 infection. AIDS *23*, 19-28.

Varadarajan, N., Julg, B., Yamanaka, Y.J., Chen, H., Ogunniyi, A.O., McAndrew, E., Porter, L.C., Piechocka-Trocha, A., Hill, B.J., Douek, D.C.*, et al.* (2011). A high-throughput single-cell analysis of human CD8(+) T cell functions reveals discordance for cytokine secretion and cytolysis. J Clin Invest *121*, 4322-4331.

Varela-Rohena, A., Molloy, P.E., Dunn, S.M., Li, Y., Suhoski, M.M., Carroll, R.G., Milicic, A., Mahon, T., Sutton, D.H., Laugel, B.*, et al.* (2008). Control of HIV-1 immune escape by CD8 T cells expressing enhanced T-cell receptor. Nat Med *14*, 1390-1395.

Velu, V., Titanji, K., Zhu, B., Husain, S., Pladevega, A., Lai, L., Vanderford, T.H., Chennareddi, L., Silvestri, G., Freeman, G.J.*, et al.* (2009). Enhancing SIV-specific immunity in vivo by PD-1 blockade. Nature *458*, 206-210.

Venturi, V., Chin, H.Y., Price, D.A., Douek, D.C., and Davenport, M.P. (2008). The Role of Production Frequency in the Sharing of Simian Immunodeficiency Virus-Specific CD8+ TCRs between Macaques. The Journal of Immunology *181*, 2597-2609.

Venturi, V., Quigley, M.F., Greenaway, H.Y., Ng, P.C., Ende, Z.S., McIntosh, T., Asher, T.E., Almeida, J.R., Levy, S., Price, D.A.*, et al.* (2011). A mechanism for TCR sharing between T cell subsets and individuals revealed by pyrosequencing. J Immunol *186*, 4285-4294.

Vingert, B., Perez-Patrigeon, S., Jeannin, P., Lambotte, O., Boufassa, F., Lemaitre, F., Kwok, W.W., Theodorou, I., Delfraissy, J.F., Theze, J., and Chakrabarti, L.A. (2010). HIV controller CD4+ T cells respond to minimal amounts of Gag antigen due to high TCR avidity. PLoS Pathog *6*, e1000780.

Virgin, H.W., Wherry, E.J., and Ahmed, R. (2009). Redefining chronic viral infection. Cell *138*, 30-50.

Visseaux, B., Hurtado-Nedelec, M., Charpentier, C., Collin, G., Storto, A., Matheron, S., Larrouy, L., Damond, F., Brun-Vezinet, F., and Descamps, D. (2012). Molecular determinants of HIV-2 R5-X4 tropism in the V3 loop: development of a new genotypic tool. J Infect Dis *205*, 111-120.

Vojnov, L., Martins, M.A., Almeida, J.R., Ende, Z., Rakasz, E.G., Reynolds, M.R., Leon, E.J., Weisgrau, K.L., Burwitz, B.J., Folkvord, J.M.*, et al.* (2011). GagCM9-specific CD8+ T cells expressing limited public TCR clonotypes do not suppress SIV replication in vivo. PLoS One *6*, e23515.

Vojnov, L., Reed, J.S., Weisgrau, K.L., Rakasz, E.G., Loffredo, J.T., Piaskowski, S.M., Sacha, J.B., Kolar, H.L., Wilson, N.A., Johnson, R.P., and Watkins, D.I. (2010). Effective simian immunodeficiency virus-specific CD8+ T cells lack an easily detectable, shared characteristic. J Virol *84*, 753-764.

Walker, C., Moody, D., Stites, D., and Levy, J. (1986). CD8+ lymphocytes can control HIV infection in vitro by suppressing virus replication

10.1126/science.2431484. Science *234*, 1563-1566.
Wang, B., Primeau, T.M., Myers, N., Rohrs, H.W., Gross, M.L., Lybarger, L., Hansen, T.H., and Connolly, J.M. (2009). A single peptide-MHC complex positively selects a diverse and specific CD8 T cell repertoire. Science *326*, 871-874.
Weiss, R.A. (2000). Getting to know HIV. Trop Med Int Health *5*, A10-15.
WHO/UNAIDS/UNICEF (2011). Global HIV/AIDS Response: Epidemic update and health sector progress towards Universal Access 2011. In Global HIV/AIDS Response (WHO/UNAIDS/UNICEF).
Wiegand, H.L., Doehle, B.P., Bogerd, H.P., and Cullen, B.R. (2004). A second human antiretroviral factor, APOBEC3F, is suppressed by the HIV-1 and HIV-2 Vif proteins. EMBO J *23*, 2451-2458.
Williams, M.A., and Bevan, M.J. (2007). Effector and memory CTL differentiation. Annu Rev Immunol *25*, 171-192.
Witvrouw, M., Pannecouque, C., Switzer, W.M., Folks, T.M., De Clercq, E., and Heneine, W. (2004). Susceptibility of HIV-2, SIV and SHIV to various anti-HIV-1 compounds: implications for treatment and postexposure prophylaxis. Antivir Ther *9*, 57-65.
Yamamoto, T., Johnson, M.J., Price, D.A., Wolinsky, D.I., Almeida, J.R., Petrovas, C., Nason, M., Yeh, W.W., Shen, L., Roederer, M.*, et al.* (2012). Virus inhibition activity of effector memory CD8(+) T cells determines simian immunodeficiency virus load in vaccinated monkeys after vaccine breakthrough infection. J Virol *86*, 5877-5884.
Yamamoto, T., Price, D.A., Casazza, J.P., Ferrari, G., Nason, M., Chattopadhyay, P.K., Roederer, M., Gostick, E., Katsikis, P.D., Douek, D.C.*, et al.* (2011). Surface expression patterns of negative regulatory molecules identify determinants of virus-specific CD8+ T-cell exhaustion in HIV infection. Blood *117*, 4805-4815.
Yang, H., Wu, H., Hancock, G., Clutton, G., Sande, N., Xu, X., Yan, H., Huang, X., Angus, B., Kuldanek, K.*, et al.* (2012). Antiviral Inhibitory Capacity of CD8+ T cells Predicts the Rate of CD4+ T-Cell Decline in HIV-1 Infection. J Infect Dis *206*, 552-561.
Yang, O.O., Daar, E.S., Ng, H.L., Shih, R., and Jamieson, B.D. (2011). Increasing CTL targeting of conserved sequences during early HIV-1 infection is correlated to decreasing viremia. AIDS Res Hum Retroviruses *27*, 391-398.
Yang, O.O., Sarkis, P.T., Trocha, A., Kalams, S.A., Johnson, R.P., and Walker, B.D. (2003). Impacts of avidity and specificity on the antiviral efficiency of HIV-1-specific CTL. J Immunol *171*, 3718-3724.
Yerly, D., Heckerman, D., Allen, T.M., Chisholm, J.V., 3rd, Faircloth, K., Linde, C.H., Frahm, N., Timm, J., Pichler, W.J., Cerny, A., and Brander, C. (2008). Increased cytotoxic T-lymphocyte epitope variant cross-recognition and functional avidity are associated with hepatitis C virus clearance. J Virol *82*, 3147-3153.
Yindom, L.M., Leligdowicz, A., Martin, M.P., Gao, X., Qi, Y., Zaman, S.M., van der Loeff, M.S., van Tienen, C., Jaye, A., Aveika, A.*, et al.* (2010). Influence of HLA class I and HLA-KIR compound genotypes on HIV-2 infection and markers of disease progression in a Manjako community in West Africa. J Virol *84*, 8202-8208.
Youngblood, B., Wherry, E.J., and Ahmed, R. (2012). Acquired transcriptional programming in functional and exhausted virus-specific CD8 T cells. Curr Opin HIV AIDS *7*, 50-57.
Zehn, D., Lee, S.Y., and Bevan, M.J. (2009). Complete but curtailed T-cell response to very low-affinity antigen. Nature *458*, 211-214.

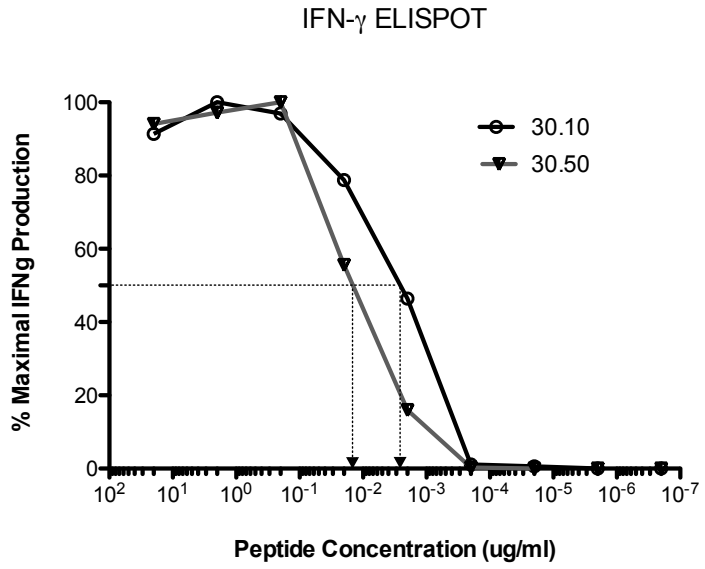
Zhai, S., Zhuang, Y., Song, Y., Li, S., Huang, D., Kang, W., Li, X., Liao, Q., Liu, Y., Zhao, Z*., et al.* (2008). HIV-1-specific cytotoxic T lymphocyte (CTL) responses against immunodominant optimal epitopes slow the progression of AIDS in China. Curr HIV Res *6*, 335-350.

Zhong, W., and Reinherz, E.L. (2004). In vivo selection of a TCR Vbeta repertoire directed against an immunodominant influenza virus CTL epitope. Int Immunol *16*, 1549-1559.

Zhou, B., Chen, Q., Mallis, R.J., Zhang, H., Liu, J.H., Reinherz, E.L., and Wang, J.H. (2011). A Conserved Hydrophobic Patch on Vbeta Domains Revealed by TCRbeta Chain Crystal Structures: Implications for Pre-TCR Dimerization. Front Immunol *2*, 5.

Appendix

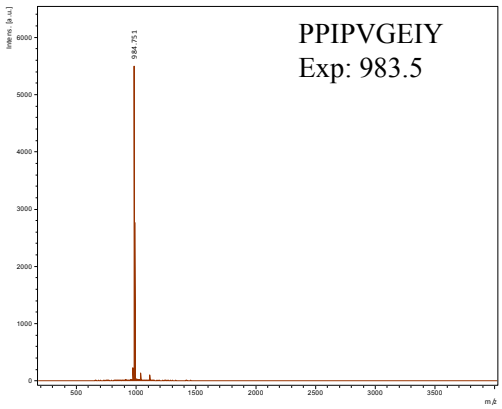
I. Chapter 4 : Functional sensitivity of CTL clones 30.10 (TRBV19) and 30.50 (TRBV20-1) as measured by IFN-γ ELISPOT. EC_{50} values were calculated from normalized values (% maximal IFN-γ production) as the concentration of peptide needed for half-maximal IFN-γ secretion.

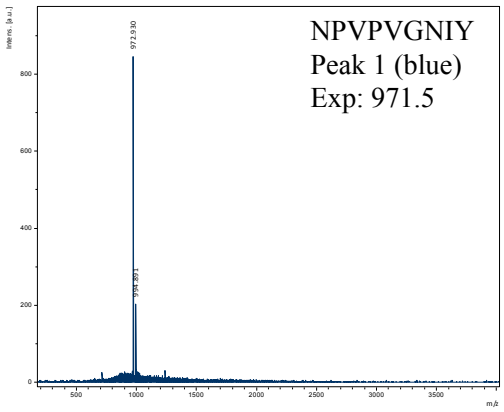


II. Chapter 4 : Production of cytokines from CTL clones 30.10 (TRBV19) and 30.50 (TRBV20-1) as assessed by cytokine bead-array analysis (Bioplex). The upregulated cytokines and their values are given in bold.

(pg/ml)	10	2	0.4	0.08	0.016	0.0032	0.00064	CTL CLONE
PDGF-b	6.84	8.87	6.14	7.19	7.19	5.43	6.14	TRBV19
	8.96	0	2.64	2.64	3.58	0	0	TRBV20-1
IL-1b	3.43	5.99	4.07	4.46	6.72	6.72	2.54	TRBV19
	6.64	6.56	5.82	7.65	8.41	5.39	6.8	TRBV20-1
IL-1ra	18.08	19.61	12.51	14.03	15.55	11	11.5	TRBV19
	15.74	12.77	9.81	17.72	8.83	9.81	7.85	TRBV20-1
IL-2	5.97	9.21	10.44	16.69	9.96	6.8	4.18	TRBV19
	5.75	3.68	5.55	8.03	6.62	2.35	3.85	TRBV20-1
IL-4	1.86	1.59	1.91	2.13	2.42	1.65	1.27	TRBV19
	1.24	0.97	1.02	1.13	1.02	0.63	0.52	TRBV20-1
IL-5	26.82	35.32	33.49	37.96	51.94	62.88	60.05	TRBV19
	27.23	22.75	39.92	50.34	32.51	45.78	43.96	TRBV20-1
IL-6	189.26	187.3	164.27	142.82	101.34	89.06	84.76	**TRBV19**
	32.5	30.28	32.2	32.2	20.33	19.1	5.62	**TRBV20-1**
IL-7	0.2	0.94	0.76	0.57	0.94	0.2	0.57	TRBV19
	0.4	0.6	0.5	0.1	0.2	0.4	0.2	TRBV20-1
IL-8	18.84	21.93	23.19	15.43	13.22	6.3	1.3	TRBV19
	28.81	31.81	38.81	14.82	8.27	9.81	8.63	TRBV20-1
IL-9	52.81	24.17	56.74	45.25	35.27	32.35	23.86	**TRBV19**
	15.48	14.69	13.78	11.59	8.32	5.64	4.93	**TRBV20-1**
IL-10	0	0	0	0	0	0	0	TRBV19
	0	0	0	0	0	0	0	TRBV20-1
IL-12(p70)	4.2	0.45	0	0	0	0	0	TRBV19
	5.91	4.88	4.37	3.79	3.5	2.78	2.2	TRBV20-1
IL-13	2496.63	1744.43	1612.63	1433.29	1256.51	1024.11	200.19	**TRBV19**
	461.6	459.43	445.15	397.01	376.31	287.03	189.99	**TRBV20-1**
IL-15	0.9	1.13	0.65	0.33	1.81	0.73	0.96	TRBV19
	1.18	0	0	0.28	0	0	0	TRBV20-1
IL-17	7.1	6.97	5.56	5.44	4.28	4.02	3.25	TRBV19
	12.35	11.96	11.45	9.62	9.36	9.36	7.24	TRBV20-1
Eotaxin	17.64	12.98	10.53	9.52	0.56	0	0	TRBV19
	3.51	2.33	0	0	0	0	0	TRBV20-1
FGF Basic	3.05	3	2.31	2.01	2.01	1.81	1.41	TRBV19
	2.03	1.93	1.93	1.83	1.63	0.92	0.21	TRBV20-1
G-CSF	8.33	7.58	7.33	7.08	6.08	4.45	2.3	TRBV19
	3.23	2.96	2.68	2.54	2.12	2.12	1.56	TRBV20-1
GM-CSF	166.16	156.97	138.16	135.57	131.37	122.14	83.73	**TRBV19**
	27.87	18.55	16.7	14.42	9.43	8.99	7.53	**TRBV20-1**
IFNg	7781.59	6555.3	6372.34	5553.37	5029.98	4917.04	4668.18	**TRBV19**
	5987.67	4829.43	4281.43	3192.1	2991.65	1726.4	1244.47	**TRBV20-1**
IP10	9.49	12.93	11.23	2.02	16.23	9.49	11.23	TRBV19
	19.73	5.09	18.68	19.73	13.1	1.83	10.63	TRBV20-1
MCP-1	53.26	41.39	39.41	17.73	11.17	7.55	1.61	**TRBV19**
	1.89	1.22	0.99	0.88	0.82	0.76	0.19	TRBV20-1
MIP-1b	4990.49	4618.87	3410.1	2179.33	1721.71	1240	935.08	**TRBV19**
	1980.39	899.24	813.19	725.55	527.2	417.28	312.97	**TRBV20-1**
RANTES	644.61	245.86	140.57	136.94	136.61	129.21	nd	**TRBV19**
	332.11	289.42	190.7	187.3	178.46	143.4	127.24	**TRBV20-1**
TNFa	915.8	746.58	744.2	693.26	637.92	537.25	440.26	**TRBV19**
	221.61	206.76	186.85	169.04	127.84	68.77	53.76	**TRBV20-1**
VEGF	54.28	29.72	28.81	28.27	19.81	16.61	13.97	**TRBV19**
	27.36	11.81	10.46	8.27	8.11	4.44	2.79	**TRBV20-1**

III. Chapter 5: Mass-spectrometry analysis of the peptides contained in the refolded NY9 and PY9 monomers





Exp: expected molecular weight. The secondary peaks observed in the NPVPVGNIY samples at molecular weight +23 represent sodium attachments from the peptide synthesis process and hence are not impurities.