HLA-B51 Associated HIV-1 Viral Control



Yanchun Peng

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Supervisors
Professor Tao Dong
Professor Sarah Rowland-Jones

MRC Human Immunology Unit
Weatherall Institute of Molecular Medicine
University of Oxford
John Radcliffe Hospital
Oxford OX3 9DS

Nuffield Department of Clinical Medicine

Declaration

I declare that the work presented in the D.Phil. thesis entitled “HLA-B51 associated HIV-1 viral control” 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 or elsewhere.

Trinity term 2013

Yanchun Peng

MRC Human Immunology Unit

Weatherall Institute of Molecular Medicine

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Abbreviations

AIDS	Acquired immunodeficiency Virus
APC	Antigen presenting cell
	Allophycocyanin
ART	Antiretroviral Therapy
bp	Base pair
β2M	β2Microglobulin
CA	Capsid
CAF	$CD8^+$ T cell antiviral factor
CDR	Complementary deterring region
CMV	Cytomegalovirus
CRF	Circulationg recombinant form
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DRiPs	defective ribosome products
EBV	Epstein Barr Virus
EC50	Effective concentration 50
ER	Endoplasmic reticulum
ERAP	ER aminopeptidase
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FPLC	Fast liquid protein chromatography
GALT	Gut-associated lymphoid tissue
GWAS	Genome-wide association study
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HTLV	Human T-lymphocyte leukaemia virus
IFN	Interferon
IL	Interleukin
KIR	Killer immunoglobulin-like receptor
LTNP	Long-term non-progressor
LTR	Long terminal repeat
MA	Matrix
MHC	Major histocompatibility complex
MHR	Major homology region
NC	Nucleocapsid
NES	Nuclear export signal
NLS	Nuclear localization signal
NK	Natural killer
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PHA	Phytohaemagglutinin
PIC	Pre-integration complex
PLC	Protein loading complex
pMHC	peptide-MHC
PR	Protease
pVL	Plasma viral load
RNA	Ribonucleic acid
RRE	Rev response element
RT	Reverse transcriptase
SFU	Spot forming unit
SIV	Simian immunodeficiency virus
SNP	Single nucleotide polymorphism
TAP	Transporter associated with antigen processing
TCID50	50% of Tissue culture infective dose
TCR	T cell receptor
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TPP	Tripeptidyl peptidase
V	variable
VL	Viral load
WHO	World health organisation

Amino Acid letter codes

A	Alanine
C	Cysteine
D	Aspartic acid
E	Glutamic acid
F	Phenylalanine
G	Glycine
H	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P	Proline
Q	Glutamine
R	Arginine
S	Serine
T	Threonine
V	Valine
W	Tryptophan
Y	Tyrosine

Nucleotide letter codes

A	Adenosine
T	Thymidine
C	Cytidine
G	Guanidine
U	Uridine

Contents

Chapter 1: Introduction 12
1.1 HIV/AIDS epidemiology 12
1.1.1 Global HIV/AIDS epidemiology 12
1.1.2 HIV/AIDS in China 15
1.1.3 HIV-1 infection in Henan Province, China 16
1.2 HIV-1 origin and Diversity 17
1.3 HIV-1 virus and genome 18
1.3.1 Structure 18
1.3.2 Genome 19
1.3.3 Viral proteins 20
1.3.3.1 Gag 20
1.3.3.2 Pol 25
1.3.3.3 Env 31
1.3.3.4 Nef 31
1.3.3.5 Tat 34
1.3.3.6 Rev 35
1.3.3.7 Vpr 35
1.3.3.8 Vif 36
1.3.3.9 Vpu 37
1.4 HIV-1 life cycle 38
1.4.1 Binding and fusion to the target cells 38
1.4.2 Uncoating and reverse transcription 38
1.4.3 Nuclear import and integration 39
1.4.4 Transcription 39
1.4.5 Nuclear export and translation 40
1.4.6 Assembly and budding 40
1.4.7 Viral maturation 40
1.5 HIV-1 pathogenesis and disease course 41
1.5.1 The disease course of HIV-1 infection 41
1.5.2 HIV-1 pathogenesis 43
1.5.3 HIV long-term non-progressors 46
1.6 Host immune responses against HIV-1 infection 47
1.6.1 Innate immune responses to HIV-1 47
1.6.1.1 DCs and IFN-α 47
1.6.1.2 NK cells 48
1.6.2 Antibody responses to HIV infection 49
1.6.3 The $CD4^+$ T-cell responses to HIV-1 51
1.6.4 The $CD8^+$ T-cell responses to HIV-1 53
1.6.4.1 Recognition of virus infected cells by $CD8^+$ T cells 53
1.6.4.1.1 Antigen processing and presentation by MHC class I molecules 53
1.6.4.1.2 Recognition of peptide-MHC class I complex by $CD8^+$ T cells 57
1.6.4.2 Mechanisms of $CD8^+$ T cell anti-vial actions 60
1.6.4.3 The role of $CD8^+$ T-cell responses in HIV-1 infection 62
1.6.4.4 The quality of $CD8^+$ T cell responses 63
1.6.4.4.1 Antigen specificity 64
1.6.4.4.2 Differentiation state 65
1.6.4.4.3 Activation and replicative senescence 67
1.6.4.4.4 Cytolytic activity 68
1.6.4.4.5 Polyfunctionality 68
1.6.4.4.6 Functional avidity 69
1.6.4.4.7 Cross reactivity 71
1.7 HLA and HIV control 72

1.8 Intra-host HIV evolution driven by CTL 73
1.9 Specific aims of this study 74

Chapter 2: Materials and Methods 76
2.1 Study subjects 76
2.1.1 SM Cohort 76
2.1.2 Patient Ya13 76
2.2 Cell lines and CTL clones 76
2.2.1 EBV-transformed B lymphoblastoid cell lines 76
2.2.2 HIV-1 permissive target cells 77
2.2.3 293T cells 77
2.2.4 Cytotoxic T cell lines 78
2.2.5 Cytotoxic T cell clones 79
2.3 Preparation of Peripheral Blood Mononuclear Cells (PBMCs) 79
2.4 IFN-γ Secretion Assay 79
2.5 Standard Human IFN-γ ELISPOT assay 80
2.6 Vβ antibody staining 81
2.7 Proteasomal digestion assay 81
2.8 *In vitro* HIV infection assays 83
2.8.1 HIV-1 virus stocks 83
2.8.1.1 HIV-1 clade B viruses 83
2.8.1.2 Wild type and Mutant HIV-1 molecular clonal viruses 83
2.8.1.2.1 HIV-1 cDNA clone pNL432 83
2.8.1.2.2 Construct of pNL432 Pol mutants 83
2.8.1.2.3 Production of viral stocks 83
2.8.2 IFN-γ Live Virus ELISPOT 86
2.8.3 Virus Suppression assay 86
2.8.4 Intracellular p24 staining 87
2.8.5 Virus Evolution Assay 87
2.8.5.1 Passage of HIV-1 under selection by CTL Clones 88
2.8.5.2 Analysis of the passaged virus for epitope mutations 88
2.8.5.2.1 Genome DNA isolation 88
2.8.5.2.2 Nested polymerase chain reaction (PCR) amplification 89
2.8.1.1.1 TA TOPO cloning 91
2.9 Preparation of HLA-B51 class I MHC tetramers 91
2.9.1 Total RNA extraction 91
2.9.2 cDNA synthesis 92
2.9.3 PCR amplifying the HLA-B51 specific gene and cloning into the expression vector 93
2.9.3.1 PCR 93
2.9.3.2 Double-digestion with restriction enzyme 93
2.9.3.3 Ligation and transformation 94
2.9.3.4 Plasmid purification and sequencing 94
2.9.4 Expressing and purification of HLA-B51 heavy chain protein and β_2-microglobulin protein 95
2.9.5 Refolding and purification of HLA-B51 peptide-MHC complex 95
2.9.6 Tetramerisation of peptide-MHC class I complex 96
2.10 Tetramer staining 97
2.11 Viral sequence analysis 98
2.12 Shannon entropy calculation 98
2.13 Statistical analysis 98

Chapter 3: HLA-B51 associated HIV-1 viral control in the SM cohort 100
3.1 Introduction 100

3.2 Results 106
3.2.1 Selection hierarchy of HLA-B51 associated mutations 106
3.2.2 Increased numbers of mutated epitopes are significantly associated with lower CD4 counts and higher viral load in the SM cohort 106
3.2.3 Patients with CTL responses detected towards at least one un-mutated HLA-B51 restricted epitope have significantly better clinical outcome 108
3.2.4 HLA-B51 restricted CTL clones 110
3.2.5 HLA-B51 restricted CTL clones recognise major epitope variants 111
3.2.6 Pol TI8 specific CTL clones lost control of viruses carrying the I8L/T mutations and the suppression of mutant virus 8V was significantly reduced 115
3.2.7 Significant reduction of the antiviral efficacy of LI9 specific CTL clones against the virus Pol743 LI9 mutants 117
3.2.8 Gag NI9, Pol TI8 and Pol LI9 specific T cell clones could effectively recognise and control IIIB and MN virus-infected target cells in Live Virus ELISPOT assays and in Virus Suppression Assays 120
3.3 Discussion 122

Chapter 4: Evaluation of the rate of epitope escape mutations driven by three dominant HLA-B51 restricted T cell responses 125
4.1 Introduction 125
4.2 Results 128
4.2.1 Selection of HLA-B51 restricted CTL clones for Virus Evolution Assay. 128
4.2.2 Virus Evolution Assay 130
4.2.3 The HLA B51 CTL clones have similar anti-viral efficacies against the WT IIIB virus in the 1st round of virus passage. 131
4.2.4 Reduced anti-viral efficacy of the three HLA-B51 CTL clones following virus passages. 133
4.2.5 Early generation of a dominant escape mutation in the epitope Pol LI9. 134
4.2.6 Escape mutations within epitope Pol TI8 occurred after the 4th round of viral passaging. 138
4.2.7 Dominant escape mutation was observed in epitope Gag NI9 after the 4th, 5th and 6th passages. 141
4.2.8 Strong correlation between the suppression of virus replication and the percentage of remaining WT viral clones under the selection of the CTL clones. 145
4.2.9 Hierarchical development of mutations within three HLA-B51 restricted epitopes 147
4.2.10 Rapid disease progression is associated with the early development of escape mutations in the Pol LI9 and Pol TI8 epitopes together with a lack of an effective response to the Gag NI9 epitope. 148
4.3 Discussion 151

Chapter 5: The role of antigen sensitivity, TCR repertoire, founder virus on HIV evolution and HIV control 159
5.1 Introduction 159
5.2 Results 164
5.2.1 Selection of HLA-B51 Pol TI8 specific CTL clones with different antigen sensitivity and T cell receptor usage 164
5.2.2 Setting up Virus Evolution Assay 165
5.2.3 The level of immune pressure amounted by CTL clones on JP-8I and SM-8V viruses at the 1st round of virus passage 166
5.2.4 Decreased suppression of JP-8I virus and better control of SM-8V virus during late virus passages. 167
5.2.5 Dominant escape mutation I8T in Pol TI8 epitope of JP-8I virus driven by Clone C5 occurred at the 4th passage 169
5.2.6 Escape mutations occurring in Pol TI8 epitope of JP-8I virus at the 3rd passage under the selection of Clone C11. 172

5.2.7 Significant correlation between the suppression of JP-8I virus replication and the proportion of remained WT viral clones under the selection of CTL clones. 174
5.2.8 Slightly faster development of escape mutations in JP-8I virus under the selection of Clone C11 175
5.2.9 No mutation observed within the epitope Pol TV8 of SM-8V virus under the selection of Clone C5. 176
5.2.10 Occurrence of a rare mutation V8A within epitope Pol TV8 of SM-8V virus after four rounds of virus passage under the selection of Clone C11 177
5.2.11 Impaired antigen processing caused by Valine at position 8 of epitope Pol TI8 179
5.3 Discussion 184

Chapter 6: The *ex vivo* Phenotype of HLA-B51 restricted antigen-specific T cells in the SM cohort 190
6.1 Introduction 190
6.2 Results.....194
6.2.1 Preparation of Class I MHC tetramers 194
6.2.1.1 Cloning modified HLA-B5101 heavy chain gene to an expression vector pGMT7 194
6.2.1.2 Refolding and purification of HLA-B51 peptide-MHC complex with D-biotin tag.....196
6.2.1.3 Staining BaF3-ILT2 cells with HLA-B51 Class I MHC tetramers 198
6.2.2 Identifying HLA-B51 restricted HIV-1-specific $CD8^+$ T cells in the SM cohort.....200
6.2.3 $CD8^+$ T cells targeting an un-mutated (WT) epitope displaying a late-differentiated phenotype 202
6.2.4 $CD8^+$ T cells targeting un-mutated HLA-B51 epitopes are more differentiated towards to replicative senescent. 206
6.2.5 Higher cytotoxic potential of CD8+ T cells targeting the un-mutated epitopes.....209
6.3 Discussion 211

Chapter 7: Overall discussion and future directions..... 217

References..... 226
Appendices……262

Chapter 1: Introduction

1.1 HIV/AIDS epidemiology

1.1.1 Global HIV/AIDS epidemiology

HIV, the virus that causes "acquired immunodeficiency syndrome" (AIDS), has become one of the most serious challenges for global health and development. The initial cases were reported in 1981, when previously rare diseases such as pneumocystis carinii pneumonia and Kaposi's sarcoma, caused by a generalised immune deficiency, were found in a cluster of drug abusers or homosexuals (Gottlieb et al., 1981; Masur et al., 1981). In 1983, the causative agent was isolated from the patients by two separate research groups in France and USA (Barre-Sinoussi et al., 1983; Coffin et al., 1986; Gallo et al., 1983). This newly identified retrovirus was then named as Human Immunodeficiency virus (HIV). The hallmark of AIDS is the eventual failure of the immune system leading to susceptibility to opportunistic infections and death.

Since 1981, HIV infection has spread across the world. According to the report published by UNAIDS/WHO (**Table 1.1**), at the end of 2011, approximately 34 million people were living with HIV worldwide. There were about 1.7 million deaths from AIDS in 2011 and nearly 30 million people have died of AIDS-related causes since the beginning of the epidemic. Although new HIV infections overall have declined by more than 20% since 2001, there were still about 2.5 million new infections in 2011 alone or more than 7,000 new HIV infections per day. Despite

the stable prevalence of HIV infection (percentage of persons infected with HIV) worldwide, the burden of the epidemic continues to vary considerably between countries and regions (**Figure 1.1**). With more than two-thirds (69%) of people with HIV living in Sub-Saharan Africa and an estimated increase in new infections in Eastern Europe and central Asia, the HIV pandemic still remains one of the most serious of infectious disease challenges to public health.

The introduction of highly active antiretroviral therapy (HAART) has had a dramatic effect on the management of HIV infection. The morbidity and mortality of HIV infection in the world, especially in the developed countries has declined significantly since then (Mocroft et al., 2000; Mocroft et al., 2003). However, problems of drug toxicity, the cost of treatment, the emergence of drug resistant HIV strains and the infrastructure required to distribute and manage their use indicate that an effective vaccine is required for controlling the epidemic.

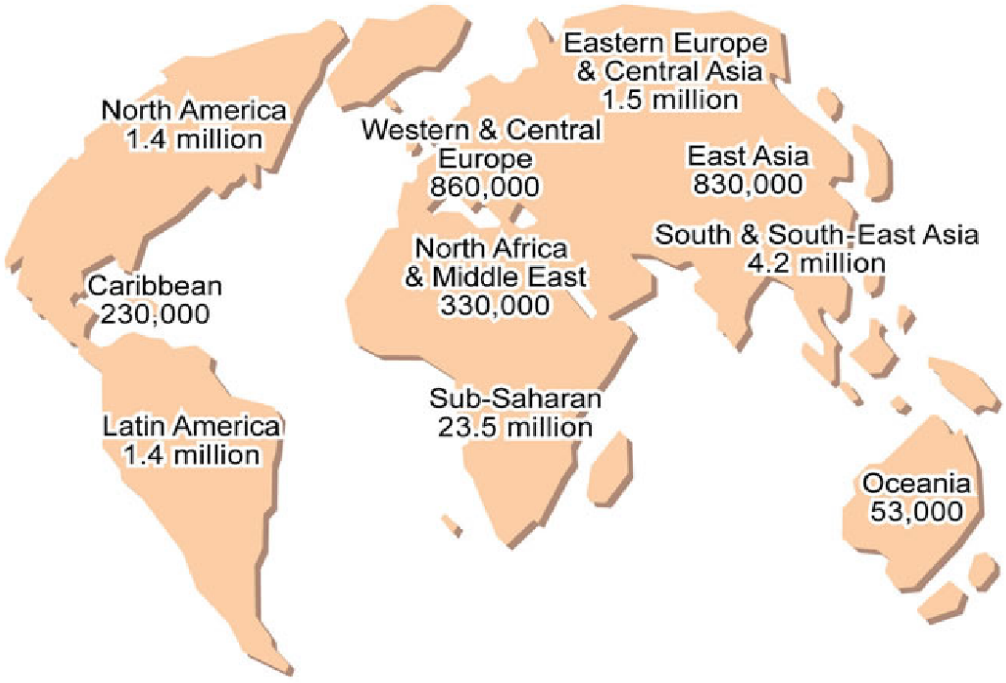

Figure 1.1 Distribution of people living with HIV worldwide. Reproduced from 2012 UNAIDS Report on the global AIDS epidemic.

Table 1.1: Global summary of the AIDS epidemic in November 2012. Reproduced from 2012 UNAIDS Report on the global AIDS epidemic.

Number of people living with HIV in 2011	
Total	34.0 million [31.4–35.9million]
Adults	30.7 million [28.4–31.5 million]
Women	16.8 million [15.8–17.6 million]
Children under 15 years	3.4 million [3.0-3.8 million]
People newly infected with HIV in 2011	
Total	2.5 million [2.2-2.8 million]
Children under 15 years	330 000 [280 000–390 000]
AIDS deaths in 2011	
Total	1.7 million [1.5–1.9 million]
Children under 15 years	230 000 [200 000–270 000]

1.1.2 HIV/AIDS in China

In 1985, AIDS was first reported in Beijing, China. The initial cases were mainly found in foreigners and Chinese who had travelled abroad (Zhang Kl and Ma, 2002). In 1989, HIV was then identified among injecting drug users in Yunnan, a province of China bordering with Myanmar, Vietnam and Laos. After 1994, the virus spread beyond Yunnan with a high number of cases reported in both drug users and commercial plasma donors. By 1996, HIV had reached all provinces in China.

The number of people living with HIV has steadily risen in China. According to the data published by Ministry of Health of the People's Republic of China, there are an estimated 780,000 people currently living with HIV in China and 28,000 people died from AIDS in 2011. Provinces Yunnan, Guangxi, Henan, Sichuan, Xinjiang and Guangdong are the ones with the highest number of HIV and AIDS cases, accounting for about 70 to 80% of the national total (**Figure 1.2**).

In addition, the rates of infection are now increasing in people outside the high risk groups such as injecting drug users, former plasma donors, sex workers and men who have sex with men (Lu et al., 2006). This became particularly evident in 2009 when AIDS had become the country's leading cause of death among infectious diseases. Nearly 50% people living with HIV at the end of 2011 were infected through heterosexual transmission, indicating HIV has started spreading into the general population. This alarming trend and the cost of life-long antiviral therapy

along with China's large population have necessitated the development of preventive means to prevent a major HIV/AIDS epidemic in China.



Figure 1.2 Reported HIV/AIDS cases by province in China. Adapted from UNGASS (2012, March) China AIDS Response Progress Report from Ministry of Health of the People's Republic of China.

1.1.3 HIV-1 infection in Henan Province, China

As shown in **Figure 1.2**, Henan province, marked with purple, has the highest number of people living with AIDS in China. During 1990s, commercial blood donation was routine practice in rural Henan Province and 42.8% of the adults below 60 years were blood donors (Dou et al., 2010). Many of the plasma donors were infected with HIV either through contaminated equipment or by pooling unscreened blood from several donors and transfusing the red blood cells back to them (Wu et al., 1995). With 10 to 20% of infection rate among the plasma donors, there were 30,820 people living with HIV in Henan, of whom 22,087 had progressed to AIDS, the highest level in China (Dou et al., 2010).

1.2 HIV-1 origin and Diversity

It is documented that HIV-1 originated from Africa and its closest ancestor is Simian immunodeficiency virus (SIV) from chimpanzees (SIVCPZ). On the basis of differences in the envelope (*env*) region, HIV-1 viruses are classified into three main phylogenetic lineages: M (Main), O (outlier), N (non-M/non-O) (Plantier et al., 2009; Robertson et al., 2000). It has been suggested that group M and group N viruses have originated by cross-species transmission from SIV-infected Pan troglodytes (Ptt) chimpanzees to human, while O group viruses can be traced back to a SIV-infected gorilla intermediate, which originally contracted the virus from chimpanzees (Gao et al., 1999; Van Heuverswyn et al., 2006). Recently, a new group P, a variant of HIV-1 group O that is more closely related to SIVgor than to group O, has been detected in a Cameroonian living in France (Plantier et al., 2009). Phylogenetic analyses suggested that HIV-1 groups M, N, O, and P were introduced independently into humans due to their phylogenetic interspersion along SIVcpz*Ptt* and SIVgor lineages (Nerrienet et al., 2005; Santiago et al., 2002).

HIV differs from other viruses by its vast genetic diversity driven by several factors, including the error-prone reverse transcription caused by its low-fidelity RT enzyme (Roberts et al., 1988), the fast turnover of HIV-1 *in vivo* (Ho et al., 1995), selective immune pressures from the host (Fellay et al., 2007), and recombination during replication in the patients with dual and triple infections (Shriner et al., 2004; Templeton et al., 2009).

Group M causes most HIV-1 infections and responsible for the current AIDS pandemic. Viruses from this group are further categorised into nine subtypes (A–D, F–H, J, and K), sub-subtypes, and 50 Circulating recombinant forms (CRFs). The subtypes and CRFs manifest the genetic diversity of HIV-1. The intra-subtype diversity is also substantial among different subtypes of HIV-1. For example, the protein sequence diversity among subtypes of HIV-1 group M viruses for Gag, Pol, and Env are reported to be 15%, 10%, and 24%, respectively (Gao et al., 1999). According to the HIV sequence compendium 2011, 414,398 HIV sequences were included in the database and 50 circulating recombinant forms (CRFs) have been reported for HIV-1 in 2012. This continual divergent evolution of the virus with its approximate 1% per year rate of evolution, poses tremendous challenge for vaccine development against HIV/AIDS (McMichael et al., 2002).

1.3 HIV-1 virus and genome

1.3.1 Structure

The mature HIV-1 virion is spherical-to-conical in shape, and 100-120 nm in diameter. It contains two identical copies of 9.2 kilo-base long single-stranded RNA genome. **Figure 1.3** illustrates the structure of a mature HIV-1 virion. The outer coat of the virus, known as the viral envelope, consists of two layers of lipids from the host cell and also a virally encoded complex glycoprotein. Each envelope glycoprotein (Env) consists of a docking glycoprotein 120 (gp120), and a transmembrane domain glycoprotein 41 (gp41). Underneath the virion envelope is a matrix protein (p17 or MA). Within the virion envelope, there is a bullet-shaped

core, called capsid protein (p24 or CA). This capsid is composed of 2,000 copies of the viral protein p24 or CA and encloses viral RNA, other viral proteins and key enzymes (Barre-Sinoussi, 1996).

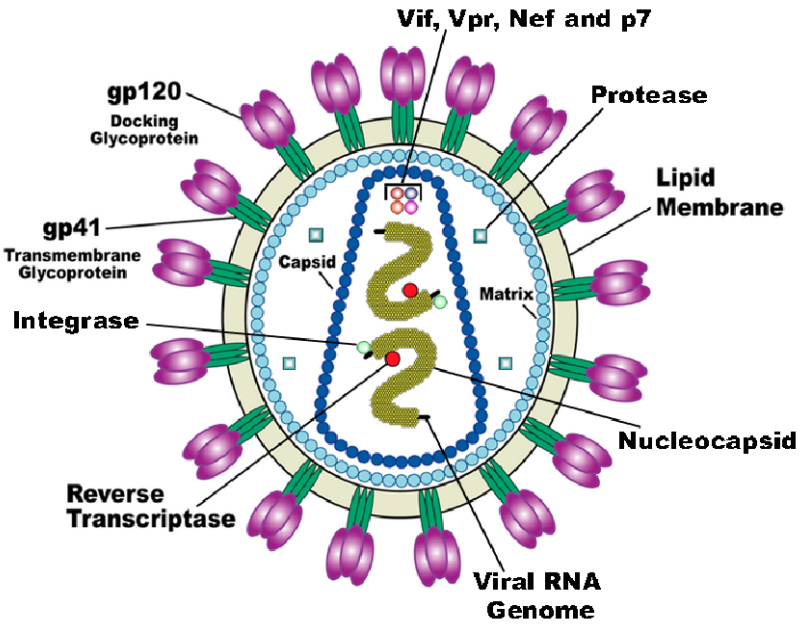

Figure 1.3 The structure of matured HIV-1 virion. Adapted from USA National Institute of Health (http://www.niaid.nih.gov/factsheets/graphics/howhiv.jpg)

1.3.2 Genome

The entire HIV-1 proviral genome, which is encoded by about 9500 RNA bases, consists of nine open reading frames that encode three structural proteins (Gag, Pol and Env) and six regulatory or accessory proteins (Tat, Rev, Vif, Vpr, Vpu, and Nef). The 5' and 3' end of the proviral genome are flanked by two long terminal repeats (LTR), which contain all the required signals for gene expression (**Figure 1.4**).

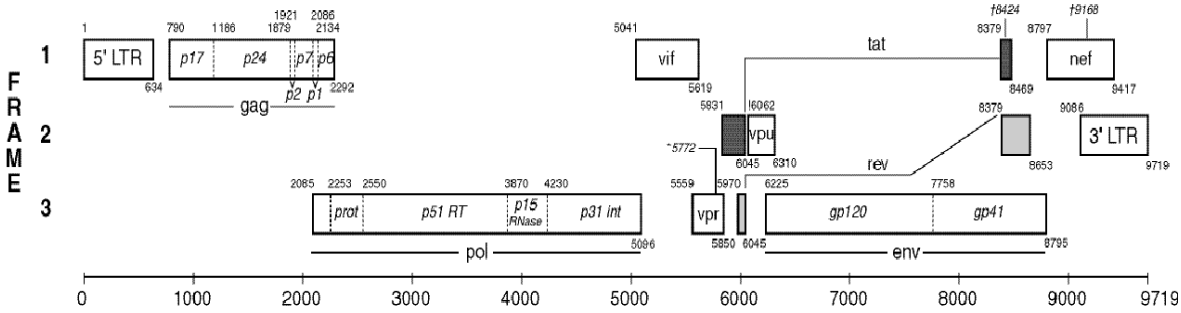

Figure 1.4 The genomic organisation of HIV-1. The HIV-1 genome is approximately 9.6 kb in length and encodes nine open reading frames, shown as rectangles. The position of each gene in the HIV-1 genome and the relevant proteins demonstrated in this figure is aligned with the HXB2 strain from the Los Alamos database. (Adapted from http://www.hiv.lanl.gov).

1.3.3 Viral proteins

1.3.3.1 Gag

The group-associated antigen (Gag) is initially synthesised as a 55kDa precursor protein (p55) and subsequently processed by the viral protease into four main structural proteins: p17 matrix (MA), p24 core or capsid (CA), p7 nucleocapsid (NC) and p6 Vpr binding protein (Freed, 1998). These gag gene products play a critical role in virus assembly and maturation.

Matrix (p17)

The matrix protein p17 consists of 123 amino acids and lies underneath the viral membrane envelope. During viral assembly, p17 plays a well-established role in targeting of p55 to the plasma membrane and in incorporation of viral envelope glycoproteins (Env) into the virion (Dorfman et al., 1994b). In addition, it is thought that the nuclear localization signals (NLS) at the N-terminal domain of

matrix may promote the rapid import of the viral pre-integration complex (PIC) in to the nucleus (Bukrinsky et al., 1993; Haffar et al., 2000).

Capsid (p24)

The p24 protein is a 24-kD structure protein forming the conical core of the virion that encloses the viral genome. With ~1500-2000 copies in each virion, p24 is the most abundant and immunogenic protein in the virus. In addition, it plays an important role in viral assembly.

P24 adopts a two-domain structure, with N-terminal and C-terminal domains (NTD and CTD) connected by a short flexible linker (Berthet-Colominas et al., 1999; Gamble et al., 1997; Gitti et al., 1996; Pornillos et al., 2009). The mutagenesis studies on HIV-1 CA functions have shown that the C-terminal domain is generally involved in viral assembly, whereas mutations in the NTD often lead to viruses that can assemble and bud but are non-infectious and typically do not form a normal capsid (Chazal et al., 1994; Dorfman et al., 1994a; Du et al., 2011; Fitzon et al., 2000; Reicin et al., 1996; Reicin et al., 1995; Srinivasakumar et al., 1995; von Schwedler et al., 2003).

As shown in **Figure 1.5**, the NTD consists of two N-terminal β-hairpins, seven α-helices, and an extended loop where the cellular protein cyclophilin A binds (CypA loop) (Gamble et al., 1996). This binding leads to the packaging of ~200 copies of CypA into each HIV-1 virion. Abolishing this binding by mutation in the loop resulted in the virions with normal appearance but impaired infectivity (Kong et al.,

1998). The smaller domain (CTD) is composed of four α-helices and an essential, highly conserved stretch of 20 amino acids (the major homology region [MHR]) that is found in all retroviruses and is critical for capsid dimerisation. Mutations in MHR lead to defects in assembly, maturation and infectivity (Gamble et al., 1997; Mammano et al., 1994). Finally, the C-terminal 11 residues of the HIV-1 CA CTD are disordered, and equivalent C-terminal tails are also disordered in other mature retroviral CA proteins.

The high-resolution CA structures have been published as full-length monomers from HIV-1 and other retroviruses ((Berthet-Colominas et al., 1999; Campos-Olivas et al., 2000; Ganser-Pornillos et al., 2007; Jin et al., 1999b; Khorasanizadeh et al., 1999; Mortuza et al., 2009; Pornillos et al., 2009). These studies collectively showed that retroviral CA proteins share a common secondary structure and tertiary fold despite having widely divergent amino acid sequences (**Figure 1.6**) and that, as a result, mature retroviral capsids are likely to be stabilised by similar quaternary interactions to the HIV-1 capsid. The hexagonal capsid lattice is composed of three different types of interfaces: a six-fold symmetric NTD-NTD interface that creates hexameric rings, an intermolecular interface between the two domains (NTD-CTD) that reinforces the hexamer, and a homodimeric CTD-CTD interface that links the hexameric building blocks into an infinite hexagonal lattice. An HLA-B51 restricted CTL Gag epitope NI9 (NANPDCKTI) located within the helix 10, as highlighted in **Figure 1.5** and **1.6**, and may be involved in the homodimeric CTD-CTD interface.

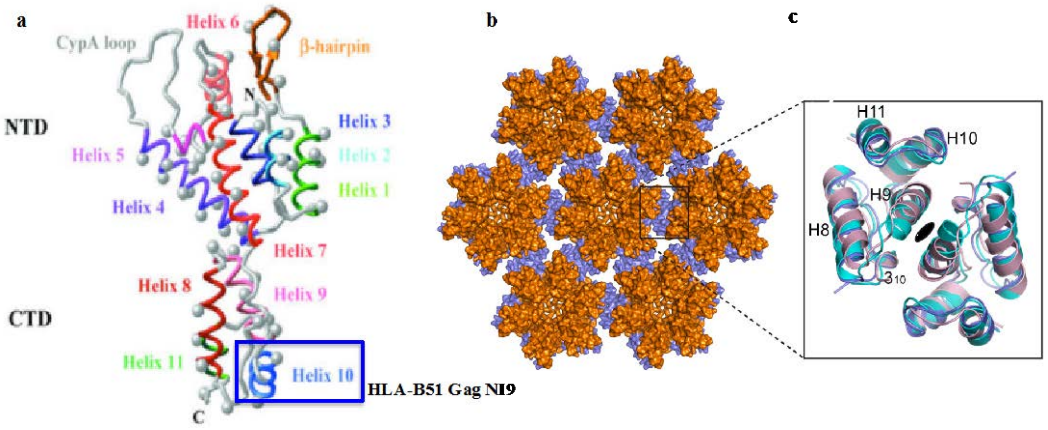

Figure 1.5 Structure of the HIV-1 CA monomer and CA Hexamer a) The structural model based upon crystal structures of the protein's NTD and CTD Secondary structures: Helixes 1-11 are displayed in different colour, the CypA loop is in grey. [Adapted from (von Schwedler et al., 2003)]. **b)** Top view of one sheet in the CcmK4-templated CA crystals. The NTDs are colored in orange, and the CTDs are in blue. c) Top view of the CTD-CTD interface that connects neighbouring hexamers, as seen in the CcmK4-templated (cyan) and crosslinked hexagonal crystals (blue), and superimposed with the isolated full-affinity CTD dimer (pink) (Worthylake et al., 1999). Neighbouring hexamers are connected exclusively by CTD contacts through the CTD-CTD dimerization interface. [Adapted from (Pornillos et al., 2009)]

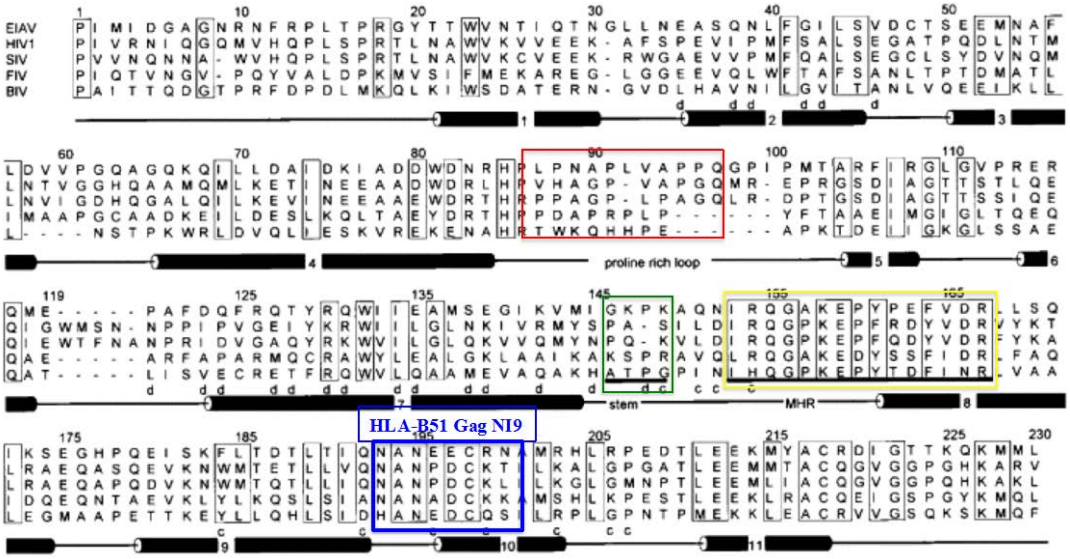

Figure 1.6 Comparison of lentivirus CA sequences and structures Alignment of five representative cone-forming lentivirus CA sequences (EIAV; SIV; HIV; FIV, feline immunodeficiency virus; BIV, bovine immunodeficiency virus). The numbering corresponds with the EIAV sequence, and positions of helices are indicated below the sequence. The proline rich loop, stem and MHR regions are boxed with red, green and yellow, respectively. An HLA-B51 restricted epitope Gag p24 NANPDCKTI is boxed in blue.

Nucleocapsid (p6 and p7)

Nucleocapsid protein p7 is the third component of Gag polyprotein and enclosed inside the virion core. The primary function of nucleocapsid protein is to bind specifically to the packaging signal and deliver full-length viral RNAs into the assembling virion (Berkowitz et al., 1995).

The function of Nucleocapsid p6 has not been well described. It is known that protein P6 plays a critical role in the binding of Vpr during viral assembly (Kondo and Gottlinger, 1996; Lu et al., 1995). Moreover, mutations in the L domain of p6

result in virions tethering to the cell membrane, suggesting its function in the late stage of budding (Gottlinger et al., 1991; Huang et al., 1995).

1.3.3.2 Pol

Pol is one of the main retroviral genes. There are three viral enzymes encoded by this polymerase gene (*pol*). They are protease (PR), reverse transcriptase (RT) and integrase (IN). These enzymes are generated from a Gag-Pol precursor polyprotein (the p160 polyprotein) that is produced by ribosome frameshifting during the translation of p55 polyprotein (Freed, 2001; Jacks et al., 1988).

Protease (PR)

Protease is an aspartyl protease with two symmetrical subunits. Each subunit consists of identical polypeptides 99 amino acids in length. HIV protease is responsible for cleaving the viral precursor proteins p55 and p160 into their functional parts, which are essential for viral assembly and subsequent activity. Blocking protease activity will prevent the Gag and Gag-Pol polyproteins from being cleaved, resulting in immature, non-infective virions being produced (Kohl et al., 1988; Krausslich et al., 1989). Thus, HIV protease has been a prime target for drug therapy and several protease inhibitors have been licensed for HIV therapy (Pomerantz and Horn, 2003; Rang, 2007). By inhibiting specific binding of its substrate to the active site, the protease inhibitors disable the enzyme activity and prevent the production of new infectious virions. This results in the substantial reduction of plasma viral load in the patients.

Reverse transcriptase (RT)

The HIV-1 reverse transcriptase (RT) is responsible for copying a single-stranded viral RNA genome into double-stranded DNA that can integrate into the host genome, potentially generating a long-term infection that can be very difficult to eradicate. It contains two main functional domains: a DNA polymerase domain and a ribonuclease H (RNase H) domain (Sarafianos et al., 2001). The DNA polymerase domain allows either a DNA or an RNA template to be copied through its functions as RNA-dependent DNA polymerase and DNA-dependent DNA polymerase. The RNase H cleaves RNA only if the RNA is part of an RNA/DNA duplex, meaning that the template RNA can be cleaved and degraded only after DNA synthesis. This allows the newly made DNA to generate a second DNA strand. In addition, HIV reverse transcriptase is error-prone and lacks proofreading function, which leads to the random incorporation of mutations in newly transcribed DNA (Roberts et al., 1988).

HIV-1 reverse transcriptase is a heterodimer consisting of two distinct, but related subunits, a 66-kD subunit (p66) and a 51kD subunit (p51). Both units are produced from a Gag-Pol polyprotein by the cleavage of the viral protease. P51 is related to p66, however, lack of the C-terminal RNase domain differentiates p51 from p66. These two domains play important role in the binding of the DNA:RNA substrate (Kohlstaedt et al., 1992; Sarafianos et al., 2001).

The crystallographic studies have elucidated important structural features of HIV-1 RT (**Figure 1.7**) (Jacobo-Molina et al., 1993; Kohlstaedt et al., 1992; Sarafianos et al., 2001). As shown in **Figure 1.7**, P66 is composed of two enzymatic functional domains, polymerase and RNase H. The polymerase domain consists four subdomains: fingers (1-85 and 118–155), palm (residues 86–117 and 156–236), thumb (237–318), and connection (319–426). P51 also folds into these four subdomains (fingers, palm, thumb, and connection) but with different positions relative to p66.

Similar to PR, RT is the targets of many drug inhibitors. Among them, Zidovudine/AZT, a nucleoside RT inhibitor (NRTI), was introduced in 1987 and is the first anti-HIV drug in therapy.

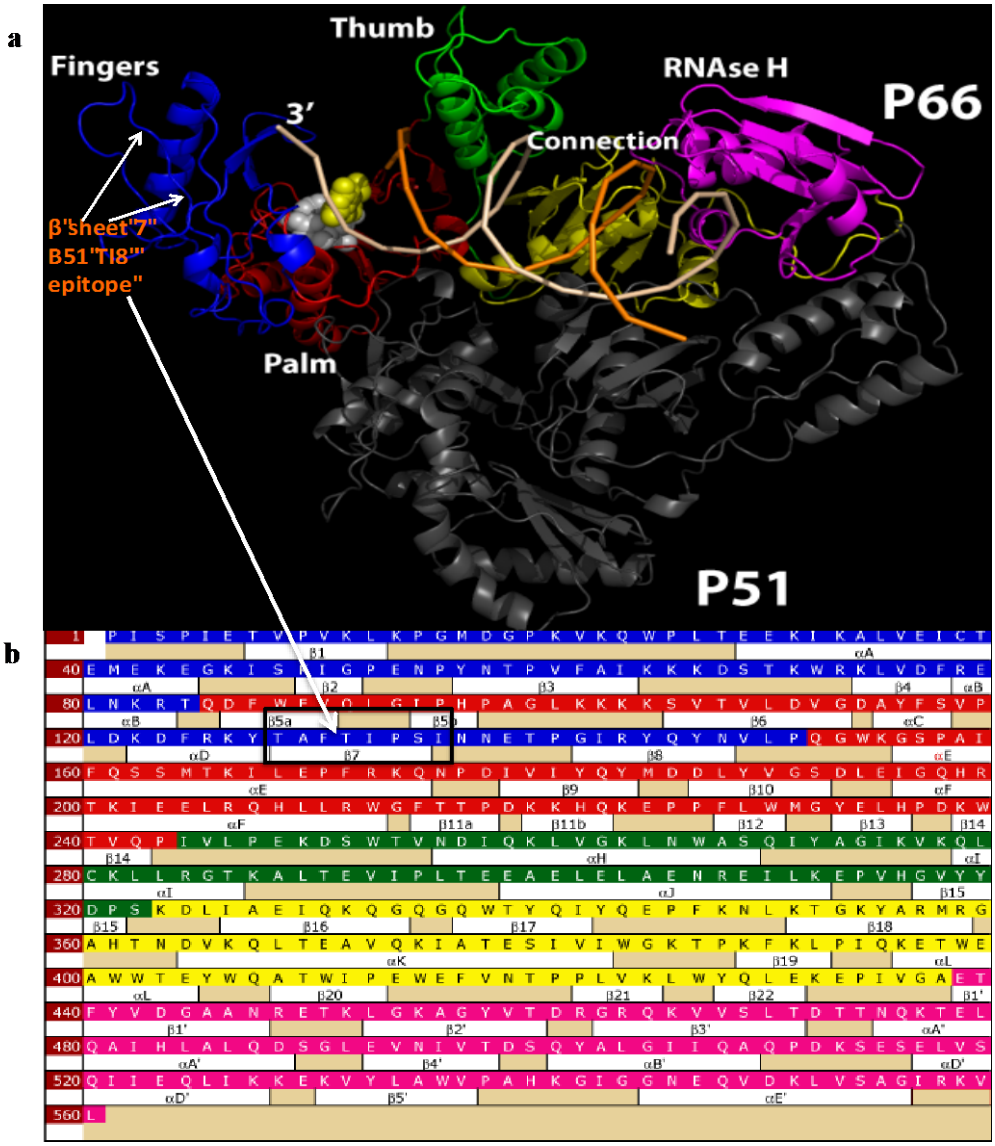

Figure 1.7 Ribbon schematic structure of catalytic heterodimer of HIV-1 RT and the amino acid sequence of p66 subunit a) The RT enzyme is co-crystallized with the nucleic acid template / primer and incoming dNTP reported by Huang et al. (1998). The enzyme active site is shown in white space-fill mode. The structural domains of p66 monomer are fingers (in cyan), palm (in green), thumb (in red), connection (in yellow) and RNase H (purple). The p51 monomer is shown in grey. b) The amino acids from different structure domains are shade in the corresponding colour as shown in a) and the positions of secondary structure are shown in white boxes. An HLA-B51 restricted epitope RT (128-135, TAFTIPSI) is boxed with black and forms the β7 strand. Adapted from http://hivdb.stanford.edu/pages/3DStructures/rt.html

Integrase (IN)

HIV-1 Integrase (IN) is responsible for inserting the double-strand viral DNA into the host chromosomal DNA during the HIV-1 replication (Bushman et al., 1990), as a result, the host cell becomes a carrier of the viral genome (provirus). IN recognises long terminal repeats (LTRs) at the 5′ and 3′ ends of the newly transcribed double-stranded viral DNA and cleaves two (or three) bases from the 3′ ends. Then the cleaved 3′ ends are ligated to the cellular DNA in the nucleus (Brown, 1997).

HIV-1 Integrase is a 32-kDa protein and consists of three functional domains: an N-terminal HH-CC zinc-binding domain, a catalytic core domain (RNaseH fold) and a C-terminal DNA-binding domain (SH3 fold). The NTD subdomains encompassing residues 1 to 49 forms an HHCC zinc binding motif and contribute to multimerisation, as illustrated in **Figure 1.9a** (Zheng et al., 1996). The catalytic core domain (CCD, residues 50–212) contains a highly conserved triad of acidic residues (D64, D116, and E152) and carries out both the 3′-P and ST reactions and contributes to both viral DNA and host DNA binding and multimerisation. The C-terminal domain (CTD, residues 213–288) is primarily involved in host DNA binding.

An HLA-B51 restricted CTL epitope LI9 (In 28-36, LPPVVAKEI), as highlighted in **Figure 1.8**, sits between helix 2 and helix 3 of the N-terminal domain.

Similar to PR and RT, Integrase is also the target of many drug inhibitors. The first Integrase inhibitor, Raltegravir, was approved for therapy in 2007. Elvitegravir as the second integrase inhibitor was approved in August 2012.

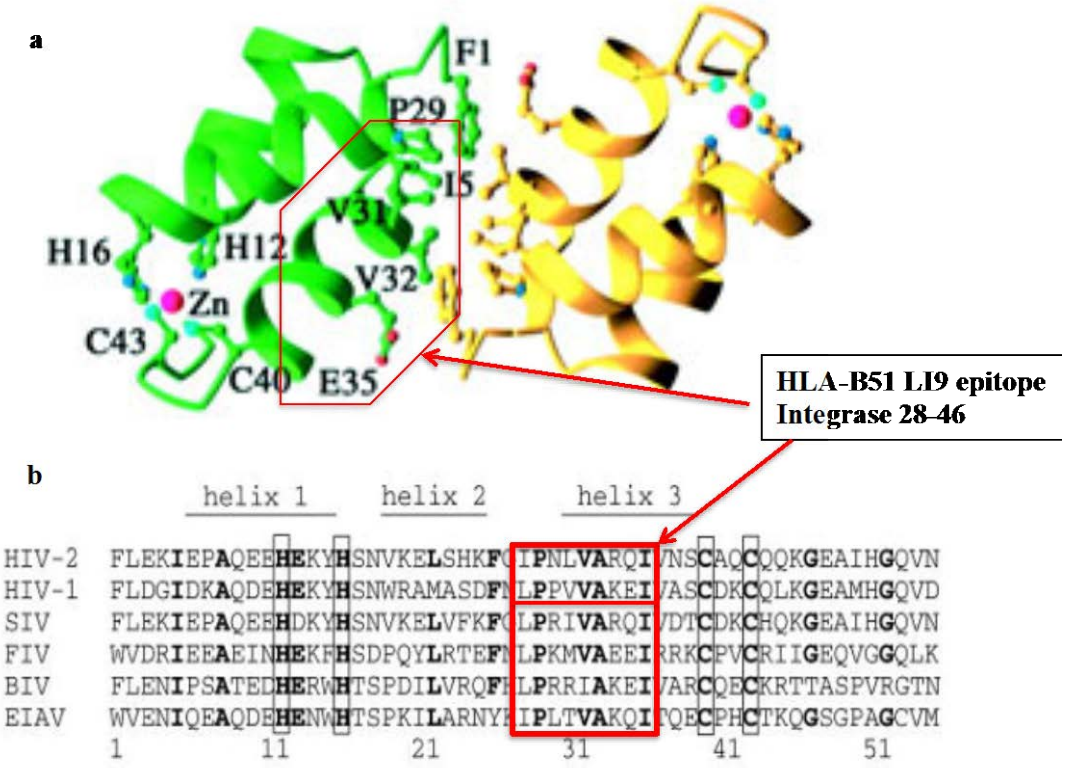

Figure 1.8 Structure and sequence of N-terminal domain of Integrase. a) Ribbons diagram of the N-terminal domain structure in the context of $IN^{1–212}$ by X-ray crystallography. Residues involved in the dimerization, which are rather hydrophobic, are shown in ball-and-stick. Zn^{2+} ions are shown as red spheres [Adapted from (Eijkelenboom et al., 1997)] **b)** Alignment of N-terminal amino acid sequence of Integrase from HIV-2, HIV-1, SIV, FIV (feline immunodeficiency virus) BIV (bovine immunodeficiency virus) and EIAV (equine infectious anaemia immunodeficiency virus). The position of the three α helices of $IN_{1–55}$ is indicated above the sequences. An HLA-B51 CTL epitope LI9 (In 28-36) is highlighted within the red box. [Adapted from (Wang et al., 2001)]

1.3.3.3 Env

The *Env* gene encodes the envelope glycoprotein of HIV-1. It is a 160 kD precursor, known as gp160, which can be cleaved by the cellular protease into the external surface unit protein gp120 (SU) and trans-membrane protein gp41 (TM) (Moulard and Decroly, 2000). Gp120 and gp41 form highly glycosylated trimeric complexes on the surface of the HIV virion, which mediate the attachment and fusion of virions to host cell.

Gp41 is a transmembrane protein that involves viral fusion and anchoring the Env to the virion surface through its binding to gp120. The external surface unit protein gp120 forms the spikes sticking out of a HIV virus particle and mediates viral entry into the host cell through its binding to specific receptors on the target cells. Gp120 binds to its major receptor, human cluster differentiation antigen 4 (CD4), and its co-receptors such as CCR5 and CXCR4 and then mediates the viral entry to the host cells (Berger et al., 1998; Dalgleish et al., 1984). In addition to its function in viral entry, as the sole HIV antigen on the viral surface, gp120 is a target for neutralising antibodies (NAbs).

1.3.3.4 Nef

Nef, initially referred to as a negative regulatory factor (Terwilliger et al., 1986), is one of the first HIV proteins that are produced in infected cells and is the most immunogenic accessory protein. Further research has revealed that this 206 amino-acids myristoylated protein plays a positive role in viral replication and

pathogenesis (Kestler et al., 1991). Although the mechanism underlying its role in AIDS pathogenesis is not well understood, it is generally assumed that Nef exerts this role via several independent activities: a) down-regulation of CD4 (Arganaraz et al., 2003; Garcia and Miller, 1991; Lundquist et al., 2002); b) down-regulation of major histocompatibility class I (MHC-I) molecules from the cellular surface (Schwartz et al., 1996); c) activation of cellular protein kinases (Renkema et al., 1999; Simmons et al., 2001); d) enhancement of viral infectivity and replication (Chowers et al., 1994; Miller et al., 1994) .

It has been proposed that HIV-1 Nef protein down regulates the CD4 co-receptor from the surface of host cells by enhancing the rate of CD4 endocytosis through a clathrin/AP-2 pathway and targeting CD4 to the multivesicular body (MVB) pathway for eventually degradation in the lysosome. Aiken et al. (1994) showed that the removal of CD4 from the cell surface can be achieved through the internalisation of CD4 by association between Nef, AP-2, and the cytoplasmic tail of CD4 in a tripartite complex. However, some studies have indicated that Nef targeting CD4 to lysosomes probably has a more profound effect on CD4 down regulation, as simply increasing the internalisation of CD4 would not significantly decrease the level of the protein on the plasma membrane unless recycling back to the cell surface is also suppressed. As a result of lysosome degradation, the total amount of CD4 in Nef expressing cells is reduced (Anderson et al., 1994; daSilva et al., 2009; Luo et al., 1996).

HIV-1 Nef protein also down regulates cell surface expression of major histocompatibility complex (MHC)-I molecules. Although the mechanism underlying this function remains to be elucidated, Nef-mediated MHC class I down regulation can be seen as another strategy to protect HIV-infected cells from CTL-mediated killing and provide a selective advantage for viral persistence and replication *in vivo* (Lewis et al., 2008; Tomiyama et al., 2002; Yang et al., 2002).

Nef mediates the enhancement of viral infectivity and replication. The replication rates of Nef-positive and Nef-defective HIV-1 clones in activated primary blood lymphocytes indicated that Nef directly promotes HIV-1 replication by enhancing the infectivity of virions (Chowers et al., 1994). However, the mechanisms underlying this effect are still unclear. It has been proposed that Nef may enhance virus entry by allowing the virus to cross the cortical actin barrier of a host cell (Campbell et al., 2004a; Fackler et al., 1999).

In addition, Nef was also found to modulate T cell function and apoptosis by engaging components of cellular signalling pathways. Nef protein can activate serine/threonine protein kinase PAK-2, which may interfere with apoptotic cell death, induces activation of infected cells (Raney et al., 2005), resulting in a remarkable increase in release of virus particles. It has been reported recently that Nef down-regulates the cell surface and total expression of the negative immune modulator CTLA-4 by enhancing protein degradation in lysosomes (El-Far et al., 2013).

1.3.3.5 Tat

Tat gene encodes an 86-101 amino acid polypeptide Tat (Trans-Activator of Transcription) that accelerates the transcription from the HIV-1 long terminal repeat (LTR) promoter (Sodroski et al., 1985). Tat binds to an RNA hairpin structure at the 5' ends of nascent HIV-1 transcripts, known as the trans-activating response element (TAR). After binding to TAR, Tat associates with the cellular transcription factors and mediates their phosphorylation, resulting in efficient transcription elongation of all HIV genes (Emerman and Malim, 1998; Kao et al., 1987).

Apart from the function as an activator of transcription, Tat also plays a more direct role in HIV disease progression. Tat can be released into supernatant and is also found in the blood of HIV-1-positive patients. This soluble Tat protein can act directly as a toxin that induces the apoptosis of uninfected T cells (Campbell et al., 2004b) and also functions as a transcription enhancer in latently infected cells (Ensoli et al., 1990), accelerating the disease progression to AIDS. In addition, it has been reported that soluble Tat, a CXCR4-specific antagonist, selectively inhibits the entry and replication of more virulent X4 viruses in the early stage of HIV-1 infection, thereby affecting the early *in vivo* course of HIV-1 disease (Xiao et al., 2000).

1.3.3.6 Rev

Rev (Regulator of Virion Expression) is another regulator protein encoded by the HIV genome. It is a 19-kDa phosphoprotein consisting of 118 amino acids and originally localised in the nucleus. Rev shuttles the un-spliced and partially spliced viral mRNA transcripts, which encode the viral genome and the structural proteins (Gag, Pol and Env), from the nucleus to cytoplasm before being spliced by the host RNA splicing machinery. Rev directly binds to a highly conserved mRNA sequence in gp41, named as a Rev Response Element (RRE), and forms a Rev-RRE complex (Malim et al., 1990). This complex is then transferred into the cytoplasm where the structural proteins and RNA genome are produced for viral assembly. Afterwards, Rev molecules dissociate from the complex and are shuttled back to the nucleus (Love et al., 1998).

1.3.3.7 Vpr

Viral protein R (Vpr) is a 96-amino-acid, 14-kDa protein that is incorporated into viral particles through an interaction with p6 (Sato A, et al., 1996). Vpr mediates multiple activities that assist HIV-1 infection, evasion of the immune system, and persistence in the host, therefore contributing to the morbidity and mortality of acquired immunodeficiency syndrome (AIDS) (Kogan and Rappaport, 2011). First, Vpr mediates rapid nuclear import of the viral pre-integration complex (PIC) and enables macrophage infection (Balliet et al., 1994; Connor et al., 1995). Secondly, Vpr has been shown to activate the HIV-1 long terminal repeat (LTR) in *trans* and other cellular promoters, which likely contributes to increased viral replication and

pathogenesis (Agostini et al., 1996; Wang et al., 1995). Furthermore, Vpr induces G_2 cell-cycle arrest by promoting phosphorylation of Chk1, which increases viral production (Li et al., 2010). In addition, Vpr molecular cause immune dysfunction that may aid HIV-1 evading immune responses (Ayyavoo et al., 2002; Muthumani et al., 2002).

1.3.3.8 Vif

Vif (Viral infectivity factor) is a highly basic, 23-kDa phosphorylated protein that plays a crucial role in the viral life cycle to facilitate viral infectivity.

It was found that Vif performs this function in a cell type-dependent way. Vif is essential for HIV-1 replication and reproduction of infective progeny in primary lymphocytes, macrophages, and some 'non-permissive' T-cell lines such as H9, PM1 and CEM (Strebel et al., 1987), whereas functional Vif is not required for the production of infective viruses in some 'permissive' T-cell lines (e.g. 293T, Hela, Jurkat) (Gabuzda et al., 1992). Numerous studies have revealed the mechanism underlying this effect. A host cellular enzyme, known as APOBEC3G, is expressed in the non-permissive' cells and inhibits HIV infection through its cytidine deaminase activity that results in lethal mutagenesis in the proviral DNA (Mangeat et al., 2003). However, Vif destroys this antiretroviral factor by preventing incorporation of APOBEC3G into HIV-1 virions and promoting destruction of the enzyme, which enables production of viable and infective HIV-1 virions in the presence of APOBEC3G activity (Pillai et al., 2008; Wissing et al., 2010).

Recent studies have reported another novel function of Vif. Similar to another accessory HIV-1 protein Vpr, Vif can also induce G2 cell cycle arrest in a p53-dependent manner in HIV-1-infected cells, which facilitates viral replication (Izumi et al., 2010; Sakai et al., 2006; Wang et al., 2007)

1.3.3.9 Vpu

Vpu gene, referred to as U ORF, is present in the genome of HIV-1 but is absent from HIV-2 and a subset of related SIVs (e.g. SIVsmm, SIVnac) (Cohen et al., 1988; Strebel et al., 1988). Vpu protein encoded by HIV-1 is a 77-86 amino-acids membrane-associated accessory protein that is found in the membranes of infected cells, but not in the viral particles themselves. Vpu exerts two primary functions during the viral life cycle. First, it functions to promote proteasomal degradation of newly synthesised CD4 molecules in the endoplasmic reticulum (ER), leading to HIV-mediated down regulation of the CD4 receptor (Schubert et al., 1998; Willey et al., 1992). In addition to its effect on CD4 degradation, Vpu enhances the release of progeny virions from HIV-1-infected cells by antagonizing Tetherin (also called BST2, CD317 and HM1.24), an interferon (IFN)-regulated host restriction factor that inhibits the release of virions from the plasma membrane of infected cells (Neil et al., 2008; Strebel et al., 1988; Van Damme et al., 2008).

1.4 HIV-1 life cycle

From infection to production, HIV life cycle proceeds in an ordered, step-wise manner and can be summarized as following seven steps (**Figure 1.9**).

1.4.1 Binding and fusion to the target cells

HIV entering the cell begins with the attachment of the virions to the cell surface through interaction between the extracellular domain of HIV-1 glycoprotein 120 (gp120) and cellular receptors on the surface of target cells (Moore J and Ho D, 1993). The initial high-affinity binding and engagement of the CD4 receptor and gp120 causes the trimeric envelope complex gp120/gp41 to undergo a conformational change, which enables the chemokine binding domains of gp120 to be exposed for interacting with the target chemokine receptor such as CCR5 or CXCR4. This stable two-pronged attachment allows the N-terminal fusion peptide gp41 to penetrate the cell membrane (Chan and Kim, 1998). The virus then fuses with the host membrane, allowing the virus core to gain access to the cytoplasm.

1.4.2 Uncoating and reverse transcription

Once virus has bound to the target cell, the virus then uncoats and the viral core is released into host cell cytoplasm (Chan and Kim, 1998). HIV RNA genome and various enzymes, including reverse transcriptase, integrase, ribonuclease, and protease, are injected into the cell. The single-stranded viral RNA is then transcribed into a partially duplex linear DNA by reverse transcriptase (RT), which involves both RNA-dependent and DNA-dependent DNA polymerisation.

1.4.3 Nuclear import and integration

The viral DNA and associated proteins are assembled into the pre-integration complex (PIC) and imported into the host cell nucleus with the assistance of HIV proteins Vpr and MA. Then the viral DNA integrates into the host genome with the help of the viral enzyme integrase. The integrated DNA, known as the 'provirus', may remain in a latent state until the host cell is activated (Stevenson et al., 1990).

1.4.4 Transcription

Proviral transcription starts when the infected cells are activated by cellular and viral factors such as TAK and HIV-1 Tat protein. The activation of transcription promoter, which is located in the 5' long terminal repeat (LTR) of HIV-1 genome, initiates the synthesis of viral RNA. Three kinds of viral RNAs are produced after transcription: a) unspliced mRNAs, encoding Gag, Pol polyprotein precursors or being packed into progeny virions as genomic RNA; b) singly spliced mRNAs, encoding Env, Vif, Vpu and Vpr; c) multiply spliced mRNAs, encoding Rev, Tat, and Nef (Freed, 2001).

1.4.5 Nuclear export and translation

The multiply spliced mRNAs are transported out easily from nucleus by cellular factors and then the accessory proteins Rev, Tat and Nef are expressed during the early stage of viral replication (Kim et al., 1989). By contrast, the unspliced and singly spliced mRNAs require the action of Rev. This small protein binds to the

Rev-Responsive Element (RRE), located within the env mRNA coding region, and forms a complex. This complex of RNA-Rev is then transported into cytoplasm by the host nuclear export factor and translated into viral proteins (Emerman and Malim, 1998; Frankel and Young, 1998).

1.4.6 Assembly and budding

The assembly of a new HIV-1 virion begins at the plasma membrane of the host cell. First, the envelope glycoproteins (gp120 and gp41) are transported to the plasma membrane of the host cell where gp41 anchors gp120 to the membrane of the infected cell. Then the Gag (p55) and Gag-Pol (p160) polyproteins also associate with the inner surface of the plasma membrane and form a capsid, where two copies of HIV genomic RNA and other associated proteins are encapsulated. This capsid then associates with the envelope proteins that are previously translocated to the cell membrane, ultimately the viral-like particle buds from the host cell membrane. This budding process often leads to the lysis of infected cells.

1.4.7 Viral maturation

During or after the budding, the viral particle undergoes a maturation process involving the proteolytic cleavage of Gag and Gag-Pol precursors by viral protease PR (Kaplan et al., 1994). After viral maturation, the newly produced viruses are ready to infect the new targets.

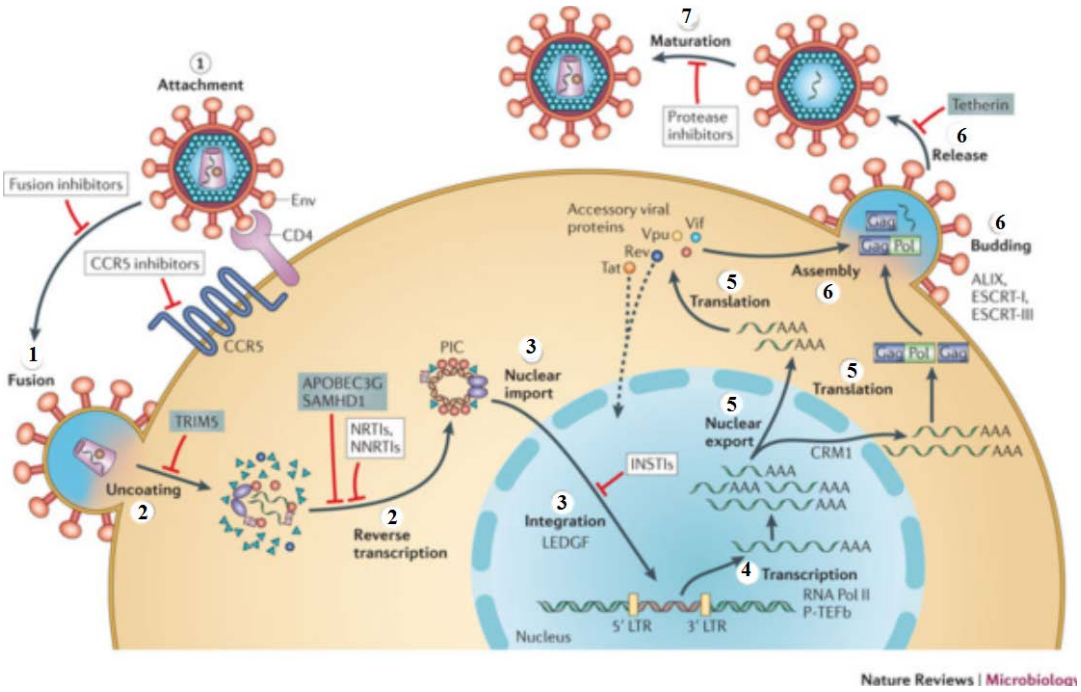

Figure 1.9 The HIV-1life cycle. The life cycle of HIV-1 from its infection to production comprises numerous key steps including viral binding and fusion into the cell, uncoating and reverse transcription, nuclear import and integrating, viral genome replication, assembly and budding, viral maturation. [Adapted from (Engelman and Cherepanov, 2012)]

1.5 HIV-1 pathogenesis and disease course

1.5.1 The disease course of HIV-1 infection

As shown in **Figure 1.10**, HIV-1 infection can be divided into three phases: an acute/primary phase with intense viral replication and spreading to lymphoid tissues; a chronic, often asymptomatic phase of sustained immune activation and viral replication; and the symptomatic phase with marked depletion of $CD4^{+}$T cells that leads to acquired immune deficiency syndrome (Pantaleo et al., 1993).

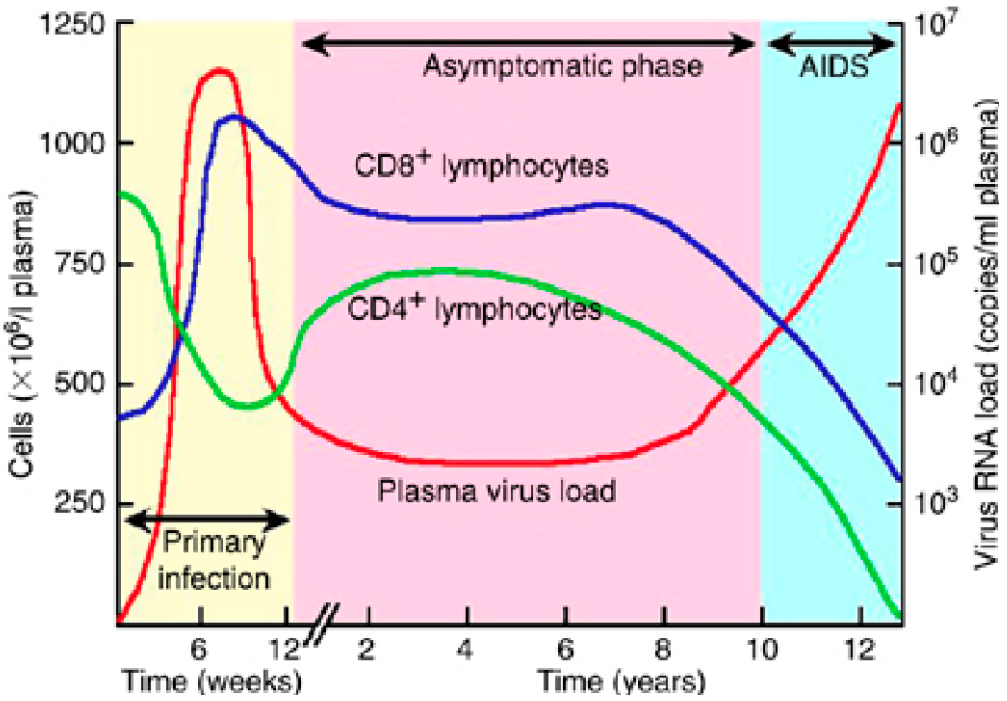

Figure 1.10 The time course of HIV infection. [Adapted from (Munier and Kelleher, 2007)]

During the first few weeks of acute infection, intensive viral replication results in sharp increases in viral load to peak level of viraemia (up to 10^7 of viral RNA molecules per ml of plasma) and is accompanied by an increase of CD8+ lymphocytes. Around the time of peak viraemia, patients may become symptomatic and symptoms may remain for 6-12 weeks (Kahn and Walker, 1998). Following the initial peak of viraemia, the plasma viral load decreases to steady-state level, referred as "viral set point", which varies between individuals and is a critical factor that determines subsequent disease progression (Mellors et al., 1996). Meanwhile, the number of circulating $CD4^+$ T cells also changes, with initial decreases and a rebound to near pre-infection levels afterwards. In addition, the HIV-specific cell-mediated immune responses are readily detected early in this phase, but neutralising antibodies are not detected until 4-6 months post-infection.

Four to six months following acute infection, the patient moves into a prolonged asymptomatic phase. Although there are detectable immune responses to HIV-1, the virus continuously replicates at a low level and the $CD4^+$ T-cell count in peripheral blood declines gradually at variable levels. During this chronic phase of infection, patients can display no symptoms for several years.

The last stage of HIV infection is the symptomatic phase or AIDS with a dramatic depletion of $CD4^+$ T-lymphocytes (<200 cells/mm^3 of blood) that leads to acquired immune deficiency syndrome (AIDS). AIDS results from the opportunistic infections or tumours that eventually lead to the death of the HIV infected individual (Samuel et al., 2002).

1.5.2 HIV-1 pathogenesis

The major hallmarks of AIDS include destruction of helper $CD4^+$ T lymphocytes and subsequent loss of immune competence.

A massive depletion of $CD4^+$ T cells in the peripheral blood has been found during the primary infection before the emergence of a detectable acquired immune response in the SIV macaque model and HIV-1-positive patients. This depletion either in primates or in humans may be associated with the massive depletion of CCR5+ memory CD4+ T cells that reside in the gut-associated lymphoid tissue (GALT) (Brenchley et al., 2004; Mattapallil et al., 2005; Veazey et al., 1998).

Several mechanisms may lead to this massive depletion of mucosal $CD4^+$ T cells and gradual decline of circulating $CD4^+$ T cells during primary infection.

First, a direct cytopathic effect of the virus on infected $CD4^+$ T cells could be the major mechanism. Infected $CD4^+$ T cells may be killed directly when large numbers of virus are produced and bud out from the cell surface, disrupting the cell membrane (Barre-Sinoussi et al., 1983; Popovic et al., 1984), or when viral proteins and nucleic acids interact with cellular machinery inside the cells (LaBonte et al., 2000; Stewart et al., 1997) . Moreover, the infected $CD4^+$ T cells can also be depleted via direct killing by antigen-specific cytotoxic T lymphocytes or natural killer (NK) cells. In addition, it has been reported that during HIV-1 infection, both the number of virus infected and uninfected $CD4^+$ T cells decreased. One possible explanation is bystander cell killing due to the upregulation of Fas ligand induced by viral protein Nef. The interaction between Fas ligands on the infected cells and Fas expressed on the neighbouring uninfected cells will trigger apoptosis, therefore leading to the depletion of uninfected $CD4^+$ T cells (Geleziunas et al., 2001; Xu et al., 1997).

Apart from the mechanisms discussed above, it is believed that the increased immune activation during HIV-1 infection may also induce $CD4^+$ T cell depletion. This immune disregulation has been observed in both SIV and HIV infections. It has been reported that SIV infected sooty mangabeys do not progress to AIDS whereas rhesus macaques develop to AIDS in spite of comparable viral load,

probably resulting from the lower level of immune activation in SIV infected sooty mangabeys (Silvestri et al., 2003). This situation is also observed in humans infected with HIV-1 and those who are infected with less pathogenic HIV-2, due to lower immune activation caused by HIV-2 (Sousa et al., 2002). Finally, recent gene-expression analysis also revealed that the inflammatory markers linked to immune activation might contribute to the pathogenesis (Bosinger et al., 2009).

By targeting the major component of immune system $CD4^+$ T helper cells, HIV-1 causes not only massive $CD4^+$ T cells depletion and disregulation, but also induces immune dysfunction of $CD8^+$ T cells, B cells, NK cells and nonlymphoid cells through increased cell turnover, activation, differentiation and homeostatic responses (Moir et al., 2011). The high level of T cell activation results in a high turnover of $CD4^+$ and $CD8^+$ T cells and impaired immune reconstitution (Douek, 2002). This physiological limit on thymic output of naïve CD4+ T cells in HIV-1 infection would severely limit reconstitution of the memory $CD4^+$ T cell pool (Teixeira et al., 2001). Moreover, the high level of immune activation also increases apoptosis of $CD4^+$ and $CD8^+$ T cells as well as B cells. More importantly, the persistent HIV-1 replication, increased immune activation and depletion of $CD4^+$ T helper cells also alters the quality of cellular and humoral responses, which probably leads to the loss of immune competence. The appearance of HIV-1-specific T cells and B cells that display markers of exhaustion and senescence is one of the examples of the ineffectiveness of immune responses against HIV-1 (Appay et al., 2008; Moir and Fauci, 2009). Another evidence of this is that $CD8^+$

T cells that primed in the absence of $CD4^+$ T cell help showed impaired proliferative capacity when reencountering the antigen (Rocha and Tanchot, 2004).

Nevertheless, in HIV-1 infection, it is generally believed that the combination of massive acute-phase depletion of mucosal $CD4^+$ T cells, chronic immune activation induced $CD4^+$ T cell depletion and $CD8^+$ T cell dysfunction result in progression to AIDS.

1.5.3 HIV long-term non-progressors

The rate of disease progression of HIV-1 infected patients varies greatly from person to person. Without ART, the majority of HIV-1 infected patients progressed to AIDS within an average time of 8-10 years after initial infection (Pantaleo and Fauci, 1996). However, there are a small number of HIV-1 seropositive patients who show a markedly slow disease progression to AIDS. These individuals, termed as long-term non-progressors (LTNPs), have been infected with HIV-1 for more than 10 years without antiretroviral therapy (Mikhail et al., 2003). They maintain low level of plasma viral load and elevated $CD4^+$ T-cell count throughout their course of infection.

There are no official standards to define the various HIV controller populations. However, it has been proposed by Easterbrook (1999) that the long-term non-progressors (LTNPs) maintain a normal $CD4^+$ T-cell count >500 cells/ mm^3 in the absence of anti-retroviral therapy (ART) for 10 years or more with a variable plasma viral load less than 10,000 copies/mm^3. Among the LTNPs, a small subset

(less than 1%) patients, referred to as elite controllers (ECs), have undetectable level of viral RNA (<50 copies/mm^3) for an unspecified period without ART (Saksena et al., 2007; Walker, 2007). Intensive research has focused on these two groups of subjects who exhibit natural resistance to HIV disease progression, in order to yield critical information on the viral, genetic and immunological factors that determine the disease non-progression, which may be beneficial to the design of new interventional strategies to control the disease (Foster et al., 2011; Walker, 2007).

1.6 Host immune responses against HIV-1 infection

1.6.1 Innate immune responses to HIV-1

Increasing evidence suggests that innate responses are key determinants of the outcome of HIV infection, influencing critical events in the earliest stages of infection and also impacting on the subsequent level of ongoing viral replication and rate of disease progression. Among them, IFN-α and natural killer (NK) cells both play an important role.

1.6.1.1 DCs and IFN-α

During the early stage of HIV-1 infection, an ordered increase in plasma levels of multiple cytokines and chemokines has been observed with an initial rapid increase and peaking of IFN-α production (Stacey et al., 2009). As the first cells to encounter HIV-1 after the mucosal transmission, activated plasmacytoid dendritic

cells (pDCs) are the main source of induced plasma IFN-α in acute HIV-1 infection. It has been shown that plasmacytoid DCs produce cytokines through interaction of toll-like receptors (TLRs) with viral ssRNA following the endocytosis of HIV virions (Beignon et al., 2005; Chang and Altfeld, 2010). The anti-viral functions of IFN-α are mediated by blocking early steps in viral replication, inhibition of HIV gene expression, and effects on viral assembly and budding (Baca-Regen et al., 1994). IFN-α was also demonstrated to potently induce APOBEC3G (Apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G) to override HIV-Vif neutralisation of APOBEC3 proteins that pose a threat to efficient macrophage HIV replication (Peng et al., 2006).

However, IFN-α may also mediate detrimental effects by promoting local immune activation and therefore enhancing virus transmission or increasing HIV replication (Fitzgerald-Bocarsly and Jacobs, 2010). Further studies are needed to explore the balance between the beneficial and detrimental effects.

1.6.1.2 NK cells

Another main cellular component of the innate immune system against tumour and virus infection is natural killer (NK) cells.

NK cells are capable of killing viral infected target cells through perforin/granzyme or death receptor dependent pathways that are also employed by $CD8^+$ T cells. It has also been reported NK cells elicit an antibody-dependent cellular cytolytic

responses (ADCC) and therefore are able to directly lyse HIV-1 infected cells (Connick et al., 1996; Forthal et al., 2001).

In addition to direct killing capability, NK cells also modulate their antiviral activity against HIV-1 infection through their germline-encoded receptors. Increasing evidence has suggested that NK cells make a significant contribution to control the virus replication in HIV-infected individuals. It was found that during the acute stage of HIV-1 infection, NK cells are expanded prior to the development of adaptive immune responses and NK cell activity is directly correlated with the level of viral replication during acute HIV-1 infection (Alter et al., 2007b). Evidence that NK cells play a role in control of HIV-1 replication comes from *in vitro* assays and the genetic studies, showing that KIR3DS1^{+} NK cells can mediate profound inhibition against HIV replication in HLA-Bw480I-positive target cells, and that KIR3DS1 in patients is associated with low-level viremia in early HIV infection and also with delayed disease progression when co-expressed with HLA-Bw480I (Alter et al., 2007a; Martin et al., 2002; Qi et al., 2006b).

1.6.2 Antibody responses to HIV infection

The anti-viral activity of antibodies can be achieved by binding to free virus particles and by several activities directed against infected cells. Binding of antibodies to free virus particles can prevent further infection of target cells, referred to as “neutralisation” and also induce complement-associated lysis of the virions. The direct recognition of infected cells by antibodies can result in cell

lysis, the inhibition of viral replication, inhibition of the release of viruses from infected cells and of cell–cell transmission of viruses (Burton, 2002).

By longitudinally monitoring B-cell responses immediately following HIV-1 transmission, Tomaras et al. (2008) showed that the first B-cell response can be detected in the form of antibody-virus immune complex eight days after detection of plasma virus load, while the first free plasma gp41-specific antibody appeared five days later. After a further 14 days, envelope gp120-specific antibodies can be detected. However, none of these antibodies are neutralising. In most patients, the neutralising antibody responses only appear three to twelve months after primary infection when the acute phase of viraemia has been controlled (Gray et al., 2007). Although the early autologous neutralising antibody responses often have high titers, the targets of the response are narrow with little to no cross-neutralising activity (Moore et al., 2008).

While most patients produce neutralising antibodies to some extent, the broadly neutralising antibodies (BnAbs) that target the conserved regions of viral envelope are rare in natural HIV-1 infection and arise too late to control viral replication. For example, the broad neutralizing antibodies (e.g. antibodies 2G12, 1b12, 2F5, Z13, 4E10, PG9 and PG16) isolated from the HIV-1 infected patients occur only after ~20-30 months (McMichael et al., 2010; Walker et al., 2009). Recently Liao et al. (2013) have shown that a CD4-binding site BnAb CH103, which neutralised approximately 55% of HIV-1 isolates, may be first detectable as early as 14 weeks

after infection. Nonetheless, no correlation between the presence of BnAbs and HIV-1 viral control has been found (Doria-Rose et al., 2010; Lambotte et al., 2009; Poignard et al., 1999; Sajadi et al., 2011). It is therefore difficult to determine their role in HIV-1 control.

However, the non-neutralising antibodies that mediate their activity through Fc-receptor, such as antibody dependent cellular cytotoxicity (ADCC), have been thought to play a role in controlling HIV-1 infection (Chung et al., 2009; Hessell et al., 2007; Stratov et al., 2008). Several studies have suggested that ADCC responses may have a contribution to viral control during the early infection (Connick et al., 1996; Forthal et al., 2001). The partial efficacy shown by the recently reported canarypox prime/protein boost vaccine trial conducted in Thailand could possibly be associated with ADCC antibodies (Karnasuta et al., 2005; Rerks-Ngarm et al., 2009). The evidence from ADCC responses forcing immune escape also suggested that ADCC responses apply significant immune pressure on the virus (Chung et al., 2011).

Nonetheless, ADCC is still an area relatively poorly explored in HIV immunology. To date, only a few ADCC epitopes have been identified within HIV and one such antibody has been isolated (Ferrari et al., 2011b).

1.6.3 The $CD4^+$ T-cell responses to HIV-1

It has been shown that $CD4^+$ T-cells are crucial to maintain anti-viral $CD8^+$ T cell memory during acute and chronic infection (Matloubian et al., 1994; Sun and Bevan, 2003; Sun et al., 2004). In the absence of $CD4^+$ T cell help, $CD8^+$ T cells can be primed, however with impaired proliferative capacity when encountering antigen again (Matloubian et al., 1994; Sun and Bevan, 2003). However, since the helper $CD4^+$ T lymphocytes are massively depleted during HIV-1 infection and HIV virus preferentially infects the antigen-specific $CD4^+$ T cells (Douek et al., 2002), limited attention has been paid to the role of $CD4^+$ T cell responses in the control of HIV-1 viraemia or prevention of infection.

Nevertheless, numerous studies have suggested that HIV-specific $CD4^+$ T-cell responses may also be important for controlling viral replication in HIV infection. After initial destruction of $CD4^+$ T cells, a low level of antigen-specific $CD4^+$ T-cell responses can be detected at all stages of HIV-1 infection using antigen stimulation cytokine production assays (Pitcher et al., 1999). Furthermore, the observation of escape mutation within $CD4^+$ T-cell epitopes in acute infection suggested immune pressure mounted by virus specific $CD4^+$ T cells (Rychert et al., 2007). In addition, $CD4^+$ T cells in HIV-1 controllers have been shown to be capable of proliferating efficiently as well as maintaining IL2 and IL21 production and consequently contributing to the viral control (Boaz et al., 2002; Chevalier et al., 2011; Potter et al., 2007; Rosenberg et al., 1997; Younes et al., 2003). Finally, a recent study by Soghoian et al. (2012) showed that the cytolytic activity of antigen-specific CD4+ T cells in acute infection is highly predictive of disease progression

and clinical outcome. In particular, the results of the recent, modestly protective RV144 vaccine trial, which induced not only non-neutralizing antibodies but also a robust HIV-specific $CD4^+$ T-cell response, raised important questions regarding the contribution of HIV-specific $CD4^+$ T cells to the initial control of HIV viraemia.

1.6.4 The $CD8^+$ T-cell responses to HIV-1

1.6.4.1 Recognition of virus infected cells by $CD8^+$ T cells

Virus-specific $CD8^+$ T cells mediate their effector functions through initial recognition of processed peptides bound to major histocompatibility complex (MHC) molecules on the virus infected cells and following the activation of the T-cell receptor (TCR) signalling complex.

1.6.4.1.1 Antigen processing and presentation by MHC class I molecules

Peptide antigen, produced by cancer cells or exogenous pathogen, can be presented by MHC class I molecules on the cell surface, which allows the immune system to discriminate them from healthy cells. This antigen processing and presentation process involves many steps as illustrated in **Figure 1.11**.

First, cytosolic and nuclear proteins, which are thought to be mainly newly synthesised yet defective ribosome products (DRiPs), are degraded by the proteasome into peptides.

This starts with ubiquitylation of DRiPs by adding a small ubiquitin "tag" to lysine residues on target proteins through ATP dependent enzymes E1, E2 and E3 (Ciechanover et al., 1994). The ubiquitin "tag" allows the targets protein to be selected by the proteasome, a ubiquitous protease that is required for generating most antigenic peptides (Rock et al., 1994). The proteasome is a 26S complex consisting of two multi-subunits: one or two 19S regulated complexes and a 20S catalytic core. The 19S subunit binds to the ubiquitinated protein and then removes the ubiquitin moieties, leading to unfolding of the protein. This process allows the substrate proteins to enter the interior of the 20S particle and contact with the proteolytic active sites to initiate the proteolysis.

There are two forms of proteasome: standard proteasome and immunoproteasome. Immunoproteasomes are constitutively expressed in immune tissues. They are also found in other cell types with a much lower level of expression, however can be induced by IFN-γ or TNF-α or other cytokines that are released in the early stages of viral infections. Compared to the standard proteasome, three catalytic subunits of immunoproteasome are replaced by homologous subunits (β1i, β2i, and β5i), also known as LMP1, LMP2 and LMP5 (Heink et al., 2005; Yewdell, 2005). Immunoproteasomes therefore generate a different spectrum of peptides from standard proteasomes by altering its preference for cleavage after hydrophobic and basic residues (Gaczynska et al., 1994), which influences the repertoire of peptides being presented by MHC class I molecules. Furthermore, immunoproteasomes enhance the rate of cleavage and increase the quantity of peptides generated in the

presence of these homologous subunits (Emmerich et al., 2000). Taken together, it is clear that immunoproteasomes can enhance antigen processing and alter the peptide-MHC repertoire available for $CD8^+$ T cell recognition.

It has also been demonstrated that the proteasomes are able to generate the correct C-terminus of epitopes with very high efficiency. This high accuracy of antigen processing can also be seen in *in vitro* experiments using purified 20S proteasomes (Eggers et al., 1995), and as a strong indicator of peptides presented by MHC class I molecules on the cell surface, this has enabled prediction of CTL epitopes (Nielsen et al., 2005).

Peptides generated by the proteasome are between 3 to 22 amino acids, with the exact C-terminal of CTL epitopes, and possibly an extended N-terminus (Kisselev et al., 1999). In order to bind to the MHC class I molecules, some of them must undergo further N-terminal trimming by aminopeptidases in the cytosol or in the ER. There are several molecules involved in this process, for example, leucine aminopeptidase (LAP) in cytosol that can be upregulated by IFN-γ; tripeptidyl peptidase II (TPPII), a cytosolic enzyme that removes three N-terminal residues in blocks; and ER aminopeptidase (ERAP1) that cleaves the peptides consisting of more than seven amino acids (Yewdell et al., 2003).

The second step of antigen processing and presentation involves translocation of peptides into the lumen of the endoplasmic reticulum (ER) by the transporter for

antigen processing (TAP). Over 99% of peptides are completely destroyed by endopeptidases and aminopeptidases in the cytoplasm (Reits et al., 2003). Less than 1% of peptides interact with TAP in the cytosol. TAP, an ER resident heterodimer peptide transporter, binds to the peptides, creates a pore in the ER membrane and then translocates the peptides into the ER lumen in an ATP-dependent way (van Endert et al., 2002).

Then in the lumen of ER, the peptides can be loaded onto the MHC class I heterodimers. Before or during the loading, peptides are trimmed further in ER by aminopeptidases such as ERAP1 and ERAP2, to ensure the correct length for MHC binding. The loading of peptides to MHC class I involves MHC class-I loading complex, which is composed of TAP, MHC class I heterodimers lacking peptide, chaperones tapasin, calreticulin and ERp60 (Ortmann et al., 1997). Tapasin interacts directly with MHC class I molecules and mediates the association with other chaperones ERp60 and calreticulin, which is required for optimal peptide loading to the MHC class I peptide-binding groove.

Finally, the correctly folded peptide-MHC class I molecule is released from the MHC class-I-loading complex, and then delivered from the ER to the cell surface through the standard secretory pathway, where the peptide is presented for recognition by $CD8^+$ T cells.

The efficiency of generating CTL epitopes from pathogen-derived antigens through this MHC class I antigen-processing pathway can be influenced by several factors. Studies of the influenza virus HA have shown that residues internal to the epitope are of primary importance for efficient presentation of epitopes (Hahn et al., 1992). Furthermore, several other studies have shown that flanking amino acids of the epitope also influence antigen presentation (Del Val et al., 1991; Eisenlohr et al., 1992; Ranasinghe et al., 2011). In addition, the intrinsic qualities of an epitope and its location within a protein influence the efficiency of epitope processing, as the efficiency of the same epitope varies when it is shuffled in to different locations within an antigen (Sijts et al., 1996). Collectively, these studies suggest that the efficiency of epitope generation is unique for each epitope, and each site within an antigen. Thus, the variation within an epitope and epitope flanking region, even in a remote area may influence the efficiency of epitope processing and presentation, and ultimately modulate the quality of $CD8^+$ T cell responses targeting this epitope.

1.6.4.1.2 Recognition of peptide-MHC class I complex by $CD8^+$ T cells

TCRs expressed on the CD8+ T cells can bind to peptide antigen presented by MHC class I molecules (Doherty et al., 1976; Townsend et al., 1989). After this TCR recognition, CD8+ T cells become activated in the presence of co-stimulation and display their effector functions.

Peptide-MHC class I complex

MHC class I molecules consist of two polypeptide chains, α and β2-microglobulin (β2M). The α chain is polymorphic and is encoded by an HLA gene on chromosome 6, while the β2M subunit is very conserved and encoded by the Beta-2 microglobulin gene. The α3 domain is the trans-membrane domain that anchors the complex within the plasma membrane and interacts with the CD8 co-receptor of T cells. The α1 and α2 domains fold to form a groove, where the peptide antigen with 8 to 12 amino acids in length is anchored at N- and C-termini as well as other key residues within the epitope. The floor of the peptide-binding groove determines which particular peptide residues the MHC class I molecules bind. Certain MHC class I molecules prefer specific residues at particular position within the epitope for the binding, which is referred as to "binding motifs" (Madden et al., 1993). Other residues that are not involved in interaction with MHC class I molecules can be altered without affecting the binding. This flexibility allows each MHC molecule to bind a variety of peptide antigens. As each individual can have up to six different MHC class I molecules, a vast array of antigenic peptides can be presented for recognition by $CD8^+$ T cells, which is important for the host defence against infection. This can be emphasised in HIV-1 infection, as it has been shown that homozygosity of HLA-locus is associated with poor clinical outcome (Carrington et al., 1999).

T cell receptor (TCR)

T cell receptors show a very high diversity in order to recognise the vast array of peptide-MHC class I complexes. This diversity can be achieved through

rearrangement of genomic DNA. TCR alpha chain and beta chain are encoded in the germline DNA by numerous non-continuous gene segments that can be rearranged before translation (Davis and Bjorkman, 1988). The recombination between different gene segments within the alpha chain and beta chain, together with the pairing of different alpha chain and beta chains, lead to a vast amount of diversity. In addition to the recombination, the addition and subtraction of nucleotides at the junctions between gene segments also contributes to the diversity of TCR repertoire.

The TCR is a heterodimer normally composed of the highly variable alpha (α) and beta (β) chains. Each chain is composed of a variable (V) region and a constant (C) region. The constant region anchors the TCR to the cell membrane, while the extracellular variable region binds to the peptide-MHC class I complex.

The variable domain of both the TCR α-chain and β-chain has three hypervariable complementarity determining regions (CDRs), i.e., CDR1 loop, CDR2 loop and CDR3 loop. As shown in **Figure 1.11b**, the CDR3 loop is the main region responsible for recognising processed antigen, although the CDR1 of Vα chain has also been shown to interact with the N-terminal of the peptide, whereas the CDR1 of the Vβ chain interacts with the C-terminal of the peptide. The CDR2 is thought to recognize the MHC (Garboczi et al., 1996; Garcia et al., 1996).

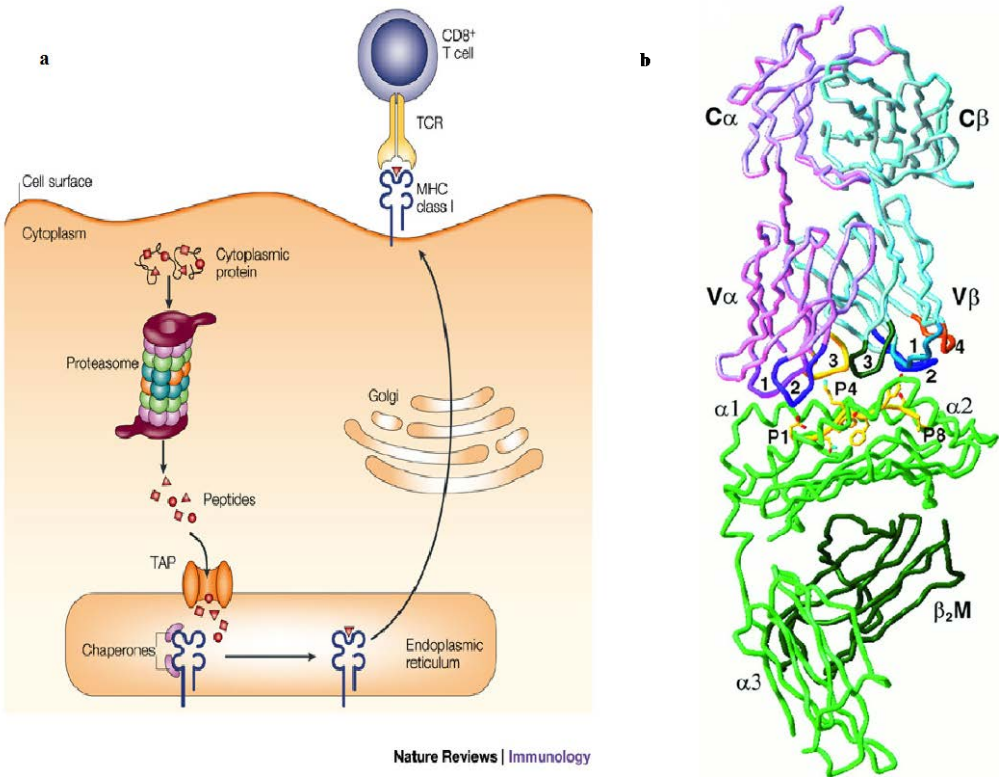

Figure 1.11 Schematic overview of antigen processing and presentation by MHC class I molecules and the interaction between TCR and peptide –MHC class I complex. a) Antigen processing and presentation by MHC class I molecules. [Adapted from (Yewdell et al., 2003)] b) Overall backbone structure of one entire TCR-pMHC complex. The TCR is on top: α chain pink, β chain light blue, CDR1$_\alpha$ magenta, CDR2α purple, CDR3α yellow, CDR1β cyan, CDR2β navy blue, CDR3β green, HV4 orange. The pMHC is on the bottom: MHC heavy chain is green, β_2-microglobulin (β_2M) is dark green, and peptide is yellow with side chains shown. [Adapted from(Garcia et al., 1998)]

1.6.4.2 Mechanisms of CD8$^+$ T cell anti-viral actions

It is well accepted that CD8+ T cells play a pivotal role in providing protection against infection with intracellular pathogens and many tumours. These functions can be acted through different mechanisms.

$CD8^+$ T cells, referred as to cytotoxic T-lymphocytes (CTLs), can directly kill the HIV-1 infected target cells preventing them from releasing viral progeny and reducing virus dissemination (Klenerman et al., 1996; Walker et al., 1987). When exposed to infected cells, CTLs release the cytotoxins such perforin, granzymes (Henkart et al., 1984; Masson and Tschopp, 1987; Shiver and Henkart, 1991). Granzymes, predominantly Granzyme A and B, are types of serine proteases that cleave at aspartate residues in the substrate. Perforin forms pores (aqueous channels) in the target cell's plasma membrane allowing granzymes to enter the target cell and induce cell apoptosis (programed cell death) (Pipkin and Lieberman, 2007). A second way to induce apoptosis is via Fas and FasL interactions between the T cells and the infected cell. When a T cell is activated, it starts to express the surface protein Fas ligands (FasL or CD95L). Binding of FasL to Fas (CD95) molecules expressed on the target cell activates caspase cascades and ultimately induces the apoptosis of target cells (Henkart, 1994).

$CD8^+$ T cells can secrete several cytokines and chemokines with anti-viral properties when they encounter virus-infected targets. $CD8^+$ T cells produce cytokines such as IFN-γ, TNF-α and IL-2 that can directly inhibit the HIV-1 virus replication or mediate other anti-viral functions through their immuno-stimulatory and immunomodulatory effects. In response to viral infection, $CD8^+$ T cells also

produce beta-chemokines (e.g. MIP-1α, MIP-1β and RANTES). These chemokines are the ligands for the HIV-1 co-receptor CCR5 and therefore reduce viral entry into new target cells through blocking the interaction of HIV-1 with CCR5. In addition to cytokines and chemokines, an undefined soluble factor, termed as T cell antiviral factor (CAF), may play a role in the antiviral effect of $CD8^+$ CTLs (Walker and Levy, 1989).

1.6.4.3 The role of $CD8^+$ T-cell responses in HIV-1 infection

Many lines of evidence demonstrate that CTL responses play an important role in controlling HIV-1 replication.

Despite the initial and persistent destruction of $CD4^+$ T helper cells by HIV-1, the robust $CD8^+$ T-cell responses to HIV can be detected in infected individuals. Those $CD8^+$ T cells responding to HIV have been shown to be capable of lysing the autologous B cells infected with recombinant vaccinia-HIV vectors or peptide-pulsed targets and inhibiting HIV replication, indicating the effective function of $CD8^+$ T-cell responses, at least *in vitro* (Walker et al., 1987). It was also observed that there is a temporal association between a rise in virus specific CTL responses and a fall in viraemia during acute HIV-1 infection, with the peak of the CTL response coinciding with the fall in viral load (Borrow et al., 1994; Koup et al., 1994). Furthermore, the escape mutations detected within the CTL epitopes revealed the evidence of immune pressure exerted by CTL responses (Phillips et al., 1991). Recent genome-wide association studies (GWAS) have shown that the

expression of certain HLA class I alleles is strongly associated with disease outcome (Gao et al., 2001; Kaslow et al., 1996; Le Clerc et al., 2011; Migueles et al., 2000), indicating that class I restricted CTLs act as a major factor modulating the disease progression.

More direct evidence for the active role of CTL in controlling HIV-1 replication has come from non-human primate models of infection. Monkeys, which were depleted of their CD8+ T cells prior to challenge with simian immunodeficiency virus (SIV), failed to control replication of the virus during the acute stage of the disease (Schmitz et al., 1999). Depletion of CD8+ T cells in chronically infected rhesus macaques also resulted in an increase in viral load that was suppressed when the SIV-specific CD8+ T cells reappeared (Jin et al., 1999a).

Taken together, these data confirm the important role of CTL in controlling HIV-1 infection and indicate that HIV-specific CTL should constitute an important component of immune responses induced by interventional strategies.

1.6.4.4 The quality of $CD8^+$ T cell responses

Given strong evidence suggesting the critical role of CTL in the control of HIV-1 infection, it is quite striking that neither the breadth nor the magnitude of HIV-1-specific T–cell responses is consistently associated with better disease outcome (either higher CD4 count or lower plasma viral load) (Addo et al., 2003; Betts et al., 2001; Klenerman et al., 2002; Ogg et al., 1998; Zuniga et al., 2006). It is now

widely accepted that the quality and not the quantity of a given CTL response is the major determinant of whether it contributes to the control of HIV-1 replication. There are several factors that influence the ability of particular CTLs to control HIV-1 replication, such as antigen specificity, the phenotypic and functional characteristics, cross reactivity. In following sections they are discussed in more detail.

1.6.4.4.1 Antigen specificity

Growing evidence has suggested that targeting a response towards a conserved protein, such as Gag and Pol, appears to be beneficial (Kiepiela et al., 2007; Payne et al., 2010; Zuniga et al., 2006). It has been shown that an increase in the breadth of Gag-specific CTL responses is correlated with a reduction in viral load whereas the broader T–cell responses to Env are associated with higher viraemia (Kiepiela et al., 2007; Ngumbela et al., 2008). It is notable that protective HLA alleles, such as B27, B57, B81, B58 and B14, have a true preference to present the epitopes from p24 Gag protein, while non-protective HLA alleles preferentially target HIV-1 Nef (Borghans et al., 2007).

The advantage of CTLs targeting epitopes within Gag and Pol may be down to the fact that Pol and Gag-specific $CD8^+$ T cells recognise infected $CD4^+$ T lymphocytes as early as two hours post-infection and eliminate infected cells 6 hours after infection. Gag and Pol are the most immunogenic HIV-1 viral proteins and also the earliest proteins to be detected during the infection. Therefore, the

epitopes from Gag and Pol can be presented and recognized by CTLs before virus integration and *de novo* synthesis of Nef and other viral proteins, more importantly prior to Nef-mediated MHC class I down-regulation. This may raise the possibility that T-cell responses targeting Gag and Pol are more effective than the ones targeting other viral proteins (Sacha et al., 2007a; Sacha et al., 2007b).

The advantage of the CTLs targeting epitopes within Gag and Pol may also be attributed to the fact that certain epitopes, especially in p24 protein, are highly conserved and that variation within these epitopes may often carry a fitness cost to the virus by interrupting the viral capsid assembly (Miura et al., 2009; Schneidewind et al., 2008; Schneidewind et al., 2007). This allows either control of the virus or escape, however, with loss of virulence (Martinez-Picado et al., 2006).

1.6.4.4.2 Differentiation state

During an immune response, $CD8^+$ T cells are recruited to provide protection and undergo a process of differentiation in order to become effector cells.

CD28 and CD27 are costimulatory receptors involved in the regulation of T-cell activation and in the generation of antigen-primed cells, respectively. Engagement of CD28 to its ligand CD80 (B7) or CD86 (B7-2) generates a potent costimulatory signal for IL-2 production and proliferation, while the engagement of CD27 and its ligand CD70 can provide a costimulatory signal for T cell activation. The differentiation process can be defined by expression of

these two markers. Based on CD27 and CD28 expression, $CD8^+$ T cells differentiate in a linear pathway from $CD28^+CD27^+$ (early-differentiated) to $CD28^-CD27^+$ (intermediate-differentiated) to $CD28^-CD27^-$ (late-differentiated) (Appay et al., 2002a). With the gradual loss of CD28 and CD27 expression, $CD8^+$ T cells differentiate to end-stage effectors.

During chronic HIV-1 infection, most HIV-1-specific CTLs are in an intermediate-differentiation stage ($CD27^+CD28^-$), an immature stage. These cells also display an impaired cytolytic activity and express lower level of perforin than fully differentiated cells (Appay et al., 2000; Appay et al., 2002b; van Baarle et al., 2002). Some HIV-1-specific CTLs can fully differentiate to $CD27^-$ effector cells, which has been shown to be associated with delayed disease progression due to their rapid effector function and high IFN-γ production, despite their limited proliferative and survival abilities (Hamann et al., 1997). Moreover, these fully differentiated effector T cells are more frequently observed in HIV controllers than viraemic subjects (Addo et al., 2007). However, Papagno et al. (2004) showed that terminally differentiated $CD8^+$ T cell ($CD27^-CD28^-$) also display a replicative senescence phenotype and are more frequently found in the patients who are progressing to AIDS.

Therefore, it remains a contentious issue whether the highly differentiated $CD8^+$ T cells represent an effector population that are capable of controlling HIV-1 replication or aging cells that have lost their proliferation capacity.

1.6.4.4.3 Activation and replicative senescence

In the presence of costimulatory molecules like (CD28, or ICOS), $CD8^+$ T cells become activated through the simultaneous engagement of the T cell receptor and its cognate peptide presented by MHC class I molecules on an antigen-presenting cell (Smith-Garvin et al., 2009). After activation, $CD8^+$ T cells display their effector functions and eliminate the virus-infected cells. T cell activation status can be assessed by measuring surface protein CD38 expression (Hultin et al., 1998).

HIV-1 induces systematic activation of immune cells, affecting many cell populations, not only those specific for HIV-1 (Finkel et al., 1995). Increasing evidence suggests an association between high levels of immune activation and poor outcome in HIV-infected individuals (Giorgi et al., 1993; Hazenberg et al., 2003; Hultin et al., 1998)

Chronic immune activation also drives HIV-1-specific $CD8^+$ T cells differentiating towards replicative senescence (Papagno et al., 2004). Expression of CD57, currently considered as a marker of replicative senescence, is found on the T cells that are highly susceptible to activation induced cell death and unable to undergo new cell-division cycles (Brenchley et al., 2003). Despite the preserved ability to secrete cytokines upon encounter with antigen, lack of proliferation capacity leads to ineffective CTL function.

1.6.4.4.4 Cytolytic activity

CTL control HIV-1 replication and limit viral dissemination mainly through the direct killing of the virus-infected cells by cytotoxines such perforin, granzymes. CTL show different ability to effectively load lytic granules and efficiently deliver the cytotoxic content to the cell membrane of target cells. It has been shown that a higher percentage of HIV-1-specific CTLs from LTNPs are perforin and granzyme B positive after *in vitro* stimulation, compared to equivalent cells from progressors (Migueles et al., 2002; Migueles et al., 2008). In addition, HIV-1-specific CTLs from LTNPs are able to deliver a substantially higher quantity of granzyme B to the target cells than the cells from progressors.

Using antibody that can detect newly synthesized perforin, some studies have demonstrated that the capability of HIV-specific $CD8^+$ T-cells to rapidly express perforin after *in vitro* antigen stimulation, rather than preloaded perforin expression, is correlated with control in HIV infection (Hersperger et al., 2008; Hersperger et al., 2010).

1.6.4.4.5 Polyfunctionality

It has been shown that the ability of an HIV-1-specific T-cell response simultaneously to produce multiple functional effectors, which is defined as the production of IFN-γ, TNF-α, IL-2, MIP-1β and degranulation of lytic granules (or CD107a expression), is associated with good viral control. In their study, Betts et al. (2006) showed that polyfunctional HIV-1-specific CTLs, which possess four or

more of these functions, are significantly enriched in non-progressors and the magnitude and proportion of CD8+ T cells with five functions is correlated with viral control. Several other studies have also established a link between polyfunctionality of the CTL and the control of viral replication. However, it is difficult to determine whether such cells are the cause or the effect of lower levels of antigen.

On the other hand, several studies have shown that polyfunctionality of CTL can be lost over the course of HIV-1 infection due to consistently high level of antigen stimulation, which resolve when antigen load is reduced through variation in the epitope or ART. These data suggested that the polyfunctional HIV-1 specific T cells are likely to be an effect of low antigen level.

1.6.4.4.6 Functional avidity

Functional avidity, also termed as antigen sensitivity, is a biological measure that indicates how well a T cell responds to a given concentration of antigen *in vitro*. Functional avidity of a $CD8^+$ T cell can be indicated by the EC50, a peptide concentration that induces 50% of the maximum response, as measured by cytokine production or specific target cell lysis. By definition, T cells with high functional avidity (lower EC50 value) are able to respond to lower does of antigen, whereas T cells with low functional avidity (higher EC50 value) require higher amount of antigen stimulation to elicit an immune response.

It is generally acknowledged that $CD8^+$ T-cell responses with higher functional avidity have higher efficacy to clear virus infections and to contain cancer cells (Berger et al., 2011; Busch and Pamer, 1999; Dutoit et al., 2001). The better anti-viral efficacy of high avidity CTL may be attributed to their ability to react to lower antigen densities and to lyse target cells more rapidly (Derby et al., 2001).

However, in HIV infection, there are conflicting reports on the relationship between the functional avidity and virus control. Some studies showed that high avidity HIV-1-specific CTL responses in non-progressors were mostly polyfunctional, with superior capacity for cross-reacting to epitope variants and associated with inhibition of virus replication (Almeida et al., 2007; Berger et al., 2011). *In vitro* assays also demonstrated that CTL functional avidity is correlated with the reduction of virus replication (Almeida et al., 2009). By contrast, other researchers observed that the HIV-1 infected patients progressed to AIDS despite the presence of high avidity HIV-1-specific CD8+ T cell responses (Chen et al., 2012; Draenert et al., 2004b). Yang et al. (2003b) have shown that the high avidity clones don't always suppress virus replication better and their anti-viral efficacy is more likely to be dependent on their antigen specificity. Furthermore, it was also reported that high functional avidity HIV-1 specific $CD8^+$ T cells preferentially display a phenotype of exhaustion and senescence as well as drive virus escape mutations (Conrad et al., 2011; Iglesias et al., 2011). In this regard, lower functional avidity T cell responses may be more important in long-term HIV control.

1.6.4.4.7 Cross reactivity

The ability of a CTL response to recognise common escape variants has an effect on its ability to contain viral replication and this cross reactivity is mainly dependent on the T cell receptor they possess (Chen et al., 2012; Ladell et al., 2013; Stewart-Jones et al., 2003).

HLA-B8 restricted Nef-specific CTL from LTNPs have been shown to bear TCRs with an extended CDR3 loop that were able to cross react to a common mutation that escapes recognition by other CTL (Dong et al., 2004). It has also been reported that in a long-term non-progressing HIV-1 infected child, the HLA-B5301 restricted Gag-specific CTL response is capable of recognising 8 out of 10 common escape variants by bearing a TCR with great tolerance to amino acid change in peptide and by generating a polyclonal T-cell population (Buseyne and Riviere, 2001).

It is difficult to elucidate which of the factors discussed above are most important determinants of the quality of a CTL response to HIV-1. Due to their highly inter related nature, no single factor could solely contribute to the antiviral efficacy of a T-cell response. Moreover, it is still under debate whether the better quality of a CTL response is the cause or the effect of low viral load, which is difficult to disentangle through observational studies. In addition, this situation is further complicated by host genetic factors, such as HLA, and viral factors, such as

founder virus and virus diversity, which also influences CTL associated viral control.

1.7 HLA and HIV control

Polymorphism in the Human Leucocyte Antigen (HLA) class I region on chromosome 6 has been consistently shown to play the major role in host genetic influences on HIV-1 disease outcome, which has been confirmed by a recent GWAS (Le Clerc et al., 2011). It has long been noted that certain HLA class I alleles are associated with Long-Term Non- Progression (LTNP) of HIV infection, whereas others are associated with more rapid disease progression. For example HLA-B58, B57, B51, B81 and B14 have been linked to late onset of disease (Kaslow et al., 1996) whilst HLA-B35 has been related to fast development of AIDS (Carrington et al., 1999). Furthermore, more rapid disease progression was observed in individuals who are HLA class I homozygous (Carrington et al., 1999) whereas rare HLA super-types are associated with relatively slow disease progression in HIV-1 infection (Trachtenberg et al., 2003). Interestingly, both positive and negative correlations of HLA with viral load are mostly observed for HLA B alleles, suggesting that an active immune control mechanism may underlie this effect.

The first large-scale study on genetic association with HIV control found two SNPs (Single-nucleotide Polymorphism) that are significantly associated with lower set point viral load (Fellay et al., 2007). One SNP is associated with HLA-B57, which

confirmed the previously findings that HLA-B57 is associated with better HIV control. Another one is associated with HLA C expression.

By analysis of more than one million SNPs in each person, recent population genetic studies revealed four SNPs that are significantly associated with viral load (Pereyra et al., 2010). Two of them are the same as those defined by Fellay et al. (2007). Further analysis of sequence in HLA region revealed that five amino acids within HLA B, all of which locate in the HLA binding groove and are involved in binding of antigenic peptides, are the major genetic influence on HIV control. In other words, the nature of viral peptide presented by HLA is the major determinant of HLA associated viral control (Pereyra et al., 2010).

1.8 Intra-host HIV evolution driven by CTL

Viral escape driven by CTL responses is a hallmark of HIV-1 infection and represents a key obstacle to the successful development of an AIDS vaccine (Goulder and Watkins, 2004). The first escape variants can replace the original founder virus sequence within days and, as a consequence of continuous immune pressure, sequential mutations follow (McMichael et al., 2010). Eventually, the frequency of such mutations correlates with the prevalence of the restricting HLA class I allele across the population as a whole (Dong et al., 2011; Kawashima et al., 2009; Moore et al., 2002). It has been acknowledged that the emergence of HIV-1 escape mutations depends upon the net balance of selective pressure and the fitness cost that potential escape mutations bear for the virus.

As discussed previously, the selective pressure exerted by CTLs may be influenced by numerous determinants, such as epitope specificity, T-cell receptor properties, and functional state of the CTL. The virus itself has also some factors that could constrain the generation of epitope mutations. First, some mutations could entail a fitness cost for the virus, leading to a direct loss of replicative capacity or they could interfere with functions important for viral persistence. Secondly, the pattern and the number of nucleotide substitutions, which is needed for the amino acid change, also play a role. In addition, the sequence of infecting virus impacts on the occurrence of epitope mutations.

1.9 Specific aims of this study

It has been long recognised that HLA-B51 is one of the protective alleles that are associated with HIV-1 virus control. However, the mechanism underlying this protection is still not clear. In order to assess the impact of HLA-B51 on viral control, we studied a unique Chinese HIV-1 patient cohort with a high prevalence of HLA-B51 and carried out the following specific studies:

1. In chapter 3, to assess the impact of HLA-B51 associated CTL responses and the epitope mutations on HIV-1 viral control, we first analysed three dominant HLA-B51 CTL responses and the corresponding epitope sequence in selected patients (*ex vivo*). Subsequently *in vitro* characterisation of those CTL responses was carried out by measuring their cross-reactivity to the epitope variants and their

antiviral efficacy against the wild type (WT) and mutant viruses, using antigen-specific CTL clones generated from the patients.

2. In Chapter 4, to verify our *ex vivo* observations of HLA-B51 associated escape mutations and to assess the impact of antigen specificity of the CTL responses on the virus evolution and viral control, we set up an *in vitro* Virus Evolution assay. In this assay, we performed six rounds of passage of lab adapted IIIB virus under the selection of HLA-B51 restricted CTL clones, and monitored the occurrence of epitope mutations as well as the inhibition of virus replication by each CTL clone.

3. In Chapter 5, to evaluate the impact of TCR, antigen sensitivity and founder virus on virus evolution and viral control, we carried out a second set of Virus Evolution assays. We co-cultured CTL clones specific for HLA-B51 epitope Pol TI8 with target cells which are infected with different founder viruses and monitored the occurrence of epitope mutations and the inhibition of virus replication by CTL.

4. In Chapter 6, phenotypic and functional characterisation of HLA-B51 restricted CTL responses was carried out to assess the effect of escape mutations on the phenotype and functionality of HLA-B51 restricted HIV-1-specific CTLs.

Chapter 2: Materials and Methods

2.1 Study subjects

2.1.1 SM Cohort

Patients who participated in this study were recruited from a Chinese cohort in Henan province. This community cohort is located in the village of ShuangMiao, China, so called SM cohort. In the SM cohort, we estimate that 407 people became HIV-infected following plasma donation in the early 1990s, based on the identification of 258 HIV positive adults in 2005 and reports of 149 premature adult deaths with symptoms compatible with HIV-1 disease before 2004. Of the surviving patients, all 258 were recruited in the study; none were treated with anti-retroviral drugs before 2004. Ethical approval was obtained from Beijing You'an Hospital and University of Oxford.

2.1.2 Patient Ya13

Patient Ya13 was recruited during an HIV-1 acute infection at Beijing You'an Hospital and was not treated with anti-retroviral drugs during the first year after infection. Ethical approval was obtained from Beijing You'an Hospital and University of Oxford.

2.2 Cell lines and CTL clones

2.2.1 EBV-transformed B lymphoblastoid cell lines

One million PBMCs were washed with RPMI1640 by centrifuging at 1500 rpm for 5 minutes and re-suspended in 100 µl of Epstein-Barr virus (EBV) supernatant (supplied by HIU Core Facility). After 3 hours incubation at 37℃/5% CO2, 100 µl of R15 (RPMI1640, with 15% Foetal Calf Serum, 2mM L-glutamine, 50 U penicillin/ streptomycin per ml) containing 2 µg/ml cyclosporine A (CsA, Sandimmun) was added and the cells were transferred into 5 wells of a round bottom 96-well plate. At Day 2, supernatant was replaced with 100 µl fresh media (CsA in R15). Two weeks later, CsA was omitted and the cells were cultured with R10.

2.2.2 HIV-1 permissive target cells

MT2 cells, a CD4 T cell line derived from an HLA-B51 patient with leukaemia and C8166 cells (stably expressing CD4) were acquired from the National Institute for Biological Standards and Control (NIBSC) and maintained in R10 media (containing RPMI 1640 with 100 U/ml of penicillin, 100 U/ml of streptomycin, 2 mM L-glutamine and 10% heat-inactivated foetal calf serum from Sigma-Aldrich.

2.2.3 293T cells

293T cells (Human Embryonic Kidney 293 cells) were kindly given by Dr Alison Simmon's group and maintained at 37℃/5% CO2 in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 100 U/ml of penicillin, 100 U/ml of streptomycin, 2mM L-glutamine and 10% heat-inactivated foetal calf serum from Sigma-Aldrich, UK.

2.2.4 Cytotoxic T cell lines

To generate short-term cytotoxic T cell lines, two-three million PBMCs were washed with 10ml RPMI by centrifuging at 1500 rpm for 5 minutes. Cells were then re-suspended in the residual volume and stimulated with the peptide at final concentration 10μg/ml for 1 hour in an incubator (37℃ + 5% CO2). 1ml of H10 (RPMI1640, supplemented with 10% human AB serum (National Blood service, UK), 2mM L-glutamine, 50 U penicillin/streptomycin per ml) was added and cells were transferred to a single well of a 24-well plate and incubated at 37℃ /5% CO2. At day 3, cells were then fed with 1ml of H10 supplemented with 200 U/ml IL-2. The antigen specificity of the cell cultures was tested by ELISPOT or tetramer staining between day 10 and day 14.

2.2.5 Cytotoxic T cell clones

Sorted (IFN- γ positive and CD8 positive) cells from IFN-γ secretion Assay (see Material and Method **4**) were plated out in 96 well U-bottom plates at limiting dilution (10, 5, 1, 0.5 cells/well) and co-cultured with 10^5 irradiated allogeneic PBMCs containing 40μg/ml PHA at 37℃ and 5% CO2. On day 3, 100μl of 200U/ml IL-2 in H10 was added to each well. After screening by IFN-γ ELISPOT at day 14, the antigen specific cells were expanded using irradiated allogeneic PBMCs containing 40μg/ml PHA.

2.3 Preparation of Peripheral Blood Mononuclear Cells (PBMCs)

Venous blood was drawn into collection tubes containing heparin and separated by Ficoll-Hypaque density centrifugation. Whole blood was diluted in the same volume of serum-free RPMI 1640 (R0) (Sigma-Aldrich, UK), and carefully layered on Ficoll-Hypaque (Sigma-Aldrich, UK). The layered blood was centrifuged without braking for 20 minutes at 2000 rpm. The cells at the interface were carefully removed and washed twice with serum-free RPMI 1640 by centrifuging at 1500 rpm for 10 minutes. Cells were then re-suspended in R10 medium (RPMI 1640 supplemented with 10% Foetal Calf Serum (FCS) (Sigma-Aldrich, UK), 2 mM L-glutamine and 50 U/ml penicillin /streptomycin).

2.4 IFN-γ Secretion Assay

10^6 cells from short-term CTL lines were washed with RPMI by centrifuging at 300×g for 10 minutes and then re-suspended with 150µl of H5 (containing RPMI1640, supplemented with 5% human AB serum (National Blood service, UK), 2mM L-glutamine, 50 U penicillin/streptomycin per ml). Cells were then incubated with corresponding peptide at final concentration of 10µg/ml in a single well of a flat bottom 96-well plate at 37℃, 5% CO2. After 3-6 hours incubation, cells were transferred to a 15ml tube and washed with 10ml of cold FACS buffer (PBS+0.5%BSA+2mM EDTA, filtered), centrifuged at 300×g for 10 minutes at 4−8 °C. The supernatant was then pipetted off completely. The cell pellet was re-suspended in 80µl cold culture medium, followed by adding 20µl of IFN- γ Catch Reagent (Miltenyi Biotec MACS®). After 5 minutes incubation on ice, 10ml of

warm H5 medium was added and cells then incubated at 37℃ for 45 minutes under slow continuous rotation. The samples were washed again with 10ml of cold FACS buffer and stained with 20µl of IFN-γ detection Antibody conjugated with PE (Miltenyi Biotec MACS®) and 5µl of Anti-CD8-APC (BD Biosciences) at final volume of 100 µl for 10 minutes on ice. Finally, cells were washed and re-suspended in 500µl of H10 for sorting.

2.5 Standard Human IFN-γ ELISPOT assay

96-well MAIP N45 Millipore plates (Millipore, MA) were coated with 50µl anti-IFN-γ mAb 1-D1k (5 µg/ml Mabtech) in PBS for 2 hours at 37ºC, washed six times with RPMI and then blocked with R10 for 1 hour. HLA-B51^{+} B cell line or MT2 cell line were stimulated with peptide at varying concentrations for 1 hour at 37℃, 5% CO2 and then washed twice with RPMI. CTL clones and peptide pulsed target cells were added to the ELISPOT plates at an E:T ratio of 400:20,000 at the final volume of 100µl/well. Negative controls included CTL clones co-cultured with target cells that have not been stimulated with peptide. Plates were then incubated for 6 hours at 37°C 5% CO_2 before being washed 6 times with 200µl/well of washing buffer (phosphate buffered saline (PBS)+0.05% Tween-20 (Sigma-Aldrich, UK). 50µl/well of biotinylated anti-IFN-γ mAb (Mabtech, Sweden) at a concentration 1µg/ml was added to plates, followed by 2 hours incubation at room temperature. Plates were then washed again six times with wash buffer and incubated for an additional one hour with 1 µg/ml streptavidin conjugated alkaline phosphatase (Mabtech, Sweden). After a final wash, plates were incubated with

100 µl/well of chromogen mix (deionised water, Bio-Rad Development Buffer, Bio Rad 5-bromo-4-chloro-3-indolyl phosphate and Bio-Rad nitroblue tetrazolium) for 10 minutes at room temperature. Spot forming units (SFUs) were counted using the ELISPOT plate reader and AID ELISPOT 4.0 software (AID, Germany). The number of specific IFN-γ secreting T-cells was calculated by subtracting the SFU value of the negative control(s).

2.6 Vβ antibody staining

CTL clones were stained with 2µl each of a panel of FITC or PE-conjugated antibodies specific for 24 different variable domains of the TCR β chain (Table 2.1). After 20 minutes incubation in the dark at room temperature the cells were washed twice in FACS wash buffer and fixed in 200µl of cell fix (BD Biosciences). Samples were read on a Cyan flow cytometer and analysed using FlowJo software (Tree Star Inc, US).

2.7 Proteasomal digestion assay

Two 25mer-peptides containing the HLA-B51 restricted epitope Pol TI8 or TV8 were synthesised by solid-phase F-moc chemistry on an automated peptide synthesiser (Advanced ChemTech) and purified to >98% by HPLC. *In vitro* proteasomal digestion of synthetic 25mer oligopeptides was conducted using immuno-20S (i20S) proteasome (Biomol International). For each oligopeptide, 1 µg of immuno-20S proteasome was incubated with 10 µg of peptide in a final volume of 100 µL of 20mM HEPES, pH 7.8, 2mM magnesium acetate and 2mM

dithiothreitol and incubated at 37°C/5% CO_2. Reactions were then terminated by the addition of 0.1 volume of formic acid (FA) after 60mins. Control reaction without the i20S-proteasome was also analysed to evaluate non-specific peptide degradation. The i20S-proteasome was pelleted by ultracentrifugation at 100000 g for 5 hours, and supernatants containing digested peptides were analysed using Nano UPLC-high/low collision energy switching MS (MS^E) as described (Xu et al., 2008).

Table 2.1 The antibodies used for testing TCR of the CTL clones.

TCR Vβ antibody	
Vβ1	FITC
Vβ2	FITC
Vβ3	FITC
Vβ4	**PE**
Vβ5.1	FITC
Vβ5.2	FITC
Vβ5.3	**PE**
Vβ7	FITC
Vβ7.2	**PE**
Vβ8 (.1 and.2)	FITC
Vβ9	**PE**
Vβ11	FITC
Vβ12	FITC
Vβ13.1	FITC
Vβ13.2	**PE**
Vβ13.6	FITC
Vβ14	FITC
Vβ16	FITC
Vβ17	FITC
Vβ19	**PE**
Vβ20	FITC
Vβ21.3	FITC
Vβ22	FITC
Vβ23	**PE**

2.8 *In vitro* HIV infection assays

2.8.1 HIV-1 virus stocks

2.8.1.1 HIV-1 clade B viruses

HIV-1 lab strain viruses IIIB and MN were obtained free from NIH AIDS Reagent Program. High titre stocks of these HIV-1 viruses were propagated by adding 250µl of the original vial stock to infect 1 x 10^7pelleted C8166 cell line for 90 minutes at 37ºC in a 5% CO2 incubator. After incubation, the infected cell pellet was washed twice with RPMI and transferred to a T25 flask for growth. Viral supernatant was taken at 4, 6, 8, 10 and 12 days post-infection and p24 antigen concentration was measured using the quantitative p24 antigen ELISA (Cambridge Bioscience, UK).

2.8.1.2 Wild type and Mutant HIV-1 molecular clonal viruses

2.8.1.2.1 HIV-1 cDNA clone pNL432

Plasmid NL4.3 was kindly provided by Prof. Takaguchi (Kumamoto University, Japan) and expanded by transforming into One Shot® Stbl3™ Chemically Competent *E. coli* (Invitrogen, Carlsbad, CA). Plasmid DNA was then extracted and purified by HiSpeed Plasmid Maxi Kit according to the manufacturer's instructions (Qiagen)**.**

2.8.1.2.2 Construct of pNL432 Pol mutants

Plasmids NL432 Pol RT pointed Mutants pNL432-8V/L/T were kindly provided by Prof. Masafumi Takaguchi (Kumamoto University, Japan). Plasmid NL432 Pol-

Integrase pointed mutants were generated by PCR-based point mutagenesis of pNL432. In brief, 3 pair of primers (Invitrogen, Carlsbad, CA, **Table 2.2**) containing one mutation, two or three mutations respectively were designed and polymerase chain reactions (PCR) were performed with PrimeSTAR HS DNA Polymerase (Takara, Japan) under the conditions shown in **Table 2.3**. Amplified PCR samples were electrophoresed through agarose gels and purified with a PCR purification kit (Qiagen) according to the manufacture's instruction. The purified PCR products were then digested by Dpn I (New England Biolab, UK) for 2 hours at 37 ºC and directly transformed to One Shot® Stbl3™ Chemically Competent *E. coli* (Invitrogen, Carlsbad, CA). On Second day, positive colonies were expanded and plasmid DNA was extracted and purified with QIAprep Spin Miniprep Kit (Qiagen). The DNA sequences of the constructs were confirmed by using an automated ABI-377 DNA Sequencer (Applied Biosciences Inc., USA).

Table 2.2 The primers were used for PCR based mutagenesis.

1	B51pol L743I Forward	GGC TAG TGA TTT TAA C**AT** **A**CC ACC TGT AGT AGC
2	B51 pol L743I Reverse	GCT ACT ACA GGT GGT ATG TTA AAA TCA CTA GCC
3	B51 pol L743I+L760I Forward	TGT GAT AAA TGT CAG ATA AAA GGG GAA GCC ATG
4	B51 pol L743I+L760I Reverse	GCT ACT ACA GGT GGT ATG TTA AAA TCA CTA GCC
5	B51 pol L743I +S754C +L760I Forward	GAA ATA GTA GCC TGC TGT GAT AAA TGT CAG ATA AAA GGG GAA GCC A
6	B51 pol L743I +S754C +L760I Reverse	GCT ACT ACA GGT GGT ATG TTA AAA TCA CTA GCC

Table 2.3 PCR conditions

Step	Temperature	Time	Number of cycles
Hot Start	96°C	120 seconds	
Denaturing	96°C	15 seconds	19
Annealing	59°C	30 seconds	
Extension	72°C	15 minutes	
Extension	72°C	20 minutes	

2.8.1.2.3 Production of viral stocks

HIV-1 molecular clone virus stocks (NL432 and NL432 pol mutants) were produced by transient transfection of plasmid DNA into 293T cells. Cells with 90% co-fluency were seeded in 6-well plates prior to transfecting with Lipofectamine-2000 transfection reagent in accordance with manufacturer's instructions (Invitrogen, Carlsbad, CA). 100µl of DNA- Opti-MEM (Invitrogen, Carlsbad, CA) mix was combined with 100µl of Opti-MEM containing 8µl of Lipofectamine 2000. The 200µl mixture was then left for 20 minutes at room temperature before being added to each well containing cells and medium. After 5 hours incubation, medium was replaced with 2 ml of fresh D10 (DMEM, 100 U/ml of penicillin, 100 U/ml of streptomycin, 2mM L-glutamine and 10% heat-inactivated foetal calf serum from Sigma-Aldrich, UK). 48 hours later, culture supernatant containing viral particles was harvested, passed through 0.45µm filter (Millipore, UK) and titered by quantitative p24 antigen ELISA in accordance with the manufacturer's instructions (Cambridge Bioscience, UK).

2.8.2 IFN-γ Live Virus ELISPOT

Differing HIV-1 strains were used to infect MT2 $CD4^+$ T- cell line pellet at 2-fold TCID50 in a 15ml round bottom falcon tube during 90 minute incubation at 37°C/5% CO2. The infected cells were then washed twice, re-suspended with 2ml of R10 and cultured in a T25 flask for 72 hours post-infection at 37ºC/5% CO2. Cells were then washed, counted and co-cultured with CTL clones in triplicate at one E: T ratio of 400:20000 on the pre-coated interferon gamma IFN-γ ELISPOT plates at a final volume of 100µl/well. Negative controls included the individual CTL clones co-cultured with uninfected target cell line whereas positive controls were each CTL clone co-cultured with uninfected target cells pulsed with 2µM of specific peptide. ELISPOT plates were incubated for 6 hours at 37°C/5% CO2 and subsequently washed and developed as described previously (see **2.5**). Spot forming units (SFUs) were counted using the ELISPOT reader system AID ELISPOT 4.0.

2.8.3 Virus Suppression assay

Differing high titre HIV-1 strains (TCID50) were used to infect MT2 cell pellets during a 90-minute incubation at 37°C/5% CO2 and were subsequently washed twice to remove free viruses. $5x10^4$ infected cells were co-cultured with HLA-matched HIV-1 specific CTL clones at differing E:T ratios of 1:1, 1:2, 1:4, 1:8 & 1:16 in H10-IL2 (200U/ml) on a flat bottom 96-well plate, in a final volume of 200µl per well, at 37°C for 4 days. Each condition was performed in triplicate, including one HLA-mismatched clone as a negative control and virus-infected cells

in the absence of CTL as a positive control. The extracellular p24 content in the supernatant was measured by quantitative p24 antigen ELISA (Cambridge Bioscience, UK) in accordance with the manufacturer's protocol. In parallel, the expression of intracellular p24 in HIV-1 infected cells was assessed using FACS staining with PE conjugated anti p24 antibody (Beckman Coulter, UK).

2.8.4 Intracellular p24 staining

The cells were washed in PBS before adding 0.5µl of the reconstituted LIVE/DEAD^{R} fixable Dead Cell Stain dye with violet fluorescent reactive (Invitrogen, Carlsbad, CA). After incubating on ice, at darkness for 30 minutes, cells were then washed with 200µl/well of PBS and stained with 2µl/well of anti-CD4-APC antibody (BD Biosciences, San Jose, CA) on ice for 20 minutes. Samples were washed again with 200µl/well of PBS prior to being fixed by 100µl/well perm/fix buffer (BD Biosciences, San Jose, CA). After 20 minutes fixation at 4°C degree, samples were then washed twice with 200µl/well of 1X perm/washing buffer (BD Biosciences, San Jose, CA) before 3µl of KC57-RD1 P24 specific antibody (Beckman Coulter, UK) being added to each sample. Samples were then incubated on ice for 30 minutes before a final wash step. Cells were fixed in 200µl of cell fix (BD Biosciences) prior to being acquired on a Cyan flow Cytometer. Data were analysed using FlowJo software (Tree Star Inc.).

2.8.5 Virus Evolution Assay

2.8.5.1 Passage of HIV-1 under selection by CTL Clones

$5x10^6$ MT2 cells were initially infected with HIV-1 IIIB virus (NIH AIDS Reagent Programme) for 90 minutes, followed by two washes and re-suspension in H10 containing 100U/ml of IL2. Cells were then co-cultured with CTL clones at an E:T ratio of 1:8 in a total volume of 10 ml 100U/ml IL2/H10 at 37°C/5% CO2. A control flask was also set up with culture of infected target cells in the absence of antigen specific CTL clones. The cultures were then fed twice over the next week, followed by harvesting of the cell pellet and supernatant virus, which were both saved at -80°C. The process was then repeated in a second round of passage using the supernatant virus, at an initial input of 500pg P24 antigen (Cambridge Bioscience, UK) per 10^6 target cells. The 3rd, 4th, 5th,and the 6th round were otherwise performed in an identical fashion to the second, by infecting target cells with the supernatant virus from the previous round. Cell pellets and supernatant virus from all 6 round passages were saved for subsequent analyses.

2.8.5.2 Analysis of the passaged virus for epitope mutations

2.8.5.2.1 Genome DNA isolation

Genome DNA was isolated from the cell pellets after each round of passage using Puregene DNA isolation kit (Gentra Systems) according the manufacturer's instructions. In brief, the cell pellet was re-suspended in 600µl cell lysis solution and then mixed well with 200µl of protein precipitation buffer by vortexing, followed by centrifuging at 13,000 g for 15 minutes. The supernatant was decanted into a clean 2 ml vial containing 600µl of 100% isopropanol (Sigma, UK).

Afterwards, the tube was gently inverted for 50 times, genomic DNA was noted floating in the solution and then the tube was centrifuged at 13,000 g for 3 minutes to pellet the genomic DNA. The supernatant in the tube was discarded and genomic DNA was washed with 70% (v/v) ethanol (Sigma, UK). After discarding the ethanol, centrifuging the tube, and removing all the solution in the tube, the genomic DNA was dissolved in 100μl hydration solution. Samples were stored at -20℃ for viral sequence analysis.

2.8.5.2.2 Nested polymerase chain reaction (PCR) amplification

Nested PCR amplification was carried out in a total volume of 50μl using AccuPrime Taq DNA Polymerase High Fidelity kit (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. The PCR primers and reaction conditions are listed below.

Primers

Table 2.4 Primers used for epitope HLA-B51 Gag 327 NI9 sequencing

	Forward	Sequence	Reverse	Sequence	Product(bp)
1st Round	1527F	GGAACTACTAG TACCCCTTCA	2229R	GGATACAGTT CCTTGTCTATC	720
2nd Round	1616F	GTAAGAATGTA TAGCCCTAC	2191R	GGAGTTGTTGT CTCTTCCCC	575

Table 2.5 Primers used for the epitope HLA B-51 Pol 283 TI8 sequencing

	Forward	Sequence	Reverse	Sequence	Product(bp)
1st Round	2611F	CCAAAAGTTAA GCAATGGCC	3229R	CCCATCCAAA GGAATGGAGG	620
2nd Round	2828F	AGTTAGGAATA CCACACCCC	3200R	GTAAATCCCC ACCTCAACAG	370

Table 2.6 Primers used for the epitope HLA B-51 Pol 743 LI9 sequencing

	Forward	Sequence	Reverse	Sequence	Product (bp)
1st Round	3918F	ATCTAGCTTTGC AGGATTCGG	4618R	CCTTAACCGTAGT ACTGGTG	700
2nd Round	4143F	AAAGGTCTACC TGGCATGGG	4402R	AGTCTACTTGTCC ATGCATGGC	260

PCR programmes

Table 2.7 Programme of the 1st round PCR for the epitope HLA-B51 Gag 327 NI9

Step	Temperature	Time	Number of cycles
Hot Start	94°C	120 seconds	
Denaturing Annealing Extension	94°C 50°C 68°C	30 seconds 30 seconds 60 seconds	34
Extension	68°C	360 seconds	

Table 2.8 Programme of the 1st round PCR for the epitope HLA-B51 Pol 283 TI8 and Pol 743 LI9 sequencing

Step	Temperature	Time	Number of cycles
Hot Start	94°C	120 seconds	
Denaturing Annealing Extension	94°C 55°C 68°C	30 seconds 30 seconds 60 seconds	34
Extension	68°C	360 seconds	

Table 2.9 Programme of the 2nd round PCR for epitope sequencing

Step	Temperature	Time	Number of cycles
Hot Start	94°C	120 seconds	
Denaturing Annealing Extension	94°C 53°C 68°C	30 seconds 30 seconds 60 seconds	34
Extension	68°C	360 seconds	

Amplified PCR products were electrophoresed through Agarose gels and purified using the PCR purification kit (QIAGEN) before cloning.

2.8.1.1.1 TA TOPO cloning

4µl of purified second-round PCR fragments was cloned into pCR™4-TOPO® TA vector in the TA TOPO cloning kit (Invitrogen, Carlsbad, CA, UK) by ligation for 10 minutes at room temperature. One Shot® TOP10 Competent E. coli were transformed with ligated plasmid and grown overnight at 37℃ on LB agar plate containing 100mg/ml ampicillin. The next day, 15 colonies were cultured overnight in 2ml LB broth containing 100mg/ml ampicillin. Purified plasmid DNA was obtained using QIAprep Spin Miniprep Kit (Qiagen) and then digested with EcoR I enzyme (New England Biolab, UK). The screening of digested plasmids for a correct insert size was conducted by gel electrophoresis. 10 clones with the inserts of correct size were sequenced with M13 forward and reverse primers by John Frankland at the Human Immunology Unit (HIU) sequencing facility in the Weatherall Institute of Molecular Medicine (WIMM), Oxford.

2.9 Preparation of HLA-B51 class I MHC tetramers

2.9.1 Total RNA extraction

Total RNA was extracted from $5X10^6$ PBMCs from an HLA-$B5101^+$ donor using RNAqueous Kit from Ambion according the manufacture instruction. Cells were collected and the medium was removed thoroughly. Samples were then disrupted in a typical high concentration guanidinium salt solution that simultaneously lyses cells

and inactivates endogenous RNases. An ethanol solution was added to the lysate to make the RNA competent for binding to the glass fibre filter in the RNAqueous Filter Cartridge. This solution is then passed through the filter pad where RNA binds and most other cellular contents flow through. The Filter Cartridge is washed 3 times to remove contaminants, and the RNA is eluted in a very low ionic strength solution.

2.9.2 cDNA synthesis

The first-strand cDNA was synthesized using Cloned AMV First-Strand cDNA Synthesis Kit from Invitrogen, Carlsbad, CA. Generally, 100ng RNA sample extracted from HLA-B51 donor's PBMCs was first denatured with 1µl of Random Hexamers and 2µl of 10mM dNTP mix together by incubating at 65°C for 5 min. During the incubation, the cDNA synthesis mix was mixed according **Table 6.1.** 8 µl of master mix was added to the denatured RNA from last step. The reaction was then transferred to a thermal Cycler and incubated at 55°C for 50 min, followed by a terminating incubation at 85°C for 5 min. The cDNA synthesis reaction can be used immediately for PCR or stored at -20°C.

Table 2.10 Reaction mix for cDNA synthesis.

Component	**1 Reaction**
5x cDNA Synthesis Buffer	4µl
0.1 M DTT	1µl
RNaseOUT™ (40 U/µl)	1µl
DEPC-treated water	1µl
Cloned AMV RT (15 units/µl)	1µl
Total volume	8µl

2.9.3 PCR amplifying the HLA-B51 specific gene and cloning into the expression vector

2.9.3.1 PCR

The polymerase chain reactions (PCR) were carried out in a total volume of 50μl using AccuPrim Taq DNA Polymerase High Fidelity kit (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. The reaction conditions are listed in **Table 2.11**. 5μl of amplified PCR samples were electrophoresed through agarose gel. A band of about 860 base pairs should be seen on the gel.

2.9.3.2 Double-digestion with restriction enzyme

The rest of the PCR products were then digested by restriction enzyme *Nde I* and *BamH I* according to the manufacture's instructions. Meanwhile, in order to obtain the vector containing the biotinylation tag, the expression plasmid containing another HLA gene was also digested by *Nde I* and *BamH I* in buffer H (Roche), followed by a 60 minutes incubation at 37°C with Alkaline Phosphatase (New England Biolab, UK) to remove the 5´ phosphate groups from vectors. Both digestion products were then electrophoresed through agarose gel and the correct bands were then cut and purified with Gel purification Kit (Qiagen) according to the manufacture's instructions. Then the purified insert and vector were again checked by running through the agarose gel before setting up the ligation reaction.

2.9.3.3 Ligation and transformation

Ligating the insert into pGMT7 vector was carried out using T4 ligase (NEW ENGLAND BIOLAB, UK) with the following recipe. The insert and the vector were added at a molar ratio of 4:1. 2µl of ligated product was then transformed to DH5α competent cells (Invitrogen, Carlsbad, CA) according to manufacture's instruction. The transformed bacteria were finally plated on an agar plate containing 100µg/ml ampicillin and the plate was incubated at 37°C over night or for at least 16 hours.

Table 2.11 Program of PCR for amplifying HLA-B51 specific gene for the expression construct.

Step	Temperature	Time	No. of cycles
Hot Start	94°C	180 seconds	
Denaturing	94°C	60 seconds	
Annealing	60°C	60 seconds	35
Extension	72°C	60 seconds	
Extension	72°C	600 seconds	

2.9.3.4 Plasmid purification and sequencing

On the second day, each colony from the agar plate was inoculated into 5ml LB medium containing 100µg/ml ampicillin and cultured at 37°C over night by shaking at 200rpm. At least 10 colonies were picked. The plasmids were then purified from the bacteria culture using Mini Prep Kit from Qiagen. The plasmids containing an insert with the right size were then sequenced and the insert was

aligned with the HLA-B51 nucleotide sequence. Only the plasmids containing the right sequence of the HLA-B51 gene were used for expression.

2.9.4 Expressing and purification of HLA-B51 heavy chain protein and β_2-microglobulin protein

Bacteria BL21 (DE) pLysS (Invitrogen, Carlsbad, CA) were transformed with the modified HLA-B51 heavy chain construct and β_2-microglobulin construct (Lab stock) according the manufacturer's protocol. A single colony from each plate was then inoculated into 200ml of low salt LB medium containing 100μg/ml ampicillin and 34μg/ml chloramphenicol. The cultures were left shaking (200 rpm) over night and then inoculated into the big flasks containing one-litre medium as required. When the optical density reached 0.6-0.9, 0.5mM IPTG was added into each flask to induce the protein expression. After 4 hours further shaking at 37°C, the cell pellet was then collected from the culture by spinning at 3600rpm at 4°C for 30 min. Since the proteins are synthesised as inclusion bodies, the cells were broken by sonication and the inclusion body protein layer was collected after centrifuging at 13,000rpm for 15min at 4°C. After three washes with Triton Wash, the inclusion body protein was finally re-suspended in the 8M Urea buffer and the protein concentration was measured using Ultrospec 2000 spectrophotometer (Pharmcia Biotech). The purified proteins were then aliquoted at 15mg and stored in -80°C for future use.

2.9.5 Refolding and purification of HLA-B51 peptide-MHC complex with a biotin tag

15mg of each peptide (HLA-B51 Pol LI9, Pol TI8 and Gag NI9), 15mg β_2-microglobulin and 30mg HLA-B51 heavy chain were then added to 1 litre of refolding buffer containing 100mM Tris (pH=8), 400mM L-arginine HCl, 5mM reduced glutathione, 0.5mM oxidized glutathione and 2mM EDTA. The refolding solution was then concentrated to 10mls using an Amicon stir cell (Millipore, UK) with a 10kD cut-off membrane after stirring at 4°C for 96 hours. Then the concentrated refolding solution was purified by gel filtration using Superdex 75 prep grade Gel filtration column (GE healthcare, UK). The fractions for the peptide-MHC complex were collected and then the buffer was changed into 10mM Tris (pH=8) by spinning in the centreprep (Millipore, UK).

After a 6-hour biotinylation reaction with BirA enzyme (Avidity, USA), the complex was then purified by gel filtration with a Superdex 200 column (GE healthcare, UK). The fractions for the complex were collected and then concentrated to an appropriate volume with a protein concentration at 1-2mg/ml. The protease inhibitor cocktail (Sigma-Aldrich, UK) containing Pepstatin A (an acid protease inhibitor), Leupeptin (both Serine and Cysteine proteases inhibitor) and Bestatin Hydrochloride (an amino-peptidase inhibitor) was then added to the solution to stabilise the complex. Finally, the complex called Class I MHC monomer is ready for tetramerisation with Extravidin-PE or aliquoted for long-term storage in -80°C.

2.9.6 Tetramerisation of peptide-MHC class I complex

ExtraAvidine-R-Phycoerythrin (PE) (Sigma Aldrich) was added into the refolded complex at the molar ratio of 1:4 and was rotated at 4°C overnight.

2.10 Tetramer staining

2µl of homemade PE-conjugated class I tetramer was added to 100µl whole blood, followed by 15mins incubation at 37°C/5% CO2. Then the red blood cells were lysed using 1X BD FACS Lysing solution (BD Pharmingen). After two washes with FACS buffer, the cell pellet was ready for staining with surface markers or intracellular staining. For surface staining, the following antibodies, including 5µl of CD8-PercP, 2µl of CD27-APC, 10µl of CD28-FITC (differentiation panel) or 5µl of CD8-PercP, 2µl of CD38-APC, 6µl of CD57-FITC (activation and senescence panel) or CD8-PercP only (intracellular staining) were added to the cells prior to incubation at room temperature in the dark for 15 minutes. After the final wash, the samples were stained with surface phenotype markers and fixed in 200µl of 1X BD Cell Fix buffer (BD Biosciences) while samples for intracellular staining were instead permeabilised with 500µl BD Perm/Fix buffer (BD Biosciences) at 4°C in the dark for 20 minutes. After washing with 1X BD Perm/Washing buffer (BD Biosciences), 5µl of anti-perforin D48 (Gen-Probe, San Diego, CA) were added to the cells for an incubation at 4°C in the dark for 30 minutes. After the final wash with Perm/Washing buffer, the cells were then fixed. All the samples were read on a BD FACSCalibur and analysed using FlowJo software (Tree Star Inc).

2.11 Viral sequence analysis

The sequence results were analysed by BioEdit software that is free downloaded from http://www.mbio.ncsu.edu/bioedit/bioedit.html. This software is a biological sequence alignment editor written for Windows 95/98/NT/2000/XP/7 and it provides basic functions for the alignment and manipulation of nucleotide and protein sequences.

2.12 Shannon entropy calculation

The Shannon entropy is a simple quantitative measure of uncertainty in a data set. This uncertainty measure can be used as a strategy to quantify sequence variability in population level, including both the frequencies and number of possibilities. For each epitope, the LANL sequence database was used to identify all known clade B sequences and 7411 of Gag NI9, 4926 of Pol TI8 and 3768 of Pol LI9 sequences were derived from the database. Data were limited to one randomly selected sequence per patient, and sequences defined as "problematic" by the LANL database search tool were omitted. The Shannon entropy of each position in the epitope was calculated with the tool of **Entropy-one** from http://www.hiv.lanl.gov. The mean of these scores was used as the epitope entropy.

2.13 Statistical analysis

Statistic analysis was carried out with GraphPad software (Prism). Graphical presentations were also performed by Prism. Correlation with HIV viral load was analysed using two-tailed Mann–Whitney *U* tests or one-way ANOVA with

kruskal–wallis test, and correlation with $CD4^+$ T cell counts was analysed using the unpaired *t* test with Welch's correction or one-way ANOVA. The significant correlation of virus suppression with mutations was determined by two-tailed Spearman rank correlation or nonparametric Pearson correlation. EC50 was calculated with a three-parameters equation (dose-response). To compare the expression of specific markers between two populations, the mean was calculated for each group, and the unpaired Mann-Whitney U test or Wilcoxon matched-pairs test was performed to determine whether the means were significantly different. Statistical test differences were considered significant if $p < 0.05$.

Chapter 3: HLA-B51 associated HIV-1 viral control in the SM cohort

3.1 Introduction

Polymorphism in the Human Leucocyte Antigen (HLA) class I region on chromosome 6 has been consistently shown to play the major role in host genetic influence on HIV-1 disease outcome, which has been confirmed by recent genetic association studies (GWAS) (Fellay et al., 2007; Le Clerc et al., 2011; Pereyra et al., 2010). It has long been noted that certain HLA class I alleles are associated with Long-Term Non- Progression (LTNP) of HIV infection, whereas others are associated with more rapid disease progression (Carrington et al., 1999; Kaslow et al., 1996). Interestingly, both positive and negative correlations are observed in HLA-B alleles. The recently reported genome-wide association studies of HIV-1–infected subjects with viral control demonstrated a major influence of HLA class I on good clinical outcome, with viral control mapping to five polymorphic positions in the peptide-binding groove of the HLA class I molecule (Pereyra et al., 2010). This indicates that the association between viral peptides-HLA is the major factor modulating durable control of HIV infection. HLA class I molecules present viral epitopes to Ag-specific T cells, and therefore, certain class I alleles could be important because of the nature of the viral peptides they select. To date, only a few distinct T cell epitopes associated with good clinical outcome have been defined for HIV-1. Nonetheless, it has been shown that multiple T cell responses

toward HIV-1 Gag are linked with lower viral loads (Borghans et al., 2007; Kiepiela et al., 2007).

HIV-specific T cell responses constitute a great pressure on HIV-1 to mutate, enabling T cell epitopes to escape from recognition by virus-specific immune cells (Goulder et al., 1997). The earliest escape mutations can be detected in the epitopes within the variable regions of the virus, such as Env and Nef, within 50 days after infection, whereas mutations develope later in the conserved regions, such as Gag (Goonetilleke et al., 2009; McMichael et al., 2010). Thus, beneficial class I molecules could confer their protective effect by presentation of conserved regions of the virus, particularly in Gag-p24, in which escape mutation is constrained by adverse consequences for the virus in terms of replicative fitness. However, the contribution of individual T cell epitope responses to disease control and the role of viral escape in disease progression still remains controversial (Rowland-Jones, 2004). In the individual patient, the outcome probably depends on the interaction between that individual's HLA molecules and the infecting viral strain (Goulder et al., 1997; Kawashima et al., 2009). This may be modified by multiple factors, such as the complexities of multiple T cell responses within the individual, the diversity of genetic background, the fitness cost to the mutated virus, the kinetics of T cell responses, the selection of escape mutants during the acute phase of infection, and the efficacy of epitope processing and presentation (Goulder and Watkins, 2004; McMichael et al., 2010).

Several HLA class I B molecules have been repeatedly shown to be associated with delayed disease progression, notably HLA-B27 and B57 in Caucasian and HLA-B51 in Asian populations (Gao et al., 2005). For HLA-B27, there is one immunodominant epitope (Gag KK10), and escape mutation in this epitope is associated with clinical decline, suggesting that T-cell responses restricted by HLA-B27 are important in protection (Goulder et al., 1997). Escape mutation and loss of immune control appears to be delayed because the critical escape mutation in the epitope requires two other compensatory mutations to occur, one being outside the epitope (Kelleher et al., 2001). Similarly, it has been argued that the reason why HLA-B57 is associated with good T cell control of HIV is because the escape mutations that occur in the three to four immunodominant epitopes all impose a fitness cost on the virus (Crawford et al., 2011; Martinez-Picado et al., 2006).

HLA-B51 is also known to be associated with low viral loads (Carrington M, 2003), but the mechanism that underlies this protection is still unclear. Indeed, a recent study of a cohort in Japan showed that this association is no longer present in Japan, because the circulating HIV strain has acquired a major escape mutation in one of the most dominant HLA-B51–restricted epitopes (Pol 283-289) in the Japanese population (Kawashima et al., 2009). In contrast, this association was observed in a cohort of haemophiliacs who had been infected in early 1980s with a non-mutated virus (Kawashima et al., 2010). These observations also could be interpreted as implicating HLA-B51–restricted T cells in control of viral

replication. HLA-B51 is a common HLA allele in China and much of Asia, and understanding the mechanism of HLA-B51 association with viral control therefore holds particular importance in the study of the pathogenesis of HIV infection in the Far East.

In most cohort studies, the analysis is complicated by the presence of individuals with different infecting viruses of different pathogenic potential for different lengths of time. In this study, we were able to access a unique cohort (SM cohort) in China where individuals had been infected with HIV through contaminated plasma in the early 1990s. All members of this cohort were infected at a similar time and by the same infection route and have subsequently progressed to diverse disease outcomes in the absence of any antiretroviral treatment during the first 10 years of infection (Dou et al., 2010). Our studies have demonstrated a narrow source clade B' HIV-1 infection in this cohort, and there are strong associations between HLA type and mutations found in the virus, without confounding founder effects (Dong et al., 2011).

In the SM cohort, over 18% of candidates (22 out of 284 patients) were HLA-B51 positive. The HLA type, clinical data and treatment information of those patients are list in **Appendix 1 (Table A.1).** Studies by a previous DPhil student in the lab showed that epitopes Pol283 TI8: TAFTIPSI, Gag327 (P24) NI9: NANPDCKTI and Pol743 LI9: LPPVVAKEI were the most immune-prevalent epitopes in the SM cohort at frequencies of recognition 42%, 64% and 72% respectively

(**Appendix 2 Figure A.1)**. Furthermore, the mutations in each of three dominant epitopes (mutation L243I at position 1 of the LI9 epitope, mutation I289X at position 8 of the TI8 epitope mutation S328A at position 2 of the NI9 epitope) were significantly associated with HLA-B51 (**Table 3.1**). In addition, the epitope Pol LI9 also developed other two mutations (X760L and C754S) in the flanking region, which are strongly linked with the intra-epitope mutation (I743L) and associated with HLA-B51 (**Appendix 3, Table A.2)**.

In this study, with the aim of understanding the association of the protective allele HLA-B51 with HIV-1 viral control in the SM cohort, we analysed the data from our *in vivo* study, which included HLA-B51 restricted CTL responses to immuno-dominant epitopes, viral mutations in these epitopes and individual clinical outcome, to identify the specific CTL responses or epitopes that are correlated with good control of HIV. To validate our observations *in vivo,* we then functionally characterised the HLA-B51 restricted CTL responses and evaluated their impact on HIV-1 virus replication *in vitro* by using CTL clones generated from the patients in the SM cohort. Ultimately we compared our findings *in vivo and in vitro* to gather more information about the nature of a potentially protective T-cell response in the Chinese population that will benefit future HIV-1 vaccine development for China.

We found that the patients with HLA-B51 in the SM cohort have benefited from having a hierarchy of protective epitopes that provide multiple layers of defence against the development of immune escape mutations, particularly when CTL target the epitope (NI9) that is relatively conserved and appears to escape slowly.

Table 3.1: HLA-B51 associated epitope mutations. Pro-viral sequences containing the three dominant epitopes (TI8, NI9 and LI9) were examined from 22 HLA-B51+ individuals and 126 HLA-B51– individuals. 18 out of the 22 HLA-B51+ individuals showed a mutation of L243I at position 1 of the LI9 epitope, 17 out of 22 showed a mutation of I289X at position 8 of the TI8 epitope and 6 out of 22 showed a mutation of S328A at position 2 of the NI9 epitope. TAFTIPSV is the SM Consensus sequence of epitope Pol TI8. **[Adapted from Yonghong Zhang's DPhil Thesis]**

Epitope	Mutant	1	2	3	4	5	6	7	8	9	B51+ donor n=22	B51- donor n=126
Pol 743 LI9	WT	L	P	P	V	V	A	K	E	I	18%	90.3%
	L1I	I	P	P	V	V	A	K	E	I	82%*	9.7%
Pol 283 TI8	WT (Database)	T	A	F	T	I	P	S	I		0	18%
	I8V SM Consensus	T	A	F	T	I	P	S	V		23%	77%
	I8L	T	A	F	T	I	P	S	L		59%*	4%
	I8T	T	A	F	T	I	P	S	T		9%*	0
	I8R	T	A	F	T	I	P	S	R		9%*	0
Gag 327 NI9	WT	N	A	N	P	D	C	K	T	I	73%	98%
	A2S	N	S	N	P	D	C	K	T	I	27%*	2%

* Statistically significant.

3.2 Results

3.2.1 Selection hierarchy of HLA-B51 associated mutations

When examining the mutations that developed in these three HLA-B51 restricted dominant epitopes in each individual, a pattern of mutations was observed **(Table 3.2),** suggesting that there may have been a sequential selection hierarchy of mutations in these three epitopes: individuals with the S2A mutation in the Gag NI9 epitope also showed mutations in the other two Pol epitopes (Pol LI9 and Pol TI8) and individuals with the V8X mutation in the TI8 epitope also showed the I1L mutation in the LI9 epitope. It seemed that the Pol743 LI9 epitope has mutated the most rapidly and is likely to be the first to mutate, followed by Pol283 TI8 epitope and then finally the Gag NI9 epitope.

3.2.2 Increased numbers of mutated epitopes are significantly associated with lower CD4 counts and higher viral load in the SM cohort

HIV viral load (**Figure 3.1a**) and CD4+T cell counts (**Figure 3.1b**) were compared amongst people in whom mutations had developed in one (Pol LI9 only), two (Pol LI9 +Pol TI8) or three epitopes (Pol LI9+Pol TI8+Gag NI9). A significantly higher viral load was observed when more than one epitope had mutated, whereas a significant decrease of CD4+ counts was observed when all three epitopes had mutated (**Figure 3.1b**)

Table 3.2 Epitope mutation selection hierarchy in HLA-B51 restricted dominant epitopes in SM cohort (0: no mutation in the epitope, 1: mutation developed within the epitope)

SM ID	NANPDCKTI (Gag327 **NI9**)	TAFTIPSV (Pol283 **TI8**)	LPPVVAKEI (Pol743 **LI9**)
SM200	0	0	0
SM379	0	0	0
SM40	0	0	0
SM166	0	0	0
SM192	0	0	1
SM446	0	0	1
SM209	0	0	1
SM349	0	0	1
SM176	0	1	1
SM64	0	1	1
SM8	0	1	1
SM55	0	1	1
SM181	0	1	1
SM215	0	1	1
SM381	0	1	1
SM350	1	1	1
SM455	1	1	1
SM47	1	1	1
SM14	1	1	1
SM342	1	1	1
SM63	1	1	1
SM353	1	1	1

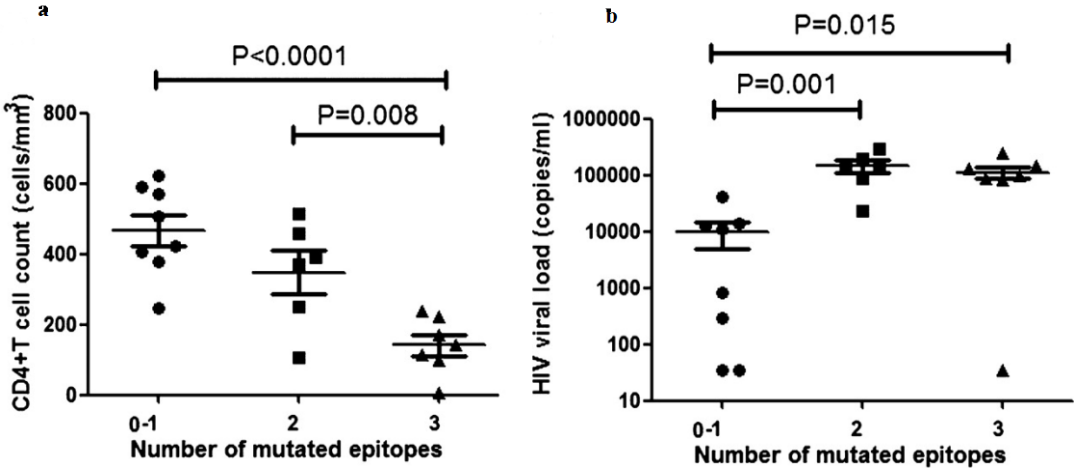

Figure 3.1 Increased numbers of mutated epitopes are significantly associated with lower CD4 counts (a) and higher viral load (b) in the SM cohort. P value was calculated with standard one-way ANOVA (CD4 count) and one-way ANOVA with Kruskal–Wallis test (viral load).

3.2.3 Patients with CTL responses detected towards at least one un-mutated HLA-B51 restricted epitope have significantly better clinical outcome

The HLA-B51 patients were divided into two groups (**Table 3.3**): Group 1 consisted of individuals with mutations in all three epitopes or who had no T cell responses against un-mutated epitopes; Group 2 consisted of individuals with at least one detectable T cell response against epitopes that had not mutated. It was found that CD4 counts were significantly higher and viral loads significantly lower in patients with no epitope mutation and with at least one detectable T cell response against the TI8 and NI9 epitopes (Group 2, p=0.001 CD4 count; p<0.001 VL) (**Figure 3.2**).

Table 3.3 Patient groups. Patients are grouped according to T cell responses detected with no epitope mutation for at least one of the epitopes (Group 2), or no T cell response detected towards any non-mutated epitopes (Group 1).

	NI9		TI8		LI9		VL-07	$CD4^+$-07
Group 1	M*	T*	M*	T*	M*	T*		
SM040	0	-	0	-	0	-	42000	248
SM215	0	-	+	+	+	+	200000	514
SM381	0	-	+	+	+	+	100000	106
SM350	+	-	+	-	+	+	200000	8
SM047	+	-	+	-	+	+	88000	143
SM014	+	-	+	-	+	+	100000	113
SM342	+	+	+	-	+	+	200000	222
SM353	0	-	+	-	+	+	100000	169
SM181	0	-	+	-	+	-	300000	252
Group 2								
SM166	0	-	0	+	0	+	11000	590
SM176	0	+	0	-	+	-	1200	621
SM446	0	+	0	+	+	+	14000	421
SM209	0	+	0	-	+	-	35	508
SM349	0	+	0	+	+	-	820	378
SM008	0	+	+	-	+	-	23000	370

M*: mutation detected in the epitope; T*: T-cell response detected

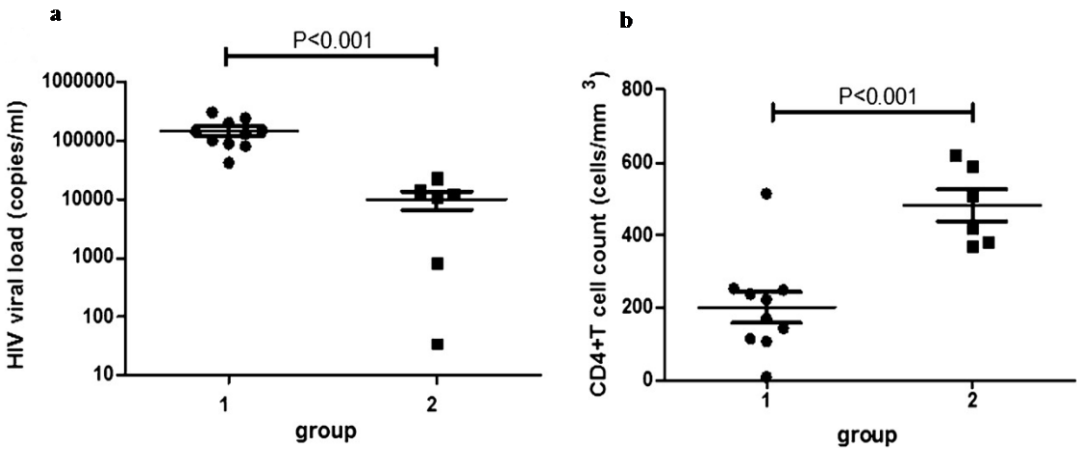

Figure 3.2: Patients with CTL responses detected towards at least one non-mutated HLA B51 restricted epitope have significantly better clinical outcome. The HIV viral load (a) and CD4+T cell counts (b) were compared between group 1 (patients did not respond to any non-mutated epitopes) and group 2 (patients responded to at least one of the epitopes without mutation). *P* value was calculated by unpaired t test with Welch's correction (CD4 count) or two tailed Mann–Whitney *U* test (viral load).

3.2.4 HLA-B51 restricted CTL clones

To evaluate the anti-viral efficacy of HLA-B51 restricted T-cell responses *in vitro*, a panel of CTL clones specific for epitopes Pol LI9, Pol TI8 and Gag NI9 were generated from HLA-B51$^+$ HIV-1 infected individuals. Antigen-specificity was examined using ELISPOT assay or tetramer staining. CTL clones were stained with a panel of Vβ antibodies to assess TCR usage, shown in **Table 3.4**.

Table 3.4 CTL clones generated and used for this study.

Patient	Clone	Epitope specific	T Cell Receptor Vβ usage
SM 215	D8	Pol743 LI9	Vβ 1
	H10	Pol743 LI9	ND
SM 196	C5	Pol283 TI8	ND
	C11	Pol283 TI8	Vβ 7.2
SM 063	G4	Pol283 TI8	Vβ 7.2
SM 342	B2	Gag372 NI9	Vβ 4
	BB9	Gag372 NI9	Vβ 4

ND: Not detected by antibody staining.

3.2.5 HLA-B51 restricted CTL clones recognise major epitope variants

The recognition of major epitope variants by HLA-B51 restricted CTL clones was examined by IFN-γ ELISPOT using titrations of peptides. Pol LI9 specific CTL clones (D8 and H10) displayed very strong cross reactivity against the WT epitope and the LI9-L1I mutant (**Figure 3.3 a**). Similarly, Pol283 TI8 specific CTL clones showed a very strong response against the WT epitope and the mutants I8V/L, with the exception of the mutants I8T/R (**Figure 3.3 b**). The Gag372 NI9 epitope variant A2S is obviously an escape mutant with no recognition by both Gag372 NI9 specific CTL clones tested (**Figure 3.3 c**).

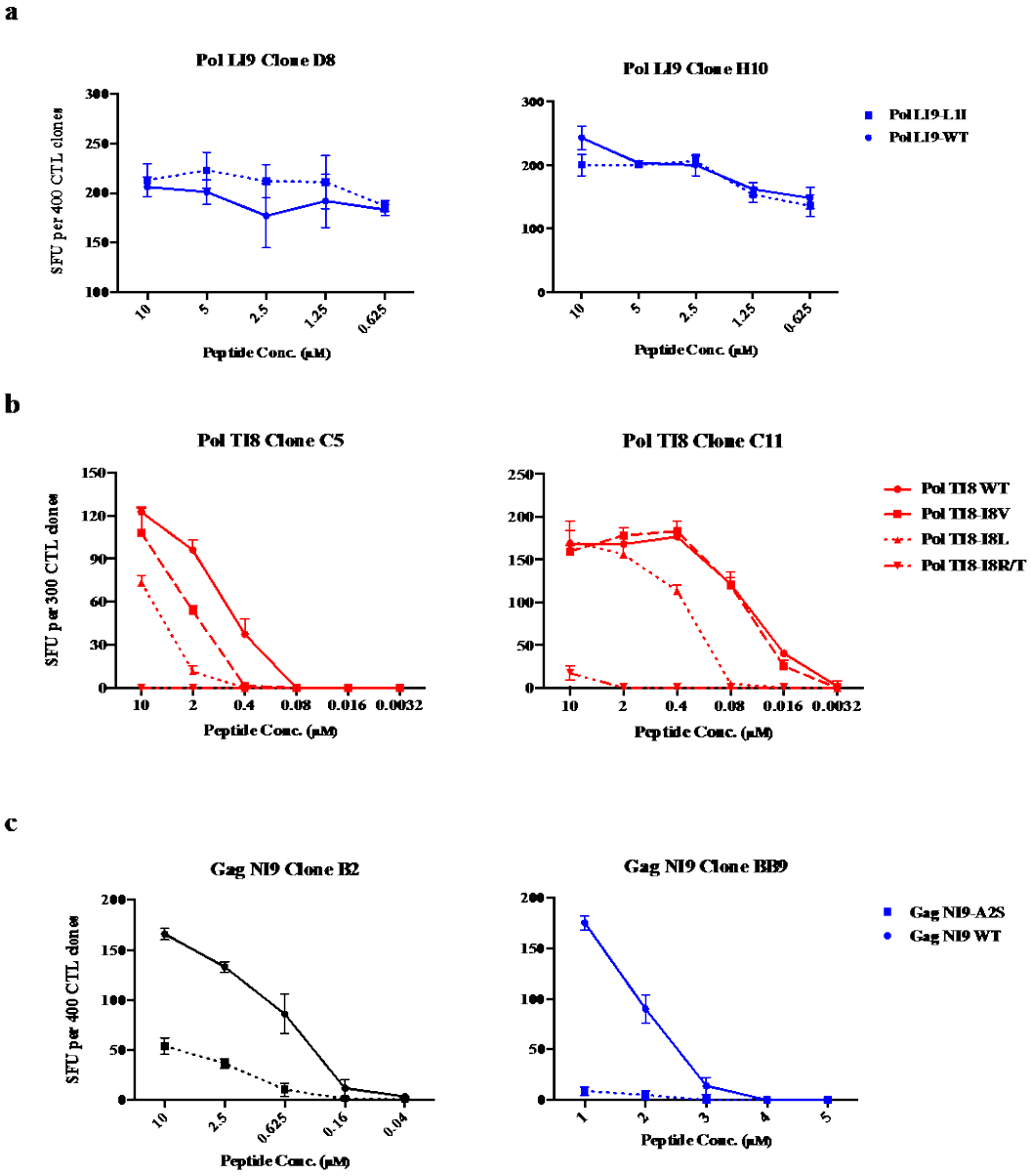

Figure 3.3 Recognition of major epitope variants by antigen-specific CTL clones. Antigen-specific T cell clones were co-cultured with target cells and IFN-γ release was measured by ELISPOT. Figure a), b) and c) show the recognition of epitope variants by Pol LI9 specific, Pol TI8 specific and Gag NI9-specific CTL clones respectively**.** N=3 (replicates)

It might be possible that WT-specific T cells have outgrown selectively by culturing with the WT epitope, which could then be further enhanced by the cloning process, whereas T cells recognising the variants have been neglected. To assess variant recognition that is relevant *in vivo*, we therefore tested *ex vivo* PBMCs that have not undergone extensive culture. The data shown in the **Figure 3.4 a, b and c** indicate that Pol TI8 variants I8T/R and Gag NI9 A2S are escape mutations, which is consistent with the data generated with the CTL clones.

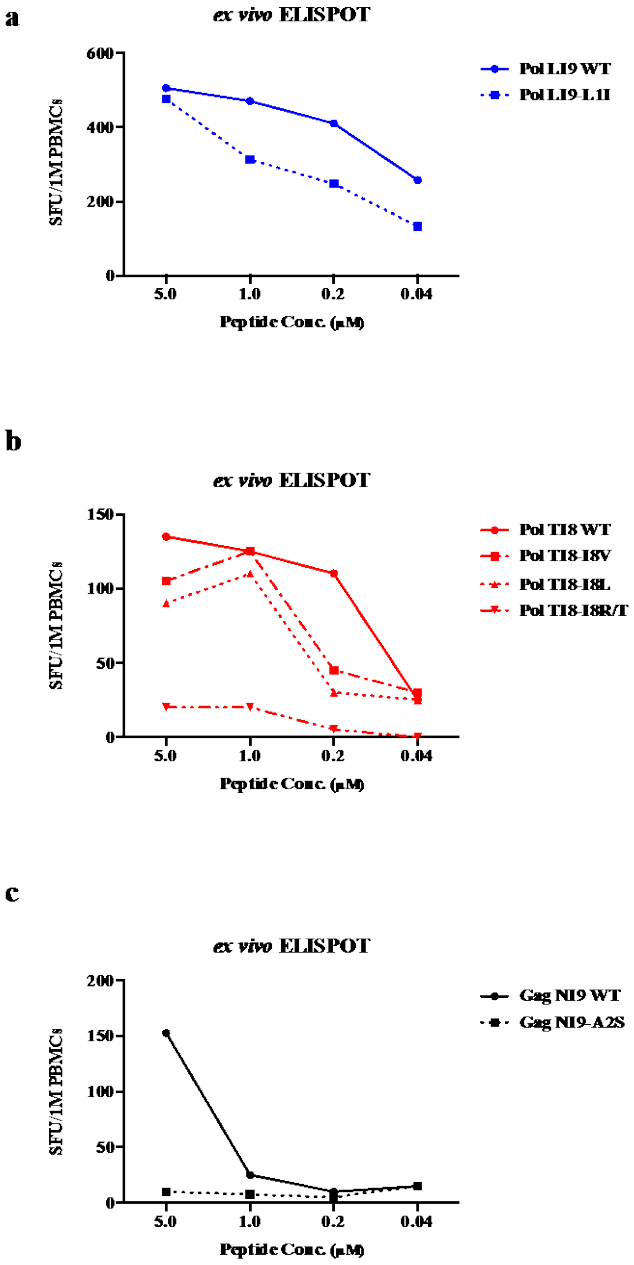

Figure 3.4 Recognition of major epitope variants *ex vivo*. IFN-γ released by HLA-B51+ patients' PBMCs pulsed with WT and variant peptides was measured by ELISPOT. Figure a), b) and c) show the recognition of epitope Pol LI9, Pol TI8 and Gag NI9 variants *ex vivo*, respectively.

3.2.6 Pol TI8 specific CTL clones lost control of viruses carrying the I8L/T mutation and the suppression of mutant virus 8V was significantly reduced

The anti-viral efficacy of the Pol TI8 CTL clones was assessed using Live Virus ELISPOT assay and Virus Suppression Assay. Target cells, MT2 cells were infected with wild type virus (NL432) or virus 8V (NL432 Pol283 TI8-I8V mutant), virus 8L (NL432 Pol283 TI8-I8L mutant) or virus 8T (NL432 Pol283 TI8-I8T mutant) and co-cultured with three Pol TI8 specific CTL clones (C5, C11 and G4). Three other CTL clones, including the B51 Pol LI9, Gag NI9 clone and an HLA mismatched clone, were chosen as controls. IFN-γ release from each clone was measured by ELISPOT (**Figure 3.5a**). The CTL clones didn't recognise target cells infected with virus 8L or virus 8T; however, they displayed decreased responses to virus 8V compared to the wild type virus. These results were further confirmed by the data generated from Virus Suppression Assay (**Figure 3.5b**), showing that Pol LI8 specific CTL clones could not inhibit the replication of viruses with mutations 8L/T and the suppression of mutant virus 8V was significantly reduced.

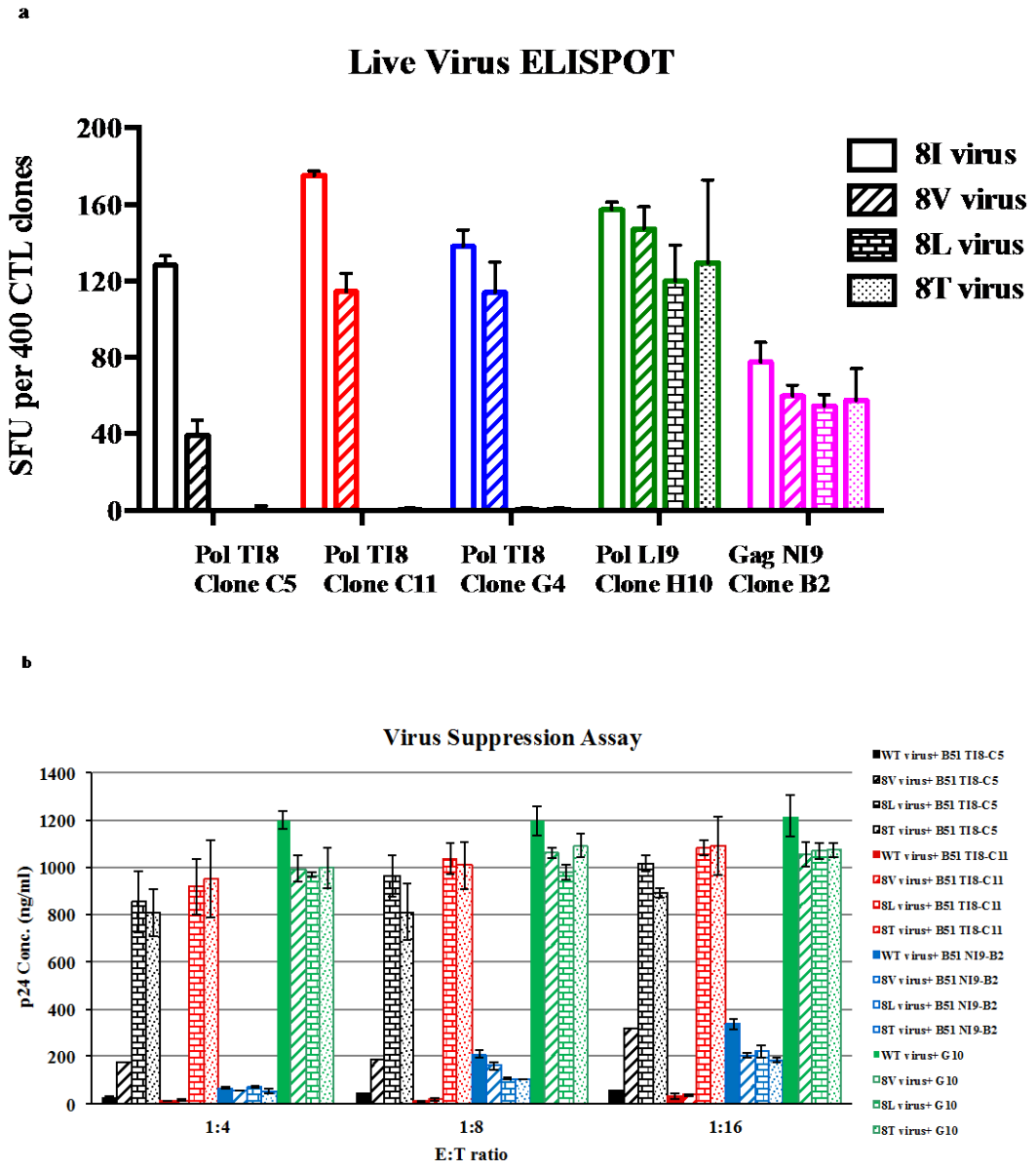

Figure 3.5 Anti-viral activities of B51 TI8 CTL clones against WT and mutant viruses. CTL clones were co-cultured with target cells infected with wild type NL432 virus and mutant viruses (NL432-8V virus, NL432-8L virus and NL432-8T virus). a) IFN-γ release was measured with Live Virus ELISPOT. b) P24 concentration in the supernatant was measured using ELISA. An HLA mismatched CTL clone (G10) is also included. N=3 (replicates)

3.2.7 Significant reduction of the antiviral efficacy of LI9 specific CTL clones against the virus Pol743 LI9 mutants

As shown in **Appendix 3**, 82% (14 out of 17) of HLA-B51^{+} patients, who developed the 743I mutation in the Pol LI9 epitope, also developed mutations in the downstream flanking region (I or V to L mutation in position 760) and 41% developed a mutation in position 754 (C to S). However, none of the B51– individuals with wild type LI9 sequences showed these mutations. To assess whether these mutations would enable the virus to escape CTL recognition, three mutant molecular clonal HIV viruses were generated with the single mutation L743I, as well the combinations L743I/L760I and L743I/L760I/S754C, which are listed in **Table 3.5**.

Table 3.5 Pol743 LI9 virus mutant generated for this study

Virus	Mutations
Cl7	Pol L743I
6G	Pol L743I/L760I
8F	Pol L743I/S754C/L760I

The replication capacity of the virus Pol 743 mutants (**Table 3.5**) in the presence of Pol LI9-specific CTL clones was assessed by virus suppression assay along with p24 intracellular staining of the infected target cells and the measurement of p24 concentration in the supernatant. **Figure 3.6** represents the data from three independent experiments of virus suppression assay. Although both Pol743 LI9

specific clones displayed strong inhibition of WT and mutant viruses, the ability to inhibit mutant viruses Cl7 (mutation L743I), virus 6G (mutations L743I/L760I) and 8F (mutations L743I/S754C/L760I) was reduced significantly, by 2 fold, 4 fold and 8 fold increments of P24 antigen level in the supernatant respectively, when compared to WT virus (**Figure 3.6 a**). The corresponding result was also seen from the intracellular p24 staining of the targets, with 5 fold, 10 fold and 25 fold of increase of p24 positive cells infected by mutant virus Cl7, 6G and 8F respectively (**Figure 3.6 b**). Both control clones showed consistent levels of inhibition of both WT and mutant viruses.

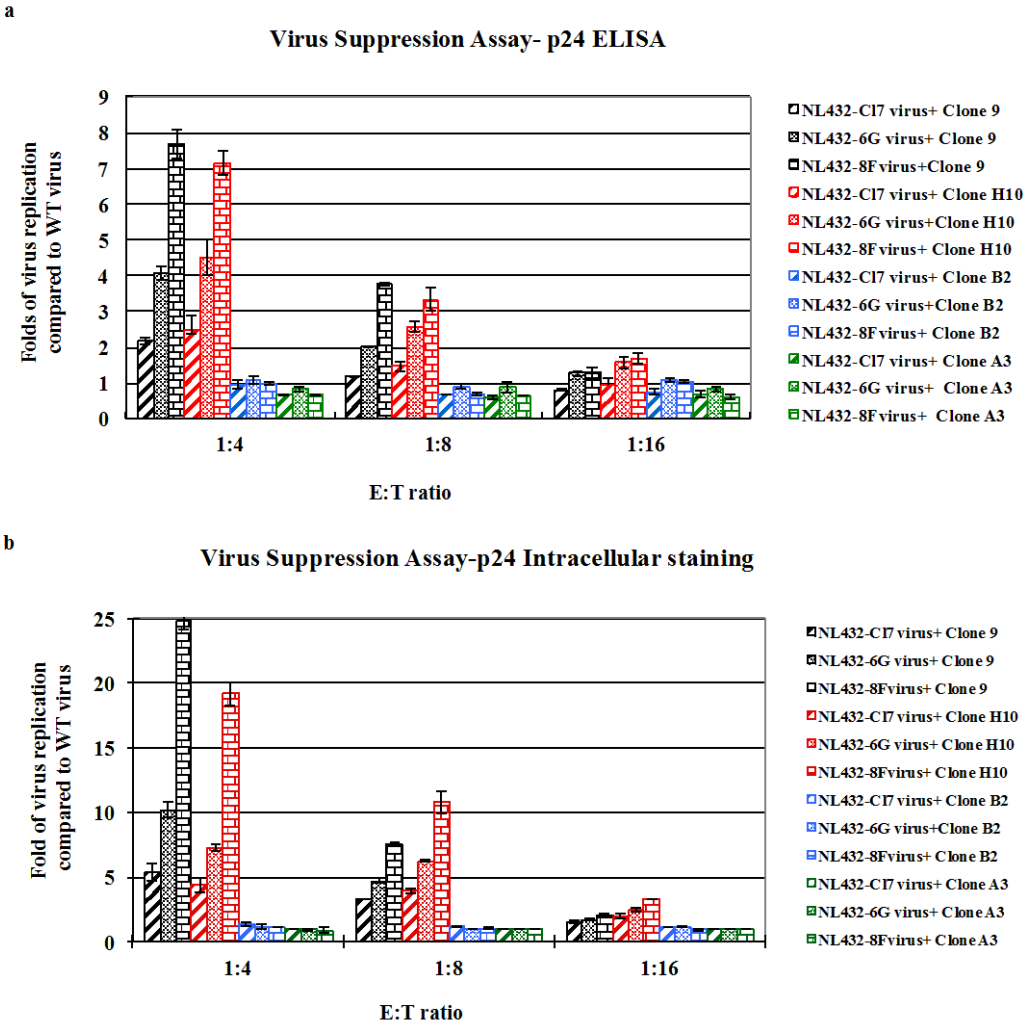

Figure 3.6: *In vitro* inhibition of WT and mutant viruses in the presence of antigen- specific CTL clones at different E: T ratios. CTL clones were co-cultured with target cells infected with mutant viruses NL432-Cl7 (Pol L743I), 6G (Pol L743I/L760I), 8F(Pol L743I/S754C/L760I). Data are expressed as fold increase of p24 level in the supernatant (a) and p24 positive cells (b) versus WT virus NL432. Clone 9 and Clone 10 are Pol LI9-specific whereas B2 is Gag NI9-specific. An HLA mismatched CTL clone (A3) is also included. N = 3 replicates

3.2.8 Gag NI9, Pol TI8 and Pol LI9 specific T cell clones could effectively recognise and control IIIB and MN virus-infected target cells in Live Virus ELISPOT assays and in Virus Suppression assays

We tested the ability of NI9, LI9 and TI8 specific T cells to recognise IIIB and MN virus-infected target cells in Live Virus ELISPOT assays (**Figure 3.7 a**). NI9 CTL showed the most effective recognition of virus-infected cells, followed by TI8 CTL. LI9 CTL showed very poor recognition of virus-infected target cells, which could be due to a mutation detected at position 4 (V4I) of epitope in the MN virus. Pol and Gag pro-viral DNA sequences were examined by extracting genomic DNA from HIV-1 IIIB and MN infected MT2 cells. Gag NI9, Pol TI8 and PolLI9 wild type sequences were confirmed in IIIB virus infected cells, whereas an intra-epitope mutation I746V at position 4 of the LI9 epitope was detected in MN virus infected cells, which explains the weaker recognition by LI9 specific T cell clones.

To confirm the anti-viral efficacy of all three epitope-specific T cells, we also performed *in vitro* Virus Suppression Assays by co-culture of Gag NI9, Pol TI8 and Pol LI9 specific T cell clones with HIV-1 IIIB or MN virus-infected MT2 cells, harvesting the culture supernatant on day 4 to measure HIV-p24 levels by ELISA. All three epitope specific T cell clones could control both viruses effectively when wild type epitopes are presented (**Figure 3.7 b and c**).

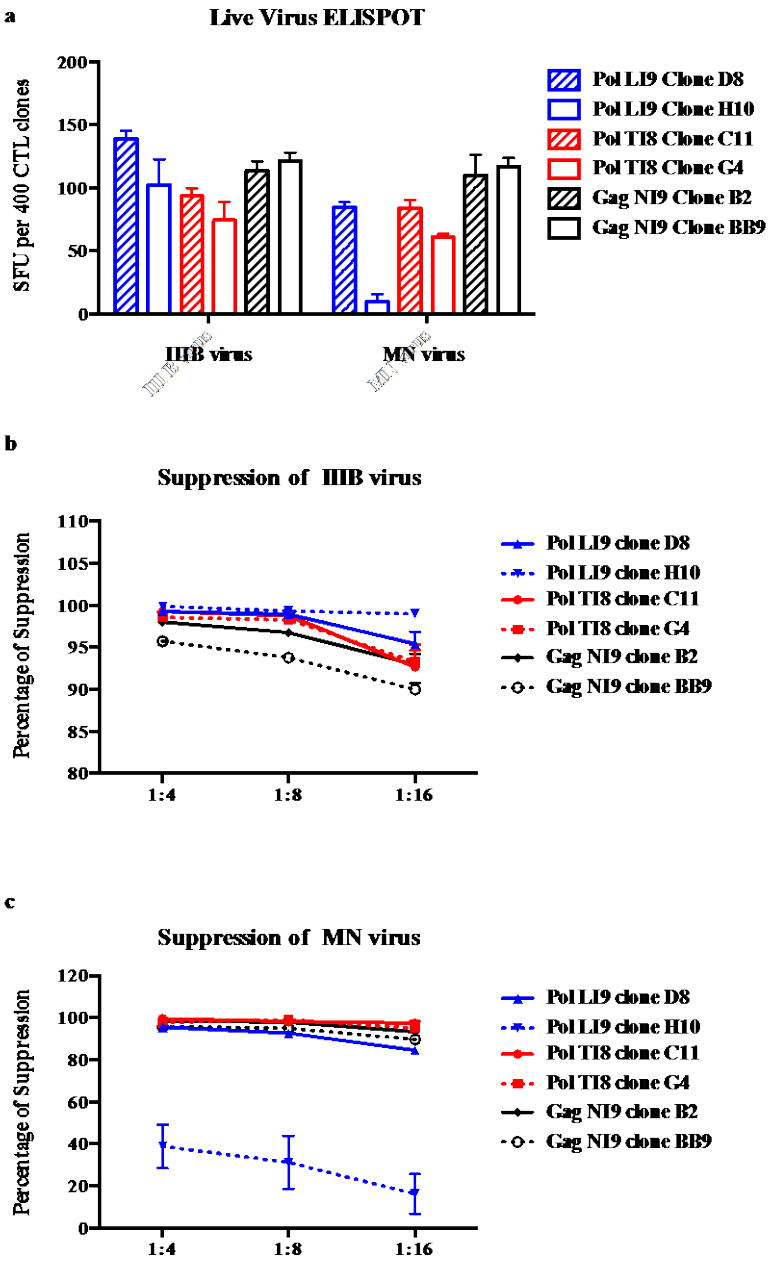

Figure 3.7 Gag NI9, Pol TI8 and Pol LI9-specific T cell clones can effectively recognise and control virus-infected target cells **a)** Anti-viral activity of CTL clones was assessed using Live Virus IFN-γ ELISPOT assay. CTL clones were co-cultured with MN and IIIB virus-infected targets. **b), c)** Ability of CTL clones to suppress HIV-1 replication in IIIB and MN infected MT2 cells at variable E: T ratios. N=3 replicates

3.3 Discussion

In most of the existing cohort studies, HIV infection route, length of infection and infecting viral strain almost always differ between individuals, leading to complexity of data analysis, in particular applied to HIV antigen-specific T cell responses, T cell escape and their association with clinical outcome. In this study, we have been able to study a unique cohort of patients who have been infected with a narrow source virus for a similar length of infection time (Dong et al., 2011). We found that HLA-B51 restricted immunodominant HIV specific T-cell responses play an important role in controlling disease progression in the SM cohort. A selection hierarchy of epitope mutations among the three most dominant HLA-B51 restricted epitopes was observed. Good control of viral load and higher $CD4^+$ counts were found in patients with T cell responses against un-mutated epitopes, whereas lower $CD4^+$ counts and higher viral loads were detected in patients with mutations in all three epitopes or with no detectable T-cell responses against epitopes that did not mutate. We conclude that the patients with HLA-B51 have benefited from having a hierarchy of protective epitopes that provide multiple layers of defence against the development of immune escape mutations, particularly when CTL target the epitope (NI9) that is relatively conserved and appears to escape slowly.

The most easily mutated epitope Pol LI9 also developed two other mutations (X760L and C754S) in the flanking region, which are strongly linked with the intra-epitope mutation (I743L) and associated with HLA-B51. We demonstrated

that CTL exhibit reduced anti-viral efficacy against viruses containing these mutations, strongly suggesting that the viruses with these mutations in HLA-B51+ individuals in our cohort will evade LI9 specific T cell responses due to impaired presentation of this epitope. The Pol TI8 CTL clones exhibited slightly reduced CTL recognition of 8V/L mutated peptide-pulsed targets, but impaired or no response to target cells infected with 8V/L mutant virus, suggesting that these mutations also had a detrimental effect on antigen processing and presentation of Pol TI8 epitope. Therefore, further experiments such as *in vitro* proteasome digestion to evaluate the impact of these mutations on antigen presentation are needed.

Unlike the Japanese cohort studied by Kawashima et al. (2009), the SM cohort showed less dependence on the Pol TI8 epitope response, presumably because this epitope already expresses a mutation in the consensus sequence, (Valine, instead of isoleucine, was dominant, found in 77% of non-B51 individuals), indicating that Pol 289V (TI8-8V) was likely to be present in the infecting virus (Dong et al., 2011). The pattern of escape mutations that developed was also very different between the two cohorts: for example, most of our patients developed an 8L mutation in this epitope. Instead, the escape mutation 8T was observed only in few individuals in our cohort, whereas it was frequently observed in the Japanese and other cohorts (The International HIV Controllers Study 2010). Overall, we have shown clear evidence that the development of epitope mutations and the sequence of the infecting virus are very different in the Chinese cohort compared to the

Japanese cohort, which might suggest the existence of different mechanisms of immune protection through HLA-B51.

Live Virus ELISPOT assays and Virus Suppression Assays showed that Gag NI9, Pol LI9 and Pol TI8 specific CTL could recognise MN or IIIB virus-infected cells efficiently, with no clear hierarchical ranking in terms of antiviral efficacy observed among these three epitope-specific clones, which suggested that these T cells could function efficiently if the epitopes were to be processed and presented properly in target cells.

Our data suggest that the presence of HLA-B51 and T cells targeting the three immunodominant epitopes (Pol LI9, Pol TI8 and Gag NI9) could be advantageous and, more importantly, that having an immunodominant response to the slowly mutating epitope Gag NI9 is beneficial for long-term control of viral replication. For future HIV vaccine design, it should be considered to elicit the most beneficial CTL responses by targeting epitopes that mutate with difficulty and are associated with viral control for common HLA alleles in a target population.

Chapter 4: Evaluation of the rate of epitope escape mutation driven by three dominant HLA-B51 restricted T cell responses

4.1 Introduction

AIDS is undoubtedly one of most serious infectious diseases to have affected mankind to date because of its high morbidity and mortality rate. Although the improvements in antiretroviral therapy have dramatically reduced the burden of morbidity and mortality (Palella et al., 1998), the rapid rate of HIV evolution poses a major obstacle for the development of successful treatments and effective HIV vaccines (Goulder and Watkins, 2004; Malim and Emerman, 2001).

The immune responses of the host are a major selective force that drives the intra-host evolution of HIV over the natural course of infection. This is because they drive HIV to evade immune responses by fixing mutations. Such immune selecting pressure could emerge from neutralizing antibodies (Frost et al., 2005; Richman et al., 2003; Wei et al., 2003), T-helper cells (Ross and Rodrigo, 2002), Cytotoxic T Lymphocytes (CTLs) (Allen et al., 2005; Liu et al., 2006) or ADCC (Chung et al., 2011; Isitman et al., 2011).

Escape mutations driven by CTLs within CTL-associated epitopes have been previously observed both in SIV (Allen et al., 2000) and HIV-1 infection (Borrow et al., 1994; Phillips et al., 1991). However, escape mutations from CTL responses in some epitopes or proteins have not been consistently observed *in vivo.* For

example, viral mutations in Gag-specific CD8 T-cell epitopes are more constrained than mutations arising in Env-specific CD8 T cell epitopes (Peut and Kent, 2007). One explanation for this phenomenon is that some mutations may bear a fitness cost for the virus, as they may lead to a decreased replicative capacity (Chopera et al., 2011; Domingo et al., 1997; Miura et al., 2009; Soderholm et al., 2006). In line with this, Ferrari et al recently reported that during the first six months of HIV-1 infection, the order of emergence of escape mutations is associated with the entropy (the population diversity) of the epitopes. Despite early T-cell responses, epitopes with lower entropy at the population level, which do not very readily tolerate sequence changes, showed delayed development of escape mutations, indicating the higher fitness cost caused by the sequence change in these epitopes (Ferrari et al., 2011a; Liu et al., 2013).

As mentioned in the previous Chapter, in our *ex vivo* study, we observed that escape mutants were sequentially selected among three dominant HLA-B51 restricted epitopes in the SM cohort. It was assumed that the mutations in epitope Pol LI9 were selected first, followed by mutations in the epitope Pol TI8. The epitope Gag NI9 on the other hand appeared to be the most conserved. The SM cohort has not been longitudinally followed. Longitudinal samples, and in particular samples from early infection when most CTL-driven mutation occurs, were not available. This fact made the task of witnessing the sequential emergence of mutations in the patients and understanding the mechanism underlying this impossible.

There are some *in vitro* experiments showing that CTL selected escape mutations for murine lymphocytic choriomeningitis virus (LCMV) (Aebischer et al., 1991), *hepatitis C virus* (HCV) (Soderholm et al., 2006) and HIV-1 (Van Baalen et al., 1998; Yang et al., 2003a). This encouraged us to set up an *in vitro* assay, called Virus Evolution Assay, to verify our *ex vivo* observations and also to investigate the impact of epitope specificity of HLA-B51 restricted T-cell responses on HIV-1 evolution. In this intensive Virus Evolution Assay, we co-cultured HLA-B51 CTL clones with an acutely infected HIV-permissive MT2 cell. Six rounds of viral passaging were performed under the selection of either a Pol LI9 specific clone (Clone H10) a Pol TI8 specific clone (Clone 3) or a Gag NI9 specific clone (Clone B2). The corresponding epitopes were then sequenced to monitor the emergence of epitope mutations. Meanwhile, the suppression of virus replication by each CTL clone from each round of virus passage was also assessed in order to evaluate the impact of those mutations on suppression of virus replication.

4.2 Results

4.2.1 Selection of HLA-B51 restricted CTL clones for Virus Evolution Assay.

In order to have a fair comparison of the development of epitope mutations driven by T cell clones with different antigen specificity, we aimed to choose clones with similar functional avidity and anti-viral efficacy, so the differences observed could be mainly contributed by antigen specificity rather than the differences in the strength of T cell pressure.

A panel of T cell clones specific for Pol LI9, Pol TI8 and Gag NI9 were tested for their antigen sensitivity using ELISPOT assay by co-culturing CTL clones with target cells stimulated with epitope peptide at different concentrations. The results in **Figure 4.1** showed that the clones with the same epitope specificity display similar recognition of epitope peptides; antiviral efficacy of those CTL clones was also assessed with the Virus Suppression Assay (**Figure 4.2**). Three clones with similar functional avidity and anti-viral efficacy were chosen for the Virus Evolution Assay, they are Pol LI9-specific Clone H10, Pol TI8-specific Clone 3 and Gag-NI9 specific Clone B2, although Pol LI9-specific Clone H10 has slightly higher antigen sensitivity and therefore better anti-viral efficacy than other two clones (Clone 3 and Clone B2).

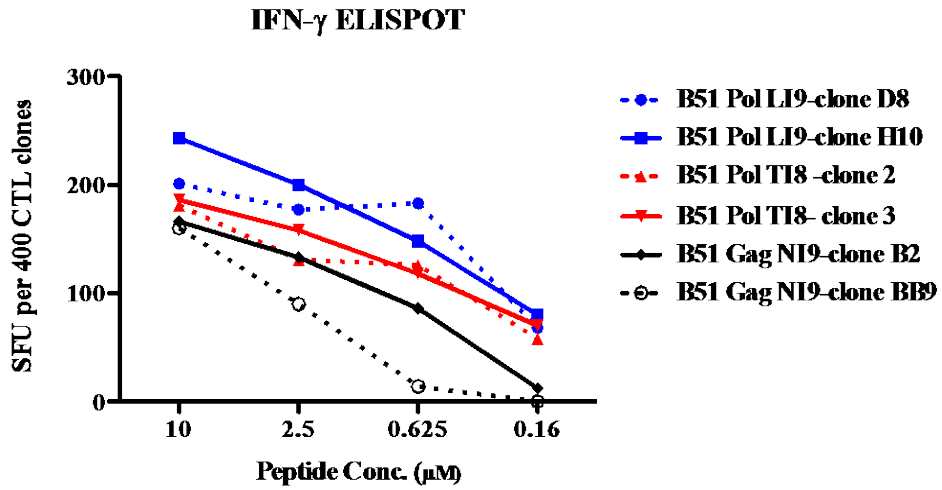

Figure 4.1 Antigen sensitivity of HLA-B51 Pol LI9 specific clones, Pol TI8 specific clones and Gag NI9 specific clones. IFN-γ ELISPOT was used to assess the antigen sensitivity of the CTL clones. The blue, red and black lines represent the recognition of the index peptide at 4-fold titrated concentrations by Pol LI9 specific clones, Pol TI8 specific clones and Gag NI9 specific clones, respectively.

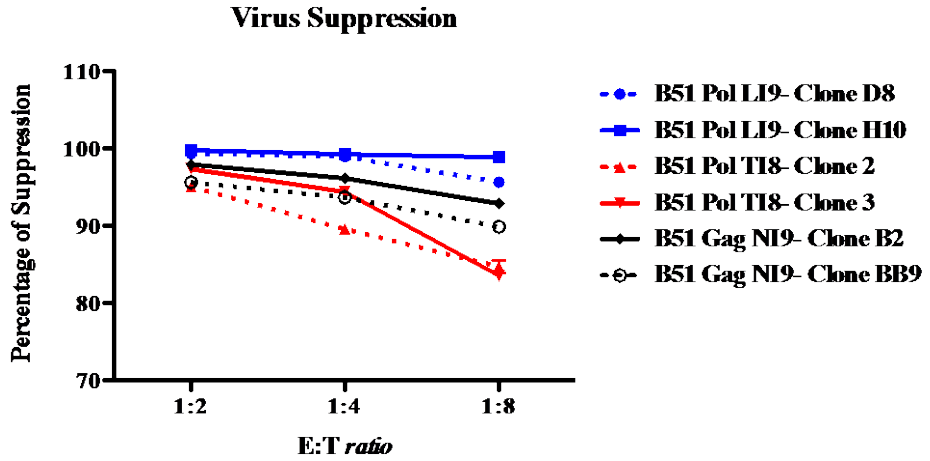

Figure 4.2 Anti-viral activities of HLA-B51 Pol LI9 specific clones, Pol TI8 specific clones and Gag NI9 specific clones against lab adapted WT IIIB virus. CTL clones were co-cultured with target cells infected with wild type WT IIIB virus at variable E:T ratios. ELISA measured p24 concentration in the supernatant. N =3 (replicates)

4.2.2 Virus Evolution Assay

As shown in Figure 4.3, $5x10^6$ MT2 cells were initially infected with HIV-1 IIIB virus (NIH AIDS Reagent Program) for 90 minutes, followed by two washes and re-suspension in RPMI 1640 with 10% of human serum and 100U/ml of IL2. Cells were then co-cultured with either an HLA-B51 restricted Pol TI8-specific clone (Clone 3), a Pol LI9 specific clone (Clone H10) or a Gag NI9 specific Clone (Clone B2) at an appropriate E: T ratio for 7 days in a total volume of 10 ml 100U/ml IL2/H10 at 37°C/5% CO2. A flask with cultured infected target cells in the absence of antigen specific CTL clones was also set up as a control. The cultures were then fed twice with 5ml of fresh medium over the next week, followed by harvesting of the cell pellet and supernatant virus, which were both saved at -80°C. The process was then repeated in a second round of passage using the supernatant virus from the flask with the corresponding CTL clone (selecting group) for next round in both selecting and control groups, at an initial input of 500pg p24 antigen per 10^6 target cells. The 3rd, 4th, 5th,and the 6th round were performed in an identical fashion to the second, by infecting target cells with the supernatant virus from the previous round, which had gone through one week of CTL selection.

After 6 rounds of virus passages, genomic DNA was extracted from cell pellets from all passages. The corresponding epitopes were then sequenced using nested PCR and TOPO TA cloning. Meanwhile, the concentration of p24 antigen in the culture supernatant from both selected flasks and control flasks of each round

passage was measured by p24 ELISA in order to monitor the suppression of virus replication by CTLs.

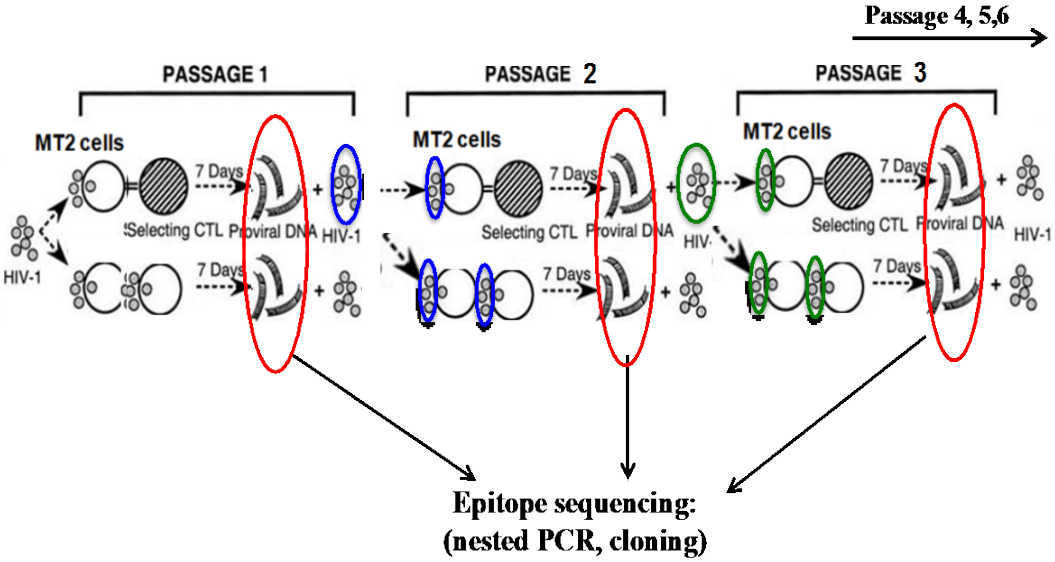

Figure 4.3 Virus Evolution Assay-passaging of HIV-1 under the selection of CTL. HLA-B51 positive MT2 cell line was infected with HIV-1 virus and then cultured with or without HIV-1 specific HLA-B51 CTL clones (Positive Control). At Day 7, the supernatant of the culture was titered by p24 ELISA and then used to infect an MT2 cell line for the next round. Meanwhile, the pro-viral DNA was extracted and the epitope was sequenced by nested PCR and cloning. [Modified from (Yang et al., 2003a)]

4.2.3 The HLA B51 CTL clones have similar anti-viral efficacies against the WT IIIB virus in the 1st round of virus passage.

The CTL clones used for this assay have different antigen sensitivities, which has been shown in Chapter 3 and is also shown in **Figure 4.1**. To achieve similar levels of CTL-mediated immune suppression for each virus passage, Pol LI9 Clone H10,

Pol TI8 Clone 3 and the Gag NI9 clone were co-cultured with the infected MT2 cells at different E: T ratios. The ratios used were 1: 16, 1:10 and 1:10 respectively. As shown in **Figure 4.4**, these three clones were found to suppress the virus replication at comparable levels during the 1st round of the viral passaging when the wild type IIIB virus was presented. This effect was seen despite their different antigen sensitivities.

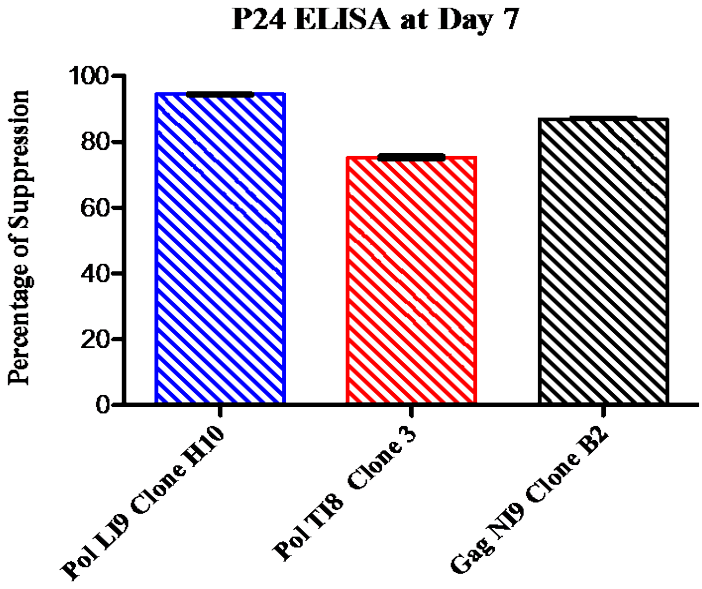

Figure 4.4 Similar anti-viral efficacies of Pol LI9 Clone H10, Pol TI8 Clone 3 and Gag NI9 Clone B2 during the 1st round of viral passaging in the Virus Evolution Assay. The percentage of suppression was calculated by the deduction of the p24 antigen in the culture at Day 7 with CTLs present compared to the control (in the absence of selecting CTLs).

4.2.4 Reduced anti-viral efficacy of the three HLA-B51 CTL clones following virus passages.

Initially, the supernatants collected at day 7 from each virus passage were titered by p24 ELISA and then the suppression of virus replication by the CTL clones after each round of replication was calculated. The hierarchy of viral suppression for all three HLA-B51 antigen specific CTL clones is shown in **Figure 4.5**.

The suppression of virus replication by all three CTL clones however, declined after sequential viral passages. As can be seen from the graph, the Pol LI9 clone H10 showed a dramatic reduction in virus suppression even during the early virus passages, tumbling from 97% of suppression at 2nd passage to 44% suppression at the 3rd passage. By contrast, the suppression of Pol TI8 and Gag NI9 clones weakened gradually after the 3rd passage.

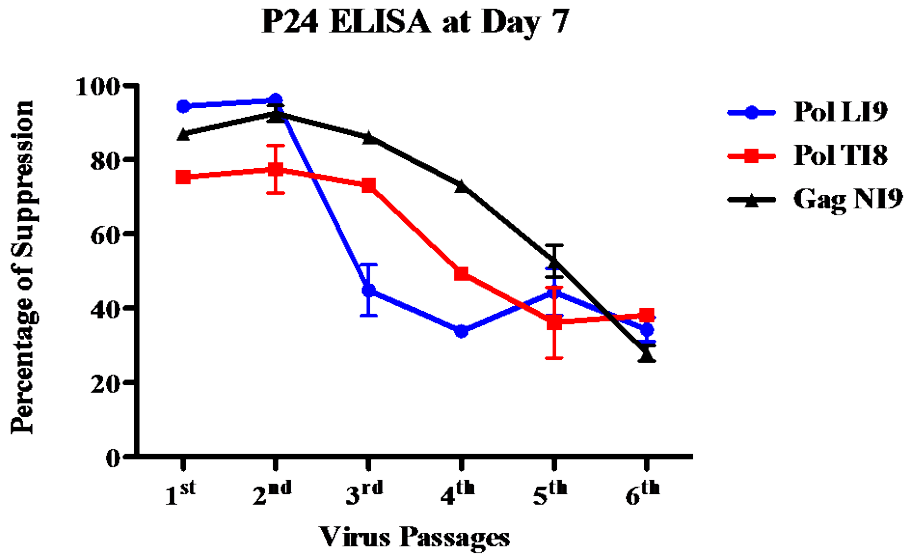

Figure 4.5 The anti-viral efficacy of the B51 CTL clones against HIV-1 IIIB virus from each passaging round under CTL selection. The percentage of suppression was calculated by the deduction of the p24 antigen in the culture with CTLs present compared to positive control (in the absence of CTLs).

4.2.5 Early generation of a dominant escape mutation in the epitope Pol LI9.

Following six rounds of virus passage, the pro-viral DNA was amplified by PCR, cloned into TOPO and then 10 clones from each epitope were sequenced. As shown in **Table 4.1**, after 2 weeks of selective passaging 60% of the virus had developed a dominant mutation (V4I) within the epitope Pol LI9. After 3 rounds of viral passaging in the presence of the Pol LI9 specific CTL clone, all the viral clones contain this single nucleotide substitution mutation (V4I). The recognition of this epitope variant by this clone was then tested by ELISPOT (**Figure 4.6**), CTL clone Pol LI9 H10 can only cross-react to this peptide variant at very high

concentration, indicating that virus with this epitope mutation escaped the recognition by this CTL clone.

To exclude the possibility of a spontaneous mutation caused by the MT2 cell line, the control virus from each round was also cloned and sequenced (Data shown in **Table 4.2**). Since the infecting virus in the controls was derived from the supernatant of the previous round of passaging under CTL selection (**Figure 4.7**), it was anticipated that there would be no differences between the epitope sequence from each round and that of the previous round. For example, it was expected that the control virus collected in the 2nd round of replication would have same sequence as the one collected after one round of passaging in the presence of the CTL clone. As can be seen from the **Table 4.1** and **4.2**, 60% of the viral clones from the 2nd virus passage have the mutation V4I in the epitope Pol LI9 whereas all the viral clones of the positive control remained unaltered when their sequences were compared to the sequence from the previous round (1st round). These data clearly demonstrate that the MT2 cell line has no impact on the occurrence of the viral mutation V4I.

Table 4.1 Epitope Pol LI9 sequences from 7-day co-culture of Pol LI9 clone H10 and HIV-1 (IIIB) infected MT2 cell line. The sequences are aligned with the infecting virus (IIIB), with the LI9 epitope in bold font.

Viral Passage	Epitope Sequence (Founder Virus: IIIB)			Clonal Frequency
	AAAFN	LPPVVAKEI	VASCD	
1st Round	———	———————	———	**(10/10)**
2nd Round	———	———————	———	**(6/10)**
	———	——I———	———	**(4/10)**
3rd Round	———	——I———	———	(10/10)
4th Round	———	——I———	———	(10/10)
5th Round	———	——I———	———	(10/10)
6th Round	———	——I———	———	(10/10)

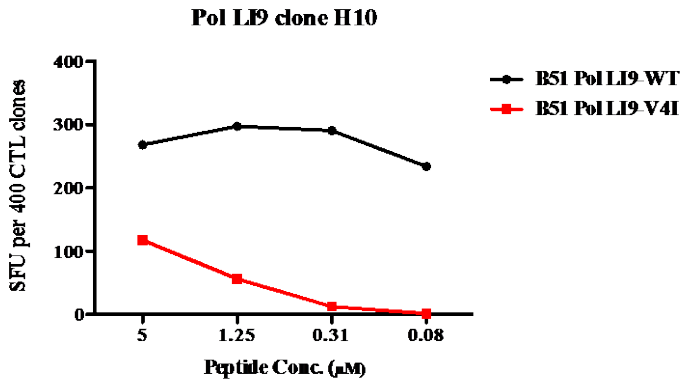

Figure 4.6 Recognition of peptide variant by the HLA-B51-restricted Pol LI9 specific CTL Clone H10. The recognition **of** the epitope variant containing the mutation selected by HLA-B51 Pol LI9 specific CTL Clone H10 in the Virus Evolution Assay was assessed by IFN-γ ELISPOT.

Table 4.2 Epitope Pol LI9 sequences from controls: 7-day culture of HIV-1 (IIIB) infected MT2 cell line without CTLs. The sequences are aligned with the infecting virus (IIIB), with the LI9 epitope in bold font.

Viral Passage	Epitope Sequence (Founder Virus: IIIB)			Clonal Frequency
	AAAFN	LPPVVAKEI	VASCD	
2nd Round Control	———	———————	———	(10/10)
3rd Round Control	———	———————	———	(5/10)
	———	——I———	———	(5/10)
4th Round Control	———	——I———	———	(10/10)
5th Round Control	———	——I———	———	(10/10)
6th Round Control	———	——I———	———	(10/10)

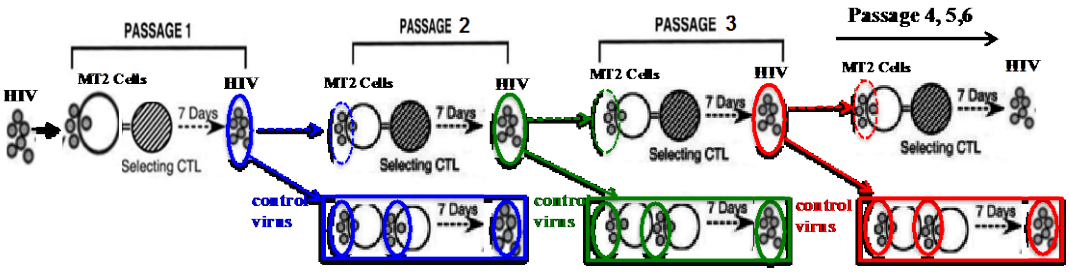

Figure 4.7 Viral passages of the controls. The virus in the controls was passaged without CTL selection. The infecting virus in each round of controls was derived from the supernatant of the previous round of passaging under the CTL selection: i.e., the infecting virus in the 2nd round of control (highlighted in blue) is the supernatant virus from the 1st round that has been through one-week of CTL selecting (highlighted in blue); the control viruses in the 3rd and 4th round of passage are highlighted in green and red, respectively.

4.2.6 Escape mutations within epitope Pol TI8 occurred after the 4th round of viral passaging.

Mutations in the Pol TI8 epitope developed slowly after 4 weeks of co-culturing with CTLs (**Table 4.3**). The mutation I8R on the other hand, arose during the 4th passage at a frequency of 20%; this mutation has also been observed in LTNPs in general and in acutely HIV-1 infected patients, where it has proved to be resistant to CTL recognition. In addition, two other mutations, S7N and S7R, occurred at slightly higher frequencies than I8R, after the 4th and 5th passage respectively. This indicates that the virus carrying mutation S7N or S7R probably has better replicative capacity than the one with I8R mutation. Though these two substitutions at position 7, together with mutation I5V, are not observed in the patients of SM cohort, they are seen in other patients infected with clade B HIV-1 virus from *LANL database*.

It was then assessed whether these two substitutions at position 7 can be recognised by the CTL clone. The results of ELISPOT assay shown in **Figure 4.8** point out that the peptide variants bearing the substitution (S7R/N) were not recognised by the Pol TI8 Clone 3. This suggests that these two mutations are escape mutants.

Epitopes from the controls were also sequenced and the results are shown in **Table 4.4**. The viral sequences from each round of passaging are quite similar to the ones from the last round passaging in the presence of CTLs. For this instance, the effect

of MT2 cell line on virus evolution can be excluded. Furthermore, there is no mutation detected in the epitope flanking regions.

Table 4.3 Epitope Pol TI8 sequences from 7-day co-culture of Pol TI8 clone 3 with HIV-1 (IIIB) infected MT2 cell line. The sequences are aligned with the infecting virus (IIIB), whereas the TI8 epitope is shown in bold.

Viral Passage	Epitope Sequence (Founder Virus: IIIB)			Clonal Frequency
	DFRKY	TAFTIPSI	NNEFP	
1st Round	———	———————	———	**(10/10)**
2nd Round	———	———————	———	**(10/10)**
3rd Round	———	———————	———	(10/10)
4th Round	———	———————	———	**(4/10)**
	———	————R—	———	(2/10)
	———	————N—	———	(2/10)
	———	————S—	———	(2/10)
5th Round	———	———————	———	**(0/10)**
	———	————R—	———	(2/10)
	———	————N—	———	(3/10)
	———	————R—	———	(3/10)
	———	————V—	———	(2/10)
6th Round	———	———————	———	**(1/10)**
	———	————R—	———	(2/10)
	———	————N—	———	(4/10)
	———	————R—	———	(3/10)

Table 4.4 Epitope Pol TI8 sequences from positive control: 7-day culture of HIV-1 (IIIB) infected MT2 cell line without CTLs. The sequences are aligned with the infecting virus (IIIB). The TI8 epitope is shown in bold.

Viral Passage	Epitope Sequence (Founder Virus: IIIB)			Clonal Frequency
	DFRKY	TAFTIPSI	NNEFP	
2nd Round Control	-----	--------	-----	(10/10)
3rd Round Control	-----	--------	-----	(10/10)
4th Round Control	-----	--------	-----	(10/10)
5th Round Control	-----	--------	-----	(5/10)
	-----	-------R	-----	(2/10)
	-----	-------N	-----	(2/10)
	-----	-------S	-----	(1/10)
6th Round Control	-----	--------	-----	(0/10)
	-----	-------R	-----	(2/10)
	-----	-------N	-----	(4/10)
	-----	-------R	-----	(3/10)
	-----	-------V	-----	(1/10)

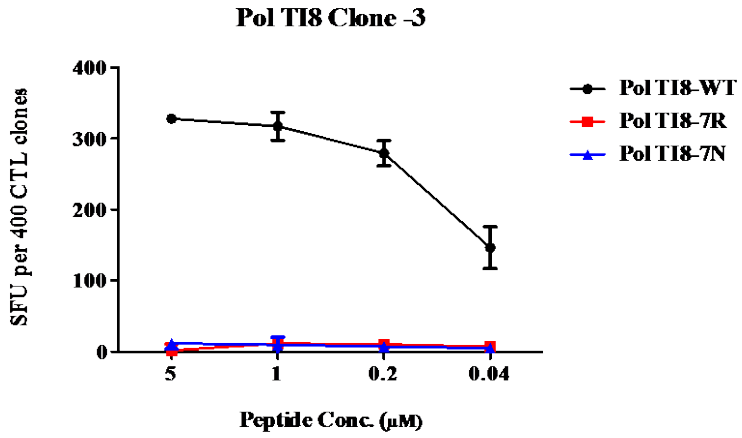

Figure 4.8 Recognition of peptide variants by the HLA-B51-restricted Pol TI8 specific CTL Clone 3. The epitope variants tested are the mutations selected by HLA-B51 Pol TI8 specific CTL Clone 3 in the Virus Evolution Assay**.**

4.2.7 Dominant escape mutation was observed in epitope Gag NI9 after the 4th, 5th and 6th passages.

The pro-viral DNA sequences of epitope Gag NI9 are shown in **Table 4.5**. After 3 rounds of viral passaging, the dominant mutation K7R was observed in 5 out of 10 sequences and its frequency increased to 80% during the 6th passage. This suggests that the K7R mutation didn't induce a significant fitness cost to the virus. On the other hand, though this mutation was not observed in our patients, the residue R is a common variation in clade B HIV-1 virus from LANL database and also the common consensus amino acid in other lentiviruses, which indicates that this substitution is not associated with a reduction in viral fitness.

The second mutation (A2T) developed in the 4th round of the passaging and was maintained at the low frequency of 10% after the 6th virus passage, indicated that this mutation may have caused more cost to the viral fitness than mutation K7R.

Recognition of these two peptide variants (K7R and A2T) by the B51 Gag-B2 clone was tested. Despite the high similarity between residues K (Lysine) and R (arginine), the peptide variant with this substitution escaped the recognition by the T cell clone, as shown in **Figure 4.9**.

On the contrary, the A2T substitution, from the hydrophobic amino acid Alanine (A) to the polar neutral amino acid Threonine (T)) at anchor position 2, may reduce or abolish the peptide interaction with MHC class I molecule, hence contributing to the escape of the virus from CTL recognition. As anticipated, the T cell clone did not recognise the peptide variant A2T, presumably due to the impaired binding to HLA-B51 molecule.

The epitope sequence of the controls from each round of virus passaging has also been monitored, shown in **Table 4.6** and the mutations detected are as expected. No mutation was observed in the flanking regions of this epitope.

Table 4.5 Epitope NI9 sequences from 7-day co-culture of Gag NI9 clone B2 and HIV-1 (IIIB) infected MT2 cell line. The sequences are aligned with the infecting virus (IIIB), with the Gag NI9 epitope shown in bold font.

Viral Passage	Epitope Sequence (Founder Virus: IIIB)			Clonal Frequency
	TLLVQ	NANPDCKTI	LKALG	
1st Round	———	———	———	(10/10)
2nd Round	———	———	———	(10/10)
3rd Round	———	———	———	(10/10)
4th Round	———	———	———	(4/10)
	———	———R———	———	(5/10)
	———	—T———	———	(1/10)
5th Round	———	———	———	(5/10)
	———	———R———	———	(5/10)
6th Round	———	———	———	(0/10)
	———	———R———	———	(8/10)
	———	—T———	———	(1/10)
	———	———N———	———	(1/10)

Table 4.6 Epitope Gag NI9 sequences from positive control: 7-day culture of HIV-1 (IIIB) infected MT2 cell line without CTLs. The sequences are aligned with the infecting virus (IIIB), with the Gag NI9 epitope depicted in bold font.

Viral Passage	Epitope Sequence (Founder Virus: IIIB)			Clonal Frequency
	TLLVQ	NANPDCKTI	LKALG	
2nd Round Control	-----	---------	-----	(10/10)
3rd Round Control	-----	---------	-----	(10/10)
4th Round Control	-----	---------	-----	(10/10)
5th Round Control	-----	---------	-----	(5/10)
	-----	-------R-	-----	(5/10)
6th Round Control	-----	---------	-----	(5/10)
	-----	-------R-	-----	(5/10)

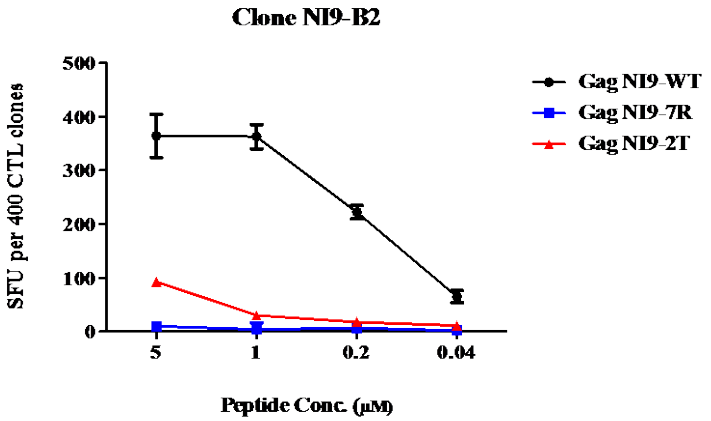

Figure 4.9 Recognition of peptide variants by HLA-B51 Gag NI9 specific CTL Clone B2 The epitope variants tested are the mutations selected by HLA-B51 Gag NI9 specific CTL Clone B2 in the Virus Evolution Assay**.**

4.2.8 Strong correlation of the percentage of remaining WT viral clones under the selection of the CTL clones with their suppression of virus replication.

To exclude the possibility that the continuous *in vitro* expanding of CTL clones may have some effect on their ant-viral efficacy, we also investigated the association between the reduced virus suppression and the emergence of the escape mutations from each virus passage.

As shown in the **Figure 4.10 a**, a significant correlation was seen between the level of the suppression of the Pol LI9 specific CTL clone with the percentage of WT viral clones remaining (*p* value =0.0056) after each selective viral passage. The higher the percentage of WT viral clones that remained, the better was the suppression of the viral replication that was detected. The same correlation was also observed when the virus was passaged under the selection of two other HLA-B51 antigen specific CTL clones, namely of Pol TI8 Clone 3 and Gag NI9 Clone B2, as shown in the **Figure 4.10 b** (*p* value <0.0001) **and Figure 4.10 c** (*p* value =0.0015)**.** In addition, across all clones, the proportion of remaining WT viral clones after each virus passage under CTL selection was strongly correlated with their virus replication suppression potential (*P*<0.0001, **Figure 4.10 d**). This observation strongly implied that the reduction in anti-viral efficacy of the CTLs was due to the emergence of escape mutations. These data also clearly demonstrated that the HLA-B51 restricted CTL responses drive the escape mutations within the epitopes that lead to the loss of viral control.

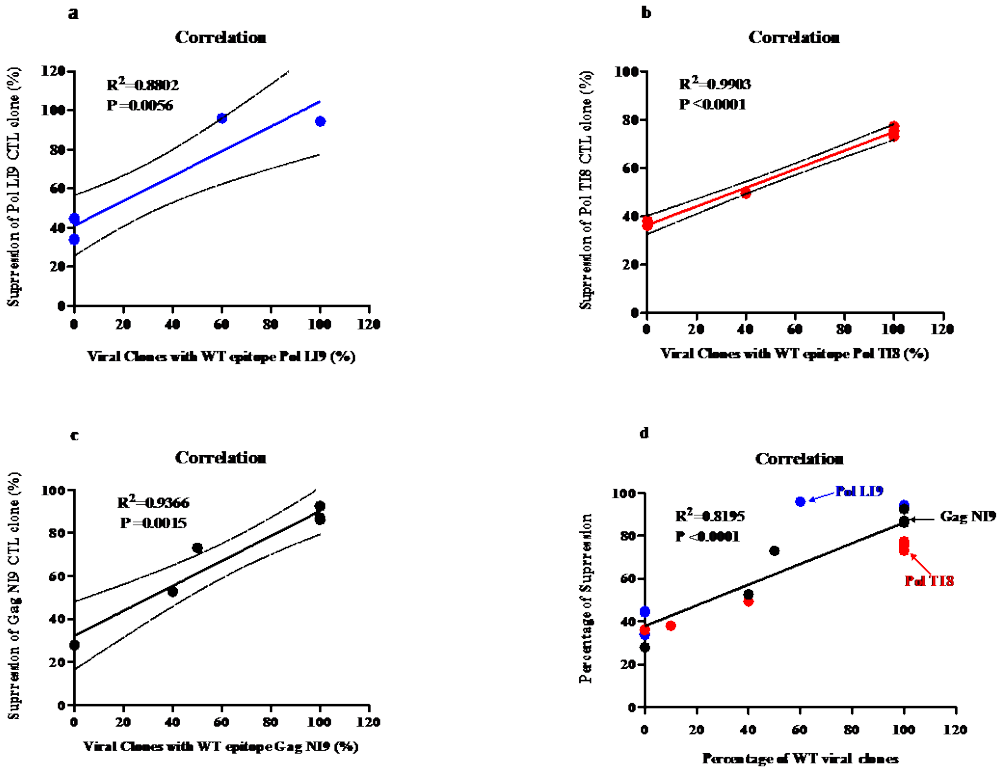

Figure 4.10 Correlation of the percentage of remaining WT viral clones with the suppression ability of the CTL Clones The Pearson correlation was analysed by Prism and *p* values are two-tailed. **a)** The correlation between the proportion of WT viral clones under the selection of Pol LI9 CTL clone and the suppression of viral replication at each round of passage. **b**) Correlation between the proportions of WT viral clones remaining under the selection of the Pol TI8 CTL clone and the suppression of viral replication at each round of passage. **c**) Correlation between the proportion of WT viral clones under the selection of Gag NI9 CTL clone and the suppression of virus replication from each round of passage. **d)** Correlation of the percentage of remaining WT viral clones under the selection of HLA-B51 specific CTL clones and their efficacy of suppression of viral replication. The blue, red and black dots represent each round of viral passage under the selection of Pol LI9, Pol TI8 and Gag NI9 specific CTL clone, respectively.

4.2.9 Hierarchical development of mutations within three HLA-B51 restricted epitopes

Then I compared the proportion of remaining WT viral clones in the culture after each round of passage under the selection of each CTL clone.

As can be seen in the **Figure 4.11**, there is a sequential decrease of the remaining WT viral clones in three HLA-B51 epitopes under the selection of the corresponding CTL. The proportion of WT viral clones was reduced most rapidly under the selection of the Pol LI9 CTL clone. The WT viral clones only account for 60% after the 2nd round virus passage and disappeared after the 3rd round of passage. In contrast, under the selection of Pol TI8 CTL clone, the percentage of WT viral clones decreased more slowly. No mutation was detected after the 3rd round of virus passage. 40% of viral clones still remained unchanged at the 4th round of passage. The WT virus disappeared after 5th round of virus passage. Compared to the selection of two Pol specific CTL clones, the reduction of WT viral clones under the selection of Gag NI9 CTL clone is even slower. There is no mutation detected after 3 round of selection. 50% of viruses are still WT even after 5 rounds of selection.

These observations suggest that under the selection of the corresponding CTL clone, the Pol LI9 epitope developed escape mutations most rapidly, followed by the Pol TI8 epitope and then epitope Gag NI9. These data paralleled the observations made in our *ex vivo* study and confirmed the sequential selection of

escape mutations within these 3 dominant HLA-B51 restricted CTL responses in the SM cohort.

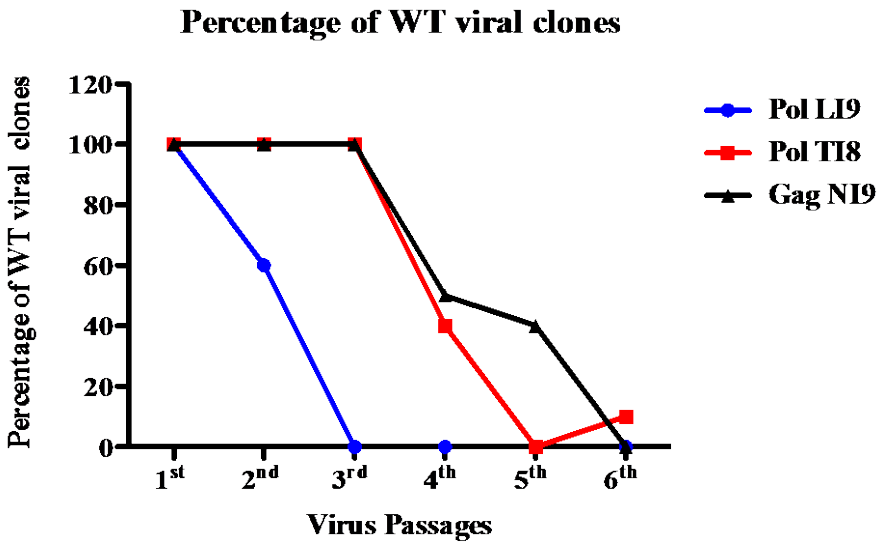

Figure 4.11 The reduction of the percentage of remaining WT viral clones under CTL selection at each viral passage The blue, red and black lines depict the proportion of pro-viral clones with WT Pol LI9, Pol TI8 and Gag NI9 epitopes respectively.

4.2.10 Rapid disease progression is associated with the early development of escape mutations in the Pol LI9 and Pol TI8 epitopes together with a lack of an effective response to the Gag NI9 epitope.

In collaboration with the You'an hospital in Beijing, we were able to have access to an HIV-1 acutely infected HLA-B51^{+} individual (YA-13) who had progressed rapidly to disease during the first year of infection. Blood samples and clinical data (CD4 count and Viral Load) were collected during three hospital visits at Day 47, Day 130 and Day 223. Meanwhile, T-cell responses against HLA-B51 restricted known epitopes were assessed by IFN-γ ELISPOT using index peptides and the

corresponding epitopes were also sequenced at all three time points. As shown in **Figure 4.12**, Pol LI9 and TI8 specific T cells were found to dominate the early stages of the acute infection, with their response magnitudes reaching to 1739 SFU and 2079 SFU/ 1M PBMCs respectively. Despite an increase in the T-cell responses against Pol LI9 and Pol TI8, the patient experienced a rapid increase in plasma viral load at 223 days post infection. This coincided with the emergence of the mutation V5I in the Pol LI9 epitope and the defined escape mutation I8T within the epitope Pol TI8. No T-cell responses against the epitope Gag NI9 were detected at all three time points. These data reiterated that early development of escape mutations in the epitopes Pol LI9 and Pol TI8, together with the lack of an effective response to Gag NI9, leads to rapid disease progression in this HLA-B51-positive patient.

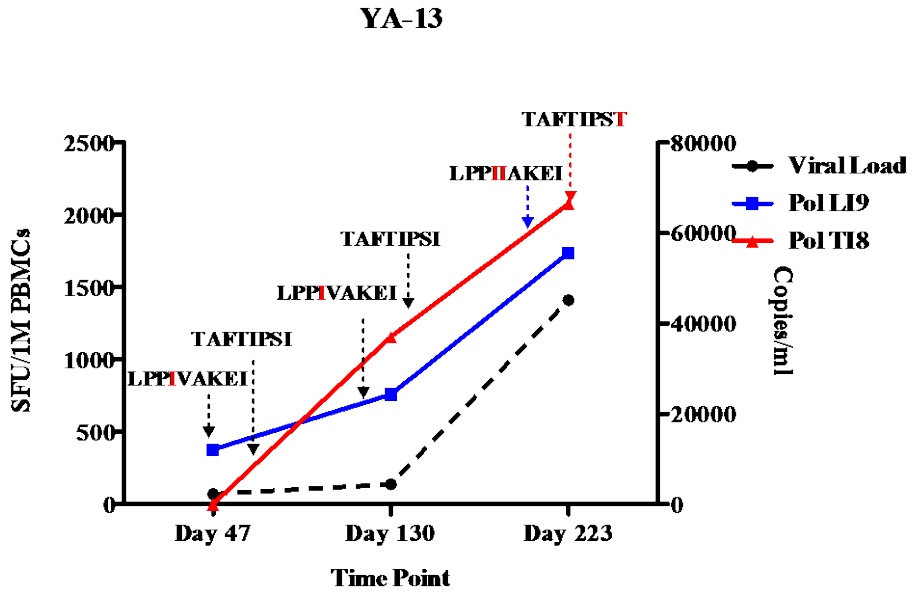

Figure 4.12 Viral Load, T cell responses and epitope sequence in an acutely HIV-1 infected HLA-B51 patient (YA-13) The Left Y Axis represents the Spot Forming Unit per one million PBMCs. IFN-γ released by PBMCs was tested by ELISPOT using index-peptides. The Right Y Axis represents the viral copies per one millilitre of plasma.

4.3 Discussion

It has been well acknowledged that CTL selection is a major force driving the development of escape mutations (Allen et al., 2005; Liu et al., 2006; O'Connor et al., 2004). The emergence of HIV-1 escape mutations depends upon the net balance of selective pressure and upon the fitness cost that potential escape mutations bear for the virus.

Although *in vitro* CTL selected escape mutations in virus infection were observed in 1990's (Aebischer et al., 1991; Van Baalen et al., 1998), the first *in vitro* assay to evaluate the ability of HIV-1 virus to escape CTL recognition was conducted by Yang et al. (2003a). The Virus Evolution Assay used in this study was adapted from their methods but is more intensive. In our assay, the input virus underwent six round passages under CTL selection so that we could have the opportunity to evaluate the impact of antigen specificity on the kinetics of development of escape mutations. Moreover, in Yang's assay, they used virus supernatant from the 1st round control to infect targets for the 2nd round control whereas the viruses from the 1st round selecting group for the 2nd round selecting. It is possible that the infected viruses in the 2nd round passage for selecting groups and control groups could be different when the viruses in selecting flask have already generated mutations after one round of CTL selection whereas viruses from control flask remain unchanged or (WT) when immune selection is absent. This might cause some bias when evaluating virus suppression by CTLs because the mutated viruses selected by CTLs may have different infectivity or impaired replication capacity,

when compared to un-mutated virus from control flasks. As shown in the **Figure 4.7**, we used the same infecting virus, virus supernatant collected from selecting flasks, for next round viral passage of both selected group (with selecting CTLs) and control group (without selecting CTLs). This feature puts our assay to good advantage in that we could evaluate the impact of emergence of the mutations on the anti-viral efficacy of the CTL clones with less bias. In addition, we also have an additional control that passaging the WT III B virus without selecting CTLs for six rounds to avoid the bias of spontaneous mutations. We sequenced all three epitopes of the pro-virus from the 6th round passage and found that there are no mutations found within the epitopes in the absence of selecting CTL clones.

In this chapter, the effect of three HLA-B51 CTL clones with different specificities (either for the dominant epitope Pol LI9, or Pol TI8, or Gag NI9) on HIV-1 evolution was carefully examined through our *in vitro* Virus Evolution Assay. It was found that the ability of the three CTL clones to suppress the viral replication decreased after a specific number of viral passages, which indicated the emergence of viral mutations and their capacity to escape the CTL recognition. We also observed that the reduction in antiviral efficacy of the Pol LI9 clone is more rapid than for that of the two other clones, suggesting that escape mutations in the epitope Pol LI9 are the most rapid to develop.

The sequences of the epitopes after each round of virus passage were also studied. As predicted, the earliest escape mutation observed was in the epitope Pol LI9. In

addition, 50% of the sequences contained the escape mutation V4I at the 2nd round of virus passage, followed by the complete escape of the virus after the 3rd round of passage. The ability of the V4I epitope mutation to arise and dominate rapidly indicates that this substitution does not carry a substantial fitness cost for the virus. This is further supported by the fact that this variant is widely accumulated in other HIV-1 virus strains (Leslie et al., 2005) and is also observed as a CTL driven mutation in our acutely infected HLA-B51 patient. It is noteworthy to mention that the patient progressed rapidly to disease, despite the high magnitude of the T-cell responses to this epitope. Altfeld et al. (2006) have also reported that Pol LI9 responses were the most immunodominant during acute infection followed by Pol TI8 responses. These observations, taken along with our *in vitro* observations, suggest that HLA-B51 restricted Pol LI9 specific CTLs could elicit the most dominant and early response in HLA-B51 positive patients during the course of HIV infection. However, under CTL selection, the Pol LI9 epitope was also the one to develop escape mutations most rapidly, leading thus to the loss of early viral control. This implies that future vaccine design approaches might need to avoid stimulating CTL responses against such epitopes.

Unlike the Pol LI9 epitope, however, the mutations that developed within the Pol TI8 epitope were multiple and highly monoclonal (multiple sequences with a single substitution), indicating the flexibility of this epitope. In terms of the I8R mutation, the substitution of an anchor residue from Isoleucine to Arginine at position 8 may have led to a lack of binding to the HLA-B51 allele and therefore

may have facilitated escape from CTL recognition. Due to the distinct structural characteristics of the residues S (Serine), R (Arginine) and N (Asparagine), the mutations S7N/R could presumably have a big influence on the interaction with HLA-B51 or on TCR recognition and would therefore escape CTL control. Indeed, when the recognition of these two peptide variants by a CTL clone was tested, the clone was not found to be reactive to neither of them, which was as expected.

Our *ex vivo* study also revealed that among the three HLA-B51 immunodominant epitopes, Gag p24 NI9 epitope is the most conserved. In addition, the rare mutation A2S in this epitope is an escape mutation. Our *in vitro* evolution assay confirmed that the mutation occurred much slower when compared with two pol epitopes indicating NI9 specific T cell may control virus better overall. Although we did not observe the A2S mutation in our *in vitro* system, a very similar substitution (A2T) occurred after the 4th passage in 10% of the viral clones. Overall, the Gag NI9-specific Clone B2 displayed a huge reduction of recognition efficacy against the dominant mutation K7R, despite the structural similarity between these two amino acids. Although this mutation was not seen in the SM cohort, it was frequently seen in other patients from the LANL database and also a consensus sequence in some other lentiviral Gag p24 proteins, for example some SIV strains including DEB.CM.99.CM5 and LST.CD.88 and EIAV (www.hiv.lanl.gov.*)*

Taking all the virus suppression and epitope sequence data of together, it was also found that the suppression of the virus replication is strongly correlated with the

proportion of WT viral clones that remain under the selective pressure exerted by the CTLs. What this observation suggests is that in this *in vitro* assay, HLA-B51 restricted CTL responses have driven the escape mutations within these three dominant epitopes, leading to the loss of viral control. When the emergence of the mutations within these three epitopes in all six rounds of viral passaging was compared, a hierarchy in the development of mutations within different epitopes was observed. The mutation to develop most rapidly was within the Pol LI9 epitope, and then mutations within the epitopes Pol TI8 and Gag NI9 followed. This mutational order was observed even when a similar level of immune pressure was mounted by all CTL clones in the *in vitro* assays. These data confirm our *ex vivo* observations, and provide evidence for the sequential selection of mutations between these three HLA-B51 restricted epitopes in the SM cohort.

It has long been proposed that any potential escape mutations selected by CTLs is largely governed by the impact of an escape mutation on relative virus fitness. Several studies have reported that CTL escape mutations in HIV-1 Gag p24 were associated with significant fitness costs (Boutwell et al., 2013; Troyer et al., 2009). The compensating mutations in HLA-B27 and HLA-B57 Gag p24 epitopes are good examples (Kelleher et al., 2001; Leslie et al., 2004). The study of the structure of lentiviral and HIV-1 Gag p24 capsid proteins revealed that the NI9 epitope residues localise in the helix 10 of the carboxyl-terminal domain of capsid protein (see **Figure 1.5** in Chapter 1) and are strongly conserved in lentiviral capsid proteins (see **Figure 1.6** in Chapter 1) (Berthet-Colominas et al., 1999; Gamble et

al., 1997; Jin et al., 1999b). So, it is possible that amino acid changes within the Gag NI9 epitope, especially in the conserved residues, such as amino acid A (P2), N (P3), D (P5) and C (P6), affect capsid dimerization and viral assembly leading to an impaired viral replication (Berthet-Colominas et al., 1999; Gamble et al., 1997; von Schwedler et al., 2003). Other studies have additionally shown that the Pol 243 TI8 epitope resides within the Reverse Transcriptase (RT 128-135) gene and lies within the finger subdomain (see **Figure 1.7** in Chapter 1) (Jacobo-Molina et al., 1993; Kohlstaedt et al., 1992). The mutagenesis study on this subdomain revealed that the mutation of conserved residues in this epitope can impair the activity of the virus DNA polymerase or RNase H (Boyer et al., 1994; Ceccherini-Silberstein et al., 2005), and thus can potentially undermine the replication of the virus. In contrast to the Gag NI9 and Pol TI8 epitopes however, the epitope Pol LI9 (In 28-36), sits within the N-terminal domain of Integrase and is not involved in enzymatic function of Integrase, and may thus pose fewer constraints on viral fitness because most residues in this epitope have naturally a high variability (see **Figure 1.8** in Chapter 1). For example position 4 in this epitope has very high entropy of 0.6. This is probably one of the potential reasons to explain why Pol LI9 mutates most rapidly and the substitution V4I is most dominant in this assay.

The studies published recently also showed that early escapes occur in epitopes with the highest nucleotide variability as estimated from the HIV sequence database, suggesting that first escapes occur in epitopes with minimal fitness cost (Ferrari et al., 2011a; Liu et al., 2013; Liu et al., 2007). Epitope entropy, reflecting

the capacity of the epitope to tolerate mutational change and defined as the diversity of epitope sequences at the population level, was also correlated with rate of emergence of escape mutants (Ferrari et al., 2011a; Liu et al., 2013). Higher Shannon entropy indicates higher variability and lower fitness cost. Then the epitope entropy was calculated for these three HLA-B51 restricted epitopes using *Shannon Entropy-One* from www.hiv.lanl.gov. (Shannon, 1997). The average entropy scores for epitope Pol LI9, Pol TI8 and Gag NI9 are 0.158, 0.152 and 0.107 respectively, indicating that the epitope Gag NI9 is the one likely to develop escape mutations most slowly.

Although our results confirmed our previous finding that sequential epitope mutations observed in a patient cohort, were associated with disease progression and viral control. However, the exact mutations that occur in our *in vitro* assay were not all the same as those in the cohort studied, which is not surprising. *Ex vivo*, the selective pressure exerted by CTLs may be influenced by numerous determinants, including frequency, T-cell receptor properties, and functional state. However, in our *in vitro* assay, only one CTL clone was used and the escape mutations selected by this CTL clone could potentially be recognised by other clones or elicit *de novo* T-cell responses, which is the case *ex vivo*. Lack of TCR diversity of CTLs in this *in vitro* system may lead to this observed bias. Moreover, the founder virus (or infecting virus) used in our *in vitro* assay could be another factor that determines the selection of virus mutations. In this *in vitro* assay, we infected the target cells with lab adapted IIIB virus rather than the autologous virus

from the patients. It is possible that unknown genetic variants, compensatory or otherwise, native to the IIIB genome may have some impact on the mutations selected by CTLs.

In conclusion, the data from our *ex vivo* study and *in vitro* Virus Evolution Assays demonstrate that three dominant HLA-B51 restricted CTL responses have driven the sequential escape mutations within the epitopes, leading to loss of viral control. Among them, Pol LI9 specific CTLs elicit highly dominant early responses in HLA-B51 positive patients during the course of HIV infection, which drives escape mutations, leading to the loss of early viral control. In contrast, the Gag NI9 epitope showed most resistance to the escape mutation, which could promote long-term protection against HIV infection. This suggests that future vaccine design may need to consider the entropy of the epitopes and induce the CTL responses against such epitopes as HLA-B51 Gag NI9, whereas avoid stimulating the responses against the epitopes like HLA-B51 Pol LI9.

Chapter 5: The role of antigen sensitivity, TCR repertoire, founder virus on HIV evolution and HIV control

5.1 Introduction

It is well established that the cytotoxic T-lymphocyte response plays a critical role in the control of HIV-1 infection both in acute (Borrow et al., 1994; Koup et al., 1994) and chronic phases (Harrer et al., 1996; Ogg et al., 1998; Rinaldo et al., 1995). In the absence of antiviral therapy, most HIV-1 infected individuals fail to contain and clear the infection despite apparently strong CTL responses (Carmichael et al., 1993; Koup et al., 1994). Goulder et al. (1997) and Borrow et al. (1997) have shown that the immune pressure exerted by CTLs has driven intra-host HIV evolution, which allows HIV carrying those mutations to escape the recognition of CTLs , leading to loss of viral control. CTL escape by HIV could be due to reduced antigen processing and presentation (Allen et al., 2004; Draenert et al., 2004a; Milicic et al., 2005; Ranasinghe et al., 2011); impairment of epitope binding to the presenting HLA molecule (Couillin et al., 1994); abrogation of the TCR-peptide interaction (McAdam et al., 1995); and antagonism (Klenerman et al., 1994; Meier et al., 1995).

The emergence of HIV-1 escape mutations depends upon the net balance of selecting immune pressure and fitness costs of potential escape mutations (Liu et al., 2007) . The immune pressure exerted by CTLs *in vivo* could be modulated by several factors, such as CTL frequency, epitope specificity, T cell receptor (TCR)

properties and functional state of the CTLs. Among them, TCR repertoire plays an important role in shaping virus evolution. There are contradictory reports on the effect of the diversity of TCR repertoire on viral escape mutations (Meyer-Olson et al., 2004; Price et al., 2004; Yu et al., 2007). It has also been reported that CTLs with a TCR repertoire that cross recognises viral epitope variants are rarely associated with mutational escape (Bhattacharya et al., 2007; Liu et al., 2007). The functional avidity of CTLs also have an impact on selecting escape mutations, however, there is no direct correlation between high functional avidity of CTL and the rapid emergence of escape mutants. While O'Connor et al. (2002) showed that CTLs with high functional avidity can drive escape mutations more rapidly, in other studies, the recognition of epitopes by high-avidity CTLs was persistently observed throughout the infection (Cao et al., 2003; Draenert et al., 2004b; Liu et al., 2007). Nonetheless, a recently reported mathematical model on acute and chronic HIV infection has shown that the strongest CTL responses are always associated with early occurring of escape mutations, when other factors, such as the fitness cost of the mutation, the replication rate of the virus and the quality of CTL responses, are equal (Ganusov et al., 2011). The reduced magnitude and increased breadth of HIV-specific CTL responses, together with increased fitness cost of the mutations, mainly contribute to a slower escape in the chronic HIV-1 infection. These data imply that an effective CTL response should control the virus replication, however, avoid escape mutations or select for the mutations with a fitness cost.

Apart from CTL pressure, the infecting virus (or founder virus) with different sequences has shown different effects on virus control and virus evolution. Some studies found that the set-point viral load could be largely determined by the virus genotype (Alizon et al., 2010; Mani et al., 2002). Another example is that many elite controllers have been infected with or carry an attenuated virus (Learmont et al., 1999; Miura et al., 2009; Miura et al., 2010). Bhattacharya et al. (2007) have shown that some HLA mediated escape mutations analysed by old methods are actually associated with the subtype of the infecting virus, meaning that sequence of the infecting virus also plays a role in shaping virus evolution.

Our *ex vivo* study (Chapter 3) demonstrated different HLA-B51 restricted CTL responses and different virus adaption, compared to a Japanese haemophiliac cohort, another HLA-B51 prevalent cohort. In the Japanese cohort, the frequency of Pol TI8 specific $CD8^+$ T cells was negatively correlated with the viral load, whereas the T cells specific to other epitopes (Pol LI9, Gag NI9 and Rev71) were not associated with the viral load (Kawashima et al., 2010). By contrast, the SM cohort showed less dependence on the T-cell response to HLA-B51 Pol TI8 epitope. As shown in Chapter 3, the patients who have T-cell responses to any of these three un-mutated HLA-B51 epitopes have a better clinical outcome. Meanwhile, comparing the viral sequences of the epitope Pol TI8 revealed different consensus sequence as well as escape mutations in these two cohorts. As demonstrated in **Table 5.1**, in the SM cohort, the consensus sequence in this epitope is TV8, Valine at position 8, and a high degree of selection for V8L was

observed, whilst in the Japanese cohort, the infecting virus has epitope sequence Pol TI8 and the dominant mutation was I8T. These findings suggest that the difference in the infecting virus, patterns of escape mutations and potentially different TCR repertoire between these two cohorts may have modulated the mechanism of protection.

Table 5.1 The consensus and dominant epitope variants of HLA-B51 Pol TI8 in the SM cohort and Japanese hemophiliac cohort. The frequency of the dominant mutation is the percentage of the dominant mutation in all the mutations in this epitope driven by HLA-B51. N represents the number of the HLA-B51-positive patients. The data of Japanese cohort are derived from (Kawashima et al., 2010)

Cohort	Consensus	Frequency of Dominant mutations
SM Cohort (N=22)	Pol TV8 (TAFTIPSV)	V8L (76%)
Japanese Cohort (N=24)	Pol TI8 (TAFTIPSI)	I8T (75%)

In this Chapter, we set up the second set of Virus Evolution Assays to evaluate the impact of "founder virus" on virus evolution driven by immune pressure at different level of strength. We selected two Pol TI8 specific CTL clones with different antigen sensitivity and two different source viruses: one virus carrying Pol TV8 epitope (SM-8V virus) and the other carrying Pol TI8 (JP-8I virus). Each virus has been through six round of passages under the selection of either clone as described in Chapter 4. The corresponding epitopes were then sequenced to monitor the virus evolution. In addition, to evaluate the impact of those mutations

on virus control, the suppression of virus replication by each CTL clone was calculated in each round of passage. We found that, although the antigen sensitivity of CTLs is important in controlling virus replication, the mutations driven by CTLs is largely determined by TCR usage of CTLs and the sequence of source virus.

5.2 Results

5.2.1 Selection of HLA-B51 Pol TI8 specific CTL clones with different antigen sensitivity and T cell receptor usage

The two HLA-B51 Pol TI8 specific CTL clones, Clone C5 and Clone C11, use distinct T cell receptors in this set of Virus Evolution Assays. As shown in **Table 5.2,** by TCR Vβ antibody staining, Clone C11 is positive whereas Clone C5 was shown to be Vβ 7.2 negative. Furthermore, Clones C5 and Clone C11 showed different antigen sensitivity to peptide Pol TI8 and Pol TV8. As shown in Figure 5.1, Clone C11 has much lower EC50 ($EC50_{TI8}$ =0.032; $EC50_{TV8}$ =0.036) when compared with clone C5 ($EC50_{TI8}$ =0.919; $EC50_{TV8}$ =3.937). Therefore, Clone C11 has better anti-viral efficacy than Clone C5, as was shown in Chapter 3. *In addition,* Clone C5 and Clone C11 exhibit differential cross recognition to peptide Pol TI8 and Pol TV8. As Figure 5.1 shows, Clone C5 displayed some cross recognition to peptide Pol TV8 with much lower antigen sensitivity ($EC50_{TV8}$ =3.937), compared to peptide Pol TI8 ($EC50_{TI8}$ =0.919). By contrast, Clone C11 can cross react to peptide Pol TV8 well, showing the similar recognition to both peptides. Therefore, these data suggested that Clone C5 and C11 could potentially present the CTLs with different TCR usage and different functional avidity *ex vivo.*

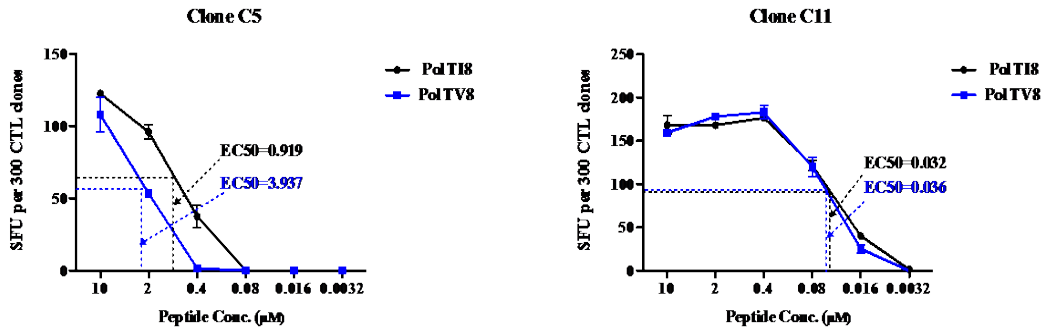

Figure 5.1 Antigen sensitivity of HLA-B51 Pol TI8 specific Clone C5 and Clone C11. IFN-γ ELISPOT was used to assess the antigen sensitivity of the CTL clones. The solid black lines and blue lines represent the recognition of peptide Pol TI8 and Pol TV8 respectively. The dashed black lines and blue lines represent the EC50 of each clone to peptide Pol TI8 and Pol TV8 respectively.

5.2.2 Setting up Virus Evolution Assay

The viruses used for this part of the study are JP-8I virus (NL432-Pol TI8) and SM-8V virus (NL432-Pol TV8). Both viruses are clone of NL432; JP-8I virus is a clone of HIV-1 virus NL432 with the index epitope Pol TI8, Isoleucine (I) at position 8, whereas SM-8V virus is the NL432 virus containing SM consensus epitope Pol TV8. Both virus constructs are the gifts from Professor. Masafumi Takiguchi (Kumamoto University, Japan).

In this part of the study, four groups of Virus Evolution Assay were set up, shown in **Table 5.2.** Both viruses, JP-8I virus (NL432-Pol TI8) and SM-8V virus (NL432-Pol TV8) underwent 6 round of passages under the selection of either Clone C5 or Clone C11 as described in Chapter 4. Epitope Pol TI8 or TV8 was then sequenced

by TOPO TA cloning. Apart from the storage of culture supernatant and cell pellet at day 7, 1 ml of supernatant was also taken from the culture at day 3 and day 5 and titred by p24 ELISA along with the samples at day 7 for these four groups. For each group, a control was also set up in parallel by co-culture of virus infected MT2 cells with an HLA mismatched CTL clone G10.

Table 5.2 Founder viruses, CTL clones and the groups of Virus Evolution Assay. Vβ antibody staining assessed TCR Vβ usage. The Vβ antibodies used for staining are listed in Chapter 2, Materials and Methods.

Group	Virus	CTL clone	TCR Vβ usage
1	Japanese (JP-8I virus*: NL432-Pol TI8*)	Clone C5	Vβ 7.2-
2	Japanese (JP-8I virus*: NL432-Pol TI8*)	Clone C11	Vβ 7.2+
3	Chinese (SM-8V virus*: NL432-Pol TV8*)	Clone C5	Vβ 7.2-
4	Chinese (SM-8V virus*: NL432-Pol TV8*)	Clone C11	Vβ 7.2+

5.2.3 The level of immune pressure by CTL clones on JP-8I and SM-8V viruses at the 1st round of virus passage

Since Clone C5 and C11 have different antigen sensitivity, the E:T ratio has been optimised to mount similar immune pressure on JP-8I and SM-8V viruses. Clone C5 was cultured with either JP-8I virus or SM-8V virus infected MT2 cells at an E:T ratio of 1:20 and 1:8, respectively, while Clone C11 was co-cultured at E:T ratio of 1:64 for both viruses. As shown in **Figure 5.2,** at the 1st round virus passage, both Clone C5 and Clone C11 showed similar suppression against JP-8I virus and SM-8V virus, although Clone C11 suppressed higher than 80% of

replication of both viruses whereas Clone C5 shows only around 50% of suppression.

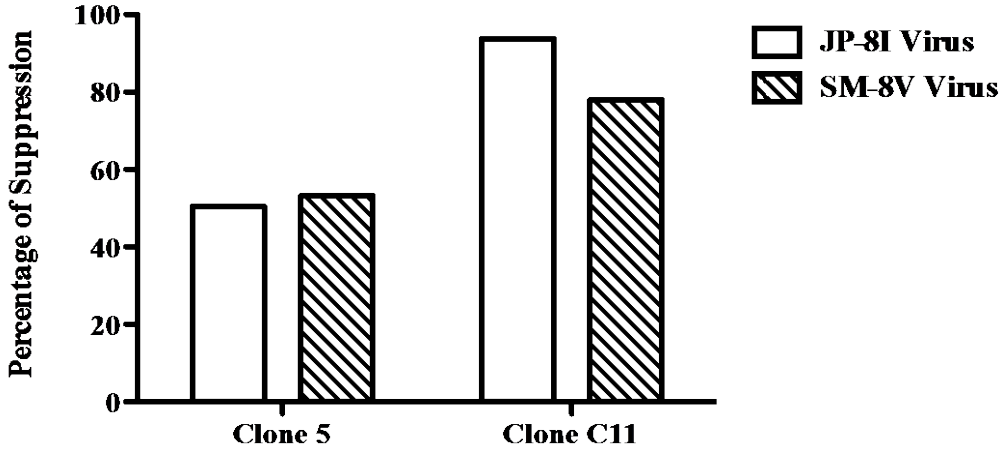

Figure 5.2 The suppression of Clone C5 and Clone C11 against WT NL432 virus and mutant NL432 Pol TI8-I8V at 1st virus passage. The percentage of suppression was calculated by the deduction of the p24 antigen in the culture at day 7 with selecting CTLs compared to the control (in the absence of selecting CTLs).

5.2.4 Decreased suppression of JP-8I virus and better control of SM-8V virus during late virus passages

Figure 5.3 shows the suppression of JP-8I virus by both CTL clones decreased during serial selective virus passages. For instance, during the 2nd round virus passage, Clone C5 and Clone C11 showed 50% and 90% suppression against JP-8I virus, respectively, whereas the suppression of this virus by both clones declined to below 10% after six rounds of selective virus passage. However, suppression of

SM-8V virus by both CTL clones remained stable across all the virus passages. When comparing the suppression against JP-8I virus and SM-8V virus, both clones showed similar suppression against both viruses during the early passages, however, the suppression against SM-8V virus was much stronger during the late virus passages (after the 4th round). For example, during the 2nd round of virus passage, Clone C11 showed better control of JP-8I virus, with 90% of suppression against JP-8I virus and 10% higher suppression than SM-8V virus. However, after 3 rounds of selective virus passage, Clone C11 suppressed SM-8V virus better than JP-8I virus, by 20% during the 3rd and 4th virus passages. This difference enlarged in following rounds of virus passage. For instance, at the end of round 6, Clone C11 was still capable of suppressing 80% of SM-8V virus replication; on the contrary, it had totally lost the control of JP-8I virus, with lower than 10% suppression. However, after four rounds of selective virus passage, Clone C5 still showed 20% higher level of suppression against SM-8V virus, compared to JP-8I virus.

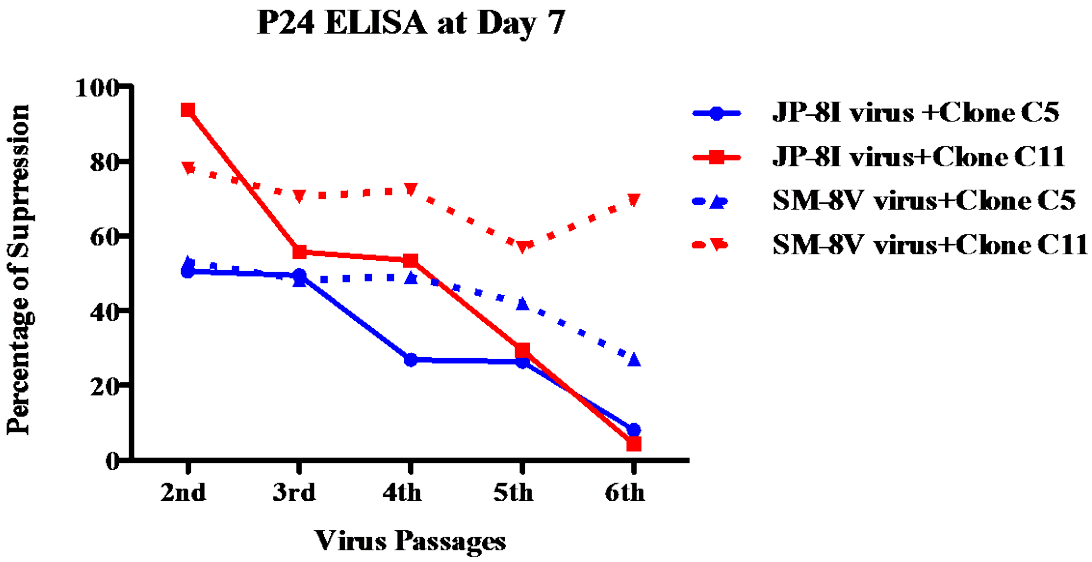

Figure 5.3 The suppression of virus replication by both antigen-specific CTL clones (Clone C5 and Clone C11) after each round of selective virus passage. The percentage of suppression was calculated by the deduction of the p24 antigen in the culture at Day 7 with CTLs present compared to the control (in the absence of selecting CTLs). The solid lines represent the suppression against JP-8I virus and the dashes show the suppression against SM-8V virus.

5.2.5 Dominant escape mutation I8T in Pol TI8 epitope of JP-8I virus driven by Clone C5 occurred at the 4th passage

As shown in Table 5.3, the first escape mutation I8T was observed at 4th passage with the frequency of 20% and accumulated vigorously at the later passages. At the final passage, the mutation frequency increased to 80%, which contributed to the vigorous virus production even after co-culture with HIV specific CTL clone for three days **(Figure 5.4)**. By contrast, the CTL clone can constrain the virus replication much better during the 3rd round virus passage when only the WT

epitope was present. Interestingly, the mutation I8V, the consensus epitope sequence of the SM cohort, was also observed but with very low frequency of 10% after 6 weeks of selection. This implies that the mutation I8V is driven by CTLs and it is more difficult to develop than other natural mutations, such as 8T/R.

Table 5.3 Epitope Pol TI8 sequences after seven-day co-culture of Clone C5 and JP-8I virus infected MT2 cell line. The sequences are aligned with the infecting virus (NL432), with the TI8 epitope in bold font.

Viral Passage (Clone C5)	**Epitope Sequence** (Founder Virus: JP-8I Pol TI8)			**Clonal Frequency**
	DFRKY	TAFTIPSI	NNETP	
1st Round	-----	--------	-----	(10/10)
2nd Round	-----	--------	-----	(10/10)
3rd Round	-----	--------	-----	(10/10)
4th Round	-----	--------	-----	**(8/10)**
	-----	-------T	-----	(2/10)
5th Round	-----	--------	-----	**(6/10)**
	-----	-------T	-----	(4/10)
6th Round	-----	--------	-----	**(1/10)**
	-----	-------T	-----	(8/10)
	-----	-------V	-----	(1/10)

As mentioned previously, the kinetics of virus replication was monitored at three time points post infection: Day 3, Day 5 and Day 7. When comparing the virus replication from each passage, it was found that before Day 5, Clone C5 showed strong inhibition, only in the presence of WT (e.g., the 3rd virus passage; **Figure**

5.4 a), whereas virus replicated vigorously when virus mutated (e.g., the 5th round virus passage; **Figure 5.4 b).** This different kinetics of replication indicates that the substitution from I to T at position 8 in the epitope Pol TI8 leads to the immune escape. Meanwhile, when comparing the kinetics of replication of the infecting virus without CTL selection, the infecting virus in the 3rd round without any detected mutations has similar replication capacity to the virus in the 5th round, by which time 40% of the virus was carrying the I8T mutation (**Figure 5.4**). These data suggested that the I8T mutation does not have any effect on virus infectivity and replication.

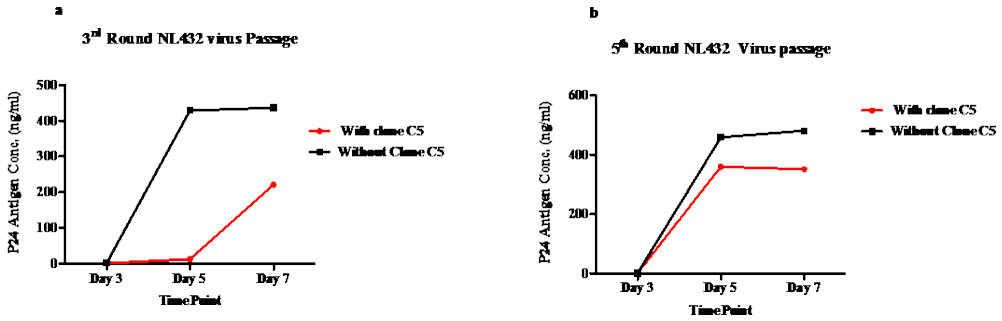

Figure 5.4 JP-8I virus replication in MT2 cell line under the selection of HLA-B51 Pol TI8 specific CTL Clone 5. a), b) show the replication of JP-8V virus during the 3rd and 4th rounds of virus passage under the selection of Clone 5, in red lines. The black lines represent the virus replication without selecting CTLs.

5.2.6 Escape mutations occurring in Pol TI8 epitope of JP-8I virus at the 3rd passage under the selection of Clone C11.

Under the selection pressure exerted by CTL Clone C11 on JP-8I virus, the first mutation (S7N) occurred at the 3rd passage (Table 5.4). This mutation was enriched during the later virus passages, starting at 30% in the 4th virus passage to 70% in the 5th and 6th virus passages. The second mutation (i.e., I8R) occurred at the 5th passage. This mutation has been observed in LTNPs (Moore et al., 2002) or during acute infection (Wang et al., 2009) and has also been proved to be an escape mutation (Sipsas et al., 1997).

The virus replication kinetics in each round of passage is shown in **Figure 5.5.** Clone C11 displayed very strong suppression against JP-8I virus in 2nd round of passage, with 90% of inhibition at an E:T ratio of 1:64. With the emergence of mutations, the suppression of virus replication declined. In the 6th round of virus passage six, when no more WT virus clones remained, there was little suppression despite the presence of CTLs.

Table 5.4 Epitope Pol TI8 sequences after seven-day co-culture of Pol TI8 clone C11 and JP-8I virus infected MT2 cell line. The sequences are aligned with the infecting virus (NL432), with the TI8 epitope in bold font.

Viral Passage (Clone C11)	**Epitope Sequence** (Founder Virus: JP-8I Pol TI8)			**Clonal Frequency**
	DFRKY	TAFTIPSI	NNETP	
1st Round	-----	--------	-----	**(10/10)**
2nd Round	-----	--------	-----	**(10/10)**
3rd Round	-----	--------	-----	**(9/10)**
	-----	------N-	-----	(1/10)
4th Round	-----	--------	-----	**(6/10)**
	-----	------N-	-----	(4/10)
5th Round	-----	--------	-----	**(2/10)**
	-----	-------R	-----	(1/10)
	-----	------N-	-----	(7/10)
6th Round	-----	--------	-----	**(0/10)**
	-----	-------R	-----	(3/10)
	-----	------N-	-----	(7/10)

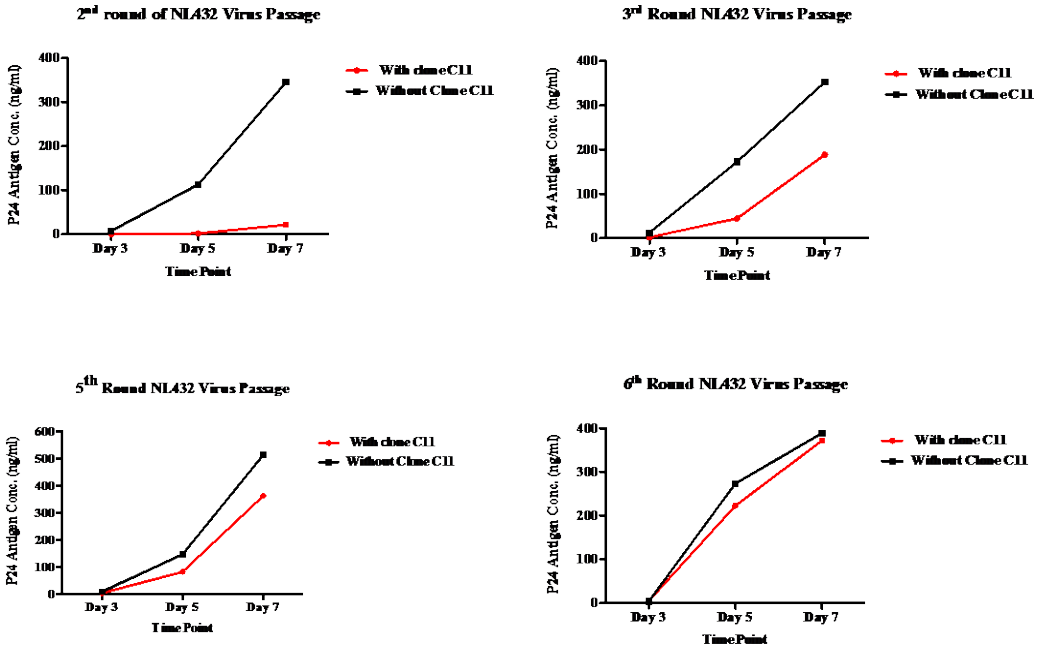

Figure 5.5 Replication of JP-8I virus in MT2 cell line under the selection of HLA-B51 Pol TI8 specific CTL Clone 11. The red lines represent JP-8I virus replication under the selection of Clone 11 at the 2nd, 3rd, 5th and 6th rounds of virus passage. The black line represented the virus replication of the controls (virus infected MT2 cell line cultured without selecting CTLs).

5.2.7 Significant correlation between the suppression of JP-8I virus replication and the proportion of remained WT viral clones under the selection of CTL clones.

As shown in the **Figure 5.6 a**, there is a significant correlation between the suppression of Clone C5 and the percentage of viral clones containing WT Pol TI8 epitope (*p* value =0.0166), which remained after each selective viral passage. Virus replication was inhibited more efficiently when higher percentage of viral clones contained the WT sequence. The same correlation was also observed when JP-8I

virus passaged under the selection of Clone C11, shown in **Figure 5.6 b** (*p* value =0.0191). This correlation indicated that the reduced inhibition of virus replication by CTLs is due to the emergence of escape mutations in the Pol TI8 epitope. This also suggested that Pol TI8 specific CTL Clones could drive epitope escape mutations, resulting in loss of viral control.

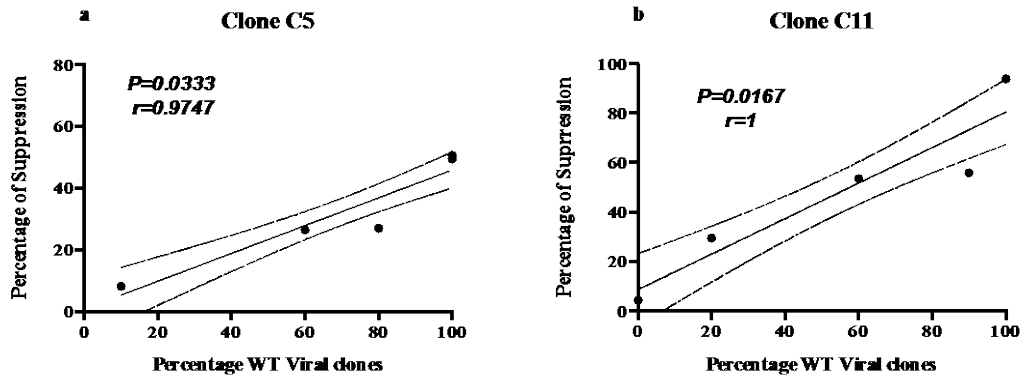

Figure 5.6 Correlation of the percentage of remained WT JP-8I viral clones with the suppression of the CTL Clones. The correlation between the proportion of WT JP-8I viral clones under the selection of Pol TI8 Clone C5 or Clone C11 and its suppression of virus replication from each round of passage. The nonparametric Spearman correlation was analysed by Prism and *p* values are two-tailed.

5.2.8 Slightly faster development of escape mutations in JP-8I virus under the selection of Clone C11

As Clone C11 has higher antigen sensitivity (EC50=0.032) than Clone C5 (EC50=0.919), it is a better 8I virus suppressor. Additionally, Clone C11 was shown to drive escape mutation at slightly faster rate than C5 (**Figure 5.7**). These

data suggested that the antigen sensitivity of the CTLs is important for virus suppression; however, it is not the major factor determining mutation rate.

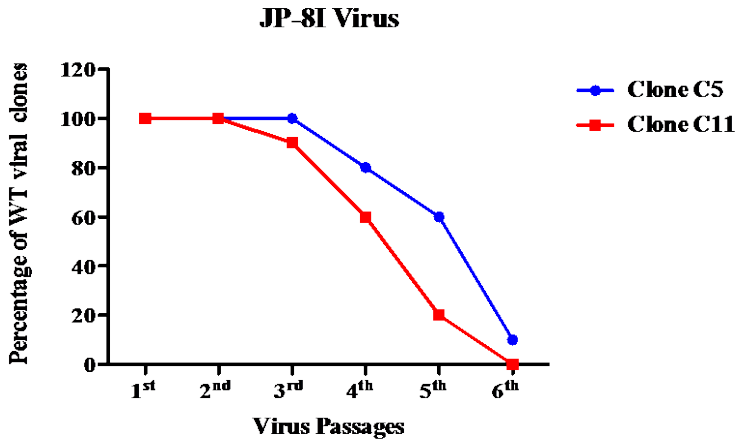

Figure 5.7 The reduction of the percentage of remaining WT viral clones under the selection of CTLs at each viral passage. The blue and red lines represent the proportion of pro-viral clones with WT Pol TI8 epitope under the selection of Clone C5 and Clone C11, respectively.

5.2.9 No mutation observed within the epitope Pol TV8 of SM-8V virus under the selection of Clone C5.

As demonstrated in **Table 5.5**, there is no mutation occurring in epitope TV8, after six rounds of selection, by Clone C5. However, there are some rare substitutions observed at the downstream of flanking region. Further investigation is required to verify whether these mutations have impact on antigen processing and presentation.

Table 5.5 Epitope Pol TI8 sequences after seven-day co-culture of Pol TI8 Clone C5 and SM-8V virus (NL432 with epitope Pol TV8) infected MT2 cell line. The sequences are aligned with the infecting virus (NL432-Pol TV8), with the TV8 epitope in bold font.

Viral Passage (Clone C5)	**Epitope Sequence** (Founder Virus: SM-8V Pol TV8)			**Clonal Frequency**
	DFRKY	TAFTIPSV	NNETP	
1st Round	-----	--------	-----	**(10/10)**
2nd Round	-----	--------	-----	**(8/10)**
	-----	--------	-S---	(1/10)
	-----	--------	--K--	(1/10)
3rd Round	-----	--------	-----	**(10/10)**
4th Round	-----	--------	-----	**(10/10)**
5th Round	-----	--------	-----	**(10/10)**
6th Round	-----	--------	-----	**(9/10)**
	-----	--------	----T	(1/10)

5.2.10 Occurrence of a rare mutation V8A within epitope Pol TV8 of SM-8V virus after four rounds of virus passage under the selection of Clone C11

As shown in **Table 5.6**, SM-8V developed V8A mutation in epitope Pol TV8 with a very low frequency of 10% after four rounds of selective passaging. However, in 6th round, number of clones with the same mutation increased by three times (i.e., mutation frequency: 30%). However, Clone C11 showed cross reactivity to this epitope variant with lower avidity than epitope TV8 (**Figure 5.8**), and the suppression of virus replication wasn't affected by the emergence of this mutation

(**Figure 5.3**). This indicates that virus carrying this mutation might partially escape the recognition of this CTL clone. On the other hand, the substitution of V8A might have better replication capability than WT virus because Valine at position 8 in Pol TI8 epitope has been reported to have a fitness cost (Kawashima et al., 2009).

Table 5.6 Epitope Pol TV8 sequences from 7-day co-culture of Pol TI8 clone C11 and SM-8V virus (NL432-Pol TV8) infected MT2 cell line. The sequences are aligned with the infecting virus (NL432-Pol TV8), with the TV8 epitope in bold font.

Viral Passage (Clone C11)	**Epitope Sequence** (Founder Virus: SM-8V Pol TV8)			**Clonal Frequency**
	DFRKY	TAFTIPSV	NNETP	
1st Round	-----	--------	-----	**(10/10)**
2nd Round	-----	--------	-----	**(10/10)**
3rd Round	-----	--------	-----	**(10/10)**
4th Round	-----	--------	-----	**(9/10)**
	-----	-------A	-----	(1/10)
5th Round	-----	--------	-----	**(10/10)**
6th Round	-----	--------	-----	**(6/10)**
	-----	-------A	-----	(3/10)
	-----	------G-	-----	(1/10)

Then IFN-γ ELISPOT was used to test the recognition of all peptide variants selected by Clone C11 in this assay. As shown in **Figure 5.8**, Clone C11 showed strong cross-reactivity to peptide variants Pol TI8-I8V and Pol TI8-I8A, whereas the substitution S7N abolished the recognition.

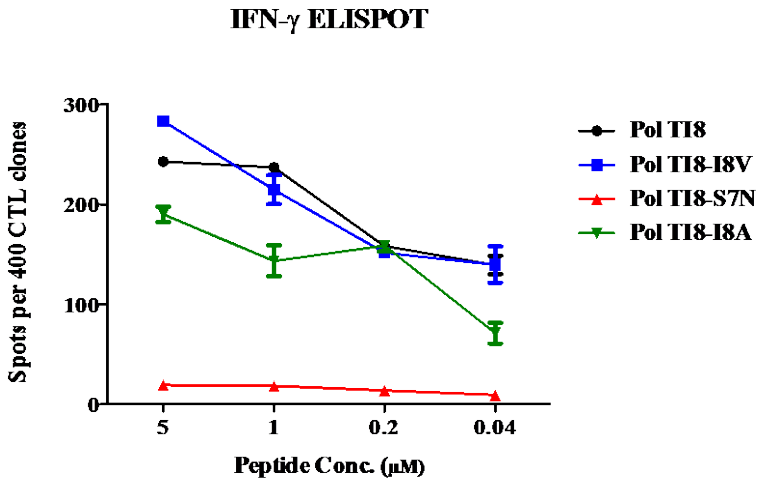

Figure 5.8 Recognition of peptide variants by HLA-B51 Pol TI8 specific CTL Clone C11. The epitope variants tested are the mutations selected by HLA-B51 Pol TI8 specific CTL Clone C11 in Virus Evolution Assay**.**

5.2.11 Impaired antigen processing caused by Valine at position 8 of epitope Pol TI8

Although Clone C11 has similar EC50 values for peptides Pol TI8 (EC50=0.032) and TV8 (EC50=0.036) (**Figure 5.1**), it displayed less capability to inhibit the replication of SM-8V virus. This suggests the possibility of other pathways through which SM-8V virus weakened CTL recognition. In order to reduce the bias caused by our selection of CTL clones, we assessed the antigen sensitivity of another Pol TI8 specific CTL clone, Clone G4, along with Clone C5 and Clone C11. Their antiviral efficacy against both 8I and 8V virus was also examined by Live Virus ELISPOT.

In **Figure 5.9**, the cross recognition of the peptide Pol TI8 and peptide Pol TV8 by the three CTL clones has been demonstrated. Similar to Clone C11, Clone G4 recognised both peptides at similar level. Furthermore, both clones showed an increase in IFN- γ by two-fold and 1.6-fold in presence of 8I virus and 8V virus, respectively (**Figure 5.10**). As a control, Clone H10 specific to another HLA-B51 Pol epitope, however, released same amount of IFN-γ against infection of both viruses.

In this study, all three Pol TI8 specific CTL clones showed less anti-viral efficacy against 8V virus. Lower antigen sensitivity (or lower avidity) against peptide Pol TV8 has contributed to less suppression against 8V virus by Clone C5. However, in Clone C11 and Clone G4, this observation could be due to impaired epitope processing and/or presentation in virus-infected cells. To verify this, we set up *in vitro* immunoproteasomal digestion assays to evaluate the impact of Valine at position 8 on epitope processing.

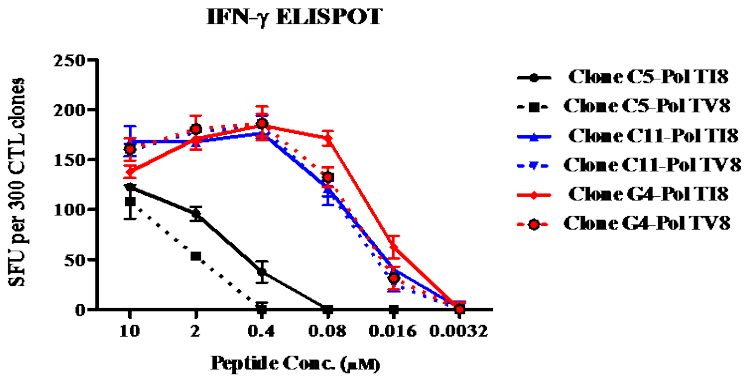

Figure 5.9 Cross recognition of peptide Pol TI8 and peptide Pol TV8 by specific CTL clones. Pol TI8 specific CTL clones were co-cultured with peptide pulsed target cells and IFN-γ release was measured by ELISPOT. The solid lines represent the recognition of peptide Pol TI8 whereas the dash lines represent the recognition of peptide Pol TV. IFN- γ production by different clones is shown in different colour.

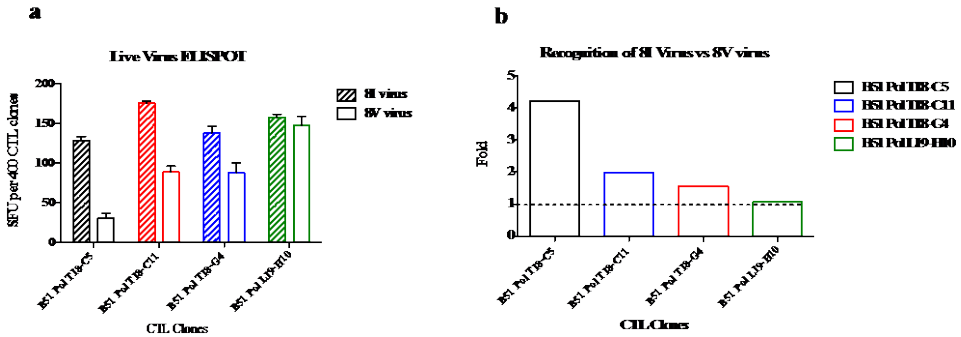

Figure 5.10 Recognition of 8I virus and 8V virus by HLA-B51 Pol TI8 specific CTL clones. The recognition of 8I virus (with Pol TI8 epitope) and 8V virus (with Pol TV8 epitope) by HLA-B51 Pol TI8 specific CTL clones was tested by IFN-γ Live Virus ELISPOT assay. **a)** IFN-γ production of CTL clones when co-cultured with virus infected targets. **b)** The comparison of IFN-γ production of each CTL clone when co-cultured with target cells infected with 8I virus and 8V virus. The recognition of both viruses by different clones is shown in different colour. Clone H10 is epitope Pol LI9 specific and was used as control.

Two 25-mer oligopeptides containing epitope Pol TI8 or Pol TV8 were synthesised with a purity of 99.9% from Advanced ChemTech. Peptide-8I has the sequence of LDKDFRKYTAFTIPSINNETPGIRTQYNVLP and Peptide-8V has the sequence of LDKDFRKYTAFTIPSVNNETPGIRTQYNVLP. Both peptides have same N-terminal and C-terminal extensions and only differ at position 8 of the epitope. The peptides were first incubated with purified immuno-20s-proteasome (i20S) at 37^0C for 60 minutes and the immunoproteasomal digests then terminated by adding Trifluoroacetic acid (TFA). Thereafter, the peptide fragments generated from immunoproteasomal digestions were identified using tandem mass spectrometry in collaboration with the Central Proteomics Facility in Oxford. Since immunoproteasomes and proteasomes rarely generate the exact epitope (8-11mer peptide), but instead produce longer peptides often with correct C-terminus cleavage, an epitope pre-cursor can be a peptide fragment containing the intact epitope with extended N (amino)-terminus and C (carboxyl)-terminus. A correct epitope pre-cursor can be only defined as a peptide only with a correct C-terminus and an extended N-terminus. The more the correct pre-cursors are produced, the more efficient the antigen processing is.

As can be seen in **Table 5.7**, with 4 hits to the exact C-terminus, Peptide-8I containing epitope Pol TI8 has more epitope precursors than Peptide-8V. The C- and N-terminal cleavage analysis (**Table 5.8**) showed that, although the exact 8mer was not observed in any of the peptides, the exact C cleavage was also found more in Peptide 8I with 6 hits, compared to 2 hits of the exact C-terminus in Peptide-8V.

These preliminary data suggested that presence of Valine at position 8 of the epitope Pol TV8 may have affected antigen digestion, which leads to less epitope presentation, and therefore reduced recognition of the virus by CTLs. As a consequence, CTLs would mount less immune pressure on 8V virus than 8I virus. This probably could explain the reason for the less frequent and slower occurrence of mutations under the selection of CTLs against cells infected with SM-8V virus.

Table 5.7 Peptide fragments identified by Tandem Mass Spectrometry after 60 minute-immunoproteasomal digestions. Exact C is the peptide fragments with exact C-terminus of epitope

Peptide	Epitope	Un-digested	Total hits*	Hits (exact C)
Peptide-8I	TAFTIPSI	YES	**21**	**4**
Peptide-8V	TAFTIPSV	YES	20	2

. * - The epitope present in digest <=25mer

Table 5.8 The analysis of C- and N-terminal cleavage by Tandem Mass Spectrometry after 60-minutes immunoproteasomal digestions.

Peptide	Exact C plus N extensions						C +1 plus N extensions						C +2 plus N extensions						C +3 plus N extensions					
	0	1+	4+	8+	13+	14+	0	1+	4+	8+	13+	14+	0	1+	4+	8+	13+	14+	0	1+	4+	8+	13+	14+
Peptide-8I				6						1						1						2		
Peptide-8V				2						1												1		

5.3 Discussion

It is well established that the CTL-mediated selection is the major force driving escape mutations (Allen et al., 2005; Liu et al., 2006). The emergence of HIV-1 escape mutations depend upon the net balance of selective pressure and the fitness costs of potential escape mutations. CTL frequency, epitope specificity, T cell receptor properties and functional state of the CTLs, affect the selective pressure exerted by CTLs on the virus. In turn, the generation of escape mutations in a virus could be constrained due to various factors. First, some mutations could have a fitness cost to the virus, leading to direct loss of replicative capacity or interference with functions important for viral persistence. Secondly, other factors, such as the pattern of nucleotide substitution or the number of nucleotide changes, may also play a role. It has been reported that the majority of mutations, induced by HIV-1 RT during reverse transcription, are transitions (Bebenek et al., 1989; Boyer et al., 2007). The most frequent transition is G→A mutation known as G to A hyper mutation. This mutation occurs more frequently than those through other nucleotide substitutions, such as transversions. Moreover, the mutations, which need multiple nucleotide changes, appeared more slowly than the ones with a single nucleotide change.

In this chapter, four Virus Evolution Assays were conducted to evaluate the impact of antigen sensitivity, the TCR repertoire of HLA-B51 CTLs, and the epitope sequence of founder virus (or infecting virus) on HIV-1 virus evolution and control.

In order to examine the effect of antigen sensitivity of CTLs on virus evolution, two CTL clones with different antigen sensitivity were chosen. Clone C11 with higher antigen sensitivity to peptides Pol TI8 and Pol TV8 ($EC50_{TI8}$ =0.032, $EC50_{TV8}$ =0.036) showed 50% higher viral suppression than Clone C5 ($EC50_{TI8}$ =0.919, $EC50_{TV8}$ =3.937), even at a lower E:T ratio of 1:64. This highlights the importance of the antigen sensitivity in anti-viral efficacy of CTLs. However, when comparing the rate of the mutations selected by these two different CTL clones, Clone C11 has driven escape mutations in JP-8I virus just slightly faster than Clone C5. Although Clone C11 has 30-fold higher EC50 (**Figure 5.1**) and shows 45% higher level of viral suppression than Clone C5 (**Figure 5.2**), the difference in the rate of escape mutations driven by the two CTL clones is insignificant. Our observations suggest that the antigen sensitivity of CTLs is an important factor for virus suppression but not for the rate of escape mutations. This is contrary to some literature, which showed CTLs with higher functional avidity drive escape mutations faster (Conrad et al., 2011; Iglesias et al., 2011).

In addition, the *in vitro* Virus Evolution Assay demonstrated the effect of the TCR repertoire on the virus evolution: clones with different TCR selected different escape mutations. Clone C5 selected I8T and I8V escape mutations whereas Clone C11 selected I8R and S7N escape mutations. This could explain the underlying reason for the occurrence of different mutations in the *in vitro* assay versus *in vivo* in the patients. As shown in Chapter 4, Pol TI8 specific CTL Clone 3 also selected

same mutations (i.e., I8R and S7N) as Clone C11, indicating that Clone 3 and Clone 11 might share some similarity in TCR. Therefore, the role of TCR repertoire in evolution of virus can be further investigated via sequencing of TCR Vβ gene, investigating in particular, the interaction of CDR3 loop of these three CTL clones with peptide- MHC class I complex.

In this Chapter, we also examined the effect of the founder virus on HIV evolution and HIV control.

In this set of Virus Evolution Assays, we observed amino acid substitutions at position 8, Valine (V), Tyrosine (T) or Arginine (R) in JP-8I virus. However, these substitutions were not developed in SM-8V virus under the selection of both CTL clones. When compared the nucleotide sequences of these substitutions, there is only one-nucleotide change from I (ATA) to R (AGA) or T (ACA) whereas two-nucleotide change is required for the mutation R (AGA) or T (ACA) in SM-8V (GTA) virus, meaning that more evolutionary strength is needed to achieve the substitution of 8R or 8T in SM-8V virus. This might explain the low frequency of 8R/T mutations in the HLA-B51 patients of SM cohort when the founder virus carries Valine (V) at position 8 of the epitope. Meanwhile, the high frequency 8L mutation in epitope Pol TV8, which has been driven by HLA-B51 in the SM cohort, is absent from our two sets of evolution assays. Previous studies on mutation patterns of HIV genes have revealed that transitions account for most of nucleotide substitutions in HIV-1 genome (Moriyama et al., 1991). The absence of

8L substitution in our assays could be due to the V8L and I8L mutations (i.e., Valine (GTA) to Leucine (TTA) and Isoleucine (ATA) to Leucine (TTA), respectively), transversion mutations. However, the substitutions observed in this study are I8T/V, S7R and V8A, which are transition mutations. The other possibility could be the higher rate of G-to-T mutation, through misincorporation of G: dATP introduced by HIV-1 RT, than the A-to-T substitution through mispairing of A: dATP (Bebenek et al., 1989) , which contribute to the higher mutation of 8L in SM cohort than in the Japanese cohort. These data suggest that, in addition to the function of CTLs, the sequence of the founder virus has a major impact on the selection of mutations by CTLs.

We also found that under the CTL selection, SM-8V virus (Pol TV8) showed greater resistance to epitope mutations. No epitope mutation was detected under the selection of Clone C5 and there is only a rare mutation selected by Clone C11 at the very late virus passages. Though some mutations occurred in the flanking region, none of them was fixed. By contrast, under the selection of same HLA-B51 Pol TI8 specific CTL clones, JP-8I virus (Pol TI8) developed escape mutations rapidly. Under the selection of Clone C11, the first escape mutation occurred after 3 rounds of viral passage and the WT virus disappeared after 6 weeks of selection. The development of escape mutations selected by Clone C5 is slightly slower than Clone C11, with emergence of escape mutations in the 4th round and 90% of virus carrying escape mutations at the end. Although each CTL clone mount comparable immune pressure on both SM-8I virus and JP-8V virus, SM-8I virus developed

escape mutations much more rapidly than SM-8V virus. This suggests that the presence of Valine (V) at position 8 in the epitope Pol TI8 could potentially delay the development of escape mutations. These data also indicate that HLA-B51^{+} patient infected with a virus with epitope Pol TV8 may have more advantages in viral control than the ones who are infected with the virus with epitope Pol TI8.

Moreover, it was also found that both CTL clones exhibit better capability to control the SM-8V virus during the later round of virus passages, even though they have better suppression against JP-8I virus during the initial virus passages (Figure 5.3). The inhibition against SM-8V virus by both CTL clones remained fairly stable across all the virus passages, the better control of SM-8V virus is instead due to the decreased suppression against JP-8I virus, as a result of development of epitope escape mutations. In addition, our data showed that SM-8V virus with epitope Pol TV8 are less efficiently processed than TI8 epitope, which resulted in lower level of immune pressure on the SM-8V virus than JP-8I virus. This reduced level of immune pressure through less efficient antigen processing is not sufficient to escape T cell recognition, but significantly slows down the rate of development of escape mutations, which led to the better viral control. This fine balance between the rate of the escape mutations and the level of immune pressure exerted by CTLs may contribute to the long-term viral control of HLA-B51 patients in the SM cohort.

It has been reported that the NL43 virus carrying mutation I8V in the epitope PolTI8, the same virus clone as SM-8V used in our study, suffered high fitness cost in *in vitro* competition assay (Kawashima et al., 2009), however, very similar replication kinetics between JP-8I virus and SM-8V virus was observed in our *in vitro* assay. Lobritz et al. (2011) demonstrated that long-term HIV control might be the result of an attenuated transmitted founder virus together with favourable host genetics factors such as protective HLA alleles HLA-B57. In this case, the potential attenuation of SM-8V virus variant may have some contribution to the long-term HLA-B51 associated viral control of in the SM cohort.

In conclusion, the data from our *ex vivo* observations and *in vitro* Virus Evolution Assay demonstrated that, although antigen sensitivity plays an important role in anti-viral efficacy of CTLs, the TCR usage of CTLs has stronger effect on virus evolution. More importantly, our study highlighted the major impact of the founder virus sequence on viral control. In the SM cohort, HLA-B51 positive patients might have benefited from having a founder virus carrying Pol TV8 epitope, which resulted in a fine balance of efficient viral control, lower level of immune pressure and the slower rate of development of escape mutations.

Chapter 6: The *ex vivo* Phenotype of HLA-B51 restricted antigen-specific T cells in the SM cohort

6.1 Introduction

Despite strong HIV-specific $CD8^+$ T cell responses during the chronic phase of HIV-1 infection, most infected individuals fail to contain and clear the infection and ultimately progress to AIDS (Carmichael et al., 1993; Klein et al., 1995). It has been shown that the failure of $CD8^+$ T cells to control the HIV-1 infection could be due to high rate of virus escape mutation (Borrow et al., 1997; Goulder et al., 1997); MHC class I down regulation in the infected cells by virus protein Nef (Collins et al., 1998; Kerkau et al., 1989; Schwartz et al., 1996); lack of $CD4^+$ T helper cells response as a result of the rapid depletion of $CD4^+$ T cells during the infection (Douek et al., 2002; McNeil et al., 2001; Palmer et al., 2002; Rosenberg et al., 1997); and the impaired function of HIV-specific $CD8^+$ T cells (Lieberman et al., 2001).

Chronic activation, caused by constant exposure to high load of antigen, may lead to abnormalities in function and differentiation of HIV-specific $CD8^+$ T cells, such as the impaired effector differentiation (Champagne et al., 2001), replicative senescence and reduced capacity to proliferate (Brenchley et al., 2003), reduced cytolytic activity (Appay et al., 2000), and the inability to produce and release different cytokines (Kostense et al., 2002). Various studies have shown that HIV-specific $CD8^+$T cells in LNTPs and ECs showed polyfunctionality (Betts et al.,

2006); greater proliferative capacity; and higher cytotoxicity (Horton et al., 2006; Migueles et al., 2002) than those in progressors. However, whether the effective function of $CD8^+$ T cells is the cause or effect of viral control remains unclear, as Tussey et al. (2003) and Rehr et al. (2008) have demonstrated that the impairment of effectiveness of HIV-specific $CD8^+$ T cells can be restored when antigen level is reduced by antiretroviral therapy (ART). In addition to viral load, Streeck et al. (2008) found that the functional quality of HIV-specific $CD8^+$ T cells could also be modulated by the sequence variation within the targeted epitope, which presumably is related to the degree of antigenic stimulation.

Over a decade ago Altman et al. (1996) developed peptide-MHC class I tetramer-staining technology for the functional study of antigen-specific T cells. As it is demonstrated in **Figure 6.1**, the peptide-MHC class I complex consists of two polypeptide chains, a polymorphic α chain encoded by a MHC class I (HLA) gene and a conserved subunit encoded by β2-microglobulin (β2m) gene. The α1 and α2 domains of the α chain fold to make up a groove, where the peptides of approximately 8-10 amino acids in length bind. Tetramers are peptide-MHC class I complexes multimerised through the binding of a biotin-tag at the N-terminal of heavy chain to the florescence-conjugated streptavidin (**Figure 6.1**). Most T lymphocytes express a clonal and highly specific antigen receptor, T cell receptor (TCR), on the cell surface. The TCR binds to the MHC-peptide complex and recognizes the antigenic peptide presented in the context of MHC molecules. Due to low avidity and fast off-rates of the binding between TCR and peptide-MHC

complexes, recombinant soluble monomeric MHC-peptide complexes could not be used to detect antigen-specific T cells (Matsui et al., 1994). However, MHC tetramers have increased avidity for their cognate TCR by binding to multiple T cell receptor molecules and are successfully used to directly visualise antigen-specific T cells *ex vivo* by flow cytometry.

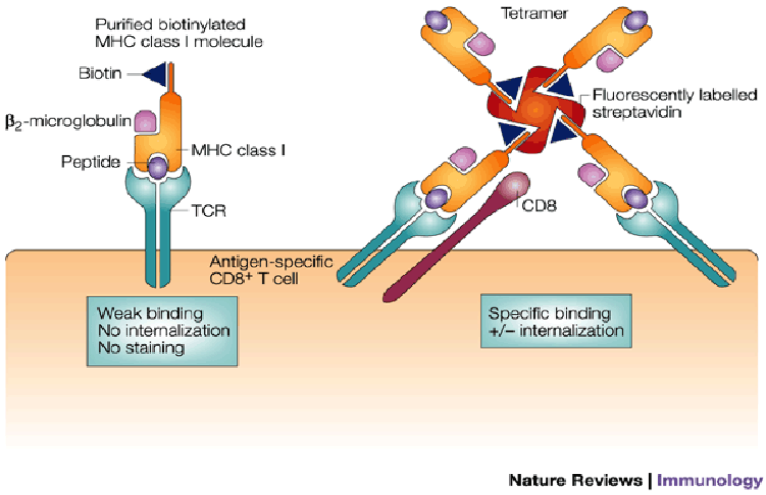

Figure 6.1. The schematic diagram of peptide-MHC complex and MHC tetramer and their interaction with TCR and CD8 Adapted from Nature Reviews Immunology 2, 263-272 (April 2002)

The *Ex vivo* phenotype and function of antigen-specific T cells can be assessed by analysing the surface expression of molecules relevant to T cell activation (CD38, HLA-DR) (Hultin et al., 1998), differentiation (CD27, CD28) (Appay et al., 2002a), replicative senescence (CD57) (Brenchley et al., 2003), and intracellular expression of molecules important for $CD8^+$ T cell effector function (perforin, Granzyme) (Henkart et al., 1984; Masson and Tschopp, 1987).

As shown in previous Chapters, in the SM cohort, there are three dominant HLA-B51 restricted T-cell responses against epitopes Pol LI9, Pol TI8 and Gag NI9. The hierarchy of epitope mutations driven by HLA-B51 restricted CTLs was observed in the HLA-B51+ patients (*ex vivo*; Chapter 3) and also in our Virus Evolution Assay (*in vitro*; Chapter 4). Mutations developed in epitope Pol LI9 first, followed by epitope PolTI8, and finally epitope Gag NI9. Efficient viral control is significantly associated with the T-cell response to un-mutated epitopes. In this study, we first characterised the phenotype and functionality of HLA-B51 restricted CTL responses by staining whole blood samples with HLA-B51 tetramers and four panels of antibodies. To assess the impact of epitope escape mutations on CTL phenotype and function, we compared the phenotypic characteristics of CTLs targeting mutated and un-mutated epitopes. The results of this Chapter showed a trend that HLA-B51 restricted $CD8^+$ T cells responding to a mutated epitope are more likely to possess an early-differentiated and less senescent phenotype than the ones responding to an un-mutated epitope, suggesting that this early-differentiated and less senescent phenotype of antigen-specific T cells is likely the effect rather than the cause of low level of antigen stimulation.

6.2 Results

6.2.1 Preparation of Class I MHC tetramers

Numerous MHC class I A or B allele restricted tetramers have been generated in a standardised procedure as described by Garboczi et al. (1992) and Reid et al. (1996). A wide range of MHC tetramers are also available commercially. However, HLA-B51 restricted MHC tetramers are not available commercially due to the low prevalence of this allele in Caucasian population (Cao et al., 2001), low peptide-binding affinity (Tomiyama et al., 1999), slow assembly, and instability of the HLA-B51 complex (Hill et al., 1993). Therefore, for this study we generated three HLA-B51 tetramers with a modified protocol and started from engineering the expression construct of HLA-B51 heavy chain.

6.2.1.1 Cloning modified HLA-B5101 heavy chain gene to an expression vector pGMT7

The coding sequence of HLA-B51 heavy chain (**Figure 6.2**) was cloned into vector pGMT7 for its expression as described by Garboczi et al. (1992) and Reid et al. (1996).

The cloned coding sequence of HLA-B51 heavy chain only includes 276 amino acids (**Figure 6.2**; highlighted in yellow), which is required for expression of class I tetrameric complex. The leader sequence, trans-membrane domain and the cytoplasmic domain (**Figure 6.2**; highlighted in grey) were not included.

MRVTAPRTVLLLLWGAVALTETWAGSHSMRYFYTAMSRPGRGEPRFIAVGYVDDTQFVRFDSDAASPRT

EPRAPWIEQEGPEYWDRNTQIFKTNTQTYRENLRIALRYYNQSEAGSHTWQTMYGCDVGPDGRLLRGHN

QYAYDGKDYIALNEDLSSWTAADTAAQITQRKWEAAREAEQLRAYLEGLCVEWLRRHLENGKETLQRAD

PPKTHVTHHPVSDHEATLRCWALGFYPAEITLTWQRDGEDQTQDTELVETRPAGDRTFQKWAAVVVPSG

EEQRYTCHVQHEGLPKPLTLRWEPSSQSTIPIVGIVAGLAVLAVVVIGAVVATVMCRRKSSGGKGGSYS

QAASSDSAQGSDVSLTA

Figure 6.2: The amino acid sequence of HLA-B51. The sequence highlighted in yellow is the class I tetramer amino acid sequence, which is cloned into pGMT7 expression vector.

Different to the standard protocol, the 5' primer for HLA-B51 cDNA amplification that is listed in the **Table 6.1,** has been modified in order to achieve the optimal expression of HLA-B51, i.e., a human gene in *E. coli*. The nucleotides encoding the first six amino acids of HLA-B51 heavy chain have been changed into *E. coli* class II codons, which are highlighted in red in **Table 6.1** (Henaut and Danchin, 1996). For example, AGG, the codon for Arginine in human HLA-B51 gene, used in *E.coli* with a frequency of 0.29%, whereas CGT is the most preferred class II codon for this amino acid in bacteria with a frequency of 65%.

Table 6.1: Modified 5' forward Primer. The primer contains the non-coding sequence, the cleavage site of *Nde I* restriction enzyme and the 5' end of HLA-B51 gene. The modified nucleotides are in red.

HLA-B51	non-coding sequen	*Nde I*		5' END OF GENE												
Amino Acid Sequence		L	T	G	S	H	S	M	R	Y	F	Y	T	A	M	S
Base Sequence	GAAGAAGTA	CAT	ATG	GGC	TCC	CAC	TCC	ATG	AGG	TAT	TTC	TAC	ACC	GCC	ATG	TCC
Modified primer 5'	GAAGAAGTA	CAT	ATG	GGT	TCT	CAT	TCT	ATG	CGT	TAT	TTC	TAC	ACC	GCC	ATG	TCC

6.2.1.2 Refolding and purification of HLA-B51 peptide-MHC complex with D-biotin tag

Purified recombinant HLA-B51 MHC (heavy chain) and human β2-microglobulin (β2m) proteins that were expressed in *E.coli* were then refolded in molar excess of the HLA-B51 epitope peptide (either Pol LI9, Pol TI8 or Gag NI9) by diluting as described by Garboczi et al. (1992) and Reid et al. (1996).

As the assembly of HLA-B51 molecule has been shown to be inefficient (Hill et al., 1993), it has to be refolded for longer period of time and in more diluted manner than HLA-A and other HLA-B molecules. Therefore, in this study, we refolded the proteins in one liter standard refolding buffer for five days and pulsed with heavy chain protein at different time points: 12 hours, 24 hours and 36 hours. In addition, prior to the addition of biotin-tag, the refolded HLA-B51 complex protein was purified using Superdex75 prep grade gel filtration. The purification step was done to increase the biotinylation efficiency of the HLA-B51 protein as the biotinylation reaction can be interfered at high concentration of salt, the aggregates and free heavy chain proteins. The collected fractions are 154-174 ml elution and are highlighted in red (**Figure 6.3**).

After adding the biotin tag to the complex using BirA enzyme, the protein was then purified by Superdex 200 column. The traces are shown in **Figure 6.4**, the collected fractions are highlighted red. The purified biotinylated complex was

concentrated to 1-2 mg/ml. At the final step, to stabilize the protein complex a protease inhibitor cocktail containing Pepstatin A (an acid proteases inhibitor), Leupeptin (both Serine and Cysteine proteases inhibitor) and Bestatin Hydrochloride (an amino-peptidases inhibitor) was added to the solution. The complex, called Class I MHC monomer, was ready for multimerisation with fluorescence-labeled extravidin or aliquoting for long-term storage.

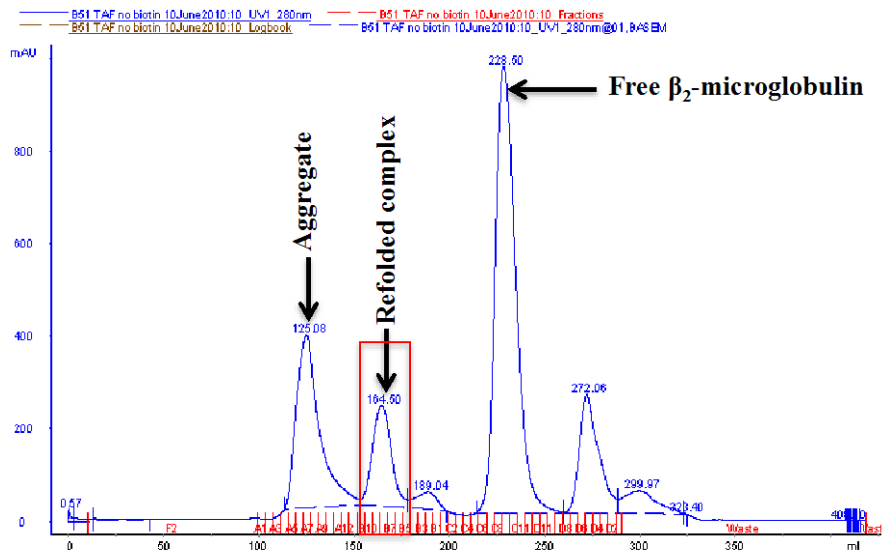

Figure 6.3 Trace picture from gel filtration using Superdex 75 to purify HLA-B51 peptide-MHC class I (TAFTIPSI) complex. The first eluted peak (at 125ml) is the aggregate. The eluted peak, highlighted with red, is the refolded complex with a size of 44kD. The protein eluted at the peak of 228ml is free β_2-microglobulin.

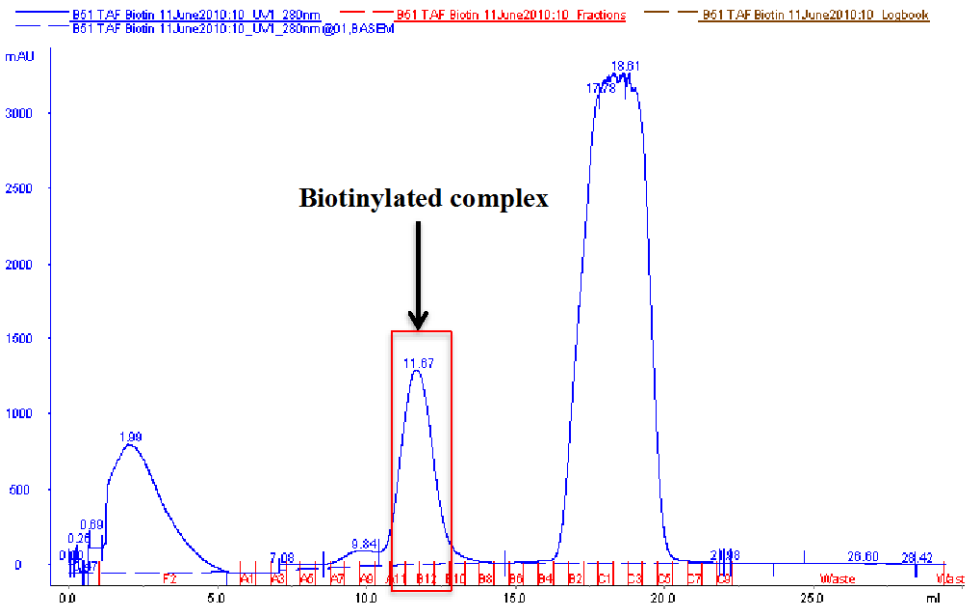

Figure 6.4 Trace picture from gel filtration using Superdex 200 to purify HLA-B51 Class I MHC-peptide (TAFTIPSI) complex. The eluted peak, highlighted with red, is the refolded complex with a D-biotin tag.

6.2.1.3 Staining BaF3-ILT2 cells with HLA-B51 Class I MHC tetramers

To verify the staining efficiency of the PE-conjugated HLA-B51 Class I MHC class I tetramers, they were used to stain BaF3 cell line expressing human inhibitory receptor Ig-like transcript 2 (ILT2). It has been reported that human ILT2 binds to most MHC class I molecules such as HLA-A and HLA-B (Shiroishi et al., 2003). This is due to the location of the binding site, which lies within the α3 domain of Class I MHC molecules, a relatively conserved region among the different Class I MHC alleles. Therefore, most fluorescence conjugated Class I MHC tetramers can bind to the cells expressing ILT2 on their surface, which can be detected by flow cytometry. Using this assay, we verified whether the tetramers

are properly refolded and tetramerised. Only properly refolded and tetramerised complexes are able to bind to the BaF3 cell line expressing ILT2 molecules, which can be detected by flow cytometry. As **Figure 6.5** shows, the HLA-B51 tetramer with peptide TAFTIPSI are in the correct conformation and therefore functional.

In this study, three HLA-B51 tetramers with peptides Pol LI9, Pol TI8 and Gag NI9 were produced to evaluate the quality and quantities of HLA-B51 restricted T-cell responses against HIV-1 infection.

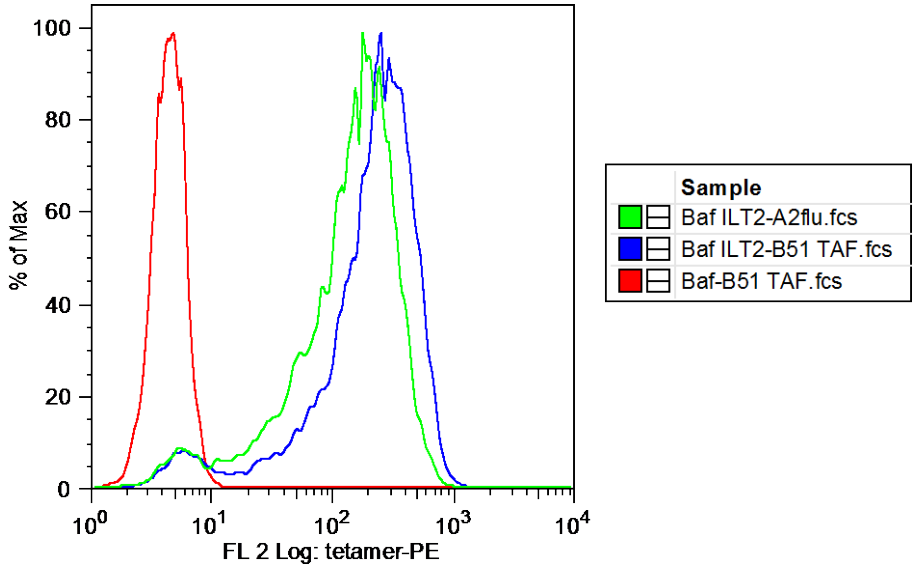

Figure 6.5 Staining BaF3 cell line or BaF3-ILT2 cell line with HLA-B51 tetramers by flow cytometry. The red line represents the negative control in which the B51 tetramer was stained with BaF3 cell line without transfecting ILT2 receptor. The green line represents the positive control in which a functional HLA-A0201 restricted flu-specific tetramer was stained with BaF3 cell line expressing ILT2 receptor. The blue line represents the cells stained with tested tetramer HLA-B51 TAFTIPSI.

6.2.2 Identifying HLA-B51 restricted HIV-1-specific CD8$^+$ T cells in the SM cohort

The whole blood samples collected from eleven HLA-B51 positive patients (Table 3) were stained with three HLA-B51 peptide-MHC tetramers (HLA-B51 Pol LI9, HLA-B51 Pol TI8 and HLA-B51 Gag NI9 tetramers). **Figure 6.6** shows the examples of identifying HLA-B51 HIV-specific CD8$^+$ T cells by flow cytometry.

The frequency of antigen-specific CD8+ T cells and the relevant pro-viral epitope sequences in each patient are shown in **Table 6.2**. Seven out 14 patients make responses to mutated Pol LI9 epitope, with one exception that the patient 196 responded to an un-mutated epitope. Six patients have T-cell responses to Gag NI9 epitope, of whom one responded to a mutated epitope. Two patients have CTLs targeting the epitope Pol TV8 with a wild type sequence. Meanwhile, there are four patients who make multiple T-cell responses to these three HLA-B51epitopes (Table 6.2, highlighted in yellow). All of them respond to epitope Pol LI9, of which two also make responses to either epitope Pol TI8 or Gag NI9. Further more, the patients' clinical data, including CD4 count, viral load and information about treatment, were also included. As shown in the table, most of patients were under antiretroviral therapy during the recruitment, making it inappropriate to link the functional data to the viral control.

Table 6.2: The frequency of antigen-specific CD8+ T cells, epitope sequence and the clinical data for each patient. The frequency of antigen specific CTLs is presented by the percentage of tetramer^{+} in CD8+ population.

Patient ID	Pol LI9		Pol TI8		Gag NI9		VL	CD4	ART
	Tet+/CD8+ (%)	epitope mutation	Tet+/CD8+ (%)	epitope mutation	Tet+/CD8+ (%)	epitope mutation			
14	0.0316	Y					88	290	Y
47	0.545	Y					218	214	Y
166	0.636	Y	1.35	N			NA	NA	N
196	0.559	N	0.764	N			<50	NA	N
215	2.45	Y					11496	280	Y
435	0.0662	Y			0.46	N	4823	872	Y
446	0.525	Y			1.8	N	7226	374	N
176					0.585	N	43569	95	Y
209					0.477	N	1195	354	Y
342					0.428	Y	406	207	Y
349					0.366	N	563	434	N

Y=Yes; N=No; NA=Not available

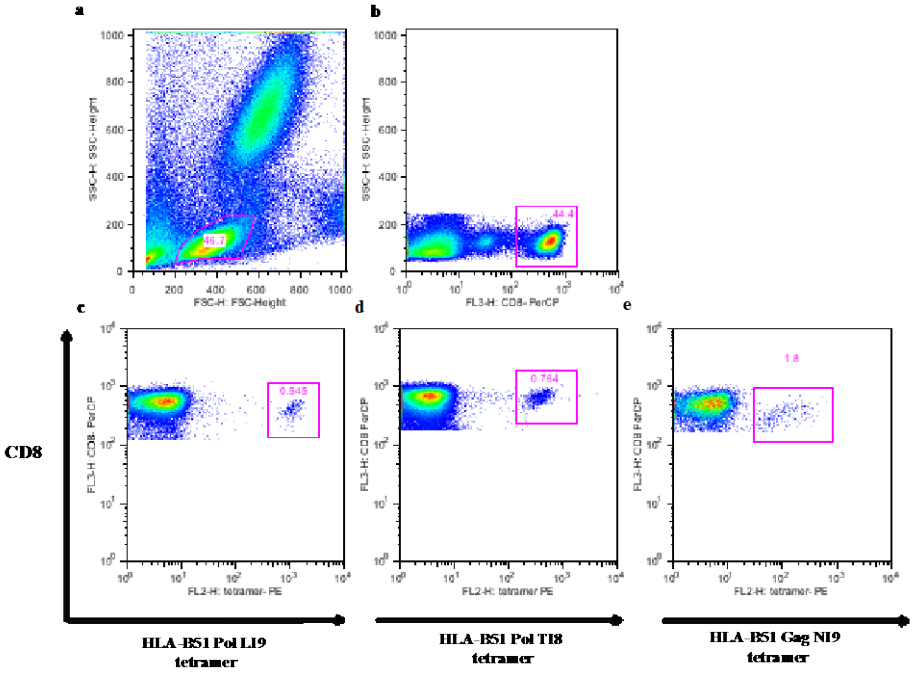

Figure 6.6 Dot plots showing whole blood from patients stained with CD8-PerCP and PE-HLA-B51 tetramers refolded with three different peptides. Cells are gated on lymphocytes (Plot a) and CD8 positive population (Plot b). HLA-B51 restricted HIV-1 specific CD8^{+} T cells are shown in Plots c, d and e.

6.2.3 CD8$^+$ T cells targeting an un-mutated (WT) epitope displaying a late-differentiated phenotype more frequently

Appay et al. (2002a) have reported that expression of co-stimulatory receptors CD28 and CD27 is associated with different stages of T-cell differentiation. CD8$^+$ T cells differentiate in a linear pathway from CD28$^+$CD27$^+$ (early-differentiated) to CD28$^-$CD27$^+$ (intermediate-differentiated) to CD28$^-$CD27$^-$ (late-differentiated). With the gradual loss of CD28 and CD27 expression, CD8$^+$ T cells differentiate to end-stage effectors. By examining the surface expression of CD27 and CD28, the differentiation state of CTLs specific to HLA-B51 restricted epitopes Pol LI9, Pol TI8 and Gag NI9 was assessed (**Figure 6.7 a**).

Comparing the phenotype of HIV-specific CTLs with the phenotype of the whole CD8 T cell population (**Figure 6.7 b**) showed, with a mean frequency of 67.73%, the majority of HLA-B51 restricted antigen specific CTLs is at an intermediate-differentiated stage (CD27$^+$CD28$^-$). A similar result was reported by Appay et al. (2002a). The CD27$^+$CD28$^-$ cell population is significantly enriched in HLA-B51 restricted antigen specific CTLs. On the contrary, the frequency of late-differentiated cells (CD27$^-$CD28$^-$) is significantly lower in HIV-specific CTLs (mean frequency=13.84%), compared to the entire CD8 T cell population (mean frequency=51.18%).

Furthermore, we showed that there is no difference in the frequency of intermediate-differentiated (CD27$^+$CD28$^-$) cells between CTLs specific to mutated

epitopes and un-mutated (WT) epitopes (**Figure 6.8 b**). In fact, most of $CD8^{+}Tetramer^{+}$ cells displayed this phenotype ($CD27^{+}CD28^{-}$), in accordance with the data in **Figure 6.7** and the findings in previous study presented by Appay et al. (2002a). By contrast, those $tetramer^{+}$ T cells appeared to differ with regard to their early and late stage of differentiation phenotype. As demonstrated in **Figure 6.8 a** and **c**, the late-differentiated ($CD27^{-}CD28^{-}$) CTLs are more frequent in T cells targeting a WT epitope, with a mean frequency of 18.5% compared to 8.5% for CTLs responding to mutated epitopes, whereas CTLs responding to mutated epitopes exhibit higher frequency of early-differentiated phenotype with a mean frequency of 26.77% compared to 17.8% for CTLs responding to WT epitopes.

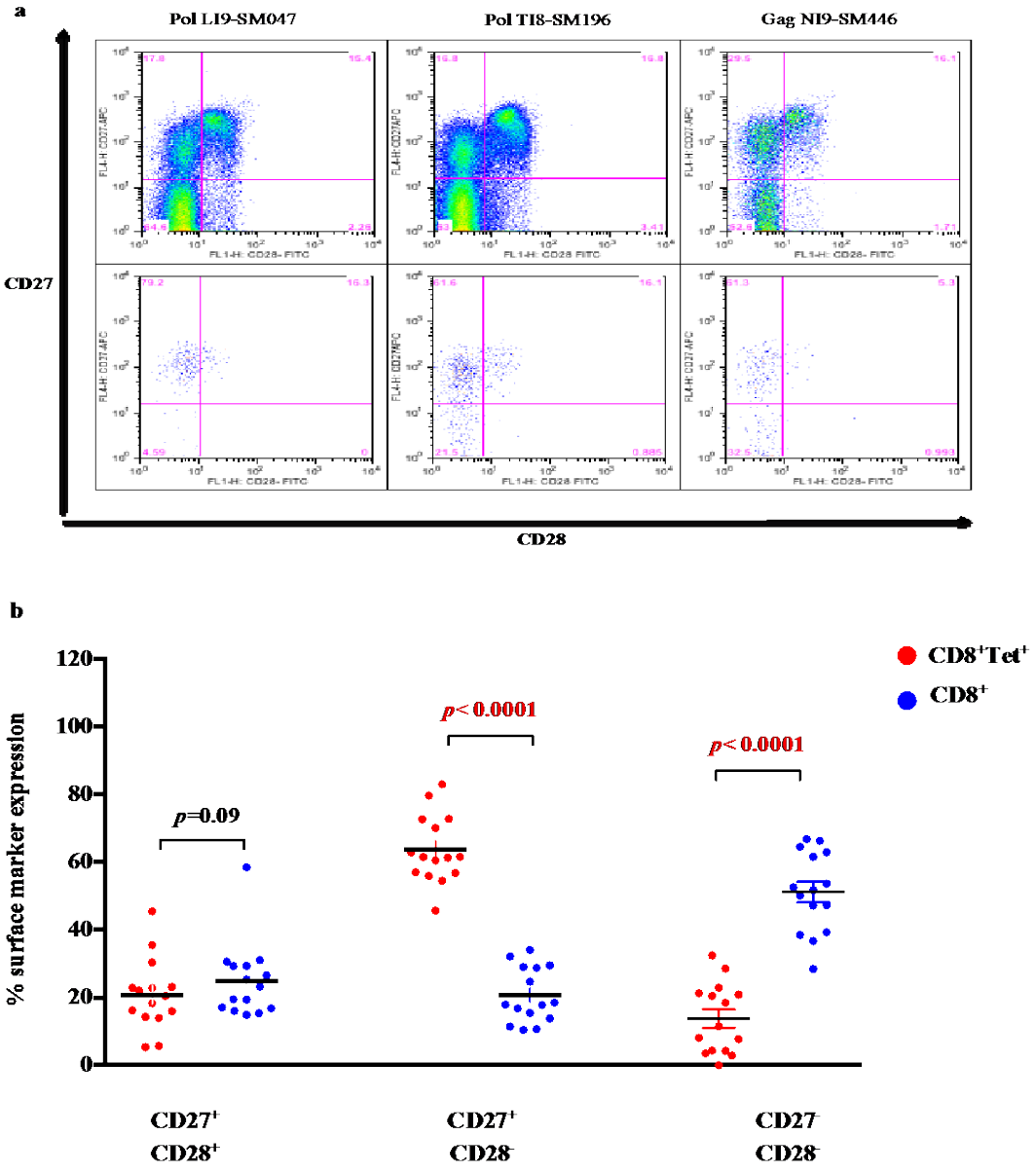

Figure 6.7 Phenotype of HLA-B51 restricted HIV-specific CD8$^+$ T cells. a) FACS plots represent CD27 and CD28 expression on entire CD8$^+$ T cells (top panel) and on tetramer$^+$ CD8$^+$ T cells (bottom panel). Tetramer restriction and patient ID are shown above each column of plots. b) Summary of the differentiation phenotype of the antigen specific CD8+ T cells (red dots) and the entire CD8+ T cell population (blue dots). Two-tailed Wilcoxon matched paired test.

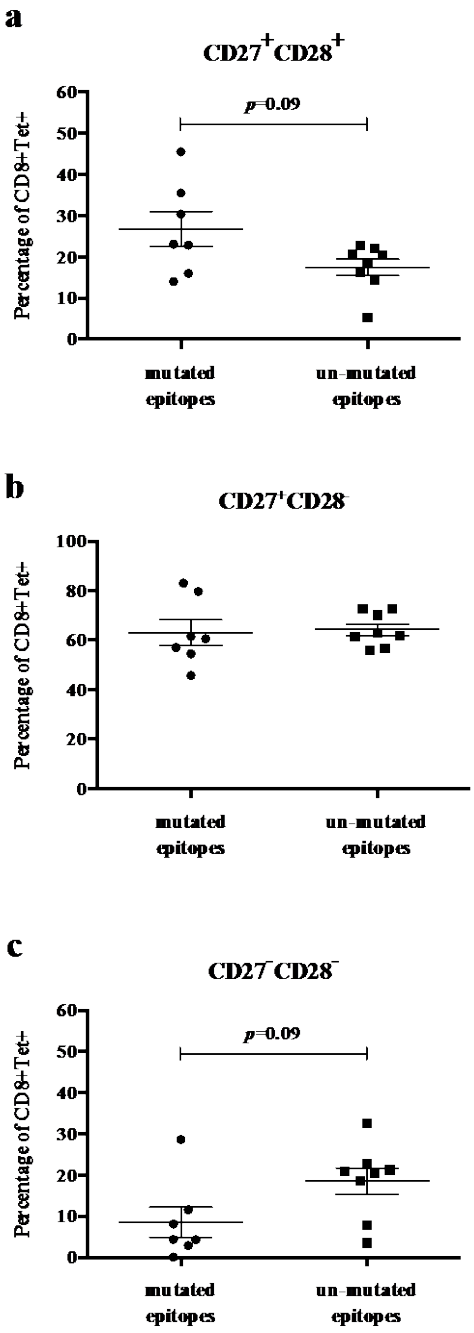

Figure 6.8: Phenotype analysis of HLA-B51 restricted antigen-specific CD8$^+$ T cells. Summary of the early (CD27$^+$CD28$^+$), intermediate (CD27$^+$CD28$^-$) and late (CD27$^-$CD28$^-$) stage of differentiation in CD8$^+$ T cells, responding to mutated and un-mutated epitopes was shown in a, b and c, respectively. P value was calculated using two-tailed Mann-Whitney U Test.

6.2.4 CD8$^+$ T cells targeting un-mutated HLA-B51 epitopes are more differentiated towards replicative senescent.

Researchers have previously noted that chronic immune activation is routinely seen in HIV infection, in which T cells, especially CD8$^+$ T cells, are inappropriately switched "on" or activated (Giorgi et al., 1993; Hazenberg et al., 2003; Hultin et al., 1998). HIV-1 induces immune activation, which leads to differentiation of CD8$^+$ T cells towards replicative senescence (Papagno et al., 2004). One of the objectives of this study was to evaluate the effect of persistent antigen stimulation on the phenotype of antigen specific CD8$^+$ T cells. To achieve this, the expression of CD38 (activation) and CD57 (replicative senescence) on CD8$^+$ T cells was studied (**Figure 6.9**). Samples from seven patients have been assessed in this part of study.

As illustrated in **Figure 6.10 a**, CD38 expression was similar on both HLA-B51 restricted antigen specific CD8+ T cells and the bulk CD8+ T cells, meaning that in HIV-1 chronic infection, all CD8 T cells are highly activated. In contrast, this molecule is expressed more frequently on CD8$^+$ T cells targeting the un-mutated epitopes than the cells specific for mutated epitopes, though the difference is not statistically significant (**Figure 6.10 b**).

CD57 is a marker of replicative inability on T cells. In HIV-1 infection, CD57$^+$CD8$^+$ T cells can expand due to the persistent immune activation (Evans et al., 1999). In line with this, CD57$^+$CD8$^+$ T cells account for 50% of CD8$^+$ T cell

population in the patients. Compared to bulk $CD8^+$ population, more antigen specific $CD8^+$ T cells express CD57 molecule, although it was not significantly different (**Figure 6.10** a). This figure also demonstrates a trend that activated antigen specific $CD8^+$ T cells ($CD38^+CD8^+$Tetramer+) co-express CD57 more frequently. In addition, as shown in **Figure 6.10 b**, with high expression of CD38 and CD57 on cell surface, $CD8^+$ T cells targeting un-mutated epitopes have an inclination towards a more activated phenotype with evidence of impaired replicative ability, compared to the $CD8^+$ T cells specific for mutated epitopes.

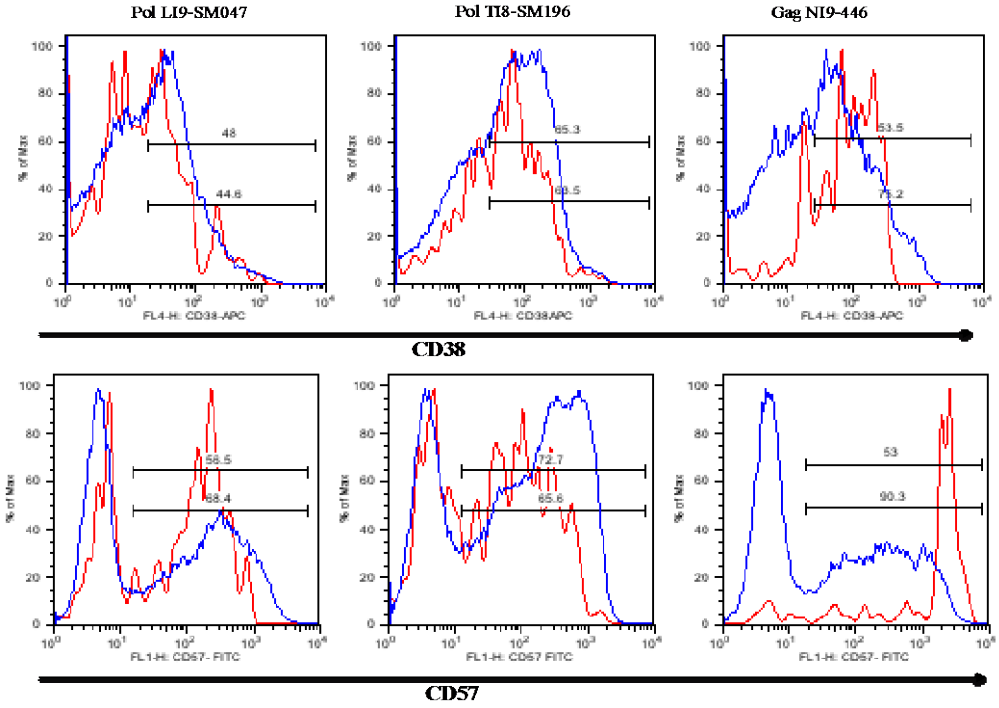

Figure 6.9 Histogram of activation marker CD38 and senescence marker CD57 expression. The top panel: CD38 expression; the bottom panel: CD57 expression. The red line represents the phenotype of $CD8^+$Tetramer$^+$ cells (lower percentage value) and the blue line represents the phenotype of bulk $CD8^+$ T cells (upper percentage value). Tetramer restriction and patient ID are shown above each column of histograms.

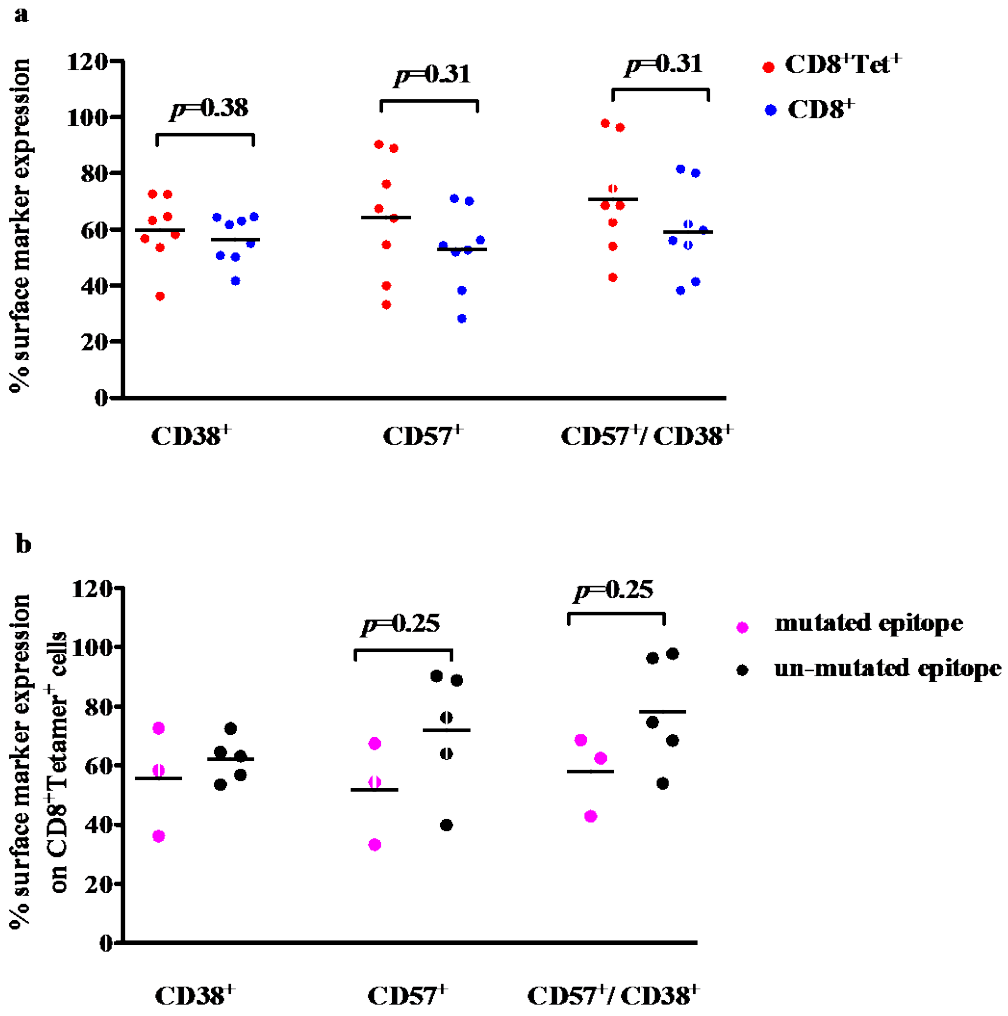

Figure 6.10 Expression of activation marker CD38 and senescence marker CD57 on HLA-B51 antigen specific CTLs. a) The frequency of $CD38^+$, $CD57^+$ and $CD57^+$ /$CD38^+$ cells in HLA-B51 antigen specific $CD8^+$ T cells (red dots) and bulk $CD8^+$ T cells (blue dots). P value was calculated by two-tailed Wilcoxon matched pairs test. b) Analysis of phenotype on $CD8^+$ T cells specific to mutated and un-mutated HLA-B51 epitopes (Pol LI9, Pol TV8 and Pol GagNI9). Two-tailed Mann-Whitney U Test.

6.2.5 Higher cytotoxic potential of CD8+ T cells targeting the un-mutated epitopes

$CD8^+$ T cells are capable of lysing virus-infected target cells by release of soluble markers such as granzyme and perforin, thereby preventing the spread of HIV to uninfected cells (Appay et al., 2000; Peters et al., 1991). The *ex vivo* cytotoxic potential was assessed by evaluating the level of pre-loaded perforin expression (**Figure 6.11 a**). The data presented in **Figure 6.11 b** and **c** suggest that perforin is expressed at significantly lower level in HLA-B51 antigen specific $CD8^+$ T cells than in the whole $CD8^+$ T cell population, while the CTLs targeting the un-mutated HLA-B51 epitopes expressed higher levels of perforin than the CTLs responding to the epitope with mutations.

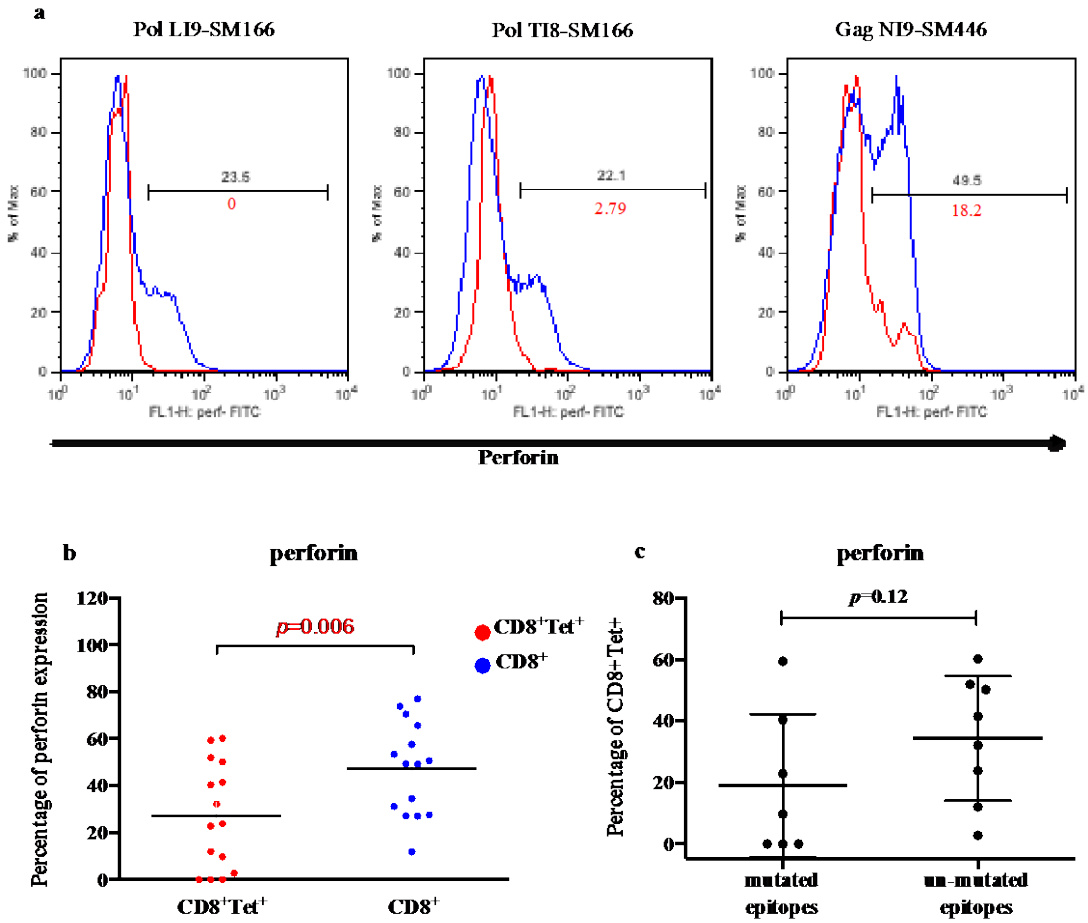

Figure 6.11 Functional analysis of HLA-B51 restricted antigen-specific CD8$^+$ T cells. a) Histogram of perforin expression on CD8$^+$ T cells and antigen specific CD8$^+$ T cells. Red line represents phenotype of CD8$^+$Tetramer$^+$ cells (lower percentage value in red) and the overlapping blue line represents phenotype of CD8$^+$ T cells (upper percentage value in black). (b) Summary of perforin expression in antigen specific CD8$^+$ T cells (red dots) and bulk CD8$^+$ T cell population (blue dots). P value was calculated by two-tailed Wilcoxon matched pairs test c) Comparison of perforin expression in CD8$^+$ T cells responding to the WT epitope and the mutated epitope, two-tailed Mann-Whitney U Test.

6.3 Discussion

Appay et al. (2002a) and van Baarle et al. (2002) showed that in different chronic virus infections, antigen specific CD8+ T cells differ in differentiation phenotype. They found that unlike CMV-specific $CD8^+$ T cells with enrichment of late-differentiation phenotype (CD27-CD28-), HIV-1-specific $CD8^+$ T cells showed a substantial enrichment of intermediate-differentiated subset (CD27+CD28-), regardless of their antigen level and disease stage. In this study we also showed that the majority of the HLA-B51 restricted HIV-1-specific CTLs displayed the intermediate-differentiated phenotype (CD27+CD28-) and this population was significantly enriched when compared to the whole CD8+ T cells (**Figure 6.7**).

Interestingly, there is a trend that CTLs responding to mutated epitopes display an early-differentiated phenotype more frequently, whereas T cells specific for the un-mutated epitope display a more late-differentiated phenotype. This observation indicates that antigen withdrawal caused by epitope mutation may have restored the early-differentiated antigen-specific T-cell population, while the persistent antigen stimulation from un-mutated epitopes has driven T cells to a late-differentiated status. As shown in **Table 6.2**, the majority of the patients in this study are under the administration of ART, which may have an impact on the differentiation of the CTLs. Various studies have shown prolonged antiviral therapy results in viral load reduction and consequently a restoration of the impaired function of HIV-1-specific CD8+ T cells, such as increased CD28 expression and decreased PD-1 expression (Rehr et al., 2008; Streeck et al., 2008). On the other hand, comparing

the viral load in treated patients (mean: 8600 copies/ml) with un-treated (mean: 4710 copies/ml) suggests that the reduced antigen load caused by escape mutations, rather than ART, may have a more profound impact on T cell differentiation in this study.

It has been reported that elevated immune activation is associated with poor outcome in HIV-1 pathogenesis (Giorgi et al., 1993; Hazenberg et al., 2003; Hultin et al., 1998). HIV-1 induces activation of the whole $CD8^+$ T cell population, not only those specific for HIV-1. The excessive level of immune stimulation drives the activated antigen-specific cells differentiated towards replicative senescence. As expected, we observed similar levels of CD38 expression on both HLA-B51 restricted HIV-1 specific CD8+ and the whole CD8+ T-cell population, whereas the expression of CD57 was shown to be more frequent on those tetramer-positive CD8+ T cells. Meanwhile, when comparing the phenotype of T cells responding to mutated and un-mutated epitopes, a clear trend was observed that T cells responding to un-mutated epitopes tends to be less capable of replication, showing higher frequency of CD57 expression. This observation indicates that antigen withdrawal, caused by escape mutations, may have reduced the immune stimulation and activation, which results in differentiation of CD8 T cells towards less replicative senescence.

Several studies have shown that HIV-specific CD8+ T cells are impaired in cytolytic activity and that HIV-specific $CD8^+$ T cells express significantly lower

levels of perforin than CMV-specific $CD8^+$ T cells (Appay et al., 2000). Similarly, in the present study, HLA-B51 restricted HIV-1-specific CTLs showed a significant decrease in expression of intracellular perforin, compared to the whole $CD8^+$ population. By contrast, our data showed that the perforin expression level in the CTLs responding to a non-mutated epitope is higher than in the cells responding to a mutated epitope. This indicates that $CD8^+$ T cells responding to un-mutated epitopes have a higher cytotoxic potential. Some studies have reported that the capability of HIV-specific $CD8^+$ T cells to rapidly express perforin after *in vitro* antigen stimulation, rather than preloaded perforin expression, is correlated with control in HIV infection (Hersperger et al., 2011; Hersperger et al., 2010). Therefore, assessing the perforin expression in tetramer-positive $CD8^+$ T cells after antigen stimulation may give us more clues on the impact of antigen load on the cytolytic function of the CTLs.

Our data show that despite the fact HLA-B51 specific CTLs displayed an impaired differentiation phenotype, HLA-B51 restricted T cell responses against un-mutated epitopes more frequently show a late differentiated (CD27-CD28-) phenotype and higher cytotoxic potential. As some studies have shown, the CD27-CD28- CD8+ T cells are specialized effector T cells because of their rapid effector function and also have high IFN-γ production despite their limited proliferative and survival abilities (Hamann et al., 1997). Moreover, these fully differentiated effector T cells are more frequently observed in HIV controllers than viraemic subjects (Addo et al., 2007). These data suggested that with efficient differentiation and higher

cytotoxic potential, the T-cell responses to un-mutated epitopes are still functional and contribute to HLA-B51 associated viral control. Further phenotypic and functional studies on this CD27-CD28- population, such as measuring proliferation ability and cytokine secretion, are needed to conform this speculation.

There are several limitations to this study, which are worth addressing and could provide clues and guidance for future studies. First, the majority of patients recruited for this study were treated with anti-retroviral dugs, making it inappropriate to link the *ex vivo* phenotype and functionality of HLA-B51 restricted HIV-1 specific T cells to clinical outcome of the patients. We also cannot exclude the effect of ART on the phenotype and function of antigen-specific T cells, as some studies showed that reduced antigen stimulation by ART can shift the phenotype of antiviral $CD8^+$ T cells from effector to memory, reduce T cell exhaustion and restore multifunctional T cells (Janbazian et al., 2012; Rehr et al., 2008; Streeck et al., 2008; Tussey et al., 2003; Vollbrecht et al., 2010). Secondly, apart from ART and escape mutations, antigen stimulation to CD8+T cells can be affected by the specificity of target epitope. The stimulatory capacity of different epitopes may differ due to their different antigenicity, which indicates that the different phenotype between CTLs specific for mutated and un-mutated epitopes might have attribute to the different stimulatory capacity of these two epitopes, as most of responses to the mutated epitope are responding to Pol LI9 whereas most responses against WT epitope are responding to Gag NI9 epitope. So examining the phenotype of T cells prior to the development of escape mutations in these

epitopes could provide more comprehensive evidence about how the epitope escape mutations impact the phenotype and function characteristics of $CD8^+$ T cells. Furthermore, to obtain more conclusive results, a larger sample size is required. The lack of statistical significance in this part of study could be due to the small sample size. Increasing patient numbers may also increase the frequency of the patients who make multiple HLA-B51 responses. This would allow us to evaluate the impact of antigen specificity and the epitope mutations on the phenotypic and functional properties of CTLs under controlled immune conditions, such as viral load, CD4+ T cell help or therapy. In addition, using multiple-parameter flow cytometer such as Cyan (9-parameters) or BD FACSAria (13-parameters) rather than BD FACSCalibur would provide a more detailed study characterising the subpopulations of antigen-specific cells. By using BD FACSCalibur with detection of four different fluorescences (FITC, PE, PerCP and APC), we were only able to combine two different surface or intracellular markers together with CD8 and Tetramer staining, which is not an ideal method to investigate the functional properties of the subpopulations. For example, analysing the expression of CD57, CD38, perforin or other phenotypes in combination with functional markers on CD27-CD28- Tetramer+ CD8+ T cells would identify the role of differentiation on the function of T cells.

In summary, our preliminary data showed that after nearly two decades of HIV-1 infection, HLA-B51 restricted HIV-1 specific CTLs displayed an overall impaired phenotype of differentiation, increased replicative senescence and reduced

cytotoxic potential. However, T cells targeting the mutated epitopes displayed an early-differentiated phenotype and better replicative capacity, presumably due to reduced antigenic stimulation. These data suggest that this early differentiated, less senescent phenotype of antigen-specific T cells in chronic HIV-1 infection is likely to be the effect, rather than the cause of low viraemia. On the other hand, although the persistent antigen stimulation has driven T cells targeting the un-mutated epitopes to a terminal-differentiation status, those T cells, with higher cytotoxic potential, may have contributed to HLA-B51 associated viral control in the SM cohort.

Chapter 7: Overall discussion and future directions

Numerous studies have shown the critical role of $CD8^+$ T cells in controlling HIV-1 infection, which led to much vaccine research aiming to elicit virus-specific $CD8^+$ T cells. However, the STEP trial, in which a HIV-1 vaccine was designed to stimulate HIV-1 specific CTL responses, was halted due to lack of efficacy (Gray et al., 2010). Although several studies have demonstrated that T-cell based vaccines could generate protective $CD8^+$ T cells in animal models (Hansen et al., 2011; Hansen et al., 2009; Liu et al., 2008), the participants, who received the STEP trial vaccine, were neither protected from acquiring HIV-1 infection nor had a good control of infecting virus, despite strong HIV-1 specific CTL responses (Priddy et al., 2008). This promoted calls for re-evaluation of the impact of different facets of the immune response to HIV-1 infection and identification of an immunological correlate of protection against HIV.

Most studies on protection mechanisms mediated by HIV-1-specific CTLs have focused on patient cohorts. These only allowed us to identify the correlative, non-mechanistic elements and it is impossible to avoid exceptions and bias in different groups of patients. In most HIV-1 cohort studies, the analysis is complicated by the presence of individuals who were infected with different viruses through different routes for different lengths of time as well as their diverse genetic backgrounds. In this study, we were able to access a unique cohort in China, SM cohort (Appendix 4), where individuals had been infected with HIV-1 through contaminated plasma in the early 1990s. All members of this cohort were infected

at a similar time and by the same infection route and have subsequently progressed to diverse disease outcomes in the absence of any antiretroviral treatment during the first 10 years of the infection. All the individuals live in an isolated rural village and therefore their genetic background are closely related. Phylogenetic analysis of proviral sequences derived from patients revealed a narrow source clade B' HIV-1 infection in this cohort (Dong et al., 2011). Taken together, with these distinctive features, the SM cohort provided us with a unique opportunity to study host-virus interactions during HIV-1 infection, avoiding many of the variables that often complicate the data analysis in other cohorts.

It has been long recognized that polymorphism of HLA class I plays an important role in host genetic influence on HIV-1 disease progression by presenting viral antigens to $CD8^+$ T cells. Some HLA class I alleles are strongly associated with slowing disease progression and lowering HIV-1 viral load. In particular, HLA-B57 and B27 in Caucasians and HLA-B51 in Asians are correlated with the control of HIV-1 infection (Carrington M, 2003; Gao et al., 2005). Unlike well-studied HLA alleles HLA-B57 and B27, the mechanism underlying the protection of HLA-B51 is still not clear. HLA-B51 is a common HLA allele in China and much of Asia; thus, understanding the mechanism of HLA-B51 association with viral control holds particular importance in the study of the pathogenesis of HIV infection in the Far East. In this thesis, taking the advantage of the unique SM cohort, we carried out very comprehensive *ex vivo* and *in vitro* studies and explored mechanisms underlying the HLA-B51 associated HIV-1 viral control.

The data, generated by a previous DPhil student in the lab, showed that there are three dominant HLA-B51 restricted T cell responses against epitopes Pol LI9, Pol TV8 and Gag NI9; and mutations developed within or around these epitopes are strongly associated with HLA-B51 (**Table 3.1**, **Appendix 2 and 3)**. Following his study, we first investigated the role of these three HLA-B51 restricted immunodominant HIV-specific T-cell responses in controlling disease progression in the SM cohort (Chapter 3). A selection hierarchy of epitope mutations among these three most dominant HLA-B51 restricted epitopes was observed. Good control of viral load and higher $CD4^+$ counts were found in patients with T-cell responses against un-mutated epitopes, whereas lower $CD4^+$ counts and higher viral loads were detected in patients with mutations in all three epitopes or with no detectable T-cell responses against un-mutated epitopes. To validate these observations *in vivo,* we generated HLA-B51 restricted HIV-1-specific CTL clones and evaluated the anti-viral efficacy of those CTL clones using Live Virus ELISPOT and Virus Suppression assay. We found that the suppression of virus replication by HLA-B51 restricted CTLs was either abrogated or significantly reduced when the virus carries mutations within or around those three HLA-dominant epitopes. However, CTLs could effectively recognise virus-infected cells and suppress virus replication when the WT epitope was properly processed and presented. The data presented in this chapter suggested that the patients with HLA-B51 have benefited from having a hierarchy of protective epitopes that provide multiple layers of defence against the development of immune escape mutations,

particularly when CTLs target the epitope (NI9) that is relatively conserved and appears to escape slowly.

CTL driven escape mutation is a hallmark of HIV-1 infection and also a major obstacle for developing an efficient T-cell based HIV-1 vaccine (Goulder and Watkins, 2004). It has been acknowledged that the emergence of HIV-1 escape mutations depends upon the net balance of selective pressure and the fitness cost that potential escape mutations bear for the virus. CTL selective pressure could be affected by several factors, such as CTL frequency, epitope specificity, T cell receptor properties, and functional state of the CTL. In turn, several viral factors also influence the development of epitope mutations, including the viral fitness cost of the mutations, the pathogenesis and sequence of infecting virus. The development of a viral escape is determined by the interaction of these multiple factors affecting immune pressure and viral fitness. However, these interactions are difficult to evaluate *in vivo.*

In Chapter 4 and Chapter 5, an *in vitro* Virus Evolution Assay was employed to assess the role of antigen specificity, TCR repertoire, functional avidity of HLA-B51 restricted CTLs as well as sequence of infecting virus in HLA-B51 associated HIV-1 viral evolution and viral control. We first evaluated the rate of escape mutations driven by the dominant HLA-B51 restricted CTL responses. In accordance with the *ex vivo* observations shown in Chapter 3, the *in vitro* assay demonstrated that three dominant HLA-B51 restricted CTL responses have driven

the sequential escape mutations within the epitopes, leading to the loss of viral control. Among them, the epitope Pol LI9, with higher epitope entropy, developed escape mutations most rapidly, leading thus to the loss of early viral control. By contrast, Gag NI9 epitope, with highly conserved sequence in viral Capsid protein and lower entropy, showed most resistance to the escape mutation, which promotes the long-term protection against HIV infection. In line with other studies, our data presented in Chapter 3 and Chapter 4 suggested that an effective T-cell based vaccine should stimulate T-cell responses targeting epitopes with low entropy or in a conserved region of virus such as Gag-p24 protein, and meanwhile avoid targeting the epitopes such as Pol LI9.

In Chapter 5, we assessed the impact of two factors including T-cell functional avidity and TCR repertoire on HIV-1 evolution and viral control. The observations suggest that the antigen sensitivity (functional avidity) of CTLs is an important factor for virus suppression but not for the rate of escape mutations. This is contrary to some literature, which showed CTLs with higher functional avidity drive escape mutations faster (Conrad et al., 2011; Iglesias et al., 2011). In addition, this *in vitro* Virus Evolution Assay demonstrated the profound effect of TCR repertoire on the virus evolution: clones with different TCR selected different escape mutations. Further study to sequence the CDR3 loop of the T cell receptors these two CTL express and assess the interaction of TCR and peptide-MHC class I complex may provide more detailed information on the role of TCR in shaping HIV-1 evolution.

More importantly, our data highlighted the major impact of the founder virus sequence on viral control. The presence of Valine (V) at position 8 in the epitope Pol TI8 could potentially delay the development of escape mutations. SM-8V virus with epitope Pol TV8 are less efficiently processed than TI8 epitope, which resulted in lower level of immune pressure on the SM-8V virus than JP-8I virus. This reduced level of immune pressure is not sufficient to escape T cell recognition, but significantly slows down the rate of development of escape mutations, which led to the better viral control. This fine balance between the rate of the escape mutations and the level of immune pressure exerted by CTLs may contribute to the long-term viral control of HLA-B51 patients in the SM cohort.

Another major obstacle for developing an efficient HIV-1 vaccine is incomplete understanding of what constitutes an effective T-cell response to inhibit HIV-1 replication.

Initial studies focused on phenotypic characterization of T cell differentiation. It has been suggested that HIV-specific $CD8^+$ T cells showed impaired efficacy with skewed maturation phenotype and failed to reach the highly differentiated effectors ($CD27^-CCR7^-CD45RA^+$). However, it is difficult to identify a specific differentiation stage that was associated solely with effective $CD8^+$ T cells. Many studies have focused on multifunctionality of T cells. Some studies showed that HIV-specific $CD8^+$T cells in LNTPs and ECs have polyfunctionality (Betts et al., 2006); greater proliferative capacity; and higher cytotoxicity than progressors

(Horton et al., 2006; Migueles et al., 2002). While Tussey et al. (2003) and Rehr et al. (2008) have demonstrated that the impairment of effectiveness of HIV-specific $CD8^+$ T cells can be restored by antiretroviral therapy (ART). This raises a question whether the effectiveness of HIV-specific T cells actually drives a low viral load in patients or if an effective functional profile is a result of a low antigenic stimulation.

Therefore, in Chapter 6, we characterised the phenotype and functionality of HLA-B51 restricted T cell responses. The preliminary data showed that HLA-B51 restricted T cells responding to the mutated epitopes tend to be more frequent in an early-differentiated stage and less replicatively senescent than the cells specific for an un-mutated epitope. This indicates that these "good phenotype" of $CD8^+$ T-cell responses in the chronic HIV-1 infection are most likely the by-product of low antigenic stimulation, rather than having been "protective".

In addition to mediating $CD8^+$ T-cell responses against HIV-1 infection, HLA-B51 also belongs to the group of HLAs that possess the 80Ile variant of Bw4 motif, which is thought to be the ligand for KIR3DL1 and KIR3DS1. The interactions between HLA-B Bw4-80Ile and KIR3DL1/KIR3DS1 have been linked to early control of HIV viral load and delayed progression to AIDS (Alter et al., 2009; Martin et al., 2002; Martin et al., 2007; Qi et al., 2006a), which is also supported by its efficient control of HIV-1 replication *in vitro* (Alter et al., 2007a). Thus, investigating the interactions between HLA-B51 and KIR3DL1/KIR3DS1 and their

impact on the development and functions of NK cells, and probably $CD8^+$ T cells, may provide us with a better understanding of protective immunity against HIV-1 infection, which is essential for rational HIV vaccine design.

To summarize, the data presented in this thesis proposed that an effective T-cell based vaccine should stimulate a moderate T-cell response targeting an epitope with low entropy or in a conserved region of virus such as Gag-p24 protein, in which an epitope mutation is difficult to develop or bears a fitness cost to the virus. In addition, the prevalence of HLA alleles in the targeted population and the circulating virus strain in the region should be taken into consideration when designing a T-cell based vaccine against HIV-1 infection. It is possible that a vaccine bearing one good epitope for each prevalent HLA allele, such as epitope Gag NI9 as to HLA-B51, could be sufficient to stimulate effective $CD8^+$ T cells against HIV-1 infection.

This study showed that under the same CTL selection pressure, different founder virus (i.e., 8I virus and 8V virus) developed different mutations, implying the sequences in the flanking regions of an epitope may influence the pattern of escape mutations. Sequencing 370 bp of *pol* gene, upstream and downstream of Pol TI8 epitope, did not show any other mutations outside this epitope. Therefore, sequencing the whole *pol* gene may identify other mutations in the epitope flanking regions which may associate with the escape mutations and affect the virus fitness. Furthermore, we showed that CTL clones with different T cell receptors selected

different virus mutations. Hence, the role of TCR repertoire in evolution of virus can be further investigated via sequencing of TCR Vβ gene, in particular, the interaction of CDR3 loops of these different CTL clones with peptide- MHC class I complex.

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Appendices

Appendix 1: HLA typing and clinical information of HLA-B51^{+} patients in the SM cohort. [Adapted from Yonghong Zhang's DPhil Thesis]

ID	A1	A2	B1	B2	Cw1	Cw2	CD4	VL	HAART
SM008	11	3303/06	440301	51	14	1302/34/41	370	23000	no
SM014	11	33	1401	51	8	01(02/04/06)	113	100000	no
SM040	24	30	3802/04/08	51	7	4	248	42000	no
SM047	2	3	47	51	06(02/03/07)	NF	143	88000	no
SM055	2	2	3501	51	3	9	203	480	yes
SM063	2	3303/06	44?	51?	7	01?	237	81000	yes
SM064	3	31	4402	51	5	15	460	86000	yes
SM166	11	31	15	51	0401N	1501/06/09/12	590	11000	no
SM176	11	32	3802/04/08	51	7	15	621	1200	no
SM181	1	2	37	51	6	1501	252	300000	no
SM192	2	02 again	46	51	1	16	na	na	yes
SM200	3	11	51	51	12	14	550	35	yes
SM209	24	31	51	54/5507	1	1501/06/09/12	508	35	no
SM215	201	3001/08/14/15	5101	51 again	602	7	514	200000	no
SM342	1	2	5701/06/08/10	51	6	7	222	200000	no
SM349	20601	11	51		14	4	378	820	no
SM350	2	33(03/06)	51	48	8	8	8	200000	no
SM353	2	68	5801	51	03(02/14)	11	169	100000	no
SM379	2	68	51	54/5507	1	15	570	35	yes
SM381	2	24	46	51	1	4	106	100000	no
SM446	2	26	38	51	1203/06	0101/05/07/08	421	14000	no
SM455	2	24	54/5507	51	1		98	35	yes

Table A.1 22 HLA B51+ Patients' HLA typing and clinical information in 2007.

Appendix 2- IFN-γ ELISPOT assay carried out by Yonghong Zhang to test 22 HLA-B51^{+} patients from the SM cohort for recognition of 17 known HLA-B51 restricted epitopes. [Adapted from Yonghong Zhang's DPhil Thesis]

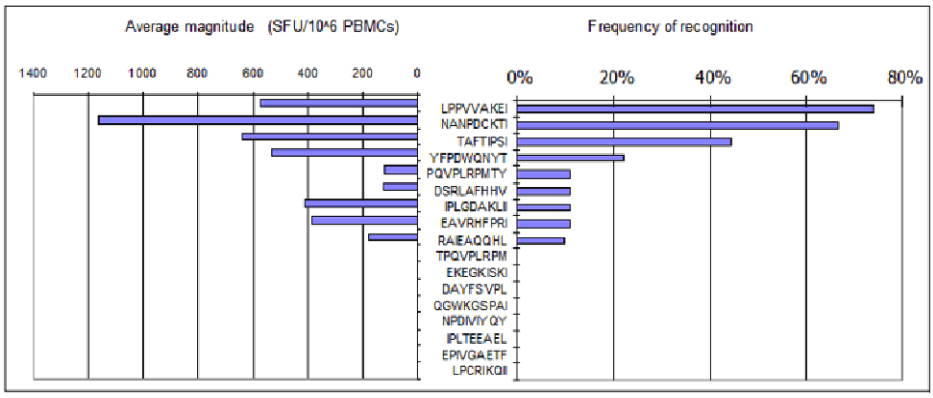

Figure A.1: The average frequency of recognition and magnitude of response to the individual epitopes restricted by HLA-B51. 17 known HLA-B51 epitope peptides from the Los Alamos database, based on consensus sequences from the cohort, were synthesized and ELISPOT assays were performed using patients' PBMCs. Epitopes Pol283 TI8: TAFTIPSI, Gag327 NI9: NANPDCKTI and Pol743 LI9: LPPVVAKEI were the most immunodominant epitopes in this cohort.

Appendix 3- Proviral sequencing carried out by Yonghong Zhang to detect the mutations within and around epitope Pol LI9 [Adapted from Yonghong Zhang's DPhil Thesis]

SM ID	740	741	742	743	744	745	746	747	748	749	750	751	752	753	754	755	756	757	758	759	760	761
cons	D	F	N	L	P	P	V	V	A	K	E	I	V	A	S	C	D	K	C	Q	L	K
B51+(N=22)																						
SM8	D	F	N	I	P	P	V	V	A	K	E	I	V	A	S	C	D	K	C	Q	I	K
SM14	D	F	S	I	P	P	V	V	A	K	E	I	V	A	C	C	D	K	C	Q	I	K
SM47	D	F	N	I	P	P	V	V	A	K	E	I	V	A	C	C	D	K	C	Q	L	K
SM55	D	F	N	I	P	P	V	V	A	K	E	I	V	A	S	C	D	K	C	Q	L	K
SM63	D	F	N	I	P	P	V	V	A	K	E	I	V	A	C	C	D	K	C	Q	I	K
SM64	D	F	S	I	P	P	V	V	A	K	E	I	V	A	S	C	D	K	C	Q	I	K
SM176	D	F	N	I	P	P	V	V	A	K	E	I	V	A	S	C	D	K	C	Q	V	K
SM181	D	F	N	I	P	P	V	V	A	K	E	I	V	A	M	C	D	K	C	Q	I	K
SM209	D	F	N	I	P	P	V	V	A	K	E	I	V	A	S	C	D	K	C	Q	I	K
SM215	D	F	N	I	P	P	V	V	A	K	E	I	V	A	S	C	D	K	C	Q	I	K
SM342	D	F	S	I	P	P	V	V	A	K	E	I	V	A	S	C	D	K	C	Q	I	K
SM350	D	F	N	I	P	P	V	V	A	K	E	I	V	A	C	C	D	K	C	Q	I	K
SM353	D	F	N	I	P	P	V	V	A	K	E	I	V	A	S	C	D	K	C	Q	V	K
SM381	D	F	N	I	P	P	V	V	A	K	E	I	V	A	S	C	D	K	C	Q	I	K
SM429	D	F	N	I	P	P	V	V	A	K	E	I	V	A	C	C	D	K	C	Q	I	K
SM446	D	F	N	I	P	P	V	V	A	K	E	I	V	A	S	C	D	K	C	Q	L	K
SM455	D	F	N	I	P	P	V	V	A	K	E	I	V	A	C	C	D	K	C	Q	I	K
SM40	D	F	N	L	P	P	V	V	A	K	E	I	V	A	S	C	D	K	C	Q	L	K
SM166	D	F	N	L	P	P	V	V	A	K	E	I	V	A	S	C	D	K	C	Q	I	K
SM192	D	F	N	L	P	P	V	V	A	K	E	I	V	A	S	C	D	K	C	Q	L	K
SM200	D	F	N	L	P	P	V	V	A	K	E	I	V	A	S	C	D	K	C	Q	L	K
SM379	D	F	N	L	P	P	V	V	A	K	E	I	V	A	S	C	D	K	C	Q	L	K
B51-(N=7)																						
SM53	D	F	N	I	P	P	V	V	A	K	E	I	V	A	C	C	D	K	C	Q	L	K
SM182	D	F	N	I	P	P	V	V	A	K	K	I	V	A	S	C	D	K	C	Q	I	K
SM188	D	F	N	I	P	P	V	V	A	K	E	I	V	A	S	C	D	K	C	Q	I	K
SM191	D	F	N	I	P	P	V	V	A	K	E	I	V	A	S	C	D	K	C	Q	V	K
SM349	D	F	N	I	P	P	V	V	A	K	E	I	V	A	S	C	D	K	C	Q	L	K
SM445	D	F	N	I	P	P	V	V	A	K	E	I	V	A	C	C	D	K	C	Q	L	K
SM168	D	F	N	L	P	P	V	V	A	K	K	I	V	A	S	C	D	K	C	Q	I	K
B51-(N=119)	D	F	N	L	P	P	V	V	A	K	K	I	V	A	S	C	D	K	C	Q	I	K

Table A.2 I1L mutation in epitope Pol LI9 is strongly linked with two downstream flanking mutations (754 and 760).

Appendix 4- HLA typing and clinical information of all the patients in the SM cohort

SM ID	Gender	Age	CD4 Count 2005	CD4 Count 2006	Viral Load 2006	CD4 Count 2007	Viral Load 2007	Viral Load 2012	CD4 Count 2012	HAART	HLA-A1	HLA-A2	HLA-B1	HLA-B2	HLA-Cw1	HLA-Cw2
SM001	1	40	182								A02	A30	B48	B13	Cw06	Cw08
SM002	2	38	45								A23	A24	B07	B13	Cw07	Cw06
SM003	1	54	287			335	180000				A02	A33	B58	B35	Cw03	Cw12
SM004	2	48	409	388	11000											
SM005	1	30	329								A03	A24	B35	B52	Cw03	Cw12
SM006	1	38	156	194	<50						A24	A24	B40	B15	Cw08	Cw08
SM007	2	52	471	370	<50	239	<50	2884	253	N	A01	A02	B40	B57	Cw06	Cw08
SM008	1	42	611			370	23000				A11	A33	B44	B51	Cw14	Cw14
SM009	2	35	583	374	29000			<50	564	N	A33	A33	B44	B39	Cw07	Cw14
SM010	2	48	324								A24	A30	B13	B46	Cw01	Cw06
SM011	1	53	1060			645	<50				A02	A29	B40	B46	Cw01	Cw03
SM012	2	38	285	276	44000	159	470				A01	A24	B57	B52	Cw06	Cw12
SM013	2	41	166								A02	A03	B40	B18	Cw07	Cw08
SM014	2	38	459	212	30000	113	100000	<50	371	2010-2012	A11	A33	B14	B51	Cw08	Cw14
SM015	2	38	1430								A03	A30	B27	B27	Cw06	Cw15
SM016	2	37	301								A01	A01	B37	B46	Cw01	Cw06
SM017	2	48	350	333	13000						A02	A30	B13	B27	Cw02	Cw06
SM018	2	40	790	337	<50	638	310									
SM019	2	40	598			698	650000	170	970	Oct-2011 till 2012	A30	A11	B13	B44	Cw04	Cw06
SM020	2	52	382					407	547	2008 till 2012	A02	A02	B46	B46	Cw01	Cw01
SM021	2	51	307	414	46000			14782	206	N	A02	A29	B44	B38	Cw04	Cw07
SM022	2	36	831			995	1400				A02	A33	B44	B67	Cw07	Cw14
SM023	1	30	792			550	28000				A02	A24	B40	B46	Cw01	Cw08
SM024	1	36	401			633	33000				A02	A24	B40	B15	Cw03	Cw03
SM025	1	34						<50	283	2009 till 2012	A02	A02	B50	B15	Cw03	Cw06
SM026	2	46	17								A30	A32	B52	B52	Cw12	Cw12
SM027	1	45	96								A30	A24	B13	B40	NA	NA
SM028	2	38	891								A01	A11	B07	B27	Cw07	Cw08
SM029	2	30	1074			127	64000				A02	A02	B40	B46	Cw01	Cw03
SM030	1	36	184			174	94000		553	2008 till 2012	A02	A24	B46	B55	Cw01	Cw01
SM031	2	59	191	133	<50						A30	A11	B40	B13	Cw06	Cw08
SM032	1	29	224								A02	A11	B40	B46	Cw01	Cw08
SM033	2	55				92					A02	A24	B40	B13	Cw03	Cw08
SM034	2	38	559	396	20000	250	5200				A24	A24	B40	B58	Cw03	Cw03
SM035	1	32	514	459	<50	424	85000				A02	A33	B40	B58	Cw03	Cw07
SM036	2	33	522	717	670	872	360				A30	A31	B13	B35	Cw04	Cw06
SM037	1	52	432					<50	361	2007 till 2012	A24	A11	B40	B40	Cw03	Cw07
SM038	2	45	192								A11	A11	B15	B52	Cw04	Cw12
SM039	2	35	105	463	21000	505	<50	1732	319	one month in 2004, 2010-2011	A02	A11	B49	B39	Cw07	Cw07
SM040	2	42	20			248	42000	<50	138	2010 till 2012	A24	A30	B38	B51	Cw07	Cw15
SM041	2	40	511			672	5700				A24	A30	B13	B35	Cw04	Cw06
SM042	2	32	759								A02	A11	B46	B15	Cw01	Cw04
SM043	2	51	487	627	390000	585	350000				A02	A24	B15	B56	Cw08	Cw01
SM044	1	30	899	715	<50											
SM045	2	40	225								A02	A02	B07	B18	Cw07	Cw07
SM046	1	42	274	168	1900			<50	350	N	A02	A30	B27	B15	Cw03	Cw15
SM047	1	40	195			143	88000	<50	296	2007 till 2012	A02	A03	B47	B51	Cw06	Cw15
SM048	1	39	14					5886	232	2009-July 2012.7	A02	A24	B13	B54	Cw01	Cw03
SM049	1	38									A30	A11	B13	B39	Cw03	Cw06
SM050	1	58	668	521	110000						A11	A11	B35	B52	Cw04	Cw12
SM051	1	42	205			294	1500									
SM052	2	48	115					<50	174	2007 till 2012	A02	A11	B15	B48	Cw08	Cw08
SM053	2	54	474			217	44000				A02	A11	B40	B48	Cw08	Cw04
SM054	1	49	625	208	260000			<50	543	2010 till 2012	A01	A24	B35	B37	Cw03	Cw06
SM055	1	42	169	203	<50	203	480	<50	291		A02	A02	B35	B51	Cw03	Cw03
SM056	2	52	50			724	35				A02	A33	B44	B13	Cw06	Cw07
SM057	1	54	567			238	7400				A32	A33	B44	B40	Cw04	Cw08
SM058	1	28	513								A02	A24	B15	B46	Cw01	Cw04
SM059	2	45	106			332	9000				A24	A30	B07	B13	Cw06	Cw07
SM060	2	59	1223			763	3100	<50	454	2002 till 2012	A11	A11	B08	B08	Cw07	Cw08

Table to be continued

Appendix 4- HLA typing and clinical information of all the patients in the SM cohort

SM ID	Gender	Age	CD4 Count 2005	CD4 Count 2006	Viral Load 2006	CD4 Count 2007	Viral Load 2007	Viral Load 2012	CD4 Count 2012	HAART	HLA-A1	HLA-A2	HLA-B1	HLA-B2	HLA-Cw1	HLA-Cw2
SM061	1	32	118								A03	A26	B08	B40	Cw03	Cw07
SM062	2	49	793	536	44000			<50	424	N	A02	A02	B54	B50	Cw01	Cw06
SM063	2	60	898		5400	237	81000				A02	A33	B44	B51	Cw07	Cw15
SM064	2	35	361	632	16000	460	86000				A03	A31	B44	B51	Cw05	Cw15
SM065	1	38	314			72	830000				A02	A29	B13	B13	Cw03	Cw03
SM121											A26	A33	B51	B39	Cw07	Cw15
SM163	1	48	330								A11	A30	B07	B13	Cw06	Cw07
SM164	2	50	327								A30	A31	B13	B35	Cw06	Cw08
SM165	1	46	333								A24	A11	B48	B58	Cw03	Cw08
SM166	2	47	1219	760	3900	590	11000	3268	746	N	A11	A31	B15	B51	Cw04	Cw14
SM167	1	50	483	776	<50	761	44000				A24	A31	B07	B46	Cw01	Cw07
SM168	2	48	257	323	<50	256	35	<50	420	N	A02	A30	B51	NA	Cw06	NA
SM169	1	55	279	333	47000	272	2900				A02	A33	B44	B40	Cw08	Cw14
SM170	2	38	267			319	<50				A29	A32	B07	B08	Cw07	Cw15
SM171	2	56	4								A02	A03	B15	B52	Cw03	Cw12
SM173	2	43	112			265	82000				A03	A33	B15	B52	Cw03	Cw12
SM174	1	45	382	477	5500						A24	A33	B44	B54	Cw01	Cw01
SM175	1	55	444	307	320000			<50	273	2009 till 2012	A11	A33	B07	B40	Cw07	Cw08
SM176	2	57				621	12000	27348	95	N	A11	A32	B38	B51	Cw07	Cw14
SM177	2	55	317								A11	A30	B13	B40	Cw06	Cw08
SM178	2	54	461			647	<50				A03	A33	B08	B58	Cw03	Cw07
SM179	1	54	45	138	72000	56	58000				A01	A24	B57	B54	Cw01	Cw06
SM180	2	54	731	830	<50	408	<50				A02	A24	B40	B40	Cw08	Cw15
SM181	2	50	504			252	300000	50435	357	2003 till 2012	A01	A02	B37	B51	Cw06	Cw14
SM182	1	51	327	355	<50			<50	134	2003 till 2012	A02	A30	B13	B40	Cw06	Cw08
SM183	2	36	34								A02	A24	B15	B46	Cw01	Cw04
SM184	2	-	1069	1024	48000						A01	A11	B08	B52	Cw07	Cw12
SM185	1	42	651	615	1200	497	1900				A01	A03	B07	B57	Cw06	Cw07
SM186	1	40	59	797	<50						A30	A01	B13	B37	Cw04	Cw06
SM187	2	-	49													
SM188	1	55	193			449	690				A02	A30	B13	B40	Cw06	Cw07
SM189	1	39	28								A01	A26	B40	B57	Cw03	Cw06
SM190	2	37	163			674										
SM191	1	32	351	455	2000	425	3200				A11	A30	B13	B40	Cw06	Cw07
SM192	1	31						5832	540	June 2011 till 2012						
SM193	1	33	38	268	11000	239	30000	<50	1315	2007 till 2012	A02	A30	B46	B13	NA	NA
SM194	2	33	1232			1039	<50				A02	A24	B38	B15	Cw07	Cw08
SM195	2	57	121								A01	A24	B37	B40	Cw03	Cw06
SM196	1	50	620	563	<50	542	8100	<50	580	N	A01	A03	B51	B57	Cw06	Cw14
SM197	1	32	791			624	<50				A01	A01	B27	B57	Cw02	Cw06
SM199	2	43	1063			513	<50				A02	A24	B08	B48	Cw07	Cw08
SM200	1	44	401		<50	406	290	<50	471	2007 till 2012	A03	A11	B51	B51	Cw12	Cw12
SM201	2	54	427								A02	A32	B48	B35	Cw08	Cw08
SM202	1	34	13	130	25000											
SM203	1	54	775	536	490			<50	240	Feb-2012 till July-2012	A02	A30	B13	B40	Cw06	Cw08
SM204	1	53	63	86	20000						A24	A33	B44	B15	NA	NA
SM205	2	38	539			559	<50	<50	175	2007 till 2012	A02	A29	B07	B39	Cw07	Cw15
SM206	1	60	41													
SM207	2	48	161	206	56000	193	1300				A32	A33	B58	B35	Cw03	Cw04
SM208	1	45	249	306	450	408	11000	<50	485	2008 till 2012	A02	A33	B44	B46	Cw01	Cw07
SM209	1	36	785	535	<50	508	<50	4034	478	N	A24	A31	B51	B54	Cw01	Cw14
SM210	1	37	5			609	<50				A02	A30	B40	B40	Cw03	Cw06
SM211	1	31	64	217	47000	911	v				A02	A02	B13	B40	Cw03	Cw03
SM212	1	46	203													
SM213	2	30									A02	A33	B51	B58	Cw03	Cw15
SM214	2	49	1348	1165	9400						A03	A24	B07	B46	Cw07	NA
SM215	2	42	430	867	7400	514	200000	<50	459	2009 till 2012	A02	A30	B51	B51	Cw06	Cw14
SM216	1	60	321					780	257	2007 till 2012	A02	A24	B13	B15	Cw03	Cw08
SM217	2	47	71	390	<50	392					A02	A31	B40	B40	Cw03	Cw15
SM218	1	78	40								A30	A11	B13	B40	NA	NA
SM219	2	40		75	1700	879	18000	9025	136		A02	A11	B39	B55	Cw01	Cw07
SM220	1	49	343								A33	A32	B40	B38	Cw07	Cw08
SM221	1	31	1020	962	40000						A02	A11	B40	B54	Cw01	Cw08
SM222	2	42	625	492	78000	679	<50				A11	A30	B13	B15	Cw06	Cw07
SM223	1	52	218								A02	A32	B44	B46	Cw01	Cw04
SM225	1	48	398								A02	A02	B40	B35	Cw03	Cw03
SM226	2	66	613								A24	A11	B40	B15	Cw03	Cw03
SM227	1	67	339			596	<50				A03	A30	B07	B13	Cw06	Cw07
SM228	2	35									A33	A33	B44	B44	Cw07	Cw14
SM229	2	33	535													
SM230	2	49						137	761	2004till 2012	A02	A02	B40	B35	Cw03	Cw03
SM231	1	36	949	1366	<50						A32	A33	B38	B40	Cw07	Cw08

Table to be continued

Appendix 4- HLA typing and clinical information of all the patients in the SM cohort

SM ID	Gender	Age	CD4 Count 2005	CD4 Count 2006	Viral Load 2006	CD4 Count 2007	Viral Load 2007	Viral Load 2012	CD4 Count 2012	HAART	HLA-A1	HLA-A2	HLA-B1	HLA-B2	HLA-Cw1	HLA-Cw2
SM232	2	33	648			1019	v				A02	A11	B07	B46	Cw01	Cw07
SM233	2	40	487								A24	A29	B55	B55	Cw01	Cw07
SM234	2	12	420								A01	A02	B57	B57	Cw06	Cw06
SM235	1	35	930	975	<50						A02	A33	B13	B35	Cw04	Cw06
SM321	2	48	204			38	12000	118	522	N	A24	A11	B54	B51	Cw01	NA
SM322	1	43	227													
SM323	2	42	496			1009	35									
SM324	2	50	218			174	20000				A11	A33	B13	B40	Cw07	Cw08
SM325	1	58	540	426	1100	340	21000				A01	A26	B27	B38	Cw02	Cw12
SM326	2	56	647													
SM327	2	50	323			658	130000	158	31	6 months in 2003	A02	A02	B15	B39	Cw07	Cw08
SM328	1	49	1030	1163	<50	976	5800				A02	A30	B51	B51	Cw06	Cw12
SM329	2	40	279					<50	302	2010 till 2012	A01	A11	B48	B35	Cw03	Cw06
SM330	2	50	238			276	9400	51	241	2006 till 2012	A02	NA	B58	B15	Cw03	Cw03
SM331	2	56	289	110	<50	157	2200				A02	A33	B51	B51	Cw03	NA
SM332	1	32	603			1322	390									
SM334	2	32	1015			700	<50				A02	A30	B13	B13	Cw03	Cw06
SM335	1	45	375	469	5500	301	79000	71263	228	6 months in 2002	A30	A31	B13	B40	Cw06	Cw03
SM336	2	43	389			454	9800	<50	415	2005 till 2012	A11	A33	B38	B58	Cw03	Cw07
SM337	2	42	656			643	35				A02	A11	B35	B39	Cw07	Cw08
SM338	1	38	593	537	79000	391	55000	<50	853	2009 till 2012	A02	A24	B52	B48	Cw08	Cw12
SM339	2	42	216	445	180000	404	19000	<50	1113	2010 till 2012	A02	A24	B40	B40	Cw07	Cw15
SM340	1	32	83	75	20000						A11	A11	B40	B52	NA	NA
SM341	1	50	48													
SM342	2	48	267	253	37000	222	150000	108	213	2010 till 2012	A01	A02	B57	B51	Cw06	Cw14
SM343	2	34	787			672	1600				A33	-	B40	B27	NA	NA
SM344	1	32	4					1162	306	2010 till 2012	A02	A24	B13	B48	Cw06	Cw08
SM345	1	35	593	568	1300	596	14000				A29	A26	B07	B39	Cw03	Cw15
SM346	2	34	1073			587	<50				A02	A02	B35	B58	Cw03	Cw03
SM347	1	34	679					<50	287	2010 till 2012	A02	A01	B57	B38	Cw06	Cw07
SM348	2	36	909								A02	A02	B40	B39	Cw03	Cw07
SM349	2	40	685	576	<50	378	820	6764	434		A02	A11	B51	NA	Cw14	NA
SM350	2	37	139			8	240000				A02	A33	B51	B48	Cw08	Cw15
SM351	1	60	758			250	280000				A02	A24	B07	B35	Cw03	Cw07
SM352	2	58	158					<50	213	2008 till 2012	A02	A33	B44	B40	Cw07	Cw07
SM353	2	42	406			169	130000				A02	A68	B58	B51	Cw03	Cw14
SM354	2	40	578	414	<50						A03	A31	B40	B52	Cw07	Cw12
SM355	1	48	332								A02	A11	B07	B40	Cw07	Cw07
SM356	1	30	563			238	200000				A02	A33	B40	B58	Cw03	Cw07
SM357	1	70	734	783	19000						A02	A24	B40	NA	Cw03	Cw08
SM358	2	34	698			736	<50	1716	387		A02	A11	B49	B50	Cw03	Cw06
SM359	1	45	727	804	3600	649	4000	26307	246	2 months in 2012	A02	A11	B13	B48	Cw06	Cw08
SM360	2	34	422	410	27000	334	110000				A01	A32	B37	B44	Cw04	Cw06
SM361	1	62	17								A01	A02	B57	B35	Cw03	Cw06
SM362	1	49	594													
SM363	1	34	45			580	640	386	507	N	A02	A31	B40	B35	Cw03	Cw08
SM364	2	27	764			1030	<50				A02	A30	B40	B13	Cw15	Cw06
SM365	2	32	808			1180	<50				A11	A29	B55	NA	Cw1	Cw15
SM366	2	36									A02	A32	B46	B52	Cw01	Cw12
SM367	1	34	61			752	<50									
SM368	2	36	8								A01	A02	B49	B48	Cw07	Cw08
SM369	2	58	28			144	1600	<50	182	2008 till 2012	A02	A24	B40	B54	Cw01	Cw03
SM370	1	40	42			894	<50									
SM373	2	55	691			464	23000				A02	A02	B40	B27	Cw08	Cw15
SM374	1	35	81	358	<50			2106	479	May 2012 till August 2012	A02	A02	B40	B15	Cw03	Cw15
SM375	2	35	607													
SM376	2	48	11			755	v				A01	A33	B57	B58	NA	NA
SM377	2	39	259			308	50000				A03	A31	B51	B44	Cw05	Cw15
SM378	1	35	402			394	<50				A30	A33	B58	B58	Cw03	Cw06
SM379	2	38	478			570	<50				A02	A68	B51	B54	Cw01	Cw12
SM380	2	43	196			519	<50	<50	532	2010 till 2012	A11	A33	B58	B15	Cw03	Cw03
SM381	2	33	244			106	140000				A02	A24	B46	B51	Cw01	Cw14
SM382	2	37	185	74	48000	10	300000				A24	A33	B48	B58	Cw03	Cw08
SM383	2	53	116			602	<50				A24	A24	B40	B51	Cw03	Cw03
SM384	2	40	917			877	<50				A03	A24	B35	B51	Cw04	Cw14
SM385	2	13	730								A02	A03	B13	B51	Cw03	Cw15
SM402	1	37	81	67	110000	160	<50				A02	A33	B44	B46	Cw01	Cw07
SM403	1	34	230			225	160000				A33	A33	B58	B52	Cw03	Cw12
SM404	2	34	384								A11	A24	B51	B27	Cw12	Cw14
SM405	1	35	274	319	100000						A02	A11	B13	B15	Cw03	Cw04
SM406	1		37					<50	946	2005 till 2012						
SM407	1	68	258								A02	A11	B27	B46	Cw01	Cw02

Table to be continued

Appendix 4- HLA typing and clinical information of all the patients in the SM cohort

SM ID	Gender	Age	CD4 Count 2005	CD4 Count 2006	Viral Load 2006	CD4 Count 2007	Viral Load 2007	Viral Load 2012	CD4 Count 2012	HAART	HLA-A1	HLA-A2	HLA-B1	HLA-B2	HLA-Cw1	HLA-Cw2
SM408	2	37	177								A11	A30	B51	B51	Cw14	Cw15
SM409	1	55	99	152	<50			<50	131	2008 till 2012	A11	A26	B38	B39	Cw03	Cw07
SM410	2	39	165	300	2700						A24	A30	B15	B15	Cw04	Cw07
SM411	1	72	1074	1133	<50	1214	<50				A02	A11	B13	B46	Cw01	Cw06
SM412	1	41	54								A02	A33	B58	B54	Cw03	NA
SM413	1	50	144													
SM414	2	40	636			683	<50	<50	532	2005 till 2006	A01	A30	B14	B27	Cw02	Cw08
SM415	2	54	1084			745	47000				A24	-	B7	B48	Cw7	Cw8
SM416	2	49	177	233			15000				A24	A33	B58	B54	Cw01	Cw03
SM417	1	34	60	68	21000	40	170000	12109	29	2010 (one year)	A01	A02	B57	B57	Cw06	Cw03
SM418	1	55	353	340	7900	338	29000				A30	A31	B13	B51	Cw6	Cw15
SM419	2	47	552								A02	A02	B46	B48	Cw01	Cw03
SM420	1	50	144													
SM421	1	29	386	285	320000											
SM422	1	45	228			362	1000				A02	A33	B40	B58	Cw03	Cw08
SM423	2	30	988								A03	A30	B27	B27	Cw02	Cw06
SM424	2	34	407	258	28000	155	11000				A29	A24	B07	B54	Cw01	Cw15
SM425	2	37	813			590	<50				A01	A30	B57	B57	Cw06	NA
SM426	2	36				765	750	<50	642	Oct-2010 till 2012	A03	A31	B46	B52	Cw01	Cw12
SM427	1	34	74			8	1100000				A02	A24	B48	B39	Cw07	Cw08
SM428	2	32	449	482	63000			<50	259	2005 till 2006						
SM429	2	43	138			87	250000	<50	216	2008 till 2012	A02	A02	B51	B58	Cw03	Cw14
SM430	2	50	342					3636	420	N	A30	A32	B13	B52	Cw06	Cw12
SM431	2	41									A02	A24	B51	B51	Cw14	Cw15
SM432	2	42						<50	419	2005 till 2012						
SM433	2	47	250													
SM434	1	41	1294			1007	35				A11	A30	B13	B13	Cw03	Cw06
SM435	2	51				238	49000	<50	966	2009 till 2012	A02	A24	B40	B51	Cw03	Cw14
SM436	2	13									A24	A33	B38	B58	Cw03	Cw07
SM437	2	38	252	226	130000	150	140000	<50	258	2010 till 2012	A11	A24	B40	B38	Cw07	Cw08
SM438	1	40	123													
SM439	1	53	282	251	21000						A03	A03	B27	B08	Cw02	Cw07
SM440	1	28	423	317	200000			<50	585	Jun-2011 till Mar-2012	A02	A32	B27	B35	Cw02	Cw03
SM441	1	60	248	233	10000	143	43000	1561	147	2009 till 2012	A11	A29	B07	B40	Cw07	Cw15
SM442	2	50	305								A24	A33	B44	B51	Cw07	Cw14
SM443	2	51									A02	A33	B46	B58	Cw01	Cw03
SM444	2	38									A11	A11	B07	B51	Cw07	Cw14
SM445	2	55	180	493		288	6800									
SM446	1	50	576	494	<50	421	14000	3217	187	N	A02	A26	B38	B51	Cw12	Cw14
SM447	2	51	573													
SM448	2	34	227	187	51000						A02	A11	B27	B54	Cw01	Cw08
SM449	1	31	214	211	7600			<50	256	2008 till 2012	A02	A02	B46	B55	Cw01	Cw01
SM450	2	37	573			406	1000				A02	A24	B44	B40	Cw07	Cw08
SM452	1	46	486	234	270000	502	13000				A32	A33	B44	B58	Cw03	Cw05
SM453	1	38	585			490	<50									
SM454	2	48	156	251	<50	316	<50				A24	A33	B48	B58	NA	NA
SM455	2	40				98	<50				A2	A24	B51	B54	Cw1	NA
SM456	2	49	294								A30	A33	B13	B58	Cw03	Cw06
SM457	1	41				397	980	902	466	N	A02	A31	B15	B15	Cw03	Cw04
SM458	2	32	1076								A11	A30	B13	B55	Cw01	Cw06
SM459	2	42	265	418	3100	222	180000				A02	A24	B40	B15	Cw01	Cw03
SM460	1	38	129	74	49000	48	310000				A02	A11	B40	B15	Cw03	Cw03
SM461	2	39	67	81	13000	29	90000				A24	A11	B15	B51	Cw04	Cw15
SM481	1	42						11200000	457							

Table A.3 258 Patients' HLA typing and clinical information in 2005, 2006, 2007 and 2012.

Gender: 1=Male; 2=Female. HAART: N= no treatment. HLA-typing: NA= not available.